INVESTIGATING GENETIC FACTORS UNDERLYING HYPOPITUITARISM AND SEPTO-OPTIC DYSPLASIA IN HUMANS

By

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DECLARATION

I, Sujatha Aneka Jayakody confirm that the work presented in this thesis is my own.
Where information has been derived from other sources, I confirm that this has been indicated in the thesis.
ABSTRACT

Differentiation of pituitary progenitors into the six different hormone-producing cell types that form the mature organ is a complex process that requires the coordinated spatial and temporal expression of multiple genes. Over the past decades, it has become apparent that mutations in a number of these developmentally important pituitary-specific transcription factors, including Hesx1 and Sox2, leads to hypopituitarism with or without syndromic features such as septo-optic dysplasia (SOD). This thesis extends previous work in further understanding basic pituitary development with the ultimate goal of increasing knowledge of hypopituitarism and SOD in humans.

Hesx1 was one of the first genes implicated in these conditions. It is a homeobox transcriptional repressor that is required for normal anterior forebrain and pituitary development in mouse and human. Here a dual approach was used to advance our understanding of Hesx1 function in the developing hypothalamic-pituitary axis (HPA). Firstly, phenotypic analysis of the neuroendocrine hypothalamus in Hesx1 mouse models has revealed an original role for Hesx1 in the development of this structure, providing a strong correlation with human SOD cases. Secondly, functional analysis of novel HESX1 mutations has showed that mutations occurring within the homeodomain impede DNA binding whilst repressor activity is maintained, providing more insight into the molecular function of HESX1.

Sox2 is a SOXB1-HMG-box transcription factor that has been recently associated with hypopituitarism, demonstrating a requirement of SOX2 for normal HPA development and function in humans. We used a condition approach in mice to investigate the pathogenesis of these defects. Our observations demonstrate that Sox2 is required for the normal proliferation of pituitary progenitors, and for the terminal differentiation of the Pit1 lineage. Furthermore, Hesx1Cre/+;Sox2fl/fl embryos exhibit reduced GnRH neurogenesis, providing insights into the characteristic hypogonadotrophic hypogonadism observed in SOX2 patients. Combined, the Hesx1Cre/+;Sox2fl/fl mouse provides a suitable model for understanding the pathogenesis of SOX2 mutations in humans.
PUBLICATIONS


*These authors made an equal contribution to this paper.
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<tr>
<td>αGSU</td>
<td>α-glycoprotein subunit</td>
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<td>αMSH</td>
<td>α-melanocyte-stimulating hormone</td>
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<td>anterior neural ridge</td>
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<td>GH</td>
<td>growth hormone</td>
</tr>
<tr>
<td>GHRH</td>
<td>growth-hormone-releasing hormone</td>
</tr>
<tr>
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<td>gonadotrophin-releasing hormone</td>
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<tr>
<td>Gnhr</td>
<td>gonadotrophin-releasing hormone receptor</td>
</tr>
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<tr>
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<tr>
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<td>HISS</td>
<td>heat-inactivated sheep serum</td>
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<tr>
<td>HMG</td>
<td>high motility group</td>
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<tr>
<td>ICM</td>
<td>inner cell mass</td>
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<td>intermediate lobe</td>
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<td>kb</td>
<td>kilobase</td>
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<td>LH</td>
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<td>magnesium chloride</td>
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<td>messenger RNA</td>
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<td>amino</td>
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<td>nitro-blue tetrazolium chloride</td>
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<td>N-CoR</td>
<td>nuclear corepressor complex</td>
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<td>optical density</td>
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<td>oxytocin</td>
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<td>P</td>
<td>postnatal day</td>
</tr>
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<td>p</td>
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<td>PBS containing Triton-X-100</td>
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<td>PCR</td>
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<td>platelet endothelial cell adhesion molecule</td>
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<td>passive lysis buffer</td>
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<td>proopiomelanocortin</td>
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<td>PP</td>
<td>posterior pituitary</td>
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<td>PRL</td>
<td>prolactin</td>
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<tr>
<td>PSIS</td>
<td>pituitary stalk interruption syndrome</td>
</tr>
<tr>
<td>PVA</td>
<td>polyvinyl alcohol</td>
</tr>
<tr>
<td>PVN</td>
<td>paraventricular nucleus</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RP</td>
<td>Rathke's pouch</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Acronym</td>
<td>Term</td>
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<td>serum-free media</td>
</tr>
<tr>
<td>Shh</td>
<td>sonic hedgehog</td>
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<tr>
<td>SOD</td>
<td>septo-optic dysplasia</td>
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<tr>
<td>TBS-T</td>
<td>tris-buffered saline plus Tween-20</td>
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<td>tetramethylethylenediamine</td>
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<tr>
<td>TGFβ</td>
<td>transforming growth factor-β</td>
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<tr>
<td>TLE</td>
<td>transducin-like enhancer of split</td>
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<td>thyrotrophin-releasing hormone</td>
</tr>
<tr>
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<td>thyroid-stimulating hormone</td>
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<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
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<td>vascular endothelial growth factor A</td>
</tr>
<tr>
<td>VMH</td>
<td>ventro-medial nucleus</td>
</tr>
<tr>
<td>YFP</td>
<td>yellow fluorescent protein</td>
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CHAPTER 1: GENERAL INTRODUCTION
1.1 INTRODUCTION

The hypothalamus and pituitary gland constitute the main axis of the neuroendocrine system and exhibit outstanding coordination in both spatial and temporal events regulating their development and differentiation. Through integrating physiological signals from peripheral structures, the instructing hypothalamus coordinates the synthesis and release of hormonal products from the pituitary, which in turn act to regulate a multitude of vital physiological functions including body growth, metabolism, sexual maturation, reproduction, stress response and immune defence [reviewed in (Andersen and Rosenfeld, 2001; Kelberman et al., 2009; Zhu et al., 2007)].

Classical embryological fate-mapping studies in frog, chick and mouse have shown that the most anterior part of the neural plate, termed the anterior neural ridge, will later give rise to non-neural structures, such as the anterior pituitary (AP), nasal cavity and the olfactory placode. In contrast, adjacent regions of the neural plate will later produce most anterior neural structures, such as the hypothalamus, posterior pituitary (PP) and the optic vesicles (Couly and Le Douarin, 1988; Dutta et al., 2005; Kawamura et al., 2002; Kouki et al., 2001; Pogoda and Hammerschmidt, 2007; Rubenstein et al., 1994). Thus, before visibly detectable, cell compartments reveal signs of pre-commitment, possessing the competency to develop into these structures. Therefore, when developmental irregularities affecting the early embryonic patterning occur, they may result in abnormalities that encompass a wide range of symptoms affecting all or many of these structures.

Over the past two decades, the era of murine transgenesis and mutagenesis has proved invaluable in our understanding of key molecules involved in the hypothalamic-pituitary axis, and indeed, mutations in these genes have been accurately associated with defects affecting the pituitary and nearby structures in humans (Andersen and Rosenfeld, 2001; Castinetti et al., 2011; Kelberman and Dattani, 2007b; Mehta and Dattani, 2008). A major class of these culprits have been found to encode transcription factors, which are proteins that bind to specific deoxyribonucleic acid (DNA) sequences in order to either activate and/or repress target genes. Transcription factors comprise a large family of proteins that are subdivided based on structure.
and function, and interestingly, they represent one of the four major groups implicated in human
disease (Chi, 2005; Jimenez-Sanchez et al., 2001).

*Hesx1* and *Sox2* are two transcription factors that have previously been shown to
participate in the development of the hypothalamic-pituitary axis, and when mutated in humans,
contribute to the pituitary conditions of hypopituitarism and/or septo-optic dysplasia (SOD)
(Dattani et al., 1999; Kelberman et al., 2006; Kelberman et al., 2008). Despite recent efforts, it
is still not fully understood how these genes participate during normal and pathological
development of the hypothalamic-pituitary axis. Consequently, this thesis aims to further
investigate the precise function of these two transcriptional mediators within the hypothalamic-
pituitary axis, using the murine system as the experimental model of choice.

In this introduction, I will briefly discuss the hypothalamic-pituitary axis, specifically
its anatomical organisation and embryonic development. This will then be followed by a
detailed review of *Hesx1* and *Sox2* function in the hypothalamic-pituitary axis, and their role in
pituitary gland development and related human conditions.
1.2 THE HYPOTHALAMIC-PITUITARY AXIS

For many years the pituitary gland has been regarded as the ‘master’ endocrine regulator, as it functions to control vast physiological processes through the secretion of polypeptide hormones into general circulation. In turn, these hormones act upon their peripheral endocrine target organs to regulate many vital processes crucial to maintain the body’s internal balance. However, it is now a widely accepted principle that the functioning of the pituitary gland is primarily regulated under the control of the hypothalamus. Consequently, the hypothalamus acts as the principal neural structure in this system by collating and coordinating complex signals from other regions of the brain and the periphery, translating them into neurosecretory products that can be recognised and processed by the pituitary (Andersen and Rosenfeld, 2001; Guillemin, 1978; Kelberman et al., 2009; Kelberman and Dattani, 2007b; Schally, 1978).

Given the intimate relationship between these two structures, it is hardly surprising that anatomically, they are situated in close proximity. The vertebrate hypothalamus encompasses the medio-basal regions of the central nervous system (CNS) and overlies the pituitary gland situated within the sella turcica, a recess within the basosphenoid bone at the base of the brain (Andersen and Rosenfeld, 2001; Markakis, 2002).

In mouse, the mature pituitary gland or hypophysis is comprised of three distinct anatomical and functional entities: the anterior lobe (AL) and intermediate lobe (IL), which collectively comprise the adenohypophysis or AP; and the posterior lobe or PP that is also referred to as the neurohypophysis (Figure 1.1). In rodents, the AL and IL are separated by a cleft, however, in humans no obvious cleft exists and therefore, a distinction between these two regions is less evident (Kelberman et al., 2009). The mature AL classically houses five different specialised hormone-secreting cell types. Namely, the somatotrophs that synthesise growth hormone (GH), the lactotrophs that secrete prolactin (PRL), the corticotrophs that produce adrenocorticotropic hormone (ACTH) by the proteolytic cleavage of proopiomelanocortin (POMC), the thyrotrophs that generate thyroid-stimulating hormone (TSH) and the gonadotrophs that release luteinising and/or follicle-stimulating hormone (LH/FSH). LH, FSH
and TSH represent a dipeptide hormone, composing of the common α-glycoprotein subunit (αGSU) and a divergent β subunit. The IL melanotrophs secrete α-melanocyte-stimulating hormone (αMSH), an additional product of POMC-mediated cleavage [reviewed in (Andersen and Rosenfeld, 2001; Kelberman et al., 2009; Treier and Rosenfeld, 1996; Vankelecom, 2011)].

In contrast, the PP contains no endocrine cell types, but is instead richly endowed with axonal projections from hypothalamic neurons, reported to comprise 42% of its total volume (Brownstein et al., 1980).

The hypothalamus shows extensive spatial patterning along the rostro-caudal axis and can be divided into four main regions; preoptic, supraoptic, tuberal and mammillary. Within these anatomical regions, many nuclei are located intermingled within less defined areas (Andersen and Rosenfeld, 2001; Markakis, 2002). The neuroendocrine hypothalamus refers to the hypothalamic neurons that are specifically involved in the regulation of the pituitary and comprises of two main subtypes, the parvocellular and magnocellular neurons, which reside in distinct and discrete nuclei (Figure 1.1).

The parvocellular neurons of the neuroendocrine hypothalamus operate to regulate the function of the AP through the synthesis and secretion of ‘releasing’ and ‘inhibiting’ factors. Specifically, the parvocellular axonal projections remain within the brain where they terminate at the median eminence, a region of the hypothalamus immediately ventral to the third ventricle that is highly vascularised by the hypophyseal portal system. Here the median eminence acts as a central ‘hub’ allowing the release of parvocellular products into the portal vasculature for delivery to the AP, and thus, provides instructive signals to specific AP endocrine cells. The five peptidic hormones produced by the parvocellular neurons are well characterised and their location within the hypothalamus defined. Both corticotrophin-releasing hormone (CRH) and thyrotrophin-releasing hormone (TRH) are produced within the paraventricular nucleus (PVN), where they stimulate the release of ACTH from corticotrophs and TSH from thyrotrophs. Similarly, gonadotrophin-releasing hormone (GnRH) is produced by neurons within the preoptic region and controls the function of gonadotrophs. Finally, growth-hormone-releasing hormone (GHRH) is synthesised by the neurons of the arcuate nucleus (Arc) and works
antagonistically with somatostatin (SS) secreted by neurons within the anterior periventricular nucleus (aPV), to regulate the release and synthesis of GH from the somatotrophs.

In contrast, the magnocellular neurons project directly to the PP, where under hypothalamic control they directly release arginine vasopressin (AVP) and oxytocin (OT) into general circulation, bypassing in this way any intermediate portal system. This projection of the magnocellular neurons provides an essential bridge between the brain and pituitary - the pituitary stalk - which is intimately associated with the hypophyseal portal system that transports hypothalamic commanding signals to the AP (HARRIS, 1955). Of worth, the pituitary stalk is not evident in mice, but very clear in humans [reviewed in (Andersen and Rosenfeld, 2001; Kelberman et al., 2009; Markakis, 2002; Szarek et al., 2010)].

Taken together, it is evident that the hypothalamic-pituitary axis represents an important system in maintaining a number of downstream physiological processes crucial for the body’s internal balance (summarised in Table 1.1). Of interest, the close association between these two structures is not merely a coincidence, but reflects an interaction between these two structures that is initiated very early during embryogenesis. In the following section, I will provide a brief overview of embryonic pituitary and neuroendocrine hypothalamic development. In particular, I will discuss factors and signalling pathways of immediate relevance to the work presented in the succeeding chapters of this thesis.
Figure 1.1 - The hypothalamic-pituitary axis
Schematic representation of the pituitary gland and its connection with the overlying hypothalamus. Two types of neurons convey signals from the hypothalamus to the pituitary gland to coordinate pituitary function. Magnocellular neurons located within the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus project their axons directly to the posterior lobe (PL) where they release AVP and OT into general circulation. In contrast, the parvocellular neurons that govern the anterior lobe (AL) project their axons to the median eminence, releasing these signals into the hypophyseal portal system that nourishes the AL. GHRH parvocellular neurons reside in the arcuate nucleus, which functions to stimulate the release of GH from the somatotrophs. SS-expressing neurons, located with the anterior paraventricular nucleus, a nucleus contiguous with the PVN, act to inhibit the release of GH. CRH- and TRH-expressing neurons are housed within the PVN and promote the release of ACTH and TSH from the corticotrophs and thyrotrophs, respectively. Lastly, GnRH neurons do not accrue in any particular nuclei, and can be found scattered along the rostro-caudal continuum of the ventral hypothalamus. These neurons act to stimulate the gonadotrophs to release FSH and LH. Melanotrophs are housed in the intermediate lobe and produce αMSH. Figure is adapted from (Andersen and Rosenfeld, 2001).
<table>
<thead>
<tr>
<th>Cell types</th>
<th>Hormone Product</th>
<th>Target</th>
<th>Tissue</th>
<th>Response</th>
<th>Positive</th>
<th>Negative</th>
<th>Regulator</th>
<th>Hypopituitarism Phenotype</th>
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<td></td>
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<td>OT</td>
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<td>Suckling activation in stretch receptors</td>
<td>Progesterone</td>
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<td>Thyroid hormone (T₃, T₄)</td>
<td>TRH</td>
<td>Thyroid hormone (T₃, T₄)</td>
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<td>GnRH</td>
<td>Gonadal steroids</td>
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<td>Sexual immaturity; infertility</td>
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</table>

* Melanotrophs have pleiotropic functions [for review, see (Strand, 1999)]. Table is modified from (Watkins-Chow and Camper, 1998).
1.3 DEVELOPMENT OF THE HYPOTHALAMIC-PITUITARY AXIS

The developing pituitary gland arises from a dual embryonic origin; the AP evolves from the oral ectoderm, whereas the PP arises from the neural ectoderm. Specifically, the AP derives from the ectodermal midline structure located immediately anterior to the developing neural plate, termed the anterior neural ridge (ANR), whereas the midline region of the neural plate, located posteriorly adjacent to the ANR, is destined to become the hypothalamus and the PP (Figure 1.2). Subsequently, during headfold stages in higher vertebrates or approximately 8.5 days post coitum (dpc) in the mouse, these two adjoining regions of the developing organism are displaced ventrally from their most anterior aspects, resulting in the developing AP occupying regions fated to give rise to the future oral cavity. At the same time, the presumptive hypothalamus and PP are relocated, becoming part of the ventral diencephalon (Kawamura et al., 2002; Kouki et al., 2001; Pogoda and Hammerschmidt, 2007; Rubenstein et al., 1994).

1.3.1 Development of the pituitary gland

1.3.1.1 Morphogenesis of the pituitary gland

Dating back to the early 1900s, Rathke and Schwind established the well-defined morphological stages of pituitary gland organogenesis (Figure 1.2) (Schwind, 1928). Initiating at 8.5 dpc in mouse, the formation of the rudimentary pouch commences as a thickening of the oral ectoderm which involutes dorsally as an epithelial bud towards the overlying ventral diencephalon. In humans, comparative stages are observed during the fourth week of gestation (Mullis, 2001). By 9.5 dpc, further invagination of this thickened oral ectoderm continues, forming a ‘definite pouch’ structure referred to as Rathke’s Pouch (RP), which results in direct cell-cell contact between RP and the nascent ventral diencephalon. This intimate positioning of
adjacent tissues is required for interactions between the oral and neural ectoderm, and is essential for progressive pituitary development. By 10.5 dpc, the first indication of an infundibular recess - the primordium of the PP - becomes evident, coinciding with a much more substantial RP (5th week of gestation in humans). By 12.5 dpc, the connection between RP and the roof of the oral cavity is lost, allowing for the subsequent fusion of the underlying developing basosphenoid bone to occur. This ultimately results in the correct placement of the pituitary within the sella turcica, beneath the overlying hypothalamus at later stages of development. Between 11.5 and 13.5 dpc, extensive proliferation of progenitor cells within the periluminal epithelium of RP occurs, giving rise to pools of progenitors of all the hormone-producing cell types (Ikeda and Yoshimoto, 1991). As these progenitors exit the cell cycle, they migrate ventrally and rostrally as they differentiate to form the AL of the pituitary (6-8 weeks in humans). The remaining dorsal region of RP generates the IL. The considerable growth of the anterior wall of the pouch results in a narrowing of luminal space to a cleft, which persists in the adult gland and acts to separate the IL and AL. Interestingly, it has been proposed that the remnant structure of RP forms the ‘periluminal zone’, providing a niche for putative pituitary stem cells during adult stages [reviewed in (Kelberman and Dattani, 2007b; Mullis, 2001)].

As evidenced from the distinct morphological events, pituitary development can be subdivided into three main phases: commitment (early induction of a rudimentary pouch), determination (progression from rudimentary status to definite pouch) and finally, cell differentiation (emergence of the specific hormone-producing cell types). These processes are highly controlled by the sequential or concomitant actions of a number of intrinsic and extrinsic signalling molecules and transcription factors, as discussed below.
Figure 1.2 - Schematic of pituitary gland morphogenesis in the mouse between 7.0 and 17.5 dpc

The primordia of the pituitary and hypothalamus are initially derived from the most anterior midline region of the developing embryo, a region contiguous with the ANR. Pituitary development commences in the mouse at 9.5 dpc, with a thickening and invagination of the oral ectoderm, which gives rise to RP, the primordium of the AP. This invagination of the oral ectoderm allows for the direct contact with the overlying ventral diencephalon, which will give rise to the infundibulum, the primordium of the PL. By 12.5 dpc, the pouch has detached from the oral ectoderm, remaining in close contact with the infundibulum. Intensified cell proliferation of RP precursors occurs, and cells migrate ventrally to populate the nascent AP, as differentiation commences. By 17.5 dpc, terminal differentiation of all the hormone-producing cell types is complete, giving rise to the ventrally located gonadotrophs and corticotrophs, the caudo-medial Pit1-dependent somatotrophs, lactotrophs and thyrotrophs and the intermediate lobe melanotrophs. The transient population of Pit1-independent thyrotrophs are located with the rostral tip of the AP. Abbreviations: ANR, anterior neural ridge; AP, anterior pituitary; BS, basosphenoid bone; C, corticotrophs; G, gonadotrophs; Inf, Infundibulum; L, lactotrophs; M, melanotrophs; OE, Oral ectoderm; RP, Rathke’s pouch; rT, rostral thyrotrophs; S, somatotrophs; T, thyrotrophs; VD, ventral diencephalon. Figure is modified from (Dasen and Rosenfeld, 1999).
1.3.1.2 Induction and formation of the definite RP: main molecular players

For many years, developmental biologists assumed that the close association between oral and neural ectoderm was more than a mere coincidence. Consequently, it was postulated that factors emanating from the neural ectoderm provided inductive signals for pituitary formation. However, it was not until 1996 that the first genetic evidence for this was established through the analysis of the *Nkx2.1*-null mouse mutant. The homeobox gene *Nkx2.1* is expressed from 8.5 dpc in the ventral diencephalon in close proximity to the pituitary anlagen. In addition, this gene is expressed within the lung and thyroid, but intriguingly, is excluded from regions of the oral ectoderm and RP. Subsequent analysis of *Nkx2.1*−/− embryos during late stages of development revealed a complete absence of pituitary tissue, providing vital clues that signals stemming from the ventral diencephalon could indeed influence pituitary organogenesis (Kimura et al., 1996).

During ontogeny, *bone morphogenic protein (Bmp)4* transcripts are expressed within the ventral diencephalon and are localised to the region immediately adjacent to the oral ectoderm destined to form pituitary. Coinciding with the initial thickening of oral ectoderm, this expression pattern peaks at 8.5 dpc and thus, it has been proposed that BMP4 signalling from the ventral diencephalon represents the first initiating factor during pituitary organogenesis (Ericson et al., 1998; Treier et al., 1998). Indeed, support for this suggestion has been provided by two main studies. In the first one, mis-expression of the BMP2/BMP4 antagonist *Noggin* within RP, mediated by the regulatory elements of the *Pitx1* gene to target its expression throughout the oral ectoderm and RP, revealed an early arrest of pouch development (Treier et al., 1998). In the second study, the analysis of pouch formation in embryos that lack *Bmp4* gene function showed no signs of a thickened ectodermal placode at 9.0 dpc (Takuma et al., 1998).

At 9.5 dpc, the expression patterns of two additional signalling molecules, *fibroblast growth factor (Fgf)-8* and -10, are restricted within the ventral diencephalon, where they partially overlap with *Bmp4* expression and persist within this region until 14.5 dpc (Ericson et al., 1998; Ohuchi et al., 2000; Treier et al., 1998). Interestingly, the onset of *Fgf8* expression
coincides with the expression of two LIM homeodomain transcription factors, $Lhx3$ and $Lhx4$ within RP, and thus it was suspected that $Fgf8$ is required to induce $Lhx3/Lhx4$ expression. Consistently, *in vitro* analysis revealed that beads coated in FGF8 were able to promote $Lhx3$ expression from RP explants (Ericson et al., 1998). Unfortunately, *in vivo* data complementing this work have been largely hampered by the early lethality of the $Fgf8^{-/-}$ mutants at gastrulation stages (Sun et al., 1999). However, analysis of $Nkx2.1$-null embryos, in which the $Fgf8$ expression domain is disrupted, has provided valuable insights as these mutants failed to promote definite pouch formation past the rudimentary stage, as evidenced by the lack of $Lhx3$ expression (Takuma et al., 1998).

$Lhx3/Lhx4$ are regarded as two of the earliest molecular pituitary markers and have been shown to act in a redundant manner in order to promote commitment to pituitary fate, as well as growth of the nascent gland. Analysis of $Lhx3^{-/-}$ and $Lhx4^{-/-}$ single mutants show no apparent defect in pouch formation, but pituitary gland development in $Lhx3^{-/-};Lhx4^{-/-}$ double mutants is halted at the rudimentary pouch stage, most likely due to ectopic apoptosis (Ellsworth et al., 2008; Mullen et al., 2007; Sheng et al., 1996; Sheng et al., 1997; Zhao et al., 2006). In addition to $Lhx3/Lhx4$, many genes are expressed throughout the region fated to become pituitary tissue at these stages, including the transcription regulators, $Pitx1/2$, $Sox2$, $Hesx1$ and $Six3$ (Gage et al., 1999; Gaston-Massuet et al., 2008; Hermesz et al., 1996; Lanctot et al., 1997; Lanctot et al., 1999; Oliver et al., 1995; Semina et al., 1996; Suh et al., 2002; Thomas and Beddington, 1996; Wood and Episkopou, 1999). The homeodomain genes $Pitx1$ and $Pitx2$ have been shown to act early in the genetic hierarchy, upstream of $Lhx3$, stimulating the early expansion of the pituitary primordium (Charles et al., 2005; Tremblay et al., 1998). Recently, a sophisticated study has shown that $Pitx2$ achieves this largely through transcriptionally activating $Cyclin D2$, a marker of the G1 phase of the cell cycle, following activation of the canonical Wnt/β-catenin pathway (Kioussi et al., 2002). Conversely, $Hesx1$ and $Six3$ are believed to counterbalance this proliferation through functioning as repressors of the Wnt/β-catenin pathway (discussed in detail in Section 1.5.2.2; (Gaston-Massuet et al., 2008)). Indeed, inactivation of $Wnt4$ within the developing pituitary gland results in a severely hypocellular gland, whereas loss of $Wnt5a$ in the
ventral diencephalon produces a highly dysmorphic pituitary gland, with no apparent effect on terminal differentiation (Cha et al., 2004; Potok et al., 2008). To date, the exact function of Sox2 during these early stages of pituitary organogenesis remains to be established (Section 1.6.3.2; (Fauquier et al., 2008; Kelberman et al., 2006)). Collectively, these data demonstrate that these genes act primarily to ensure correct size and shape of the gland by controlling the main phase of proliferation. Of value, with the exception of Hesx1, many of these genes continue to be expressed throughout pituitary development, persisting through to adult stages, when they are re-utilised for later functions in specific cell type determination, as exemplified by the subsequent role of Pitx1 in corticotrophs differentiation (Section 1.3.1.3; (Lamolet et al., 2001)).

1.3.1.3 Commitment and terminal differentiation of hormone-producing cells

As introduced above, proliferation of progenitors in the nascent pituitary gland is achieved primarily through the expansion of periluminal progenitor cells surrounding the lumen of RP. Consistent with this, Cyclin D2, a cell cycle regulator promoting cell cycle progression, appears to be predominantly expressed in these progenitors (Ward et al., 2005). Interestingly, recent work by Bilodeau and colleagues demonstrated that cells exiting the cell cycle initially express the cell cycle inhibitor, p57Kip2, representing a non-cycling, undifferentiated transient population. Consequently, upon differentiation, p57Kip2 expression is downregulated and is replaced by the G0 stabiliser p27Kip1, which acts to prevent the cells from re-entering the cell cycle (Bilodeau et al., 2009). As cells exit the cell cycle and initiate the process of cellular differentiation, they are excluded from the periluminal area and are incorporated into the distending lobe, where they finally reach their characteristic locations (Ikeda and Yoshimoto, 1991). At present, it has not been established whether this process is consequential of an active migration or of a passive translocation (Section 1.3.1.4).

The process of cellular differentiation during pituitary organogenesis occurs between 13.5-16.5 dpc and is defined by the appearance of one or more specific polypeptide hormones (Bilodeau et al., 2009; Davis et al., 2011; Japon et al., 1994). Conserved within species, this
process occurs in a well characterised temporal and spatial sequence (Figure 1.3). Corticotrophs are one of the first AP cell types to differentiate at 13.5 dpc and are noted to occupy ventro-lateral regions of the AP (Begeot et al., 1982; Japon et al., 1994). They are identified through the presence of ACTH, which is the proteolytically-cleaved product of POMC (Lamolet et al., 2004). NEUROD1, a member of the basic helix-loop-helix (bHLH) family of transcription factors and TPIT, a T-box transcription factor, act in synergy along with PITX1 to transcriptionally activate the expression of the Pomc1 gene (Lamolet et al., 2001; Lamolet et al., 2004; Lamonerie et al., 1996; Pulichino et al., 2003). Somatotrophs, lactotrophs and thyrotrophs emerge within the caudo-medial region of the AP at 14.5 dpc and are transcriptionally dependent upon the preceding expression of Pit1 (Pou1f1), a POU homeodomain transcription factor, which normally starts to be expressed at 13.5 dpc and is maintained throughout AP development (Camper et al., 1990; Dasen et al., 1999; Li et al., 1990; Lin et al., 1994; Nasonkin et al., 2004). Pit1, in turn is initiated through the expression of Prop1, a member of the paired-like class of homeodomain transcription factors, whose expression pattern commences in the dorsal region of the pouch at 10.5 dpc and persists until 15.5 dpc (Andersen et al., 1995; Nasonkin et al., 2004; Sornson et al., 1996). Finally, gonadotrophs are the last cell type to appear in ventral regions of the pituitary prior to birth and are likely to undergo a stepwise progression of differentiation through expressing a number of gonadotropin-specific markers; initially GnRH receptor (Gnrhr) at 14.5 dpc, closely followed by the orphan nuclear receptor Sf1 expression at 15.5 dpc, and finally are defined through the expression of LHβ or FSHβ between 16.5 and 17.5 dpc (Barnhart and Mellon, 1994; Holley et al., 2002; Japon et al., 1994). Melanotrophs that occupy the IL are identifiable by the presence of αMSH, another cleaved product of POMC at 15.5 dpc. Similar to corticotrophs, Pomc1 transcription within these cells is dependent upon the synergism between Pitx1 and Tpit. Expression of Tpit within the primitive IL is evident at 14.5 dpc (Lamolet et al., 2001; Lamolet et al., 2004; Lamonerie et al., 1996; Pulichino et al., 2003). Of note, a subset of Pit1-independent thyrotrophs can be detected within the most rostral and ventral region of the developing AP at 13.5 dpc. These thyrotrophs, or rostral-tip thyrotrophs as termed because of their location, have been shown to be a transient
population of cells that disappear at birth. Given their fleeting expression following birth, they are believed to be non-functional (Lin et al., 1994).

Combined, these data illustrate two points: (1) periluminal progenitors are exposed to a dorsal to ventral morphogenetic gradient that may control terminal differentiation, and (2) particular cell types are programmed to emerge and occupy distinct regions of the AP gland. The mechanisms underlying these developmental processes are discussed below.
Figure 1.3 - Schematic representation of developmental genes required for normal pituitary gland development, with particular reference to pituitary cell differentiation

A cascade of transcription factors result in a stepwise appearance and determination of all pituitary cell types. All of these cell types are derived from a common progenitor cell type. Many genes, including Pitx1/2, Lhx3/4, Hesx1, Six3 and Sox2, are expressed within these cells at these very early stages of pituitary development. The subsequent development of somatotrophs (GH), lactotrophs (PRL) and thyrotrophs (TSH) is dependent on Pit1, which in turn, relies on the preceding expression of Prop1. Note that rostral tip thyrotrophs (rTSH) are independent of Pit1 expression and are derived from αGSU-expressing cells. In addition, αGSU-expressing cells give rise to gonadotrophs (LH/FSH), which are also dependent upon the prior expression of Sfi. Both ACTH and αMSH hormones, that label corticotrophs and melanotrophs respectively, are derived from the proteolytic cleavage of POMC1, which is dependent upon NeuroD1 and Tpit expression.
1.3.1.4 A gradient model for specification of specific committed progenitors

Since the identification of extrinsic signals influencing initial steps in pituitary development, it has been proposed that similar signals emanating from both the oral ectoderm and ventral diencephalon function to pattern the developing pituitary and control cell fate specification. *Bmp2* transcripts are detectable from 10.5 dpc within the boundary of the pouch and oral ectoderm, and by 12.5 dpc, they are progressively incorporated into the pouch following a ventral to dorsal pattern. Classical co-culture experiments of pituitary tissue with surrounding mesenchyme demonstrated that BMP2 signalling from this region is sufficient to maintain expression of *Isl1*, another LIM homeodomain transcription factor required for the generation of ventral αGSU (gonadotrophs and thyrotrophs) precursors (Ericson et al., 1998; Treier et al., 1998). During this period, FGF8 signalling emanating from the ventral diencephalon is still active and thus, it has been speculated to induce a dorso-ventral polarity. In this case, co-culture experiments with pituitary explants and infundibular tissue have shown that FGF8 was able to inhibit the expression of *Isl1* *in vitro* (Ericson et al., 1998; Takuma et al., 1998). In addition, ectopic expression of *Fgf8* under the control of αGSU, which targets *Fgf8* expression in RP and ventral cell types, leads to the absence of ventral and intermediate cell types (Treier et al., 1998). Thus, it has been proposed that ventral cell types (gonadotrophs and corticotrophs) are likely to derive from ventral locations in RP, which are subject to high levels of BMP2 signalling, whereas dorsal cell types (somatotrophs and melanotrophs) may arise from regions closest to the ventral diencephalon that are consequently under the influence of high Fgf signalling. *Pit1*-dependent thyrotrhps located within caudo-medial regions of the gland have been suspected to form from regions that receive intermediate levels of both signals. To summarise, it has been proposed that cells exiting the cell cycle at discrete timepoints are likely to be influenced by differing levels of Bmp and Fgf signalling, ultimately resulting in a stratified appearance of cell types within the AP.

Although initially widely accepted (for just over a decade), this cell specification model has recently been challenged. Classic birthdating studies performed by Davis and colleagues
revealed that enrichment of cell types within discrete regions of the AP is not consequential of an ordered cell cycle exit. Instead, it was shown that all cell types of the AP exit the cell cycle concurrently and thus, it has been suggested that cell types reach their final destination within the AP through active directed migration (Davis et al., 2011).

Given this finding, close analysis of the opposing ventral (Bmp) to dorsal (Fgf) signalling has been conducted through the temporal and spatial analysis of their downstream effectors, phosphorylated (p)-SMAD1,5,8 and p-ERK, respectively. Surprisingly, the only time that a gradient of these intracellular factors has been found is at 10.5 dpc, a timepoint prior to cell cycle exit (11.5-13.5 dpc). Although these are not the sole mediators of these two signalling pathways, these data strongly imply that factors intrinsic to the pituitary may also be determinant for cell specification (Davis et al., 2011).

Over the last few years, Notch signalling has been identified as a pathway required for the correct numbers and specification of cell types (Raetzman et al., 2004; Zhu et al., 2006). In the absence of Hes1, an intracellular mediator of Notch signalling, cells prematurely exit the cell cycle, ultimately leading to a hypocellular AP. In addition, somatotrophs are ectopically formed within the IL at the expense of melanotrophs (Raetzman et al., 2007). From the birthdating data, it is evident that melanotrophs are the last cell type to form, and thus, it is believed that cells fated to become melanotrophs are incorrectly converted to somatotrophs due to a depleting progenitor pool. From this, it is clear that Notch signalling is required within the AP to maintain a progenitor state, ultimately resulting in the appropriate numbers of AP cells permissive for the formation of all cell types.

In conclusion, there is an emerging view that pituitary organogenesis is not simply orchestrated by a sequential series of extracellular factors, but a dual process initially coordinated by extrinsic factors that are temporally succeeded by intrinsic mechanisms. It is clear that Bmp and Fgf signalling emanating from the ventral diencephalon are required for the early stages of pituitary induction and proliferation. Subsequently, cell fate specification appears to require the presence of additional intrinsic signals, such as Notch, to allow for an appropriately-sized and fully functional gland.
1.3.2 Development of the neuroendocrine hypothalamus

Despite large interest in the physiological functions of the hypothalamus throughout the 20th century, knowledge of the mechanisms underpinning hypothalamic development remains basic. The hypothalamus, due to its multitude of functions, is comprised of a diverse collection of cell groups and neuronal subtypes. Consequently, the lack of well-defined markers, coupled with its highly complex anatomy, has traditionally disadvantaged studies into the formation and development of this structure. Nevertheless, progression in this field has subsequently been aided by the advancement of developmental genetics over the past two decades. Of note, much of this early work was conducted in rat; when extrapolating from rat to mouse, the timepoint difference for the same developmental processes has been defined to be 1-1.5 days less (Markakis, 2002).

1.3.2.1 Morphogenesis of the hypothalamus

The forebrain arises from the anterior neuroectoderm during gastrulation stages, and by the end of somitogenesis, this region comprises the hypothalamus and floor plate ventrally, and the telencephalon, eye and diencephalon, dorsally (Wilson and Houart, 2004). Despite this complexity, all forebrain structures derive from a simple sheet of neuroepithelial cells, which undergoes regionalisation and dramatic morphogenesis during early development. In fish, fate-mapping studies have shown that cells destined to contribute to the hypothalamus are initially located caudally to the prospective dorsal forebrain (telencephalon and eye fields), in close proximity to the organiser. From this location, prospective hypothalamic cells move rostrally within the neural plate, displacing more dorsal forebrain structures laterally where they occupy their final destination (Mathieu et al., 2002; Woo et al., 1995). Morphologically, hypothalamic development is evident by a ventral expansion of the anterior neural tube at 11 dpc in rat, which coincides with initial stages of pituitary gland organogenesis. Concomitantly, the first in a series of neuromeric folds appear, creating the anterior diencephalic folds. By 12-13 dpc, additional neuromeric folds emerge, which include the middle and posterior diencephalic folds. At this
stage, the wall of the diencephalon has thickened considerably due to the proliferation of neural progenitors in the ventricular and subventricular zone. At 14 dpc, two hypothalamic masses appear, the anterior of which will give rise to the ventro-medial region, whilst the posterior mass forms the mammillary regions. By 15 dpc, the first signs of the primitive nuclei are distinguishable [reviewed in detail in (Cavodeassi et al., 2005; Markakis, 2002; Wilson and Houart, 2004)].

1.3.2.2 Induction and formation of the hypothalamus: main molecular players

Similarly to the pituitary gland, the formation of the hypothalamus requires the concerted actions of numerous signalling pathways, as discussed herein. Comparable to the role of the ventral diencephalon in inducing pituitary fate, the axial mesendoderm is required for the induction of the hypothalamus. This tissue, which includes the notochord and the prechordal plate, derives from the organiser and is situated beneath the presumptive midline of the neuraxis (Michaud, 2001). Conserved between fish, chick and mouse, from 7.5 dpc in mouse, the axial mesendoderm is a major source for (sonic hedgehog) SHH secretion and is believed to provide initial cues for hypothalamic fate (Manning et al., 2006; Mathieu et al., 2002; Szabo et al., 2009). Indeed, all hypothalamic tissue is absent in mice lacking SHH activity (Chiang et al., 1996). Furthermore, in vitro assays have shown that SHH protein, when cultured with prospective forebrain regions of the neural tube, can induce ventral neuronal subtypes as deemed through the expression of Nkx2.1, a marker commonly used to determine hypothalamic fate (Ericson et al., 1995; Pabst et al., 2000). Following the initial induction of the hypothalamus, members of the transforming growth factor-β (TGFβ) superfamily are expressed, and downregulate SHH expression to confer regional identity. For instance, Bmp signalling has been shown to downregulate SHH in floor plate-like cells of the forebrain in chick, which transforms them into hypothalamic ventro-tuberal mammillary progenitor cells - a process mediated through the expression of the T-box transcriptional repressor, Tbx2 (Manning et al., 2006). Furthermore, experiments conducted in zebrafish have shown that Nodal signals are
necessary cell autonomously for the specification of the posterior-ventral region, whereas the actions of SHH promote the specification of anterior-dorsal regions. Interestingly, when cells are compromised to receive both Nodal and Shh signals, they are excluded entirely from a hypothalamic fate (Mathieu et al., 2002).

In addition to the TGFβ superfamily, it has been shown that further signals are necessary to provide subregional information which includes the canonical Wnt pathway. As such, a Wnt activity gradient, from posterior to ventral appears to bestow further regional identity. Experiments in zebrafish have demonstrated that inhibition of Wnt signalling from within the ectoderm is required for the correct specification of rostral hypothalamic destiny, rather than caudal floor plate tissue. Clear evidence for this has been demonstrated in zebrafish; mutants for Axin1, a negative regulator of the canonical Wnt/β-catenin pathway, results in the expansion of floor plate tissue at the expense of the developing ventral hypothalamus (Kapsimali et al., 2004).

To summarise, it is apparent that the acquisition of hypothalamic fate requires SHH signalling from the underlying mesendoderm. However, further regionalisation and patterning are dependent upon exclusion of SHH from the more posterior-dorsal regions, a developmental event largely mediated through the combined actions of Bmp/Nodal and Wnt signals.

1.3.2.3 Commitment and terminal differentiation of the neuroendocrine hypothalamus

Despite recent efforts to understand hypothalamic induction and patterning, a gap still remains as to how these subregional patterns govern specification of distinct neuronal subtypes required for a functional hypothalamus. Nonetheless, characterisation of various mouse mutants has revealed the novel transcriptional pathways that demarcate the development of neuroendocrine nuclei, providing insight into the transcriptional code required for each neuroendocrine subtype.
Cellular differentiation of the neuroendocrine hypothalamus is a process that occurs between 10.5-18.5 dpc in mouse and is dependent upon a genetic hierarchy of transcription factors (Figure 1.4). Detailed analysis from birthdating studies has revealed that in a similar mechanism to the development of the pituitary gland, parvocellular and magnocellular subtypes are released from the proliferating ventricular zone concurrently between 12.5 and 13.5 dpc in rat in an outside-to-inside pattern (Karim and Sloper, 1980). Thus, neurons born earlier occupy a more lateral position in the mantle zone, whereas late-born neurons reside in more medial locations. Furthermore, it is believed that this process is largely passive in nature, and ultimately results in the condensation of the third ventricle as these nascent neurons accumulate in a lateral-to-medial sequence. Support of this outside-inside process has come from gene expression analysis within the developing hypothalamus, which demonstrates that layered gene expression may, to some extent, correspond with waves of neurogenesis (Caqueret et al., 2006).

However, this finding appears to be an oversimplification, as these waves of gene expression are not sufficient to explain the complex generation of the neuroendocrine nuclei regionalisation. Detailed birthdating studies have demonstrated that neuroendocrine neurons are born earlier than their prospective nuclei, which are birthdated between 13-15 dpc in rat and therefore has been proposed that a period of migration of the neuroendocrine subtypes occurs, in order to give rise to their ultimate location within the hypothalamus. Apart from the GnRH neurons, which undergo extensive migration from their original source in the olfactory placode, little is known regarding this process (Karim and Sloper, 1980; Markakis, 2002; Szarek et al., 2010).

From our current understanding, the homeodomain transcription factor Otp and the bHLH-containing factor Sim1 represent the first two genes in the genetic cascade that are absolutely required for the terminal differentiation of the neuroendocrine hypothalamic neurons (Acampora et al., 1999; Michaud et al., 1998; Wang and Lufkin, 2000). Furthermore, it has been shown that heterodimerisation with ARNT2 is required for SIM1 function within the developing hypothalamus (Hosoya et al., 2001; Michaud et al., 2000). Expression analysis of these genes has shown that they are expressed from as early as 9.5 dpc in mouse, in regions
immediately adjacent to the ventricular zone and persist within regions of the PVN, SON and aPV from late development through to postnatal stages. As such, these genes function to promote differentiation of the varying cell types within these nuclei, namely AVP- and OT-expressing magnocellular neurons, as well as TRH-, CRH- and SS-expressing parvocellular neurons. Furthermore, given their early expression, loss-of-function mouse models have demonstrated that both Otp and Sim1 are not only involved in the terminal differentiation of these cell types, but also in the initial expansion of the hypothalamic progenitors destined to give rise to these neurons, and in the migration of these neurons to their ultimate destination. Despite the common function between these two genes, Caqueret et al. (2006) showed that OTP does not interact with SIM or ARNT2 and moreover, that these two genes appear not to regulate each other’s expression, suggesting that they may act in convergent or parallel pathways (Acampora et al., 2000; Caqueret et al., 2006; Michaud et al., 1998; Michaud et al., 2000; Wang and Lufkin, 2000).

Downstream of these two genes, the POU domain transcription factor Brn2 and the Sim1-paralog Sim2 act to further define the initial Otp/Sim1 domain. Analysis of the Brn2 mutant mice has shown that this factor functions relatively late during neuroendocrine development, being required for the terminal differentiation of CRH-, AVP- and OT-expressing neurons. In contrast, analysis of the Sim2-deficient mice has shown that this factor is required for the terminal differentiation of the more anteriorly located SS- and TRH-expressing parvocellular neurons. Thus, within the initial Otp/Sim1 domain, downstream factors act to further refine microenvironments for the generation of particular neuronal subtypes. As with SIM1, it is postulated that SIM2 interacts with ARNT2, however, other factors may be involved in this process (Goshu et al., 2004; Nakai et al., 1995; Schonemann et al., 1995).

Interestingly, the deficiencies displayed in all mouse lines discussed above exclude abnormalities of the GHRH-expressing neurons located within the Arc nucleus, suggesting that this neuronal population may arise from independent genetic pathways. The homeobox gene Gshl is expressed within the presumptive Arc from 9.5 dpc in mouse, where it persists until at least 13.5 dpc (Valerius et al., 1995) and is essential for the terminal differentiation of GHRH
neurons within this nucleus (Li et al., 1996). Targeted disruption for Gsh1 in mice leads to an absence of GHRH neurons which ultimately results in extreme dwarfism and sexual dysfunction. Furthermore, two homeobox genes, Hmx2 and Hmx3, are also essential for the development of these neurons. Overlapping in expression with Gsh1, they are expressed from 10.5 dpc and double mutants for these genes have shown an identical phenotype to Gsh1-null mutants. In addition, analysis of Arc neuronal numbers in these mice has shown that they remain unchanged, suggesting that although these genes are expressed early during ontogenesis, they are not required for the early determination of the neuroprogenitors in this region of the hypothalamus (Wang et al., 2004).
Figure 1.4 - Transcriptional regulation of the hypothalamic neuroendocrine cell types

*Nkx2.1* functions early during hypothalamic development to specify the ventral region of the prospective hypothalamus. **SIM1:**ARNT2 and **OTP** function in parallel to influence the development of most cell types, including the AVP- and OT-expressing magnocellular neurons, as well as the CRH-, TRH- and SS-expressing parvocellular neurons. These two complexes are important for the maintenance of **Brn2** expression, which in turn is required for the terminal differentiation of AVP-, OT- and CRH-neuroendocrine neurons. These two complexes are also required for **Sim2** expression, which is needed for the development of the SS- and TRH-expressing neurons. Independent of this cascade of transcription factors, **GHRH**-expressing parvocellular neurons required the preceding expression of both **Hmx2/3** and **Gsh1**.
Finally, the last population of neuroendocrine neurons to be mentioned is the GnRH-expressing parvocellular neurons. These represent a unique cohort of neuroendocrine cells that are born outside of the brain, within the olfactory epithelium between 10.5-11.5 dpc in mouse and subsequently migrate into the brain, where they reach their final location within the preoptic area of the hypothalamus at 18.5 dpc (Schwanzel-Fukuda and Pfaff, 1989). To date, their embryonic origin remains controversial; the olfactory placode, AP, neural crest cells and respiratory epithelium have all been implicated as their source [as reviewed in (Whitlock et al., 2006)]. In mouse, it is postulated that GnRH neurons derive from both neural crest and ectodermal progenitors through lineage tracing studies with the Wnt1-Cre (a neural crest marker) and Crect (ectodermal marker) mouse lines (Forni et al., 2011).

Irrespective of the origin of these neurons, the transcription factors and signalling molecules that impart GnRH identity onto neuronal precursors remain elusive. This is mainly due to the fact that except for GnRH transcripts/peptides, no identifiable markers are specific for this population. Thus, prior to the onset of this hormonal marker, olfactory placode progenitor cells fated to become GnRH neurons are not distinguishable. Nevertheless, many studies have implicated FGF signalling in the fate specification of these neurons, as the murine olfactory placode has been shown to express FGF receptors 1-3 during the developmental window of 10.5-11.5 dpc, when GnRH specification occurs (Gill et al., 2004). Amongst all the FGF members, FGF8 appears to be the most likely candidate, as Fgf8 messenger ribonucleic acid (mRNA) is most abundant within the developing olfactory placode at 9.5 dpc. Moreover, analysis of Fgf8 hypomorphs, where homozygotes display a 58% reduction of Fgf8 mRNA, has revealed complete absence of GnRH neurons (Chung et al., 2008; Falardeau et al., 2008).

In addition to its role in specification of GnRH neurons, FGFs have also been implicated in their migratory properties (Chung et al., 2008), along with hepatocyte growth factor (Giacobini et al., 2007) and secreted-class 3 semaphorins (Cariboni et al., 2011). Since their discovery within the nasal placode, GnRH neurons are known to migrate along olfactory/vomeronasal nerves towards the cribiform plate where they enter into the forebrain, a process occurring between 12.5-14.5 dpc. Recent studies in mouse have shown that disruption
of this axonal projection, as demonstrated by the loss of SEMA3A signalling, alters the migration of these neurons into the brain, ultimately leading to dysfunction of the reproductive axis (Cariboni et al., 2011).

Combined, the data discussed in this section illustrate that the developmental programs of these essential components of the hypothalamic-pituitary axis are interdependent, and closely linked to that of the forebrain. Therefore, it is conceivable that any disorders that occur during these crucial stages of embryogenesis can result in dire downstream effects affecting all or many of these structures that rely on the normal development of the anterior midline. Conveniently for developmental biologists and clinicians alike, pituitary and hypothalamic embryogenesis is similar in all vertebrates, including humans, thereby endorsing the mouse as a useful model for exploring normal and pathogenic hypothalamic-pituitary axis development. Indeed, spontaneous and genetically engineered mouse models have greatly enhanced our understanding of congenital endocrine disorders and related conditions in humans (Dattani et al., 1998; Kelberman et al., 2006; Pulichino et al., 2003; Sheng et al., 1996; Sheng et al., 1997; Sornson et al., 1996).
1.4 MALFORMATIONS OF THE HYPOTHALAMIC-PITUITARY AXIS IN HUMANS

Pituitary dysfunction can manifest as pituitary hyperplasia, which results in the overactivation of the pituitary axis (hyperpituitarism). These are often caused by lesions, and can result in a multitude of outcomes based on the founding cell type (Davidoff, 1940; Levy, 2004). Of significance to this study, pituitary dysfunction may manifest as pituitary hypoplasia, which results in varying levels of endocrine deficits (hypopituitarism). Hypopituitarism resulting from developmental abnormalities of the hypothalamic-pituitary axis is termed congenital hypopituitarism and can manifest solely as hormonal deficiencies or in conjunction with extra-pituitary defects and midline abnormalities as part of a syndrome. For the scope of this work, I will only discuss conditions of congenital hypopituitarism, with or without syndromic features.

1.4.1 Congenital hypopituitarism

Congenital hypopituitarism varies widely in presentation, with the most common form of this disorder being isolated GH deficiency, which is reported to occur in 1 in 3,500-10,000 live births (Bao et al., 1992; Parkin, 1974; Thomas et al., 2001a; Vimpani et al., 1977). The loss of more than one pituitary hormone results in combined pituitary hormone deficiencies (CPHDs) which are less common, however, are strongly linked with considerable morbidity, especially if diagnosis is delayed (Thomas et al., 2001b). Following GH deficiency, TSH and ACTH insufficiencies are the most common endocrine pathological features (Cameron et al., 1999; Costin and Murphree, 1985). Additionally, it is not uncommon for pituitary hormone deficits to evolve over time, indicating that long-term medical follow-up of patients is a necessity. Diagnosis can occur early during neonatal periods, however, hypopituitarism may remain undetected until later in development with the failure of growth or abnormal pubertal development [reviewed in (Alatzoglou and Dattani, 2009; Kelberman and Dattani, 2007b; Mehta and Dattani, 2008)]. Interestingly, a correlation between neuroradiological abnormalities
of the pituitary gland and the severity of the endocrinopathy is proposed. This includes the size of the AP gland, as well as the exact position of the PP (e.g., ectopic/undescended). For instance, the risk of hypopituitarism in patients with abnormal PP is ~27 times greater than in those who retain a normally located PP (Mehta et al., 2009). Although the majority of cases are idiopathic in origin, there have been familial reports of both dominant and recessive inheritance, which are postulated to account for between 5-30% of all cases (Phillips, III and Cogan, 1994). To date, mutations in the pituitary specific transcription factor $PROP1$ are the most common genetic root of CPHD, accounting for roughly 50% of familial cases (Mody et al., 2002). Furthermore, given the close link between the hypothalamus and pituitary gland, dysfunction of the hypothalamus has also been documented to be causative in certain hypopituitarism cases (Lam et al., 1986; Roessmann et al., 1987).

1.4.2 Septo-optic dysplasia

On the graver spectrum of congenital hypopituitarism lies the syndromic condition of SOD. SOD is a rare congenital anomaly, with a reported incidence of 1 in 10,000 live births and is equally prevalent in males and females (Webb and Dattani, 2010). The first report of SOD was originally described by Reeves in 1941 as an absence of the septum pellucidum in combination with optic nerve abnormalities (Reeves, 1941). Subsequently, an association with pituitary dysfunction was reported (Hoyt et al., 1970). Typically, a clinical diagnosis of SOD is made when two or more features of (i) optic nerve hypoplasia, (ii) pituitary dysfunction and/or (iii) midline defects (including agenesis of the corpus callosum and absence of the septum pellucidum) are present. There is a wide variation in the severity of features presented. Previous studies have shown that approximately 30% of patients manifest the complete triad (Morishima and Aranoff, 1986). The main reported clinical findings are hypopituitarism (62-80%), followed by visual impairment, usually bilateral optic nerve hypoplasia (23%) and developmental delay (Haddad and Eugster, 2005).

Several aetiologies have been associated with sporadic cases of SOD, which remain a subject of much debate. These include viral infections, environmental teratogens (e.g., alcohol
abuse during early pregnancy) and vascular or degenerative damage. In addition, infants born to young mothers appear to be a common preponderance (Lippe et al., 1979). Familial cases of SOD are infrequent and are believed to be more commonly associated with autosomal recessive modes of inheritance [reviewed in (Dattani et al., 2000; Kelberman et al., 2009; Kelberman and Dattani, 2007a; McCabe et al., 2011; McNay et al., 2007; Webb and Dattani, 2010)].

To date, the quest for understanding normal midline development, has led to the identification of two genes, *Hesx1* and *Sox2*, that when mutated cause SOD and in milder cases, congenital hypopituitarism. I will now move to a detailed discussion of these early developmental transcription factors, which are the main focus for the work presented in this thesis.
1.5 HESX1

A milestone in our current understanding of developmental biology came from the identification of homeobox genes, more than four decades ago. Homeobox genes represent an important class of transcription factors that function to regulate developmental decisions in metazoans (Gehring, 1992). They encode a 60 amino acid conserved DNA binding protein, termed the homeodomain that mediates specific interactions between homeodomain proteins and DNA. Members of this family were originally identified in *Drosophila*, as the *Hox* sub-group of homeobox genes, found to carry crucial roles in defining the embryonic body plan in the fly. Great promise came when their structural and functional counterparts were identified in vertebrates, playing analogous roles in establishing positional identity along the rostro-caudal axis (Duverger and Morasso, 2008; Galliot et al., 1999; Wigle and Eisenstat, 2008; Wilson et al., 1995).

Following this revelation, the search for homeobox genes during various phases of embryonic development commenced. Consequently, the isolation of *Hesx1* (Homeobox expressed in ES cells 1) was achieved through a screening for homeobox genes expressed in murine embryonic stem (ES) cells, the pluripotent cells derived from the inner cell mass (ICM) of the early embryo (Thomas and Rathjen, 1992). Unlike the vast majority of homeobox genes, *Hesx1* fell into a relatively small group of homeobox genes expressed by undifferentiated pluripotent stem cells, suggesting a possible role for *Hesx1* in early embryogenesis and in the regulation of cell pluripotency. Consistently, further analyses revealed that this gene maps to chromosome 14 in the mouse, validating that this homeobox gene does not belong to the well-characterised *Hox* gene clusters located on chromosomes 2, 6, 11 and 15. Given its early expression during mouse embryogenesis, it has been proposed that *Hesx1* is unlikely to contribute to anterior-posterior patterning, a common function of the *Hox* homeobox genes, but is rather involved in earlier developmental events (Thomas et al., 1995; Webb et al., 1993).

Independent to the above study, Mahon and colleagues screened for homeobox genes expressed during murine gastrulation events, where they also managed to identify *Hesx1*
Evaluation of *Hesx1* gene expression throughout embryogenesis revealed that this gene was expressed from the early stages of gastrulation in a broad pattern, where it was later restricted to the developing neuroectoderm and subsequently, the primordium of the anterior pituitary, RP. As a result, they termed this homeobox gene as *Rpx*, Rathke’s pouch homeobox, (for clarity *Rpx* will be referred to as *Hesx1* hereafter). Close examination of the putative amino acid sequence of the *Hesx1* homeodomain has revealed that *Hesx1* belonged to the subfamily of the *paired*-like homeodomains. This class of homeobox genes is defined by their close resemblance to the *Drosophila* *paired* gene, and represent a class of homeodomain proteins that bind to their target sites cooperatively as homodimers. As a result, protein-protein interactions appear a requirement for *Hesx1* function (Galliot et al., 1999; Hermesz et al., 1996).

In addition to the homeodomain, analysis of the predicted *Hesx1* protein suggested the presence of another highly conserved octapeptide sequence at the amino (N)-terminus, close to the initiator methionine sequence (Hermesz et al., 1996). Further analysis of this sequence revealed close homology to the *Drosophila* engrailed protein, and as such, was termed engrailed-homology 1, (eh1). In *Drosophila*, the eh1 sequence has been shown to be necessary for the recruitment of the corepressor, Groucho (Smith and Jaynes, 1996). Faithful to this function, murine HESX1 has also been shown to interact with Transducin-like enhancer of split (TLE)-1 and -3, mammalian homologues of the *Drosophila* corepressor Groucho, and this interaction is mediated through eh1 (Brickman et al., 2001; Carvalho et al., 2003; Carvalho et al., 2010; Dasen et al., 2001). Furthermore, in addition to facilitating DNA binding, the homeodomain has been shown to interact with cofactors, such as the nuclear corepressor complex (N-CoR) exerting repressor function (Dasen et al., 2001; Xu et al., 1998). As such, HESX1 contains two repressor domains, located both at the N-terminus and carboxyl (C)-terminus. Indeed, *in vitro* analysis of both the eh1 and homeodomain regions of HESX1 show that these two domains work independently as repressors of the *tk* promoter when expressed as Gal4 fusions (Dasen et al., 2001). Finally, DNA methyltransferase 1 (DNMT1) also withholds the ability of partnering with HESX1 to repress transcription, an action believed to be mediated...
through CpG methylation of HESX1 target genes (Sajedi et al., 2008a). Collectively, these data suggest that HESX1 acts as a potent transcriptional repressor in vivo.

Interestingly, both Mahon and Rathjen revealed close homology of murine Hesx1 to Xenopus Xanf1 gene, a homeobox gene reported to be expressed in the anterior neural folds and pituitary primordium of the frog embryo, implying that these two genes may have evolved from a common ancestral gene (Hermesz et al., 1996; Mathers et al., 1995; Thomas et al., 1995; Thomas and Rathjen, 1992; Zaraisky et al., 1992). Subsequently, orthologues have been identified in other vertebrate species; GANF in Chick, Danf in zebrafish and HESX1 in humans, where they collectively comprise the family, ‘Anf’ (anterior neural folds), due to their restricted expression pattern (discussed in depth in Section 1.5.1; (Dattani et al., 1998; Knoetgen et al., 1999; Reim and Brand, 2002; Spieler et al., 2004)). Of worth, Hesx1 orthologues are absent in non-vertebrate groups, including the close vertebrate relatives, amphioxus and ascidians, alluding to a role of Hesx1 and orthologues in the development of structures, such as the forebrain, which are responsible for higher-order functions (Hermesz et al., 1996; Knoetgen et al., 1999; Thomas and Beddington, 1996).

1.5.1 Expression of Hesx1

Much insight into the role of Hesx1 during embryonic development has come from expression study analyses. In mouse, expression of Hesx1 is initially detected during the early stages of gastrulation (6.5 dpc) in a small subset of cells in the anterior midline of the endoderm layer, termed the anterior visceral endoderm (AVE). Comparison between the expression patterns of Brachyury (T), a gene strongly activated along the entire primitive streak during gastrulation, demonstrated that Hesx1 expression is initiated at the time of streak formation, remaining in this anterior-restricted pattern during primitive streak elongation. By 7-7.5 dpc, Hesx1 is clearly expressed in the axial mesendoderm of the prechordal plate primordium, a region believed to be involved in the induction of anterior head structures, such as the forebrain. Subsequently, Hesx1 expression broadens to encompass the anterior neural ectoderm overlying the endodermal expression domain. At 8-8.5 dpc, expression of Hesx1 is limited to the anterior
neuroectoderm, slowly spreading laterally during the formation of the anterior folds, to include the developing prospective forebrain region. Half a day later, transcripts are also detectable within the ventral diencephalon and in oral ectoderm. Fascinatingly, Hesx1 expression is suddenly extinguished within the neuroectoderm, and is exclusively restricted to a layer of oral ectodermal cells fated to form RP. Here, strong expression persists in the developing pituitary until 12.5-13.5 dpc, when finally Hesx1 transcripts are undetectable in the developing embryo (Figure 1.5) (Hermesz et al., 1996; Thomas and Beddington, 1996).

Close scrutiny of Hesx1 expression within the developing pituitary revealed that at 11 dpc, when the nascent pituitary gland begins to separate away from the oral ectoderm, virtually all cells within the pouch expressed Hesx1. This is consistent with subsequent genetic fate-mapping experiments that clearly demonstrated that all terminally differentiated cells within the mature pituitary gland derive from Hesx1-expressing cell types (Gaston-Massuet et al., 2011). Furthermore, initial reports of Hesx1 within the pituitary gland have revealed that its transcripts are spatially and temporally downregulated as cellular differentiation progresses. Therefore, it is believed that Hesx1-expressing cells represent embryonic progenitors within the developing RP (Gaston-Massuet et al., 2011; Hermesz et al., 1996).
**Figure 1.5 - Hesx1/HESX1 expression during mouse and human embryonic development**

(a-e) Whole-mount *in situ* hybridisation with *Hesx1* in the developing forebrain between 6.5 and 9.5 dpc in the mouse. (f-m) Section *in situ* hybridisation with *Hesx1* highlighting the expression of *Hesx1/HESX1* within the mouse developing pituitary gland from 9.5-14.5 dpc (f-i) and human pituitary gland between CS11 and CS17 (j-m). (a-c, e-k) are sagittal views, whereas (d, l-m) represent frontal views. (a) *Hesx1* transcripts are initially detected with the AVE at the onset of gastrulation at ~6.5 dpc. (b) By 7.0 dpc, *Hesx1* is expressed in the anterior axial mesendoderm. (c) By 7.5 dpc, *Hesx1* expression broadens to encompass the anterior neural ectoderm, and (d) at 8.5 dpc, *Hesx1* transcripts are very strong within the prospective forebrain region. (e) *Hesx1* expression is restricted to the ventral diencephalon by ~9.0 dpc and (f-g) By 9.5 dpc, *Hesx1* is only expressed within the primordium of the anterior pituitary gland, Rathke’s pouch (RP). (h) Expression of *Hesx1* persists within this structure until 12.5 dpc, where a high dorsal to low ventral gradient of *Hesx1* transcripts is evident. (i) Subsequently, by 14.5 dpc, *Hesx1* expression is no longer detectable within the nascent pituitary gland. A similar expression pattern of *HESX1* transcripts is observed within the ventral forebrain and developing RP of human embryos. (j) At CS11, *HESX1* transcripts are detectable within the ventral forebrain (arrowhead) and the incipient RP (arrow). (k-l) By CS12-CS13, *HESX1* transcripts are only confined to the developing RP. (m) *HESX1* expression is not detectable at CS17. Scale bars: a-e, 200 µm and j-m, 125 µm. Images a-d are taken from (Martinez-Barbera and Beddington, 2001); e from (Thomas and Beddington, 1996); f-i from (Dasen et al., 2001) and (j-m) from (Sajedi et al., 2008b).
This dynamic spatial and temporal expression pattern during development is, at least in part, controlled by conserved cis-elements in the 3’-, 5’- and intragenic sequences of the Hesx1 locus. Elegant studies using transgenic mice carrying the LacZ reporter gene fused to different fragments of Hesx1 genomic sequences clearly demonstrated that early expression in the anterior neural plate was determined by regulatory factors separable from those controlling late Hesx1 expression in RP. As little as 568 base pairs (bp) of upstream sequence coupled with the intragenic sequence containing the first exon and intron were sufficient to recapitulate proper spatial and temporal transgenic expression in the anterior endoderm, prechordal plate and anterior neural plate, but not in RP (Hermesz et al., 2003). Subsequent investigation revealed that regulatory elements in the 3’-region of the gene were both required and sufficient for the late expression of transgenes in RP. Interestingly, this degree of regulation appears conserved, at least between Xenopus and mouse. Injections of constructs containing a reporter gene fused to the murine Hesx1 promoter into Xenopus embryos revealed a pattern of reporter gene expression similar to that of endogenous Xanf1 (Chou et al., 2006). This data demonstrates that the regulation of mouse Hesx1 and Xenopus Xanf1 is highly conserved at both the primary sequence and mechanistic level, further reiterating a common ancestral denomination for these two orthologues.

Of relevance to this study, as with the other Anf members, in situ hybridisation experiments on human embryonic samples revealed that human HESX1 follows a close expression pattern to the mouse (Ermakova et al., 1999; Ermakova et al., 2007; Hermesz et al., 1996; Knoetgen et al., 1999; Sajedi et al., 2008a; Thomas and Beddington, 1996). At Carnegie Stage (CS) 11, corresponding to 8.5-9.0 dpc in mouse, HESX1 transcripts are localised to the ventral diencephalon and oral epithelium of the developing RP. By CS 12 (~9.5-10.25 dpc), neural expression of HESX1 is no longer detectable, however transcripts are abundant in the developing RP, remaining until CS 15 (~11.0-11.5 dpc). By CS 17 (~12.0-12.5 dpc), no HESX1 transcripts are identified (Figure 1.5) (Sajedi et al., 2008b). Owing to the difficulty of obtaining earlier-staged human embryos, this analysis has not been extended to include the developing neural plate. However, the identical resemblance of Hesx1 and HESX1 transcripts in the ventral
diencephalon and developing RP, strongly suggests conserved expression within the presumptive anterior forebrain during human embryonic development.

The spatial restriction and transient expression of *Hesx1* and its orthologues during embryogenesis further implies a developmental role for *Hesx1*, a feature that is conserved throughout vertebrate evolution. Accordingly, it has been suggested that *Hesx1* initially acts to define a larger embryonic field that is later restricted to smaller developmental units through the interaction with other developmental control genes, a trait characteristic of many developmental repressor genes. Specifically, *Hesx1* may be employed to help pattern the developing embryo, where later it has more specific roles in forebrain and finally, pituitary development.

### 1.5.2 Role of *Hesx1* during embryonic development

#### 1.5.2.1 The role of *Hesx1* in forebrain development

The first indication of *Hesx1* requirement during forebrain development came from classical embryological studies performed by Thomas and Beddington in 1996 (Thomas and Beddington, 1996). However, it was not until the subsequent generation of the *Hesx1*−/− mouse model that the true picture emerged (Figure 1.6) (Dattani et al., 1998). Despite the early expression of *Hesx1* during mouse embryogenesis, the first morphological defects were only evident at 8.5 dpc. *Hesx1*-deficient embryos display a significant reduction of prospective forebrain tissue and absence of developing optic vesicles, rostral to the zona limitans intrathalamic. Commonly, one side of the forebrain appears more affected than the other, and by 9.5 dpc, the severity of the *Hesx1* phenotype is evidently variable. Consequently, embryos have been classified into two groups: class I embryos, equating to roughly 5%, which show severe truncations or absence of telencephalic vesicles, olfactory placodes, eyes and frontonasal mass; and, class II mutants that are less affected with an overall reduction in forebrain tissue. Initial analysis of *Hesx1*−/− mutants demonstrated that only a small minority of mice reach adult stages,
proving both viable and fertile (Dattani et al., 1998). However at present, Hesx1-null mice fail to reach weaning stages, dying perinatally, a finding believed to be background dependent.

Until recently, the mechanism with which Hesx1 exerts its function within forebrain structures remained elusive. With the use of genetic fate-mapping techniques, it has been shown that descendants of Hesx1-expressing cells are destined to colonise the anterior forebrain, including the cerebral cortex, basal ganglia, ventral diencephalon and eyes. Only a rare minority of these descendants are found within regions of the posterior forebrain. Conversely, cells deficient for Hesx1 change their fate to colonise posterior forebrain regions, as well as the neural crest lineage (Andoniadou et al., 2007). The function of Hesx1 in maintaining anterior neural identity appears conserved between species, as knockdown experiments of Xanf1 in Xenopus result in a similar phenotype to that of mouse (Ermakova et al., 1999). Continuing investigation into the underlying mechanism of Hesx1 in anterior forebrain precursors revealed that Hesx1 acts as an antagonist of the canonical Wnt/β-catenin signalling pathway in the mouse and zebrafish forebrain, maintaining a low-anterior gradient of signalling throughout the developing neural plate (Andoniadou et al., 2011).
Figure 1.6 - Forebrain and pituitary defects associated with Hesx1<sup>−/−</sup> mutant mouse embryos
(a) Lateral view of a wild-type 9.5 dpc embryo, with anterior to the left. (b-c) Hesx1<sup>−/−</sup> embryos showing (b) mild and (c) severe forebrain truncations. In the more severely affected embryo (c), a complete truncation of telencephalic and optic vesicles is noticeable. (d-g) Magnetic Resonance Imaging (MRI) of (d, f) wild-type and (e, g) Hesx1<sup>−/−</sup> embryos at 18.5 dpc. (d) Upper panel shows a sagittal view of the midline region, highlighting the location of the pituitary. Lower panel displays a frontal view, showing the pituitary gland situated between the hypothalamus and basosphenoid bone. (e) In the Hesx1<sup>−/−</sup>, the pituitary gland is severely hyperplastic, and remains embedded within the oral cavity. In this case, the normal fusion of the basosphenoid bone is inhibited (lower panel). (f) The eyes and olfactory bulbs are fully evident in a wild-type embryo. (g) In contrast, the Hesx1<sup>−/−</sup> embryo demonstrates a complete absence of eyes, and the olfactory bulbs are abnormal, with the right side of the embryo more affected than the left. Scale bar in a-c, 200 µm, and in d-g, 2 mm. Abbreviations: BS, basosphenoid bone; Hypo, hypothalamus; OB, olfactory bulbs; Pit, pituitary. Panel a-c is taken from (Martinez-Barbera et al., 2000); d-e upper panel from (Cleary et al., 2011) and lower panel d-e and f-g are unpublished data from Jayakody and Cleary.
1.5.2.2 The role of *Hesx1* in pituitary development

Similar to the forebrain, insights into the role of *Hesx1* within the developing pituitary gland have been established through the analysis of the *Hesx1* 

mutant mouse. Initial analysis of the pituitary gland in these mutants revealed that in the severely affected class I embryos, histological experiments failed to identify any RP structure at 12.5 dpc (Dasen et al., 2001). However, recent findings revealed that mice homozygous for a point mutation (R160C), hypothesised to render a null mutation, show all telencephalic, eye and pituitary defects observed in the *Hesx1* 

, but the pituitary tissue is ectopically located within the nasopharyngeal cavity (Figure 1.6) (Dattani et al., 1998; Sajedi et al., 2008b). In contrast, in the more mildly affected class II subtype of *Hesx1* 

mutants, all embryos display ventral midline defects in the developing ventral hypothalamus, with abnormal morphogenesis of RP. Specifically, the floor of the hypothalamus appears expanded, and RP presents as multiple oral ectoderm invaginations (Dattani et al., 1998; Sajedi et al., 2008b). Comprehensive analysis of the pituitary phenotype in these mutants revealed dramatic cellular hyperproliferation in RP between 13.5 and 15.5 dpc, corresponding to the time of differential cellular commitment (Dasen et al., 2001). Consequently, it is believed that this overgrowth of the developing pituitary gland leads to the failure of the underlying mesenchyme to condense and form the basosphenoid cartilage, ultimately resulting in the incomplete separation of the pituitary gland from the oral cavity. Despite the observed severe pituitary dysmorphogenesis, expression of both pituitary determination and subsequent terminal differentiation markers was detectable, appearing increased when compared to control littermates, most likely reflecting the overall increase in tissue size (Dasen et al., 2001; Sajedi et al., 2008b). Similar pituitary dysmorphic phenotypes have been reported in the *Sox3*, *Tcf4* as well as the *Wnt5a* mutants, which are all factors that are exclusively expressed within the developing ventral diencephalon rather than the pituitary, implicating that the defects observed within the *Hesx1* could be hypothalamic in origin (Brinkmeier et al., 2007; Cha et al., 2004; Rizzoti et al., 2004).
However, further knowledge into the role of Hesx1 during pituitary development has been facilitated by the generation of two mouse models harbouring missense point mutations occurring at two highly conserved residues within the two functional HESX1 domains, eh1 (I26T) and the homeodomain (R160C). As briefly mentioned, homozygous mutants bearing the R160C mutation revealed forebrain and pituitary abnormalities equivalent to the Hesx1-null mouse model, whereas mice homozygous for the I26T displayed only pituitary and ocular defects comparable to Hesx1<sup>-/-</sup> mice, without any apparent telencephalic anomalies. These observations suggest that the I26T mutation in the eh1 domain yields a hypomorphic allele, whereas the R160C occurring within the homeodomain results in a null allele (Sajedi et al., 2008b). This finding was corroborated by human data: two human patients harbouring the R160C mutation resulted in the more severe clinical phenotype of SOD, whereas the I26T mutation was identified in a young girl only presenting with pituitary dysfunction (Carvalho et al., 2003; Dattani et al., 1998). In vitro analysis of these two mutations revealed that the I26T mutation allowed the DNA-binding to be mediated through the intact homeodomain, however, protein interactions with the TLE1 was impaired (Carvalho et al., 2003). Collectively, these data illustrate that the repressor function mediated by the TLE1:HESX1 interaction is absolutely essential for normal pituitary development, a result that is further endorsed by the coexisting expression of Tle-1 and -3 with Hesx1 in the developing pituitary (Carvalho et al., 2010; Dasen et al., 2001).

As previously mentioned, expression analysis suggests a potential role for Hesx1 in maintaining a progenitor-like state in the developing pituitary (Gaston-Massuet et al., 2011; Hermesz et al., 1996; Thomas and Beddington, 1996). Interestingly, during normal pituitary development, it has been demonstrated that the diminishing expression of Hesx1 coincides with the activation of Prop1, a pituitary specific paired-like homeodomain transcription factor (Gage et al., 1996; Sornson et al., 1996). As such, a potential regulatory relationship between these two factors has been suggested. Indeed, subsequent analysis of the Ames dwarf mouse, a mouse model harbouring a hypomorphic mutation in the Prop1 gene, demonstrated the persistent expression of Hesx1 in the AP until 15.5 dpc (under normal circumstances, Hesx1 expression is
extinct by 13.5 dpc) (Gage et al., 1996; Sornson et al., 1996). Additionally, co-transfection experiments revealed that HESX1 is able to antagonise PROP1 in vitro. Thus, it is accepted that as PROP1 and HESX1 bind cognate DNA elements as heterodimers, HESX1 is able to suppress the activity of PROP1, in order to maintain progenitor status during earlier pituitary development. Subsequent extinction of HESX1 in the developing anterior lobe then allows for PROP1-dependent cellular differentiation (Gage et al., 1996; Sornson et al., 1996).

Despite this data, the reasons underlying the hypercellular phenotypes in *Hesx1* mouse mutants remains unanswered. As highlighted in Section 1.3.1.2, pituitary induction is initiated and maintained through signals originating from the infundibular region of the ventral diencephalon. In accordance, early analysis of *Hesx1*+/− embryos suggested that the pituitary hypercellular phenotype was due to an over-recruitment of oral ectoderm resulting from increased *Fgf8* signalling within the overlying ventral diencephalon (Dasen et al., 2001). However, this mechanism has been challenged by the successive analyses of the *Hesx1*R160C/R160C mice, where little or no difference in *Fgf8* expression domain was noted in these embryos when compared to wild-type controls (Sajedi et al., 2008b). Instead, successive analyses of heterozygous mice for *Hesx1* and *Six3*, another transcription factor shown to have overlapping expression with *Hesx1* in the forebrain and pituitary, has proposed that the hypercellular phenotype is a consequence of de-repression of the Wnt/β-catenin pathway (Section 1.3.1.2), consistent with previous research showing that both *Hesx1* and *Six3* antagonise ectopic activation of the Wnt/β-catenin pathway in the anterior forebrain (Andoniadou et al., 2007; Andoniadou et al., 2011; Gaston-Massuet et al., 2008; Lagutin et al., 2003). These double heterozygous mice display a comparable pituitary phenotype to the *Hesx1*-null mutants, in which terminal differentiation of pituitary cell types occurs normally, however morphogenesis is severely affected. To date, the molecular mechanism underlying this genetic interaction remains unclear, as it was shown that a protein/protein interaction between HESX1 and SIX3 is unlikely. Furthermore, *Six3* expression in the *Hesx1*+/− appears unaffected (Gaston-Massuet et al., 2008). Nevertheless, it is clear that both *Hesx1* and *Six3* play analogous roles in maintaining...
the correct number of progenitor cells within early pituitary development through repressing the Wnt/β-catenin pathway, which is later opposed by Prop1 activity.

Taken together, all these analyses reveal a crucial function for Hesx1 in the development of forebrain structures (including the telencephalon and eyes) and the pituitary gland. Conserved between tissues, Hesx1 exerts its function largely by acting as a negative modulator of the Wnt/β-catenin signalling pathway. Interestingly, the pituitary gland appears to be more sensitive to lessening levels of Hesx1, a mechanism that appears to be conserved between mice and humans.

1.5.3 Human HESX1 and disease

As evidently demonstrated throughout the previous section, Anf genes share a vast array of conservation in gene regulation, expression and function. With this in mind, and given that Hesx1-deficient mouse mutants show a plethora of forebrain and pituitary defects (Figure 1.6), it is hardly surprising that human patients with mutations in HESX1 have subsequently been associated with hypopituitarism and related conditions, such as SOD (Dattani et al., 1998). At present, the Hesx1 mouse model represents the only mouse model of this highly devastating condition.

To date, a total of 16 mutations in HESX1 have been identified (Table 1.2). These HESX1 mutations cause both missense and frameshift changes in the protein and include both dominant and recessive modes of inheritance. As in the mouse, a broad spectrum of phenotypes has been reported, varying in penetrance and severity. Consistently, pituitary abnormalities appear to be the most frequent, ranging from the loss of one hormone through to a complete endocrine malfunction, and can occur in association with structural abnormalities including an undescended/ectopic PP. Similarly to the mouse, ocular insufficiencies are the most common forebrain presentation (Brickman et al., 2001; Carvalho et al., 2003; Cohen et al., 2003; Corneli et al., 2008; Coya et al., 2007; Dattani et al., 1998; McNay et al., 2007; Reynaud et al., 2011; Sobrier et al., 2005; Sobrier et al., 2006; Tajima et al., 2003; Thomas et al., 2001b).
<table>
<thead>
<tr>
<th>HESX1 mutation</th>
<th>Inheritance</th>
<th>Endocrine Deficit</th>
<th>Neuroradiological findings</th>
<th>Reference</th>
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<tr>
<td>p.Q6H</td>
<td>Dominant</td>
<td>GH, TSH, LH, FSH</td>
<td>AP hypoplasia, ectopic PP</td>
<td>(Corneli et al., 2008; Thomas et al., 2001b)</td>
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<tr>
<td>p.I26T</td>
<td>Recessive</td>
<td>GH, LH, FSH; evolving ACTH, TSH</td>
<td>AP hypoplasia, ectopic PP, normal ON</td>
<td>(Carvalho et al., 2003)</td>
</tr>
<tr>
<td>c.306_307insAG</td>
<td>Dominant</td>
<td>GH, LH, FSH; hypothyroidism</td>
<td>AP hypoplasia, ON hypoplasia</td>
<td>(Tajima et al., 2003)</td>
</tr>
<tr>
<td>p.R109X</td>
<td>Recessive</td>
<td>GH, ACTH; evolving panhypopituitarism</td>
<td>AP hypoplastic, ectopic PP, normal ON</td>
<td>(Reynaud et al., 2011)</td>
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<tr>
<td>p.Q117P</td>
<td>Dominant</td>
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<td>AP hypoplasia, ectopic PP</td>
<td>(Coya et al., 2007)</td>
</tr>
<tr>
<td>c.357 + 3G&gt;A</td>
<td>Dominant</td>
<td>GH</td>
<td>Normal AP and PP</td>
<td>(Vivenza et al., 2011)</td>
</tr>
<tr>
<td>c.357 + 2T&gt;C</td>
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<td>GH, TSH, ACTH, PRL</td>
<td>AP aplasia, normal PP, normal ON</td>
<td>(Sobrier et al., 2006)</td>
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<tr>
<td>Alu insertion (exon 3)</td>
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<td>Panhypopituitarism</td>
<td>AP aplasia, hypoplastic sella, normal PP</td>
<td>(Sobrier et al., 2005)</td>
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<td>GH</td>
<td>AP aplasia, ectopic PP</td>
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<td>c.449_450delCA</td>
<td>Recessive</td>
<td>GH, TSH, ACTH</td>
<td>AP aplasia, normal PP and ON, thin CC, hydrocephalus</td>
<td>(Sobrier et al., 2006)</td>
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<td>p.R160C</td>
<td>Recessive</td>
<td>GH, TSH, ACTH, LH, FSH</td>
<td>AP hypoplasia, ectopic PP, partial ACC</td>
<td>(Dattani et al., 1998)</td>
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<tr>
<td>p.R160H</td>
<td>Recessive</td>
<td>Panhypopituitarism</td>
<td>AP aplasia, normal PP and a thin pituitary stalk</td>
<td>(Durmaaz et al., 2011)</td>
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<td>p.S170L</td>
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<td>GH</td>
<td>Normal AP, ON hypoplasia, ectopic PP, partial ACC</td>
<td>(Thomas et al., 2001b)</td>
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<td>Ectopic PP</td>
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<td>p.T181A</td>
<td>Dominant</td>
<td>GH</td>
<td>AP hypoplasia, normal ON, absent PP</td>
<td>(Thomas et al., 2001b)</td>
</tr>
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</table>

ACC, agenesis of the corpus callosum; AP, anterior pituitary; CC, corpus callosum; ON, optic nerve; PP, posterior pituitary.
Despite the clear association between HESX1 and SOD, information derived from a systematic mutational screen has shown that mutations in HESX1 are a rare cause of SOD and hypopituitarism (McNay et al., 2007). Certainly, it remains possible that homozygous mutations affecting the function of this gene during early stages of development could result in embryonic lethality, as observed in the mouse (Dattani et al., 1998). Nonetheless, it is evident that other genes may be implicated in the aetiology of this complex and variable condition. Indeed, over the recent years, mutations in SOX2 have been linked to conditions of hypopituitarism and SOD in humans (Bakrania et al., 2007; Kelberman et al., 2006; Kelberman et al., 2008; Sato et al., 2007; Williamson et al., 2006; Zenteno et al., 2005).
1.6 SOX GENES

Concurrent with the discovery of homeobox genes, the SOX (Sry-related box) transcription factor family was first discovered in 1990 as a group of genes closely related to the mammalian sex determining gene, Sry (Gubbay et al., 1990). Sequence homology between the mouse and human protein sequences was poor, but was restricted to a small 79 amino acid region. Close examination of this region demonstrated that it is present in other genes and thus, it has been revealed that Sry is a member of a substantial group of embryonically expressed genes, the Sox family (Collignon et al., 1996). It is now known that the Sox family belongs to the high motility group (HMG) superfamily of transcription factors, which all harbour the 79 amino acid HMG domain. Members are designated to the Sox family if they carry a single HMG with >50 amino acid residues identity with the HMG domain of Sry, in addition to the sequence motif of at least RPMNAF (positions 5-10) within the HMG domain. Moreover, the extended version common to all non-SRY SOX members (RPMNAFVW) appears to be the most reliable signature of the Sox family. To date, a total of 20 Sox genes have been identified in mouse and humans. Based on sequence alignments, the Sox family is categorised into at least 10 subgroups; Group A solely contains Sry and is specific to eutherian mammals, Groups B-F are found in all higher metazoans and Groups G-J are limited to particular lineages. SOX proteins within the same group share a high degree of identity within and around the HMG domain (usually 70-95%) whereas members from differing groups share partial identity (≥46%) (Bowles et al., 2000; Lefebvre et al., 2007; Schepers et al., 2002).

SOX proteins are transcription factors and mediate their DNA binding through the HMG domain. Structural analysis of the HMG domain has demonstrated that it consists of three α-helices that conform into a twisted L-shape. This overall structure is maintained by a highly conserved internal hydrophobic core, which recognises a common consensus sequence (A/T-A/T-CAA-A/T-G) located within the minor groove of target DNA. Upon DNA binding, little alteration is made to the HMG domain, however a large conformational change is induced to the target DNA, resulting in a 70-85° bend in the DNA molecule. Consequently, it is hypothesised
that SOX proteins facilitate part of their function as ‘architectural’ proteins by organising local chromatin structure and assembling other DNA-bound transcription factors into active multi-protein complexes [reviewed in (Lefebvre et al., 2007; Pevny and Lovell-Badge, 1997; Prior and Walter, 1996; Wegner, 1999)].

In addition to their N-terminal HMG domain, SOX proteins can function as transcriptional activators or repressors, a function that is largely mediated through the C-terminus. Close scrutiny of the Group B family of SOX proteins by Uchikawa and colleagues, led to the suggestion that members of this group would be more correctly partitioned into two distinct subgroups, B1 and B2 (Uchikawa et al., 1999). The most compelling reason for this was based upon functional in vitro analyses, which demonstrated that the B1 subgroup, comprising of Sox1, Sox2 and Sox3, functioned as transcriptional activators, whilst Sox14 and Sox21 (B2 subgroup) acted as transcriptional repressors. The amino acid sequences encompassing the HMG domain and its immediate C-proximal domain, deemed the ‘Group B homology’, are well conserved between all SOX proteins belonging to these groups (B1 and B2), however, the remaining portions only display similarities within the subgroups (Uchikawa et al., 1999).

As deduced from all SOX HMG domains examined (SRY, SOX5, SOX9 and SOX17), individual HMG domains of the SOX proteins appear similar to each other in their sequence preference and their DNA-bending activity. Despite this, individual SOX proteins interact selectively and regulate a unique set of target genes. Cumulative evidence suggests that target specificity and regulatory function of SOX proteins is dependent upon the interaction with specific partners expressed within a specific cell type [reviewed in (Kamachi et al., 2000; Wilson and Koopman, 2002)].

Following the identification of natural SOX target genes, many insights into the molecular action of SOX proteins have been gained. A classic example is provided. The SoxB1 factors are involved in the early specification and development of the lens. All three SoxB1 members, Sox1-3, have been shown to bind to the 5’ sequence of the DC5 enhancer of the chicken δ-crystallin gene to coordinate lens-specific gene expression (Kamachi et al., 1998). Importantly, yeast chromatin immunoprecipitation studies demonstrated that the activation of δ-
crystallin expression by SOX2 is reliant upon the direct association of SOX2 with a second lens-specific protein, PAX6. Consequently, PAX6 is required to bind to the 3’ distal region of the DC5 enhancer. Kamachi et al. (2001) have revealed that when either SOX2 or PAX6 are expressed alone in yeast cells, neither protein is capable of binding to the DC5 enhancer, but when expressed together, both are bound in vivo. From this, it has been proposed that in order for SOX2 to bind to 5’ DC5 enhancer with high specificity and ultimately trigger δ-crystallin expression, the close association with its adjacent DNA bound partner PAX6, is required (Kamachi et al., 1999; Kamachi et al., 2001).

The requirement of partner proteins is not essential for the function of the C-terminal regulatory domains. In vitro studies have shown that when the C-terminal domain is transplanted onto a GAL4-DNA-binding domain, thus bypassing HMG-induced DNA binding, this region is able to confer its regulatory action, in a manner independent of cell context. However, when the C-terminus of SOX2 is replaced with the potent activator domain of VP16, the SOX2-VP16 fusion protein still requires the binding of PAX6 to activate δ-crystallin. Thus, the highly potent activator VP16 is not sufficient to overcome the requirement of the partner factor when DNA binding is mediated through the HMG domain (Kamachi et al., 1999).

Finally, a member of the divergent group E, Sox9, that retains 60% homology within its HMG domain with Sox2, was shown to be capable of binding to the DC5 enhancer. However, it was unable to interact with PAX6. This resulted in the inability of SOX9 to activate δ-crystallin transcription, illustrating that the activation of δ-crystallin is a specific action of SoxB1 subgroup. From this, it is believed that in addition to SOX2 and PAX6 binding to the DC5 enhancer, a direct interaction between both proteins is required to allow the formation of a ternary complex, reinforcing high-affinity binding of SOX2 to DNA (Kamachi et al., 1999).

Similar partnerships between SOX proteins and tissue specific transcription factors have been noted. For instance, SOX2 synergistically interacts with OCT3/4 in regulating Fgf4 expression in ES cells, whereas SOX9 coordinates with Tip60, a Tat interactive protein-60, in activating transcription of type II collagen, Col2a1, during the regulation of chondrogenesis (Hattori et al., 2008; Yuan et al., 1995).
Taken together, these experiments illustrate that with the assistance of co-factors, SOX proteins are able to maintain strong and stable binding, subsequently allowing for the C-terminal regulatory domain to exert its transcriptional function. In addition, it is highlighted that the cell specific action of SOX proteins depends on the presence of distinct combinations of protein partners.

1.6.1 Expression of SoxB1 subfamily

The focus of this study is Sox2, a member of the SoxB1 subfamily, and its role during hypothalamic-pituitary development. As referred to in the previous section, during lens development the employment of SoxB1 proteins is interchangeable, and it appears that, within the same tissue, these factors can function redundantly. This seems to be a common function of SOX proteins within the same subgroup, and thus it is important to detail the current knowledge regarding the SoxB1 family, with emphasis placed on Sox2.

Sox2 expression in the mouse is detectable prior to gastrulation in few cells at the morula stage (2.5 dpc) and within the ICM of the blastocyst at 3.5 dpc (Figure 1.7). During early stages of gastrulation, expression is restricted to anterior ectoderm, which is fated to become neuroectoderm and anterior surface ectoderm. By 9.5 dpc, Sox2 transcripts are visualised throughout the developing CNS in a uniform pattern. At this stage, it appears that most of these cells are pluripotent. Subsequently, as the columnar epithelium of the developing neural plate develops a more complex, stratified appearance, expression of Sox2 is limited to the germinal layer, where the pluripotent cells persist. Consequently, Sox2 expression within the CNS is maintained throughout adult stages, largely confined to neurogenic regions, such as the subventricular zone and hippocampus dentate gyrus. Additionally, at 9.5 dpc Sox2 transcripts are present within the sensory placodes, branchial arches and gut endoderm. Of the sensory placodes that will give rise to the eye, Sox2, along with Sox1 and Sox3 are initially expressed within the developing optic cup. However, it is apparent that only Sox2 is maintained throughout neural retinal development (Kamachi et al., 1998). At 11.5 dpc, Sox2 mRNA can be detected uniformly within RP and its expression persists in a few scattered cells within the
proliferating periluminal space and AP through to 18.5 dpc. This expression of Sox2 is maintained within the adult pituitary in a similar distribution around the cleft (Avilion et al., 2003; Collignon et al., 1996; Ellis et al., 2004; Taranova et al., 2006; Wood and Episkopou, 1999) and reviewed in (Alatzoglou et al., 2009; Uchikawa et al., 2011).
Figure 1.7 - Sox2/SOX2 expression during mouse and human embryonic development, with particular emphasis on hypothalamic-pituitary development

(a-e) Whole mount in situ hybridisation with Sox2 during early mouse embryogenesis. (f) Section in situ hybridisation with Sox2, and (g-h) immunofluorescence staining against SOX2 on mouse tissue, highlighting the expression of Sox2/SOX2, within the developing pituitary gland. (a-g, j) are sagittal views, whereas (h-i, k) represent frontal views. (i-k) Section in situ hybridisation with Sox2 revealing the expression of SOX2 during human pituitary gland development. (a) Sox2 mRNA is seen in the ICM of the mouse blastocyst, (b) throughout the epiblast and extraembryonic ectoderm of a 6.5 dpc embryo, and (c) in the chorion and anterior region of the presumptive neuroectoderm of a 7.5-8.0 dpc embryo. (d) At 8.5 dpc, Sox2 expression persists in the chorion, headfolds, and neural tube. (e) At 9.5 dpc, Sox2 transcripts are detectable throughout the nervous system, sensory placodes, branchial arches, and gut. (f) At 11.5 dpc, Sox2 transcripts are detectable within the developing hypothalamus (Di) and RP. (g) At this stage, SOX2 is ubiquitous within RP. (h) In the adult, SOX2+ve cells are mostly localised to the periluminal region, as outlined by the dashed line. Few SOX2+ve cells can be found scattered through the anterior pituitary. During human embryonic development, SOX2 expression is detectable within RP and overlying neural ectoderm at (i) CS16 and (j) CS19. (k) At fetal stage 2, SOX2 transcripts continue to be detected in the cells lining the lumen of the anterior pituitary and within the overlying hypothalamus. Scale bar in (a) represents 25 µm in a, and 60 µm in b; scale bar in c represents 60 µm in c, 120 µm in d and 435 µm in e. Scale bars: f, 100 µm; g-h, 15 µm; i, 100 µm; j-k, 300 µm. Abbreviations: AP, anterior pituitary; C, chorion; Di, diencephalon; Ep, epiblast; ExE, extraembryonic ectoderm; Hf, headfolds; Hyp, hypothalamus; Ne, neuroectoderm. Images a-e are taken from (Avilion et al., 2003), f from (Kelberman et al., 2006), g-h from (Fauquier et al., 2008), and i-k from (Kelberman et al., 2008).
Although not as early during embryonic development as for Sox2, the expression of Sox3 commences between 5.5-6.5 dpc and can be seen throughout the epiblast. Following gastrulation, its transcripts are localised to prospective anterior neural plate. Slight variations in the expression patterns of Sox2 and Sox3 are observed; Sox2 is more strongly expressed in the anterior half of the anterior neural plate, whereas Sox3 in the posterior end. By 9.5 dpc, Sox3 is detected in the neuroectoderm, coupled with some expression within the olfactory placode and optic vesicle, as noted above. Expression of Sox3 mRNA is maintained within the developing CNS, however, is gradually excluded from the optic cup. In a similar pattern to Sox2 within the CNS, transcripts are confined to the ependymal layer of the CNS, where progenitor cells are actively dividing. By neonatal and adult stages, high expression of Sox3 can be found within the neurogenic regions of the subventricular zone and subgranular zone of the hippocampal dentate gyrus. Its expression is also maintained, albeit at lower levels, within non-neurogenic regions of the brain, including the ventro-medial hypothalamus (Alatzoglou et al., 2009; Collignon et al., 1996; Uchikawa et al., 2011; Wood and Episkopou, 1999).

Sox1 expression initiates later than the rest of its B1 members within the developing embryo. In mouse, Sox1 expression is completely absent until 7.5 dpc. Following somitogenesis at 8.0 dpc, strong Sox1 expression initiates in the closed posterior neural tube region first, whereas low levels of transcripts are subsequently detectable within the anterior region of the neural plate. By 8.5 dpc, Sox1 expression persists throughout the neural tube with clear gaps in rhombomeres 3 and 5 (Uchikawa et al., 2011; Wood and Episkopou, 1999). In addition to the CNS, Sox1 is also expressed within the lens vesicle commencing from 12.5 dpc and persists thereafter (Nishiguchi et al., 1998).

To complement the expression analysis conducted in mouse, detailed expression of SoxB1 homologues have been noted in chick. cSox1, cSox2 and cSox3 largely recapitulate the expression of these factors observed in mouse. However, slight interspecies differences are identified prior to gastrulation, when cSox3 is the first B1 gene to be expressed prior to gastrulation (Rex et al., 1997). Detailed expression analysis of cSox1, cSox2 and cSox3 within the brain at early head fold stages has revealed that cSox2 expression is most widely distributed
within the brain with the highest levels of expression within the forebrain and midbrain. Of relevance, *cSox2* transcripts have been identified within the diencephalon, with strong expression within the ventral thalamus and rostral hypothalamus, with weak expression within the mammillary region. In contrast, *cSox1* and *cSox3* have been shown to be expressed in subsets of *Sox2*-expressing domains within the rostral to the mid-brain. Thus, it is evidenced that as the brain develops, expression of B1 Sox genes seems to create newer subdivisions within a pre-existing region, through locally increasing or decreasing expression levels of SOX proteins (Uchikawa et al., 1999).

Of importance to my study, the data collated so far from the mouse and chick appears to correlate with expression analysis of *Sox2* during stages of human development. *In situ* hybridisation studies on human embryonic samples, from CS14 through to fetal stage (F) 2, have been conducted by Kelberman *et al.* (2008). At all stages analysed, strong expression of SOX2 mRNA and protein is detected throughout the CNS. Strong expression is maintained within the forebrain at CS16 (~11.0-11.5 dpc in mouse) and subsequently, SOX2 transcripts are observed within the cortex and neuroepithelium of the lateral ventricles. In addition, SOX2 expression has been identified throughout the entire length of the developing neural tube between CS14 through to CS20. SOX2 expression is also present during stages of eye development between CS16 and F2. Of value, SOX2 expression is detected throughout RP at CS16, and it has been noted that its expression is maintained within the RP and AP, in addition to the overlying hypothalamus (Figure 1.7). As in the mouse, this expression pattern persists through the fetal stages analysed (Kelberman et al., 2008).

Combined, these expression data suggest that SOXB1 factors have evolutionary conserved roles. Specifically, it is evident that the main site of expression of the SoxB1 subfamily of genes is within the developing CNS. With this, it is not surprising that SoxB1 factors have been reported to have functional roles during various stages of neural development, as well as during the formation of the sensory placodes, fated to become eye. Finally, although a large degree of overlap is detected between members of this group, there are also cases in which a single B1 Sox family member is uniquely expressed, such as *Sox2* in the pituitary.
1.6.2 SOXB1 mouse mutants

In stark contrast to the analysis of the *Hesx1*-null mouse mutant, investigating the requirement of SOXB1 genes during development using murine loss-of-function mutants has been less fruitful. Mouse embryos, homozygous for targeted deletion of *Sox2*, show peri-implantation lethality, most likely due to the unique functional role of *Sox2* within the blastocyst (Avilion et al., 2003). Although viable, *Sox1* homozygous mutants display minimal neural defects, with reports only of spontaneous seizures (Nishiguchi et al., 1998). Gross morphological malformations have been only notable within the lens of these mutants. This is presumably because *Sox3* is never expressed within the lens, whilst *Sox2* expression is restricted to an earlier stage of lens development, prior to the onset of *Sox1* expression (Miyagi et al., 2009). Finally, two reports of *Sox3*-deficient mice have demonstrated that similarly to the analysis of the *Sox1*-deficient mice, *Sox3* mutants are viable, displaying relatively mild CNS effects. The most common phenotype reported is abnormal craniofacial development, presenting largely as overgrowth of teeth due to jaw misalignment and malformation (Rizzoti et al., 2004; Weiss et al., 2003). Of value, both groups reported low body weight and infertility in a minority of mutants, indicative of a possible hypothalamic-pituitary defect. Histological examination of these mutants revealed midline defects, coupled with AP hypoplasia at birth. Detailed investigation into this phenotype revealed that the pituitary defects observed in these mutants are an indirect effect of early abnormal hypothalamic development, consistent with previous expression analysis that *Sox3* transcripts are excluded from the pituitary gland at all stages studied (Rizzoti et al., 2004).

As illustrated from these works, despite the overt expression of SOXB1 factors within the developing CNS, deletion of these genes, with the exception of *Sox2*, has relatively mild phenotypic effects, most likely due to adduced functional redundancy with other members of the SOXB1 family. Together, efforts to clarify the role of SoxB1 genes have been clouded by functional redundancies and embryonic lethality.
1.6.3 Role of Sox2 during embryonic development

To circumvent the lethality observed in the Sox2-null mutants, researchers have made use of sophisticated gene targeting to generate hypomorph and conditional/null knockouts to provide insights into the function of Sox2 within the developing embryo. To complement this work, classical in vitro studies have also provided much value to deducing the role of Sox2 during embryogenesis.

1.6.3.1 Role of Sox2 in early embryogenesis and neural development

As highlighted through expression analysis, Sox2 expression is largely localised to regions retaining stem cell characteristics. At early stages of embryogenesis, this is exemplified through high levels of expression within the ICM of the blastocyst and the epiblast. A functional role for SOX2 within this crucial time period is highlighted by the peri-implantation lethality of the Sox2 null mutant (Avilion et al., 2003). In vitro studies have also highlighted that a minimum of four transcription factors, namely Sox2 along with Oct3/4, c-Myc and Klf4, are sufficient to revert both embryonic and adult fibroblast cells to induced pluripotent states (Takahashi and Yamanaka, 2006). With this, Sox2 has often been referred to as a ‘stemness master gene’ (Lefebvre et al., 2007).

Following initial stages of embryogenesis, Sox2 expression marks the developing CNS, where later it universally marks neural stem/progenitor cells throughout the CNS, including the neural retina (Section 1.6.1). Crucial insights into the function of Sox2 within the CNS have been provided by studies in chick. Utilising SoxB1 mutants functioning as chimeric transcriptional repressors results in delamination of neural progenitor cells from the proliferating ventricular zone, coupled with a concomitant exit from the cell cycle. Conversely, the overexpression of Sox2 results in the inhibition of neuronal differentiation, preserving a neural progenitor-like state. Moreover, in the absence of Sox2, Sox1 overexpression is able to restore neuronal differentiation, indistinguishable from endogenous Sox2, further endorsing functional redundancy amongst SoxB1 proteins (Bylund et al., 2003; Graham et al., 2003).
From this, it appears that Sox2 functions in part to maintain neural progenitor status within the developing CNS. Two independent studies revealed that Notch signalling acts downstream of Sox2 to achieve this function. Chromatin immunoprecipitation studies, complemented with transfected experiments, identify Notch1 as a direct SOX2 target. This has been confirmed in vivo through the analysis of Sox2-deficient retinas, which demonstrate reduced expression of both Notch1 and its downstream effector, Hes5 when compared to controls (Taranova et al., 2006). This is further corroborated by in vitro data obtained from subventricular zone progenitors, that overexpression of Sox2 results in the concomitant upregulation of Notch1, as well as of its downstream effectors (Bani-Yaghoub et al., 2006).

To complement these works, targeted deletion of a telencephalic enhancer of Sox2 has been conducted in mouse, resulting in a knockdown allele expressing only 25-30% of wild-type levels of Sox2 in the brain. Heterozygous mice for this hypomorphic allele are phenotypically comparable to wild-type littermates. However, compound knockout/knockdown mice reveal brain abnormalities, including reduced cortex volume, thalamo-striaral parenchymal loss, epilepsy and motor/neurological deficits (Ferri et al., 2004). In addition, analysis of the mouse neocortex in Sox2-deficient mice has been conducted by Miyagi et al. (2008). To achieve this, conditional deletion was generated through the employment of a Nestin-Cre transgene. Despite perinatal lethality, minimal defects during embryonic neural development and neurogenesis have been observed. Importantly, these mice display elevated levels of Sox3, and thus it has been postulated that the mild CNS impairment is the result of Sox3 compensation (Miyagi et al., 2008), further supporting the in vitro data aforementioned. Similarly, the use of an independently generated Sox2 conditional mouse mutant, which results in complete pan-neural deletion commencing at 12.5 dpc, results in minor brain abnormalities at birth; however of value, these mice survive to postnatal week 4. Analyses of postnatal brain regions in these mutant mice demonstrate a severe reduction of hippocampal neural stem cells, which ultimately results in dentate gyrus hypoplasia, highlighting a unique role for Sox2 within this structure postnatally (Favaro et al., 2009). Lastly, in an attempt to investigate the role of Sox2 in the developing eye, Taranova and colleagues (2006) created an allelic series, comprising both
hypomorphic and conditional null mutants. Complete ablation of Sox2 utilising the retina-specific Pax6-Cre mouse line, results in a dramatic reduction of neural progenitor competence, in a function that appears to be dosage-dependent (Taranova et al., 2006).

Accumulating data suggest that Sox2 may have a dual function within the development of the CNS; at first, Sox2 is required for maintaining and controlling a neural progenitor-like state whereas later, it is re-deployed for the differentiation of specific subtypes. Within the developing CNS, the neuronal subset of GABAergic neurons appears to be most sensitive to altering levels of Sox2. In addition to initial cellular changes, migration from their origin within the ganglionic eminences to their final destination within the cortex and to the adult olfactory bulb is disrupted (Cavallaro et al., 2008). This phenomenon is also apparent within the retina, as diminishing levels of Sox2 appears to result in differentiation defects in the retinal ganglion cell, with negligible effects on other retinal subtypes. At present the mechanisms underlying this remain to be identified (Taranova et al., 2006).

Collectively, these findings conclude that Sox2 is absolutely required for the maintenance of progenitor type status, both within the developing early embryo and within the CNS. However, despite the widespread expression of Sox2 in the neural plate from very early stages of embryogenesis, analyses of Sox2 hypomorphs and conditional mutants result in minimal defects in the CNS embryonically. Given the overlapping expression of both Sox1 and Sox3 with Sox2 (Section 1.6.1), it is plausible that these closely related family members may compensate for much of its function in the embryonic CNS. Finally, there is an emerging view that Sox2 also participates later during development in the process of terminal differentiation.

1.6.3.2 Role of Sox2 in pituitary gland development

The first indication that Sox2 may be important during hypothalamic and pituitary development came from the analysis of Sox2βGeo heterozygotic animals. One third of these mice die between birth and weaning, with the remaining survivors demonstrating growth retardation and reduced fertility, suggestive of a hypothalamic-pituitary defect (Avilion et al., 2003;
Kelberman et al., 2006). Consistent with this, analyses of pituitary hormone levels at 2 months have shown a reduction in GH and LH content in the Sox2<sup>βGeo</sup> heterozygotes when compared to their wild-type counterparts (Kelberman et al., 2006). Accordingly, further in-depth analysis has demonstrated a variable degree of pituitary dysmorphology during embryonic development. At 12.5 dpc, approximately a third of these embryos display a bifurcated RP, which is believed to display as extra clefts at 18.5 dpc. In addition to this subtle morphological defect, the proportion of mutants analysed has also revealed AP hypoplasia. In agreement with this latter finding, adult pituitaries exhibit combined hypopituitarism, postulated to be a reflection of overall reduced-tissue size. Interestingly, no overt differences have been found in the levels of ACTH between controls and mutants at adult stages. However, at postnatal day (P)7, a significant reduction of ACTH has been noted. As a result, it is speculated that surviving mutants represent a population of mildly affected animals, and that the observed neonatal death corresponds to the ACTH-deficient cohort identified at P7. As it is known that Sox2 is expressed within the hypothalamus, it still remains unclear whether the hypoplastic pituitary documented is a result of aberrant hypothalamic function, as identified in the Sox3 mutants (Rizzoti et al., 2004). However, despite this, these findings collectively allude to a possible role for Sox2 in the development of either or both the pituitary and hypothalamus.

The requirement of Sox2 in the pituitary has been further illustrated through the work of Fauquier et al. (2008). It is well established that the pituitary gland adapts the proportion of endocrine cell types to meet varying hormonal requirements throughout life. Thus, it has been hypothesised that these cellular fluxes in the pituitary are driven, at least in part, through the recruitment and differentiation of progenitors/stem cells intrinsic to the gland. Given the requirement of Sox2 in the maintenance of several stem cell populations (as exemplified by pluripotent cells of the early embryo and their in vitro counterpart, ES cells), SOX2<sup>+</sup> cells were analysed within the pituitary to investigate whether these cells could represent a pool of progenitors/stem cells within the adult gland. As determined through the generation of SOX2<sup>+</sup> floating spheres (pituispheres), the authors report that all SOX2<sup>+</sup> cells withhold the ability to differentiate into all endocrine cell types of the mature pituitary, potentially representing a
progenitor/stem cell niche within the mature anterior lobe (Fauquier et al., 2008). Accordingly, one could hypothesise that the SOX2$^{+ve}$ embryonic counterpart may play a similar role maintaining cells in a progenitor like state for correct pituitary development.

Taken together, these analyses demonstrate a role for Sox2 in the development of the anterior neural structures, as well as the pituitary gland. Conserved between structures, SOX2 is sustained within regions that retain progenitor capacities, suggesting a shared role of SOX2 in maintaining a progenitor/stem cell niche within these structures. Furthermore, given the similarity of Sox2/SOX2 expression in mice and humans within these regions, it is likely that the function of Sox2 is conserved between species.

1.6.4 Human SOX2 and disease

The eye and brain defects observed in the Sox2-deficient mouse models, reported above, reproduce defects observed in human patients carrying heterozygous de novo mutations in SOX2 (Fantes et al., 2003; Sisodiya et al., 2006). Importantly, a broad spectrum of severity is displayed in these patients, both in the eye and brain defects, similar to that observed in mice.

Historically, SOX2 mutations were initially described in patients harbouring the most severe forms of eye defects, bilateral anophthalmia/microphthalmia (Bakrania et al., 2007; Fantes et al., 2003; Ragge et al., 2005; Williamson et al., 2006). Accordingly, it has been reported that 3% of unilateral and approximately 10% of bilateral eye abnormalities were associated with SOX2 (Fantes et al., 2003; Ragge et al., 2005). To date, SOX2 mutations have also been linked to other abnormalities, including developmental delay, spastic diplegia, oesophageal atresia, sensorineural hearing loss, as well as male genital abnormalities (Chassaing et al., 2007; Faivre et al., 2006; Hagstrom et al., 2005; Sisodiya et al., 2006; Williamson et al., 2006; Zenteno et al., 2005).

Consistent with SOX2 expression within the hypothalamus and pituitary gland during human embryonic development, recent works have now correlated SOX2 mutations with hypopituitarism. Kelberman and colleagues first reported this association in 2006 and subsequently, a total of 10 mutations have been identified. Phenotypically, all patients present a
hypoplastic AP and with variable abnormalities of the midline, including hippocampal abnormalities, hypoplasia of the corpus callosum and hypoplasia/absence of optic nerves (summarised in Table 1.3). Intriguingly, despite the invariable hypoplasia, the only endocrine disorder observed is isolated gonadotropin deficiency, which is also referred to as hypogonadotrophic hypogonadism (HH) (Bakrania et al., 2007; Kelberman et al., 2006; Kelberman et al., 2008; Sato et al., 2007; Williamson et al., 2006; Zenteno et al., 2005).

HH is defined by impaired gonadotrophin release without any other abnormal function of the pituitary gland and results in abnormally low serum levels of LH, FSH in addition to sex steroids. Clinically, this is usually characterised by bilateral cryptorchidism in males, absent or incomplete puberty with amenorrhea in females and infertility [reviewed in (Beate et al., 2012; Hayes et al., 1998; Seminara et al., 1998; Seminara et al., 2000; Tziaferi et al., 2008)]. In many cases, the underlying cause is resultant of abnormal GnRH neurons. As GnRH neurons are sourced from the olfactory placodes, GnRH can occur in association with anosmia and is then regarded as Kallmann syndrome (Schwanzel-Fukuda et al., 1989).
<table>
<thead>
<tr>
<th>SOX2 mutation</th>
<th>Eye Phenotype</th>
<th>Endocrine Deficit</th>
<th>Neuroradiological findings/ Other</th>
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<td>p.Y160X</td>
<td>Bilateral anophthalmia</td>
<td>HH</td>
<td>AP hypoplasia, severe developmental delay, cryptorchidism, micropenis</td>
<td>(Kelberman et al., 2006; Williamson et al., 2006)</td>
</tr>
<tr>
<td>p.Q177X</td>
<td>Bilateral anophthalmia</td>
<td>HH</td>
<td>Severe developmental delay, cryptorchidism, micropenis</td>
<td>(Kelberman et al., 2006)</td>
</tr>
<tr>
<td>SOX2 deletion</td>
<td>R anophthalmia, L microphthalmia</td>
<td>HH</td>
<td>AP hypoplasia, thin corpus callosum</td>
<td>(Bakrania et al., 2007; Kelberman et al., 2008)</td>
</tr>
</tbody>
</table>

*: this deletion is due to a de novo chromosomal translocation

DD, developmental delay; HH, hypogonadotrophic hypogonadism; R, right; L, left
1.7 THESIS AIMS

*Hesx1* and *Sox2* are two early developmental transcription factors that are expressed during early stages of neural and pituitary organogenesis. Accordingly, disruptions in these two genes, both in mice and humans, have highlighted important roles in the morphogenesis of the hypothalamic-pituitary axis. Despite this, many questions remain unanswered. The work presented in this thesis primarily investigates the role of these two transcription factors during normal hypothalamic-pituitary development, contributing to the previous knowledge, with an ultimate aim of proving our understanding of hypopituitarism and related conditions in humans.

Since the association of *HESX1* and SOD, great progress has been made in elucidating the role of this transcription factor in this complex condition. Despite this, a true association between the mouse and human phenotypes remains incomplete. Chapters 3 and 4 utilise two different but complementary approaches to resolve this issue. In light of the clinical presentation of patients harbouring *HESX1* mutations, Chapter 3 investigates a possible hypothalamic contribution to this particular cohort of patients through the analysis of the developing neuroendocrine system in various *Hesx1* mouse models. Relatedly, Chapter 4 employs an *in vitro* approach to analyse the functional consequences of three recently identified *HESX1* variants, with the dual aims of improving our understanding of *HESX1* function, as well as in gaining insight into the contribution of *HESX1* variants in the formation of disease states.

Similarly, despite the recent connection between SOX2 and hypopituitarism/SOD, little is known about the function of *Sox2* within the developing pituitary gland. To date, progression within this field has been inhibited due to the early lethality of the *Sox2*−/− mice and has largely relied on the phenotypic analysis of *Sox2* heterozygous mice (Avilion et al., 2003; Kelberman et al., 2006). Using a conditional approach, Chapter 5 specifically explores the role of *Sox2* within the developing pituitary gland. In addition, the reasons underlying the invariable HH observed in patients with *SOX2* haploinsufficiency is investigated using this mouse model.
CHAPTER 2: MATERIALS AND METHODS
All chemicals were obtained from Sigma, unless stated otherwise.

2.1 MICE

2.1.1 Maintenance of colonies

All animal procedures were performed in accordance to the Animals (Scientific procedures) Act 1986 of the UK Government, and under guidelines issued by the Medical Research Council, UK ‘Responsibility in the use of animals for medical research’. All mice were housed in compliance with the Home Office Code of Practice. Mice were kept on a 12 hour light:dark cycle and fed ad libitum with a complete pelleted mouse diet and with constant access to water. C57Bl6 and CD1 mice used in this work were obtained from Harlan and Charles River.

2.1.2 Mouse Lines

2.1.2.1 Hesx1-null mouse line

The Hesx1<sup>-/-</sup> mouse line, in which the entire coding sequence of Hesx1 was removed by homologous recombination in ES cells, was maintained on a C57Bl6 background for more than 40 generations. As homozygous mice died perinatally, Hesx1<sup>-/-</sup> embryos were derived from heterozygote intercresses, as previously described (Dattani et al., 1998).

2.1.2.2 Hesx1-Cre mouse line

The Hesx1<sup>Cre/+</sup> was previously generated in our lab through replacing the Hesx1 coding region by a cassette containing a Cre recombinase gene (Andoniadou et al., 2007). Mice were maintained on a C57Bl6 background for more than 25 generations. Heterozygous mice were used for intercrossing and for generation of embryos.
2.1.2.3 *Hesx1-I26T* mouse line

Site directed mutagenesis was used to introduce the I26T mutation into a wild-type mouse *Hesx1* sequence, converting the triplet codon ATT at position 26 to ACC (Sajedi et al., 2008b). Mice for this work were maintained on a C57Bl6 background for at least 25 generations. Embryos and neonates used in this thesis were either derived from crosses between *Hesx1*\textsubscript{I26T/+} heterozygotes (*Hesx1*\textsubscript{I26T/I26T}), or between *Hesx1*\textsubscript{I26T} and *Hesx1*\textsuperscript{+/+} animals (*Hesx1*\textsubscript{I26T/-}).

2.1.2.4 *R26R\textsuperscript{YFP}* mouse line

*R26R\textsuperscript{YFP}* mice contained a *Yellow Fluorescent Protein* (YFP) gene inserted into the ubiquitously active *ROSA26* locus. Expression of YFP was inhibited through the presence of an upstream STOP polyadenylation sequence, which was flanked by two *loxP* sites (Srinivas et al., 2001). When bred to mice with a Cre recombinase gene under the control of the promoter of interest, such as the *Hesx1*\textsubscript{Cre/+} mouse, the STOP sequence upstream of the YFP gene is removed, and YFP expression is observed. *Hesx1*\textsubscript{Cre/+} mice were crossed with this reporter mouse to obtain *Hesx1*\textsubscript{Cre/+};*R26R\textsuperscript{YFP/+} mice and embryos, which were used in this thesis to lineage trace *Hesx1* cells (Andoniadou et al., 2007).

2.1.2.5 *Sox2-flox* conditional mouse line

*Sox2\textsubscript{fl/fl}* mice were kindly provided by Dr. L. Pevny (North Carolina, USA) (Taranova et al., 2006). This conditional allele contains the *Sox2* open reading frame flanked by two *loxP* sites. Upon Cre-mediated recombination, *Sox2* coding region is excised and a null allele is generated. *Sox2\textsubscript{fl/+}* were crossed with *Hesx1*\textsubscript{Cre/+} animals in order to generate *Hesx1*\textsubscript{Cre/+};*Sox2\textsubscript{fl/+} mice. These mice were used for mating with either *Sox2\textsubscript{fl/fl}* or *Sox2\textsubscript{fl/+}* animals.
2.1.3 **Collection of embryos and dissections**

Experimental litters were generated through timed matings, which involved the pairing of male and female mice overnight. The subsequent morning, females were separated from the males and were checked for vaginal plugs. The presence of a copulation plug was designated 0.5 dpc. Pregnant females were sacrificed at the relevant timepoint by cervical dislocation and embryos were dissected in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal calf serum (FCS) under a dissecting microscope (ZEISS, Semi SV6). Embryos were separated from the muscular wall of the uterus, Reichert’s membrane and visceral yolk sac, using fine forceps. For embryos older than 16.5 dpc, each embryo was cut at the level of the stomach and a hole was made at the hindbrain region, in order to aid penetration of the fixative. For bromodeoxyuridine [BrdU (Invitrogen)] labelling experiments, each pregnant female was weighed and injected intraperitoneally with 0.1 mg/g body weight BrdU at the relevant timepoint prior to collecting the embryos.

Embryos were briefly washed in phosphate-buffered saline (1x PBS) and fixed in cold, fresh 4% paraformaldehyde (PFA) in 1x PBS at 4°C overnight. For 18.5 dpc and older embryos, tissue was incubated for an additional day in fresh PFA. Subsequent to fixation, embryos were dehydrated to 70% ethanol in double distilled water [ddH2O (MilliQ)] through a graded ethanol series at room temperature (1x PBS washes twice, 25% ethanol, 50% ethanol and 70% ethanol, diluted in ddH2O) where they were stored at 4°C for subsequent paraffin embedding. Timings of each change of ethanol varied according to the size of the embryo and thus were determined by the developmental stage of the embryo. As a general guide, 9.5-10.5 dpc followed 25 minute incubations, 11.5-13.5 dpc, 45 minute incubations and 14.5-18.5 dpc, 1 hour incubations.

2.1.3.1 **MRI analysis of embryos**

For MRI analysis of 18.5 dpc embryos, embryos were harvested and placed immediately into pre-warmed 37°C Hanks (GIBCO)/5 mM ethylenediaminetetraacetic acid (EDTA). Each embryo was cut at the level of the stomach and washed for 15-20 minutes in
warm media to remove any blood remnants. A piece from the left forelimb was taken for
genotyping as described below. Embryos were then fixed for approximately three weeks in cold
4% PFA with the addition of 8 mM Magnevist (Schering, UK), a contrast agent used for
enhancement of the MRI image. Embryos used for analysis were embedded in 1% low melting
agarose in 1x PBS, again with the addition of 8 mM Magnevist. Subsequent imaging was
conducted by the UCL Centre for Advanced Bioimaging, UK (Cleary et al., 2011).

2.1.4 Paraffin embedding and sectioning of embryos

Embryos for paraffin embedding (stored in 70% ethanol in ddH2O at 4°C) were further
dehydrated to 100% ethanol (80% ethanol, 90% ethanol, 95% ethanol in ddH2O) at room
temperature. Duration of ethanol incubations were depicted through the size of the embryo, as
described previously. Embryos were left overnight in 100% ethanol at 4°C on a roller to ensure
complete dehydration. The following morning, embryos were once again washed for an hour in
100% ethanol. For embryos older than 16.5 dpc, the skin was removed to allow complete
penetration of clearing solutions and wax.

Both the clearing agent used for embedding, as well as the timing of washes, varied
based on the size and density of the tissue. For embryos between 9.5 and 11.5 dpc, a 20 minute
incubation of HistoClear (National Diagnostics) was performed at room temperature, followed
by an additional 20 minute incubation at 60°C. Embryos were then washed at least 4 times in
wax at 60°C before orientation. For older embryos, 2 changes of xylene (VWR International)
were performed at room temperature (12.5-13.5 dpc, 25 minutes; 14.5-15.5 dpc, 30 minutes;
17.5-18.5 dpc, 45 minutes). Subsequent to xylene washes, embryos were placed in xylene:wax
(1:1) at 60°C for 35 minutes. This was followed by at least 4 wax 1 hour washes before
orientation. In both cases, during the final wax wash, embryos were placed in plastic moulds
with warm wax and orientated using a heated needle and a microscope. Embryos were left
overnight to set at room temperature.

Sagittal, coronal and transverse sections (7-8 μm) were cut using a rotary microtome.
Serial sections were prepared in a series of 4 for embryos younger than 12.5 dpc and a series of
7 for older embryos. Wax sections were mounted onto superfrost slides by floating the sections on ddH$_2$O placed on the slides. Slides were then gently heated on a heat block to 40°C allowing the wax to flatten. Excess water was then removed carefully and mounted wax slides were allowed to dry completely overnight at 37°C. Slides were stored at room temperature until required for further analysis.

2.1.5 Haematoxylin and eosin staining

Paraffin sections were de-waxed and rehydrated by placing slides into HistoClear for 10 minutes and 100%, 75%, 50%, 25% ethanol in ddH$_2$O and ddH$_2$O for 2 minutes each. Slides were stained with haematoxylin for roughly 5 minutes in order to visualise the nuclei structures. Once the desired stain was achieved, slides were placed under running water for 5 minutes. Slides were then counter-stained with eosin for 2 minutes to visualise cytoplasmic structures. Slides were again rinsed in running water for 2 minutes, before dehydrating through the same ethanol series, cleared with HistoClear for 10 minutes and mounting with VectaMount (Vector Laboratories).

2.1.6 Microscopy, Imaging and Statistical Analyses

All fluorescent images were visualised on a Ziess Axiovert 135 microscope, and images were captured using a Hamamatsu ORCA-ER digital camera attached to Leica software. Brightfield light images were visualised on a Ziess Avioplan 2 microscope and captured using a Ziess AxioCam HRc camera connected to Axiovision 4 software. All images presented in this thesis were processed using Adobe Photoshop CS3 software. Statistical analyses were performed using SigmaStat v3.5 software. All graphs were compiled using GraphPad Prism 4.

2.1.6.1 Cell count analysis

Slides containing mid-sagittal sections from 12.5 or 14.5 dpc wild-type or mutant embryos were stained and imaged as described. All images taken for analysis were photographed at x20 magnification. Cells that were solidly stained in nature were counted as
positive. 4',6-diamidino-2-phenylindole (DAPI)-counterstained cells were counted to obtain a total cell count for the 7 µm thick pituitary section. For each embryo at 12.5 dpc, 2-3 sections were examined, and at 14.5 dpc, 5-6 sections were analysed, in triplicate for each genotype. The proportion of immunoreactive cells was compared with the total number of DAPI-positive cells within the pituitary. The mitotic index was then determined and analysed for statistical significance. For BrdU stained sections, PITX1 immunoreactive cells were quantified to give a total number of pituitary specific cells, as DAPI staining was less effective following BrdU labelling protocol.

2.2 DNA METHODS

2.2.1 Genotyping mice and embryos

Genomic DNA from mice was either extracted from tail or ear biopsies approximately one week prior to weaning. Embryos were genotyped from a piece either taken at time of storage in 70% ethanol, or at time of harvest.

Each sample was digested with 20 µl of 0.2% DNAreleasy™ (Anachem) in ddH₂O according to the manufacturer’s instructions. Following lysis, samples were vortexed thoroughly and pulse-spun before use in genotyping.

2.2.2 Standard PCR for genotyping of embryos and mice

Genotyping of embryos and neonates was carried out by polymerase chain reaction (PCR) amplification on DNA samples purified as described below using specific primers; Table 2.2 contains a list of primers and PCR conditions used for genotype analyses.

For standard diagnostic PCR reactions, DNA sequences were amplified using Taq polymerase (Bioline). Reaction mixtures were generally made up to 25 µl total volume, containing 200 µM deoxynucleotide triphosphates [dNTPs; (Roche)], 1.5 µM magnesium chloride [MgCl₂ (Bioline)], 0.5 µM of forward and reverse primers, and 1 U of polymerase
enzyme in either 1x Taq polymerase buffer or JD buffer (3x JD Buffer: 60 mM K-glutamate, 24 mM Hepes, 90 mM Tris, 15 mM MgCl$_2$, 6 mM DTT, 180 mM NH$_4$Ac, 3% DMSO, 24% glycerol). JD buffer contains DMSO and helps to reduce any non-specific background. As MgCl$_2$ is also included in this buffer, additional MgCl$_2$ did not need to be specifically included in the reaction mixture (Table 2.1). General primer stock solutions were prepared to 100 µM in Molecular Grade Water. Working stocks were either prepared to 25 µM or 10 µM for further use. dNTPs working stocks were made to 25 µM. For every PCR reaction, three control samples were included; a negative control including the lysis buffer without any biopsy extract (T-), an additional negative control containing only the PCR reaction mixture (C-), and a positive control containing 1 µl of template DNA to ensure the quality of the reagents (C+). Reaction mixtures were either scaled up or down according to the final application.

Two general strategies were employed for the PCR reaction; cycling reactions with and without the incorporation of the ‘hot-start’ protocol. The hot-start method was generally used to increase the specificity of the reaction, and thus reduce any potential background. The majority of genotyping performed in this thesis incorporated the hot-start protocol. For reactions without hot-start, all reagents, apart from the DNA template, were pooled together, and then aliquots were divided evenly between the numbers of samples. 1 µl of DNA template was added to each reaction mix, with care taken not to contaminate the samples.

For the hot-start protocol, the PCR components were divided into two pools; one for the DNA samples, and another for the enzyme (Table 2.1). For this method, components of the DNA mixture were added to each sample tube, followed by a drop of mineral oil. 1 µl of template DNA was carefully added to each tube and samples were spun briefly to ensure mixture of both the template DNA and DNA reaction mix. Samples were then placed in the PCR machine, with each lid of the tube remaining open and the specific PCR program initiated. Following the initial denaturing step of 2 minutes at 94°C, the samples were held at 85°C, whilst the enzyme mixture was carefully added to the bottom of each tube. Sample tubes were closed and the PCR program resumed.
Table 2.1 - Hot-Start PCR reaction mix

<table>
<thead>
<tr>
<th>Component</th>
<th>DNA Mix (µl)</th>
<th>Enzyme Mix (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Taq Buffer</strong></td>
<td><strong>JD Buffer</strong></td>
</tr>
<tr>
<td>H₂O</td>
<td>7.08</td>
<td>1.7</td>
</tr>
<tr>
<td>Taq Pol Buffer (x10)</td>
<td>1.2</td>
<td>-</td>
</tr>
<tr>
<td>MgCl₂ (25 mM)</td>
<td>0.72</td>
<td>-</td>
</tr>
<tr>
<td>JD Buffer (x3)</td>
<td>-</td>
<td>2.5</td>
</tr>
<tr>
<td>Primer 1</td>
<td>1 (of 25 µM)</td>
<td>0.5 (of 10 µM)</td>
</tr>
<tr>
<td>Primer 2</td>
<td>1 (of 25 µM)</td>
<td>0.5 (of 10 µM)</td>
</tr>
<tr>
<td>dNTPs (25 mM)</td>
<td>-</td>
<td>0.3</td>
</tr>
<tr>
<td>Taq Pol (5U/µl)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The cycling reaction involved an initial denaturation step at 94°C for 2 minutes; followed by 30-35 amplification cycles each comprising of a denaturation step at 94°C for 30 seconds, a primer annealing step and a primer elongation step at 72°C for 1 minute. This was then followed by an extended elongation step at 72°C for 5 minutes to ensure that all PCR amplified products were at full length. These conditions were optimised according to the specific amplification and application. Total PCR products were resolved on an agarose gel, as described in 2.2.5.
<table>
<thead>
<tr>
<th>Mouse line</th>
<th>Primer sequences</th>
<th>Annealing temperature</th>
<th>Conditions</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hesx1-null</td>
<td>OL1A: cccagatctccccagtgagacttc</td>
<td>58°C for 30 seconds;</td>
<td>JD Buffer;</td>
<td>WT: 590 bp</td>
</tr>
<tr>
<td></td>
<td>OL2A: gattctgtctctctctaagtttagc</td>
<td>30 cycles</td>
<td>Hot start</td>
<td>Mutant: 650 bp</td>
</tr>
<tr>
<td></td>
<td>3'KO: cacctgtgaagggtttcaaga</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PGK2: accaatattaaggccagctc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hesx1-Cre</td>
<td>Fwd: ggagacaattctttttgaaac</td>
<td>58°C for 30 seconds;</td>
<td>JD Buffer;</td>
<td>WT: 500 bp</td>
</tr>
<tr>
<td></td>
<td>Rev: ccagagtctctggtctctctcac</td>
<td>30 cycles</td>
<td>Hot start</td>
<td>Mutant: 300 bp</td>
</tr>
<tr>
<td>Hesx1-I26T</td>
<td>Fwd: tgaagtctcactgggaagatctgg</td>
<td>58°C for 30 seconds;</td>
<td>JD Buffer;</td>
<td>WT: 590 bp</td>
</tr>
<tr>
<td></td>
<td>Rev: acagacacctgtgcaacttcag</td>
<td>30 cycles</td>
<td>Hot start</td>
<td>Mutant: 650 bp</td>
</tr>
<tr>
<td>R26R</td>
<td>R1: aagtctctcagtttgttat</td>
<td>60°C for 1 minute;</td>
<td>Taq Poly. Buffer;</td>
<td>WT: 500 bp</td>
</tr>
<tr>
<td></td>
<td>R2: gcgaagcatttcctcaacc</td>
<td>35 cycles</td>
<td>Hot Start</td>
<td>Mutant: 250 bp</td>
</tr>
<tr>
<td></td>
<td>R3: ggagcggagaatggatag</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YFP</td>
<td>Fwd: tgaaccgcatcagctgaagg</td>
<td>65°C for 30 seconds;</td>
<td>Taq Poly. Buffer</td>
<td>Single band: 350 bp</td>
</tr>
<tr>
<td></td>
<td>Rev: tccagcagcactgtgctgatgc</td>
<td>35 cycles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sox2 flox</td>
<td>Fwd: cagagactcgttctggaaga</td>
<td>57°C for 30 seconds;</td>
<td>Taq Poly. Buffer</td>
<td>WT: 307 bp</td>
</tr>
<tr>
<td></td>
<td>Rev: tcttgatacataagggtgatag</td>
<td>35 cycles</td>
<td></td>
<td>Flox: 345 bp</td>
</tr>
</tbody>
</table>
2.2.3 PCR site directed mutagenesis

2.2.3.1 Primer design

Mutations were introduced into wild-type HESX1 constructs by PCR site directed mutagenesis using specific primers (Figure 2.1). To incorporate the single base-change mutations, reverse primers were designed to contain 28-32 bp in length, incorporating the specific base-pair mutation approximately half-way through the primer sequence. Forward primers used consisted of 19 bp nucleotide sequence, consisting of the single base change mutation towards the beginning of the primer sequence, usually within the first three nucleotides. A forward and reverse primer sequence was also designed to amplify the full length human HESX1 sequence harbouring the individual mutations. This forward primer (hHESX1-fwd) was designed to include an EcoRI restriction enzyme sequence immediately upstream to the hHESX1 ATG start site, whereas the reverse primer (hHESX1-rev) contained a SalI restriction enzyme site immediately downstream to the TAG stop sequence.
Figure 2.1 - Primers used for the site directed mutagenesis

Schematic shows the open reading frame of human HESX1. Mutations (highlighted in yellow) are introduced to the wild-type HESX1 sequence through site directed mutagenesis using the primers sets denoted in the figure. For each mutation, reverse primers are designed to incorporate the single base change approximately halfway through the primer sequence. In contrast, the forward primer for each mutation is designed to contain the single base change towards the beginning of the primer sequence. Forward and reverse primers are also designed to generate the full length sequence of HESX1. The forward primer contains the EcoRI site (underlined), whereas hHESX1-rev primer contains the SalI sequence (underlined) to facilitate cloning into the vector (Section 2.2.7).
2.2.3.2 PCR site directed mutagenesis

To generate complementary DNA (cDNA) for subcloning and expression studies, template DNA was amplified using *Pfu* DNA polymerase (Stratagene), due to its high proof-reading activity.

To produce mutant fragments, the PCR reaction was conducted according to the ‘hot-start’ protocol, as described in Section 2.2.2. The final reaction mix consisted of 1x *Pfu* buffer, 200 µM dNTPs, 1.25 µM forward and reverse primers, and 1 µl (2.5 Units/µl) of *Pfu* polymerase in a total reaction volume of 50 µl. Approximately 3 ng of DNA was used as a template. The cycling reaction involved an initial denaturation step at 94°C for 1 minute; followed by 30 amplification cycles each comprising of a denaturisation step at 94°C for 30 seconds, a primer annealing step at 55°C for 30 seconds and a primer elongation step at 72°C for 1 minute. This was then followed by an extended elongation step at 72°C for 5 minutes. Total PCR products were resolved on an agarose gel and purified by gel extraction as described in Sections 2.2.5 and 2.2.6.2. Estimation of total purified DNA samples was determined through comparison to hyperladder standards (Bioline).

To generate full length sequences incorporating each mutation, *Pfu* reaction mixture was made as outlined. Template DNA, however, comprised of a mixture of newly synthesised beginning and end mutant fragments. Specific amounts of each DNA templates were determined through calculating equal ratio of molecules. The cycling reaction was performed as specified above, but the elongation step within the amplification cycles was increased to 90 seconds to favour amplification of the full length sequence. Total PCR products were again resolved by gel electrophoresis and purified by gel extraction.

2.2.4 Restriction enzyme digestion of DNA

Restriction digestion of DNA was performed in accordance to the specific manufacturer’s conditions (Promega or Roche). Final volume of the digestion varied according
to the specific purpose of the digest. For digestions to obtain large amounts of linearised plasmid for in vitro transcription of RNA probes, the total volume was scaled to 100 µl, whereas for diagnostic purposes, a final volume of 20 µl was sufficient. Typically, restriction digests consisted of 1x relevant restriction enzyme buffer, proportional quantities of the restriction enzyme according to the amount of DNA being cut, in ddH$_2$O. The combined reagents were mixed thoroughly and incubated at the appropriate temperature (as denoted by the manufacturer’s instructions), usually for 2 hours. Digestions either to check for the presence of a cloned insert in a recombinant plasmid, or for the orientation of an insert were usually performed for shorter incubation times as complete digestion of the plasmid was often not required.

If two or more enzymes were required for digestion which were not compatible for simultaneous digestion, digests were performed sequentially. After the initial digestion, the primary enzyme was heat inactivated, usually by incubation at 65°C for 10 minutes, followed by incubation with the second enzyme. For this method, the final volume was scaled up to ensure that neither the enzymes nor buffers exceeded 10% of the total volume.

2.2.5 Agarose gel electrophoresis

DNA fragments obtained from PCR reactions and DNA digests were separated by horizontal agarose gel electrophoresis. Gels were prepared through dissolving high quality agarose in 1x Tris-acetate-EDTA (40 mM Tris-acetate, 1 mM EDTA pH 8.0; TAE) buffer, aided by microwaving. Once at room temperature, 2.5 µl of Ethidium bromide (10 mg/ml stock solution) was added to 100 ml of gel and the agarose was left to solidify further in casting trays. The specific percentage of the agarose gel was determined by the size of the DNA samples and resolution required to visualise the DNA products. For high resolution of larger DNA fragments, lower percentage gels (1% w/v) were prepared, whereas 2-3% agarose gels were sufficient to accurately separate DNA products between 200 – 500 bps in size. 1x loading buffer (50% v/v glycerol:50% v/v 1x TAE, 0.1% Orange G) was added to each DNA samples prior to
loading. Product sizes were determined through running a DNA ladder [usually 1 kb HyperLadder1 (Bioline)] alongside the samples. Following electrophoresis at 120V for time periods between 30 minutes to 2 hours, DNA gels were visualised and photographed under ultraviolet (UV) light using a trans-illuminator.

2.2.6 Purification of DNA

2.2.6.1 Purification of DNA fragments using spin columns

QIAquick PCR Purification Kits (Qiagen) were used to purify both single and double-stranded DNA samples [100 bp – 10 kilobase (kb)] derived from amplification reactions and DNA clean-up from other enzymatic reactions. The propriety names for all buffers obtained from these kits are used herein. For purification, 5 volumes of QIagen binding buffer PB was added to each sample and thoroughly mixed, allowing for the efficient binding of DNA as well as the concomitant removal of primers up to 40 nucleotides in length. QIAquick spin columns, which contain a specialised silica membrane to purify DNA fragments from both aqueous and agarose membranes, were placed into 2 ml collection tubes and entire samples were directly loaded onto the membrane and spun at 13,000 rpm for 1 minute to bind the DNA to the membrane. For sample volumes greater than 800 µl, columns were loaded, spun, and reloaded again with remaining sample. Eluate from each spin was discarded and the columns replaced to the collection tubes. For samples that would be subsequently used for sequencing and in vitro transcription, 500 µl of buffer QC was added to the membrane, spun at 13,000 rpm for 1 minute and the flow-through discarded. Membranes were then washed by the addition of 750 µl of QIAGEN wash buffer PE, spun at high speed and eluate discarded. Columns were once again spun at high speed for 1 minute to remove any residual wash buffer. Columns were then transferred to new clean 1.5 ml microcentrifuge tubes. To elute the DNA, 40 µl of pre-warmed buffer EB was added to the membrane, left to stand for a minute to increase final DNA yield, and then spun for 1 minute a high speed.
2.2.6.2 Purification of DNA fragments from agarose gels

DNA PCR products, ranging between 70 bp and 10 kb, were excised from 1% low melting point agarose gels using the Qiagen® Gel Extraction Kit according to the manufacturer’s instructions. Bands were precisely excised from gels using a sharp scalpel under UV light, with care taken not to contaminate samples if more than one product was run on a single gel, as well as ensuring that as much agarose was removed from the sample. The weight of the sample was then determined and 3 volumes of solubilisation and binding buffer QG were added to 1 volume gel. Samples were incubated at 50°C with intermittent shaking for approximately 10 minutes, or until the agarose had fully dissolved, before proceeding further. Once the gel had completely dissolved, the colour of the sample mix was determined; if samples ranged between orange or violet, 10 µl of 3 M sodium acetate, pH 5.0 was added and mixed, until the sample returned to yellow in appearance. 1 gel volume of isopropanol was then added to the sample and mixed. Entire samples were directly loaded onto QIAquick Spin Columns and DNA fragments were then purified from agarose components using the QIAquick Spin protocol (Qiagen), as described above (Section 2.2.6.1).

2.2.7 Cloning

2.2.7.1 Preparation of Vector and Inserts

Approximately 1.5 µg of high quality DNA plasmid was digested with the appropriate enzyme for 2-3 hours in a total volume of 50 µl (Section 2.2.4). Following digestion, 2 µl of reaction was analysed by agarose gel electrophoresis to ensure complete digestion of the plasmid. The linearised vector was then treated with 1 µl (20 Units) of alkaline phosphatase (Roche) for 1 hour at 37°C. As DNA possesses a phosphate group on the 5’ end, treatment with an alkaline phosphatase prevents the self-ligation of the DNA plasmid. Following digestion and alkaline phosphatase treatment, enzyme and buffer components were removed from the linearised vector
sample by using the QIAquick PCR Purification protocol (Section 2.2.6.1). Vector was either used immediately, or stored at -20°C for future use.

cDNA inserts were prepared using two methods dependent on whether they were derived from PCR amplification, or from a previously cloned product. For inserts obtained from PCR amplification, approximately 25% of PCR product was further digested with the appropriate enzymes, however, the incubation time was increased to 4 hours to ensure complete and clean digestion. Enzyme and buffer reagents were removed from the cDNA using the QIAquick PCR Purification kit. For subcloning of inserts, plasmids were digested with the appropriate enzymes for 2 hours and then purified using the QIAGEN gel extraction kit (Section 2.2.6.2).

For the mutational analysis, human HESX1 inserts (Section 2.2.3) were cloned into the pM vector [Figure 2.2; (Clontech)], which is used to generate a fusion of the GAL4 DNA binding domain (BD), or into the HA-pcDNA3 [Figure 2.3; (Invitrogen)], allowing for HA-tagged constructs.
Figure 2.2 - pM vector used to generate GAL4 DNA binding domain fusion proteins used for the mammalian one hybrid assays
Figure 2.3 - pcDNA3.1 vector used to generate HA-tagged constructs
2.2.7.2 Ligation

2 µl of prepared vector and inserts were electrophoresed on a 1% agarose gel and DNA quantities estimated by comparison with a DNA standard (HyperLadder I). The ligation reaction was composed in a final volume of 15 µl using approximately 50 ng of vector and the appropriate mass of insert DNA to give a molar ratio of 3:1 insert to vector. Three units of T4 DNA Ligase (1 µl) and 1.5 µl of 10x T4 ligase reaction buffer (Roche) were added, components vortexed and incubated at room temperature for 2 hours. In parallel, a reaction control containing vector alone, T4 DNA ligase and 1x T4 ligase reaction buffer was included, to determine the numbers of colonies obtained following transformation by self-ligation of the plasmid, either due to insufficient alkaline phosphatase treatment, or insufficient restriction digestion.

Following ligation, 5 µl of reaction from each sample, including the vector alone, was transformed into competent E. coli cells by heat-shock (Section 2.2.8). Success of the experiment was determined by the ratio of colonies on the experimental plates compared with the number of colonies on the control plate (vector alone); ideally the vector alone plate contained little or no colonies, whereas the experimental plates contained considerably more. If deemed successful, a number of recombinant colonies were selected, DNA isolated using the QIAprep Miniprep Kit (Section 2.2.9.1), and positive clones were determined by restriction enzyme digestion. Positive clones were further cultured for high quality DNA isolation using the QIAfilter Plasmid Maxi Kit (Section 2.2.9.2).

2.2.8 Transformation of foreign DNA into bacterial cells by heat-shock

Transformation of plasmid DNA into bacterial cells was achieved by heat-shock. E. coli strains used in this thesis were selected by their acquired resistance to either ampicillin or kanamycin.
A 50 µl aliquot of DH5α cells were briefly thawed on ice. 50 ng of plasmid DNA was added to each tube, swirled gently to ensure sufficient mixture of the DNA, and incubated on ice for 45 minutes. Cells were heat-shocked through incubation at 37°C for 5 minutes, immediately followed by incubation on ice for 2 minutes. 500 µl of L-Broth was added to each vial and the bacterial samples were further incubated at 37°C for 45 minutes to allow for the expression of the antibiotic resistant proteins. Bacterial cells were pelleted through a centrifugation of 2,000 rpm for 5 minutes. Generally, cell pellets were re-suspended in 200 µl of L-Broth and plated onto pre-warmed agar plates containing either 50 µg/ml of ampicillin or kanamycin. Plates were inverted and incubated overnight at 37°C.

2.2.9 Plasmid DNA isolation

2.2.9.1 Small scale isolation of plasmid DNA: mini prep

Small scale isolation of plasmid DNA was achieved through using the QIAprep Miniprep Kit according to the manufacturer’s guidelines. The propriety names for all buffers obtained from these kits are used herein. This protocol allowed for the quick and convenient processing of numerous samples within a short time frame. According to this protocol, a 1.5 ml overnight culture can yield between 5 to 15 µg of plasmid DNA. A single colony from a freshly streaked selective plate was inoculated in 3 ml of L-Broth supplemented with the appropriate selection antibiotic (50 µg/ml) overnight at 37°C with vigorous shaking. 1.5 ml of culture was then transferred to a 1.5 ml microcentrifuge tube and pelleted through a centrifugation step of 8,000 rpm for 3 minutes. Pelleted bacterial cells were subsequently re-suspended in 250 µl of chilled buffer P1. 250 µl of lysis buffer P2 was added to each sample and tubes were inverted gently 4-6 times to ensure sufficient mixture of the two solutions. 350 µl of neutralisation buffer P3 was added to each tube and mixed immediately, and precipitated by centrifugation at full speed for 10 minutes. The supernatant containing the DNA was carefully removed and
transferred to a QIAprep Spin Column. Plasmid DNA was then purified as previously described (Section 2.2.6.1).

2.2.9.2 Large scale isolation of plasmid DNA: maxi prep

Large scale (usually between 100 to 500 µg) and high quality DNA, for use in transfections and *in vitro* transcriptions, was purified using the QIAfilter Plasmid Maxi Kit (QIAGEN), according to the manufacturer’s instructions.

A single colony from a freshly streaked plate was selected and grown overnight in 100 ml of L-Broth with the appropriate selection antibiotic at 37°C with vigorous shaking. The subsequent day, the entire bacterial cell culture was harvested by centrifugation at >6000 g for 30 minutes at 4°C, and the supernatant discarded. The bacterial pellet was then re-suspended in 10 ml of chilled buffer P1. Bacterial cells were then lysed by the addition of 10 ml of buffer P2 lysis buffer, followed by 4 gentle inversions to ensure thorough mixing, before incubation for 5 minutes at room temperature. During this incubation, QIAfilter cartridges were prepared by screwing the caps onto the outlet nozzle. Following incubation, 10 ml of chilled buffer P3 was added to the lysate, mixed immediately through vigorous inversion, and transferred to the prepared QIAfilters. Samples were incubated for 10 minutes on ice. Equilibration of QIAGEN-tip 500 was achieved through the addition of 10 ml of solution QBT to the columns and allowing the solution to empty by gravity flow. Following incubation of the lysate and equilibration of the QIAGEN-tip 500, the cap was carefully removed from the QIAfilter Maxi cartridge, and the cell lysates were filtered through the QIAfilter directly into the QIAGEN-tip 500. The cleared lysate was allowed to enter the tip by gravity. Columns were subsequently washed twice by the flow-through of 30 ml of buffer QC. The columns were then transferred to new, clean 50 ml tubes and the DNA was eluted with 15 ml of buffer QF. DNA was precipitated by the addition of 10.5 ml (0.7 volumes) of room temperature isopropanol, which was mixed and centrifuged immediately at ≥15,000 g for 30 minutes at 4°C. The DNA pellet washed with
10 ml of room temperature 70% ethanol, air-dried for 5-10 minutes and re-dissolved in 1 ml of pre-warmed buffer EB.

2.2.10 DNA quantification

1.2 µl of DNA sample was loaded onto a NanoDrop 1000 Spectrophotometer which allowed the quantification of nucleic acid concentration as well as providing the 260/280 values, to determine the integrity and purity of the samples.

2.2.11 DNA sequencing

All DNA clones generated for further in vitro studies were sequence analysed to confirm that no additional mutations were introduced during the PCR amplification step, as well as to ensure the inserts were in-frame. Plasmids, diluted to a 100 ng/µl in a final volume of 10 µl, were sent for sequence analysis to Source Bioscience LifeSciences, along with 3 pmol/µl specific primers (Figure 2.1). This allowed for the complete reading of both the whole insert, as well as areas both upstream and downstream. Sequence analysis was performed using the Sequencher 4.6 software, which allowed for both the visualisation of the electropherogram, as well as giving the sequence in a FASTA format. Sequences were manually checked against the wild-type HESX1 open reading frame (Figure 2.1).

2.3 RNA METHODS

General laboratory practice whilst working with ribonucleic acid (RNA) was to wear gloves and clean protective outer-coats at all times and clean (RNA free) equipment was used. All solutions were treated with diethylpyrocarbonate (DEPC), a potent inhibitor of ribonucleases, to prevent degradation of cellular or newly synthesised RNA. DEPC water was prepared through the addition of 0.1% v/v DEPC in ddH₂O, which was shaken vigorously, left ajar overnight at room temperature, and autoclaved prior to use. Filtered RNAsé-free pipette tips
were used throughout. Glassware and metal racks were rinsed with MilliQ water and baked at 280°C for 8 hours. All reagents were freshly prepared and were used only for RNase-free work.

2.3.1 Riboprobe synthesis and preparation

Up to 10 µg of vector was linearised in a reaction volume of 100 µl with the appropriate enzyme. Upon digestion, the linearised plasmid was purified using the QIAquick PCR Purification kit (Qiagen) according to the manufacturer’s instructions. Purified product was re-suspended in 40 µl of EB buffer and used for in vitro probe transcription.

For in vitro probe transcription, 1 µg of purified linearised DNA template was incubated with 0.5 µl RNAse Inhibitor (Roche), 2 µl Transcription Buffer (Roche), 2 µl 10x Digoxigenin (DIG) labelling mix (Roche), and 1 µl of either T3/T7/Sp6 (Roche) in a total volume of 20 µl, for 2 hours at the appropriate temperature (37°C for T3 and T7; 42°C for Sp6). 1 µl of the reaction was analysed by gel electrophoresis to ensure efficient labelling of probe, and the remainder of reaction was cleaned using CHROMA SPIN™-100 Columns (Clontech) according to the manufacturer’s instructions. Cleaned probe was eluted in 50 µl of DEPC-treated ddH₂O and 50 µl of formamide was added to the reaction for long-term storage at -20°C. 5 µl of final probe was once again analysed by gel electrophoresis to ensure no degradation of probe during column purification. Probes used in this thesis have been previously been described: Ot, Ss, Ghrh, Avp, Otp, Sim1 (Acampora et al., 1999); Nkx2.1, (Dattani et al., 1998); Lhx3, Prop1, Pomc1, (Simmons et al., 1990); Prl, (Sajedi et al., 2008b); Bmp4, (Furuta et al., 1997); Fgf8, (Crossley and Martin, 1995; Martinez-Barbera et al., 2000); Fgf10, (Min et al., 1998); NeuroD1, (Lee et al., 1995); Sf1, (Barnhart and Mellon, 1994).

2.3.2 Section in situ hybridisation

All solutions used prior to hybridisation were made using DEPC-treated ddH₂O. Slide sections were de-waxed in HistoClear for 10 minutes and rehydrated through a graded ethanol series for 2 minutes (100%, 75%, 50%, 25%, 1x PBS, all diluted in DEPC-treated ddH₂O),
before fixation in 4% PFA for 20 minutes. Slides were then treated with Proteinase K (20 µg/ml in 1x PBS-DPEC treated ddH₂O) for 8 minutes and re-fixed in 4% PFA for 5 minutes. Slides were incubated in 0.1 M triethaloamine with 0.25% acetic anhydride for 10 minutes at room temperature whilst stirring. Slides were then dehydrated using the same ethanol series and hybridised on the same day. Hybridisation was carried out overnight at 65°C in 50% formamide, 0.3 M sodium chloride, 20 mM tris-hydrochloric acid (Tris-HCl), 5 mM EDTA, 10% Dextran sulphate, 1x Denhardt’s reagent supplemented with 1 µl/ml RNase Inhibitor (Roche), 0.5 mg/ml tRNA and the relevant DIG-labelled riboprobe at a 1:100 dilution. Following overnight incubation, slides were subjected to stringency washes consisting of 2x saline-sodium-citrate (SSC) (twice for 20 minutes), 1:1 formamide:2x SSC (twice for 20 minutes), 2x SSC (twice for 30 minutes) and 0.2x SSC (twice for 30 minutes) at hybridisation temperature. Slides were then briefly washed in buffer 1 (0.1 M Tris-HCl pH 7.5, 0.15 M sodium chloride) before an hour incubation with blocking solution (10% FCS in buffer 1). Slides were then incubated in a humid chamber overnight at 4°C with sheep anti-DIG antibody conjugated with alkaline phosphatase [1:1000 (Roche)] in 2% FCS in buffer 1. Subsequent to overnight incubation, slides were washed with buffer 1 thrice for 5 minutes, and then twice with 1x buffer 2 (0.1 M Tris-HCl pH 9.5; 0.1 M sodium chloride; 0.05 M magnesium chloride) for 5 minutes each. Staining of the slides was carried out through incubation with nitro-blue tetrazolium chloride (NBT; 4.5 µl/ml)/5-bromo-4-chloro-3-indolyl phosphate (BCIP; 3.5 µl/ml) reagents (Roche) in 1:1 ratio of 2x Buffer 2 and 10% polyvinyl alcohol (PVA) in a light-sensitive manner. Once sufficient staining was achieved, the reaction was stopped through continuous washing with running tap water for 10 minutes and then incubation at 50°C for 15 minutes to loosen any residual PVA. Slides were then dehydrated through a graded ethanol series for 2 minutes each, incubated in HistoClear for 10 minutes and then mounted with VectaMount.
2.4 PROTEIN METHODS

2.4.1 Protein quantification

Protein concentrations were determined using the BradfordUltra™ reagent (Expedeon), which provides a quick and convenient colorimetric method for determining total protein concentrations within a given sample. A standard curve was generated through preparing a series of dilutions of bovine serum albumin (BSA) in the specific lysis buffer used to extract the protein samples. 300 µl of BradfordUltra reagent was added to 20 µl of standards, blanks (lysis buffer alone) and samples in a microtiter plate, and the absorbance read at optical density (OD)\textsubscript{595}. All measurements were performed in duplicate. An average measurement of 595 nm readings from the blank samples was subtracted from the average measurements of the standard and samples, and total protein concentrations were calculated accordingly.

2.4.2 In vitro translation

\textit{In vitro} translation of mutant cDNA was performed using the TNT® Quick Couple Transcription/Translation kit (Promega) as recommended by the manufacturer. HA-tagged wild-type and mutant inserts (cloned into the pcDNA3.1 vector) were \textit{in vitro} transcribed using this system based upon the presence of an upstream T7 promoter. 1 µg of wild-type and mutant plasmid templates were individually mixed with 1 µl Methionine (1 mM, Promega) and 20 µl of TNT® Quick Master Mix in a total volume of 25 µl. Samples were thoroughly mixed, pulse spun to recover all components, and incubated at 30°C for 90 minutes. Samples were immediately stored at -20°C for future use. 10 µl of lysate was resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

2.4.3 Western blot analysis

Approximately 3 x 10\textsuperscript{5} of 293T cells were seeded in 6-well tissue culture wells, a day prior to transfection. Between 1-2 µg of \textit{HA-HESX1} expression constructs were transfected into
293T cells using a 3:1 dilution of FuGENE® 6 (Section 2.5.4). Approximately 48 hours post-transfection, cells were placed on ice and rinsed carefully with ice cold 1x PBS. Cells were then vigorously scraped using the end of a p1000 pipette tip to detach the monolayer of cells and harvested into pre-chilled microcentrifuge tubes. Cells were pelleted through centrifugation at 4,000 rpm for 3 minutes at 4°C. Supernatant was discarded and cell pellets were lysed in 100 µl lysis buffer [20 mM Tris pH 8.0, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, supplemented with protein inhibitor cocktail (Roche)] for 30 minutes rotating at 4°C. Protein extracts were separated from cell debris by centrifugation at 15,000 g for 10 minutes at 4°C and quantified by performing a Bradford assay, as outlined in 2.4.1. A total of 25 µg of protein extracts were resolved by SDS-PAGE.

2.4.3.1 SDS-Page electrophoresis

SDS-PAGE is a method implored to resolve protein extract according to size. Plates, customised with either 0.75 mm, 1 mm or 1.5 mm spacers, were thoroughly cleaned with detergent prior to use, rinsed with ddH₂O and wiped dry with 70% IMS. Plates were assembled according to Bio-Rad instructions. Care was taken to ensure a good seal was achieved. 10 ml of resolving gel was prepared according to Table 2.3. Tetramethylethylenediamine (TEMED) and ammonium persulphate (APS) were added immediately prior to pouring. Once all reagents were added, the solution was vortexed thoroughly and poured in a single effort between the plates, until the meniscus reached approximately 2.5 cm from the top. The concentration of acrylamide used in the resolving gel was determined upon the size of proteins wishing to be visualised; higher percentage gels were prepared as to achieve high resolution of smaller proteins, whereas lower percentage gels were prepared for larger proteins. Once poured, a small volume of isopropanol was added to achieve a flat, even surface for the stacking gel. Following polymerisation of the resolving gel, the isopropanol layer was carefully decanted and the upper surface of the gel was rinsed with ddH₂O. Components of the stacking gel were prepared, vortexed and pipetted on top of the resolving gel until reaching the top of the plates. A comb
was sandwiched between the plates, and the stacking gel was left to set. Once completely set, the plates were removed from the casting apparatus and secured to the central unit of the tank. The comb was gently removed and running buffer (30.2 g Tris base, 144 g glycine, 10 g SDS up to 1 l in ddH$_2$O) was poured into the wells, the inner component of the reservoir and to the level of the lower electrode wire within the outer reservoir. Samples prepared for electrophoresis by the equal addition of 2x Laemmli buffer (25 mM Tris-HCl pH6.8, 2% SDS, 25% glycerol, 0.1% bromophenol blue, 5% β-mercaptoethanol (added fresh)), denatured at 100°C for 5 minutes and loaded carefully into the wells alongside a protein standard (Promega). Gels were electrophoresed at 180 V (50 mA) for approximately one hour at room temperature or until the bromophenol blue dye front reached the bottom of the gel. Following electrophoresis, the gel was carefully dismantled, the stacking gel discarded and a small corner of the resolving gel was removed to provide orientation.

Table 2.3 - Composition of SDS-PAGE resolving and stacking gels

<table>
<thead>
<tr>
<th>Component</th>
<th>Resolving gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15%</td>
<td>12.5%</td>
</tr>
<tr>
<td>30% acrylamide</td>
<td>5.0 ml</td>
<td>4.2 ml</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>2.8 ml</td>
<td>3.7 ml</td>
</tr>
<tr>
<td>Tris-HCl buffer*</td>
<td>2 ml</td>
<td>2 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>10% APS</td>
<td>50 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µl</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

*: 1.5 M Tris pH8.8 is used for resolving gels; 1 M Tris pH 6.8 is used for stacking gels

2.4.3.2 Western Blot transfer

Western blot analysis was conducted using a horizontal semi-dry blotting apparatus (Bio-Rad). Following electrophoresis, the gel was pre-equilibrated for 15 minutes in 1x transfer buffer (10x: 58.2 g Tris-base, 3.75 g SDS, 2.94 glycine, 20% methanol up to 1 l with ddH$_2$O). A piece of Hybond-C nitrocellulose membrane was cut to the area size of the gel and pre-wet in
methanol for 5 minute with agitation. The membrane was subsequently rinsed in ddH₂O and pre-equilibrated in 1x transfer buffer. Four pieces of pre-equilibrated Whatman 3MM filter paper was layered onto the blotting apparatus, followed by the membrane, gel and a further 4 pieces of pre-equilibrated filter paper. Air bubbles were gently removed by gently rolling a glass pipette over the layered stack. The lid of the blotting apparatus was then attached and the transfer was run at 20 V (400 mA) for 30 minutes at room temperature.

2.4.3.3 Immunoblotting

Following transfer, the membrane was washed in Tris-buffered saline plus Tween-20 (TBS-T; 40 g NaCl, 1 g KCl, 0.25 M Tris pH7.5 in 500 ml water plus 0.1% Tween-20) for 5 minutes, and blocked in 5% milk (Marvel) diluted in TBS-T, overnight at 4°C. Membranes were then incubated with rat anti-HA-peroxidase antibody [1:1000 (Roche)] diluted in blocking buffer for one hour at room temperature. Membranes were thoroughly washed for 15 minutes with agitation in block buffer, 5 times. Immunoreactive proteins were visualised using the ECL Detection Reagent System (Amersham) according to the manufacturer’s instructions. Solution A and B were mixed at a ratio of 1:40, in a total volume of 1 ml per membrane. The A:B reagent was evenly distributed onto the membrane surface, incubated for 5 minutes and the bands visualised by autoradiography. Membranes were stored for future use, carefully sealed in cling-film to avoid drying, at 4°C.

In some instances, following detection of an antibody, membranes were stripped for re-probing with a different antibody. This was particular useful to detect levels of the protein of interest in relation to a loading control. For this method, membranes were wetted in methanol for 5 minutes, and washed twice in TBS-T for 5 minutes with agitation. They were then incubated in pre-warmed 0.2 M NaOH for 20 minutes at 37°C, and then transferred to a roller for a further 10 minutes. Membranes were then washed thrice in TBS-T for 5 minutes with gentle agitation, blocked as outlined above and incubated with mouse anti β-actin antibody [1:10,000 (Sigma)] for 1 hour at room temperature. Membranes were then thoroughly washed 3
times for 15 minutes with agitation in block buffer, before incubation with a polyclonal goat anti-mouse-HRP antibody [1:10,000 (DakoCytomation)], again for one hour at room temperature. Detection was performed as described above.

2.4.4 Slide immunostaining

Specific conditions for each antibody were tested in order to determine the antigen retrieval method (Declere and 10 mM citric acid) that provided optimal staining. For antibodies requiring Declere, slides were immersed in 1x Declere and steamed for a total of 40 minutes using a conventional steamer. Following 30 minutes of steaming, a new container containing fresh 1x Declere was placed into the steamer and allowed to equilibrate for 5 minutes, prior to transferring slides into fresh solution for the ultimate 5 minutes. For citric acid antigen retrieval, embryo sections were deparaffinised in HistoClear for 10 minutes, and rehydrated in decreasing concentrations of ethanol (100%, 75%, 50%, 25% ethanol in ddH$_2$O and ddH$_2$O) for 2 minutes each. Slides were subjected to antigen retrieval using 10 mM citrate buffer (pH 6.0) for 10 minutes in a microwave.

For both methods (Declere and citric acid), slides were allowed to cool thoroughly before blocking in 5% heat inactivated sheep serum (HISS) diluted in TBS-T for 1 hour. Slides processed for diaminobenzidine (DAB) staining were subjected to an additional 10 minute wash with 3% hydrogen peroxide diluted in ddH$_2$O prior to blocking in order to quench endogenous peroxidase activity. Primary antibodies were diluted in block solution and slides were incubated overnight at 4°C in a humid chamber. Slides were washed in TBS-T thrice for 10 minutes each in order to ensure complete removal of excess antibody. In most cases, biotin-conjugated secondary antibodies against the primary species (DakoCytomation) were employed (Table 2.5). Secondary antibodies were diluted in block solution and incubated with sections for one hour at room temperature. Following TBS-T washes, slides were incubated with tertiary antibodies, either streptavidin conjugated to cy3 fluorophores for one hour for fluorescence (Molecular probes), or with ABC reagent (two drops of Reagent A [Avidin DH] and Reagent B
[Biotinylated Horseradish Peroxidase H] diluted in 5 ml of 1x PBS) (Vector Laboratories) for 30 minutes. For fluorescent staining, slides were washed with TBS-T before mounting in VectaShield containing DAPI. For DAB staining, slides were washed thoroughly with 1x PBS and incubated with peroxidase substrate solution (Vector Laboratories) until the desired stain was achieved. The reaction was stopped by immersing the slides in ddH$_2$O. Slides were briefly counterstained with haematoxylin, dehydrated (25%, 50%, 75%, 100% ethanol in ddH$_2$O) and cleared (HistoClear for 10 minutes) before mounting with VectaMount (Vector Laboratories). For GnRH DAB staining, nickel solution was added to the peroxidase substrate solution to increase the contrast of the staining. Under these conditions, counterstaining with haematoxylin was omitted.

**2.4.4.1 BrdU staining**

For double immunostaining against PITX1 and BrdU, slides were treated to citric acid retrieval and incubated with primary antibody (PITX1) overnight, as described above. Following tertiary antibody incubation, slides were washed with TBS-T and the staining was fixed with 4% PFA for 50 minutes at room temperature. Fixative was subsequently removed through washes with TBS-T and slides were incubated with 0.1 M hydrochloric acid (HCl) for 30 minutes at room temperature, 2 M HCl for 30 minutes at 37°C and then with 0.1 M borate buffer for 10 minutes at room temperature. Slides were extensively washed with TBS-T and re-blocked with 5% HISS for one hour at room temperature prior to overnight incubation with rat anti-BrdU antibody at 4°C. Slides were then washed with TBS-T and then incubated for one hour with fluorescein anti-rat secondary antibody. Slides were washed and mounted in VectorShield containing DAPI mounting medium as outlined above.

Table 2.4 contains a list of antibodies used in this thesis along with their specific conditions.
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host Species</th>
<th>Method</th>
<th>Dilution</th>
<th>Secondary Antibody</th>
<th>Supplier of Primary Antibody</th>
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<tr>
<td>GNRH</td>
<td>Rabbit</td>
<td>Citric Acid</td>
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<td>Goat α-rabbit biotinylated (IF:+streptavidin 555)</td>
<td>Immunostar</td>
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<td>Chicken</td>
<td>Citric Acid</td>
<td>1:300 (IF)</td>
<td>Goat α-chicken fluorescein</td>
<td>Abcam</td>
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<td>Rabbit</td>
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<td>Millipore</td>
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<td>Rabbit</td>
<td>Citric Acid</td>
<td>1:200 (IF)</td>
<td>Goat α-rabbit biotinylated+streptavidin 555</td>
<td>Cell signaling</td>
</tr>
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<td>GH</td>
<td>Rabbit</td>
<td>Dec蝶ere</td>
<td>1:1000 (DAB)</td>
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<td>Developmental Studies Hybridoma Bank</td>
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<tr>
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<td>Rabbit</td>
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<tr>
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<td>Cell signaling</td>
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DAB, diaminobenzidine staining; IF, immunofluorescence staining
2.5 CELL CULTURE METHODS

2.5.1 Maintenance of cultured cells

All cells were grown at 37°C in a 5% CO₂-incubator at 100% relative humidity. All cell types were grown in DMEM, supplemented with 10% FCS, 2 mM L-glutamine (Invitrogen), 100 units/ml penicillin G (Invitrogen) and 100 µg/ml streptomycin (Invitrogen). All cell work was performed in a tissue culture hood under sterile conditions. All equipment and reagents used for cell culture work was kept separately in sterile conditions. All materials were cleaned thoroughly with 70% IMS prior to use.

2.5.2 Splitting cells

For passaging, cells were briefly washed in PBS and harvest with pre-warmed 0.25% trypsin-EDTA and pelleted through a centrifugation step of 1,000 rpm for 5 minutes. The pellet was then re-suspended in 10 ml of complete medium and plated as required. For all experiments, 10 µl of cell suspension was mixed with 10 µl of trypan blue and live cells were counted using a haematocytometer. Accurate numbers of cells were then plated into dishes in growth medium and were incubated at 37°C until further use.

2.5.3 Freezing and thawing of cells

To prepare frozen aliquots of a cell line, a healthy low-passive number sample was grown in a 75 cm² flask until at optimal confluence. Fresh medium was added and cells were harvested with 0.25% Trypsin-EDTA as described above. Following centrifugation, cells were gently re-suspended in 3 ml of freezing medium (90% FCS and 10% DMSO). 1 ml aliquots were transferred into 2 ml cryovials, and directly transferred to a Mr Frosty storage container where they were stored at -80°C for a few days. Subsequently, vials were stored indefinitely in liquid nitrogen.
When required, cells were thawed in a 37°C water bath for a few minutes. As soon as fully thawed, cells were transferred into 10 ml of fresh medium and gently re-suspended with a p1000 pipette. Cells were then spun at 1,000 rpm for 5 minutes. The supernatant was discarded and the pellet was re-suspended in 10 ml of fresh growth medium and plated in a 75 cm² flask. The following day, an additional 5 ml of supplemented DMEM was added to the flask and cells were grown to optimal confluency. Cells were then passaged as described above.

2.5.4 Transfection of cells

Cells were accurately plated the day before transfection at a confluence of 70-80% in complete medium (precise numbers of cells used in each experiment are denoted under specific techniques). Transient transfection assays were performed using FuGENE® 6 transfection reagent (Roche) according to the manufacturer’s instructions; the exact amount of FuGENE® 6 transfection reagent used was optimised according to the experiment. On the day of transfection, FuGENE® 6 transfection reagent was diluted in serum-free media (SFM) through the careful addition of the FuGENE® 6 reagent directly to medium, followed by an incubation of 5 minutes. In the meantime, DNA constructs were compiled according to each experimental condition. Following the 5 minute incubation, the diluted FuGENE® 6 reagent was added to each DNA condition, vortexed briefly to ensure mixture of the contents and then incubated for a further 15 minutes at room temperature. Plated cells were removed gently from the incubator and fresh pre-warmed media added. Following incubation of the transfection reagent and DNA, the complex was added to each well in a drop-wise manner and the culture plates were lightly swirled to ensure even distribution of the transfection:DNA complex over the entire well surface. Cells were further incubated until required for gene expression assay.

For all transfection experiments, a GFP-expression vector was used in order to determine the efficiency of transfection. Experiments with a 70% or higher transfection efficiency were used for further analysis.
2.5.5 Immunocytochemistry

Approximately $7.5 \times 10^4$ HEK 293T cells were seeded onto coverslips placed in 12-well tissue culture plates and allowed to grow for approximately 24 hours prior to transfection. Cells were transfected with either 150 ng or 300 ng of HA-HESX1 expression constructs (wild-type or mutant) and the total amount of DNA transfected per well was fixed to 380 ng with empty vector (pBluescript, Stratagene). One day post-transfection, cells were gently washed with 1 ml of PBS and then fixed in 1 ml 4% PFA for 20 minutes at room temperature. Cells were then washed three times in PBT (PBS containing 0.1% Triton X-100), and permeabilised with PBS containing 0.5% Triton X-100 for 15 minutes at room temperature. Cells were washed again with PBT and then blocked with 10% FCS in PBT, for an hour at room temperature. Following overnight incubation at 4°C with rat anti-HA antibody [1:500 (Roche)], cells were washed in PBT for 5 minutes with gentle agitation and incubated with 594-conjugated anti-rat secondary antibody [1:200 (Invitrogen)] for 1 hour at room temperature. This was followed by a further four washes as described. Nuclear counterstaining was performed using VectaShield containing DAPI. Fluorescence microscopy was performed as detailed in Section 2.1.6.

2.5.6 Luciferase assays

$5 \times 10^4$ cells were seeded into each well of a 24-well tissue culture plate 24 hours prior to transfection. For the mammalian one-hybrid system, Chinese Hamster Ovarian (CHO) cells were co-transfected with 80 ng of Renilla control vector, 100 ng of the appropriate firefly luciferase reporter and 100 ng of Gal4-HESX1 expression vectors. For mammalian two-hybrid assays, CHO cells were co-transfected with 40 ng of Renilla control vector, 100 ng of PIII-reporter containing six paired-class binding sites (PIII), 200 ng of Tle1-VP16 and 50 ng of Gal4-HESX1 expression vectors. The total amount of transfected DNA per well was normalised to 240 and 390 ng per well, respectively, by addition of empty vector (pBluescript).

Cells were lysed and assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) 48 hours post-transfection. Growth medium was carefully aspirated.
from each well and cells were gently washed in 1 ml PBS. 100 µl of 1x Passive Lysis Buffer (PLB) was dispensed into each well and culture plates were placed on a rocking platform with gentle rocking for at least 30 minutes. Culture plates were sealed with parafilm and frozen at -20°C for future analysis.

Luciferase Assay Reagent was prepared prior to initial use by re-suspending the lyophilised Luciferase Assay Substrate in 10 ml of the Luciferase Assay Buffer II. Substrate and Buffer mixture was stored for further use at -20°C for one month or at -80°C indefinitely. Stop & Glo® reagent was always prepared immediately prior to use; for 100 reactions, 200 µl of 50x Stop & Glo® substrate was added to 10 ml of Stop & Glo® Buffer. Luciferase activity was measured using a BMG FLUOstar Optima multiplate reader (BMG LABTECH GmbH, Offenburg, Germany) according to the manufacturer’s General Injector Wash Protocol. Experiments were performed in triplicate and repeated independently, at least three times. All data was normalised to *Renilla* expression and results shown in this thesis represent the means ± SEM of each condition.
CHAPTER 3: THE INVOLVEMENT OF

*HESX1* IN THE DEVELOPING

NEUROENDOCRINE HYPOTHALAMUS
3.1 INTRODUCTION

The hypothalamus is the central organiser of endocrine and autonomic function within the brain. Therefore, in addition to its aforementioned role in the regulation of the endocrine system, the hypothalamus governs a diverse range of functions, including temperature regulation, feeding behaviour and circadian rhythms. It has also been associated with much diverse emotional and social behaviour, such as fighting, mating and parental care. Taken together, it is not surprising that developmental defects affecting the hypothalamus may act as the aetiological underpinning of multiple diseases, such as obesity, mood disorders and abnormal sleep patterns, in addition to hypopituitarism (Arborelius et al., 1999; Blackshaw et al., 2010; Lugaresi et al., 1998; Rubenstein et al., 1994; Shimogori et al., 2010; Stoving et al., 1999).

Despite our sparse understanding of the highly variable condition of SOD, meticulous observations of patients diagnosed with this condition have proved of useful value. Further to endocrine deficits, it has been noted that patients presenting with SOD can harbour associated features, such as temperature instability, excessive appetite, sleep arrhythmicity, as well as autistic traits, implying a possible hypothalamic component to this complex disease (Mehta et al., 2009; Parr et al., 2010; Rivkees, 2001; Webb et al., 2010; Webb and Dattani, 2010).

3.1.1 Mouse models for SOD and hypopituitarism

Given the close resemblance of the Hesx1-null mutant phenotype and SOD, as well as the conserved expression of HESX1 during human embryonic development, the Hesx1-deficient mouse has been a useful model for investigating the aetiology, pathogenesis and variable nature of human SOD. To exploit this tool further, our lab previously generated a knock-in mouse model harbouring a formerly identified human HESX1 mutation, namely Hesx1-I26T, which occurs in the highly conserved and functionally important eh1-domain, as described in the general introduction (Chapter 1).
To briefly reiterate, the analysis of Hesx1<sup>I26T/I26T</sup> mouse model has shown that this mutation yields a hypomorphic allele, which is consistent with in vitro studies (Carvalho et al., 2003; Sajedi et al., 2008b). Hesx1<sup>I26T/I26T</sup> embryos show very mild defects in the forebrain that are restricted to the eyes, but normal telencephalic development. Conversely, pituitary defects caused by the I26T mutation are fully penetrant. Pituitaries in these mutants are highly dysmorphic and characterised by bifurcations, multiple lumens and expanded anterior lobes. Despite the abnormal morphology, terminal differentiation of all pituitary cell types occurs as normal. Of interest, Hesx1-<i>I26T</i> homozygous mice are viable and fertile (Figure 3.1) (Sajedi et al., 2008b).

This is in contrast to the phenotype to the Hesx1-null mutant mouse. Phenotypic analyses of these mutants reveal variable abnormal forebrain phenotypes, which are commonly displayed as severe forebrain defects including absence of eyes, reduced telencephalic vesicles and frontonasal mass, as well as abnormalities of the septum pellucidum and corpus callosum. In addition, these mice show pituitary defects that are fully penetrant and identical to the Hesx1-<i>I26T</i> mouse embryos. Hesx1<sup>+/−</sup> mutants die perinatally (Figure 3.1) (Andoniadou et al., 2007; Dasen et al., 2001; Dattani et al., 1998).
Figure 3.1 - Forebrain and pituitary defects associated with \textit{Hesx1}-deficient mouse models

(a-d) Lateral views of 12.5 dpc embryos, with anterior to the left. (e-h) Haematoxylin and eosin staining and (i-l) \textit{in situ} hybridisation against pituitary terminal differentiation on frontal sections of 17.5 dpc wild-type and \textit{Hesx1} mutant embryos. Note that the defects observed in the \textit{Hesx1}^{R160C/R160C} mouse mutant phenocopies the \textit{Hesx1}-null mouse. (a) Wild-type embryo highlights the normal development of the eyes (black arrowhead), telencephalic vesicles (white arrowhead) and frontonasal mass (white arrow). (b) Development of the telencephalon and frontonasal mass appears to be grossly unaffected in the \textit{Hesx1}^{I26T/I26T} mutant embryo, but the eye of this mutant displays signs of microphthalmia. (c) Similarly, a mildly affected \textit{Hesx1}^{R160C/R160C} embryo reveals normal telencephalic development, but severe microphthalmia. (d) Conversely, a severely affected \textit{Hesx1}^{R160C/R160C} embryo displays an absence of eyes, reduced telencephalic vesicles and frontonasal mass. (e) At 17.5 dpc, the wild-type pituitary gland is placed between the basosphenoid bone and hypothalamus. (f) \textit{Hesx1}^{I26T/I26T} embryos typically display anterior pituitary enlargement and bifurcation. This often results in the disruption of basosphenoid bone development. (g) In very mild circumstances, \textit{Hesx1}^{R160C/R160C} embryos show minimal defects in pituitary development, with normal development of the underlying basosphenoid bone. (h) However, in the majority of cases, development of the pituitary gland is severely affected in the \textit{Hesx1}^{R160C/R160C} mutants, which results in an ectopic pituitary gland, embedded within the nasopharynx. (i-l) Despite these defects in morphogenesis, all anterior pituitary cell types are correctly specified, as highlighted through the expression of \textit{αGsu}, \textit{Gh} and \textit{Pomc1}. Abbreviations: BS, basosphenoid bone; np, nasopharynx. Scale bars: a-d, 940 µm; e-l, 110 µm. Figure adapted from (Sajedi et al., 2008b).
Hesx1^{+/+}  Hesx1^{126T/126T}  Hesx1^{-/-}=Hesx1^{R160C/R160C}

12.5 dpc

Viable and fertile

Perinatal lethal

17.5 dpc

H&E  αGsu  Gh  Pomc1
3.1.2 Working hypotheses

At present, the reasons underlying the perinatal lethality of the \textit{Hesx1}^{−/−} mouse mutant neonates remain elusive. Indeed, the severe craniofacial defects, as observed in the most severe cases of \textit{Hesx1}^{−/−}, can account for a proportion of the lethality; however, mutants with mild defects also die. Given that pituitary defects are fully penetrant and present to a similar degree, in both the \textit{Hesx1}^{−/−} and the \textit{Hesx1}^{I26T/I26T} mutants, it is unlikely that this alone can account for the discrepancies between the genotype and postnatal phenotype observed. Consequently, in light of the hypothalamic contribution to SOD postulated through human patient data, it has been hypothesised that the differences in the postnatal phenotype within the various \textit{Hesx1} mouse models could be due to defects within the hypothalamus of these mutants. Specifically, a loss of the neuroendocrine nuclei governing the pituitary gland could obviously lead to severe hypopituitarism in the various mutants. Furthermore, a disrupted pituitary stalk could equally compromise contact between the neuroendocrine hypothalamus and pituitary gland. In fact, previous analyses of mouse mutants harbouring null mutations in transcription factors involved in the cellular differentiation of the neuroendocrine neuronal subtypes, result in neonatal death (Acampora et al., 1999; Goshu et al., 2004; Hosoya et al., 2001; Michaud et al., 1998; Nakai et al., 1995; Schonemann et al., 1995; Wang and Lufkin, 2000).

In this chapter, I have investigated the integrity of the neuroendocrine hypothalamus in the \textit{Hesx1} mouse mutants, with the ultimate intention of shedding more light into the highly variable condition of SOD and various pituitary disorders in humans. Given that human mutations in \textit{HESX1} vary in both penetrance and severity, I have utilised all available mouse models for \textit{Hesx1}, namely the \textit{Hesx1}^{I26T/I26T} and \textit{Hesx1}^{−/−} mutants, to gain a more complete insight and thus, to compare and deduct conclusions for the range of phenotypes illustrated in humans. Analysis of the \textit{Hesx1}^{I26T/−} was also conducted in sight of revealing an intermediate phenotype between the \textit{Hesx1}^{I26T/I26T} and \textit{Hesx1}^{−/−} mutants.
3.2 RESULTS

3.2.1 Functional anatomy of the neuroendocrine hypothalamus

The vertebrate hypothalamus is located ventrally to the thalamus, dorsally to the pituitary and encompasses the medio-basal region of the CNS. It is best anatomically described in terms of its four rostro-caudal levels, as originally proposed by Swanson (1987), which are largely identifiable by prominent ventral structures, such as the optic chiasm, tuber cinereum and mamillary bodies (Andersen and Rosenfeld, 2001; Markakis, 2002; Swanson, 1999). For an acquaintance with the neuroendocrine hypothalamus, haematoxylin and eosin staining was performed on coronal sections of an 18.5 dpc wild-type embryo. Identification of the four rostro-caudal regions was achieved: the preoptic region was located immediately rostral to the supraoptic region, which was recognised by the presence of the optic chiasm; the tuberal region was determined at the level of the median eminence and infundibulum; and the mammillary region was located immediately caudal to the infundibular sulcus (Figure 3.2a-d).

Initial work to identify the precise location of these nuclei was conducted in wild-type embryos at 18.5 dpc by in situ hybridisation using specific anti-sense riboprobes against all the hypophysiotrophic factors. As anticipated, expression of Ss transcripts revealed the exact location of the aPV, immediately adjacent to the ventricle, most rostrally within the supraoptic region of the hypothalamus (Figure 3.2b, e, g). Caudal to this, expression of Avp- and Ot-transcripts were identified within the PVN and SON (Figure 3.2b, e, h, i). Finally, expression of Ghrh transcripts identified the location of the Arc, at the level of the median eminence and pituitary gland, within the tuberal region of the hypothalamus (Figure 3.2c, f, j).

Detection of Trh, Crh and Gnrh expression using our riboprobes and standard protocols did not reveal the presence of these transcripts. This result could have been due to inadequate sensitivity of our technique. Previous work by Wang and Lufkin (2000) was able to show the presence of Crh and Trh transcripts in the developing hypothalamus of wild-type embryos, at stages as early as 15.5 dpc, by the use of radiolabelled probes (Wang and Lufkin, 2000). It is
well established that radiolabelled probes are much more sensitive than the alternative use of non-radiolabelled DIG-labelled probes employed in this study, which could account for this discrepancy (Sajedi et al., 2008b). As a consequence, the neuroendocrine hypothalamus in our mouse models was initially investigated by the analysis of AVP- and OT-expressing magnocellular neurons and GHRH- and SS-expressing parvocellular neurons.
Figure 3.2 - Morphological examination of the neuroendocrine hypothalamus

(a-f) Haematoxylin and eosin (H&E) staining and (g-l) in situ hybridisation on frontal sections through the hypothalamus of a control 18.5 dpc embryo. (a-d) Low magnification images identify the rostro-caudal regions of the hypothalamus based upon their proximity to neighbouring ventral structures. (e-f) Higher magnification images of the (e) supraoptic region and (f) tuberal region of the hypothalamus depicting the location of the neuroendocrine nuclei. (g) In situ hybridisation against Ss reveals the location of the aPV. (h-i) Ot transcripts reveal the location of the (h) SON and (i) PVN. (j) Lastly, the Arc is defined through the expression of Ghrh transcripts in the tuberal region of the hypothalamus at the level of the median eminence. Abbreviations: 3V, third ventricle; BS, basosphenoid tissue; LaV, Lateral ventricle; MB, mammillary bodies; Or, oral cavity; To, tongue; V, trigeminal nerves; WT, wild-type. Scale bars, (a-d) 200 μm (e-j) 100 μm.
3.2.2 Hesx1 is required for terminal differentiation of the neuroendocrine hypothalamus

To investigate the integrity of the neuroendocrine hypothalamus in mouse mutants carrying the various Hesx1 alleles (Hesx1<sup>I26T/I26T</sup>, Hesx1<sup>I26T/-</sup> and Hesx1<sup>-/-</sup>), in situ hybridisation using the anti-sense riboprobes aforementioned was performed at late gestation. Coronal sections from the eye through to the mammillary region of the hypothalamus were mounted serially for use in subsequent analyses. One slide per series was used for analyses to exclude the possibility of mislocalised neurons. Therefore, the quantification analyses described in this section represents the number of positive neurons identified within the entire rostro-caudal axis of the hypothalamus in control and mutant embryos.

For this study, the more severe, rarer form of the Hesx1-null phenotype (Class II; Chapter 1) was omitted from the analyses, as complete truncation of anterior forebrain structures will mask any potential neuroendocrine defects (Dattani et al., 1998). Moreover, as this study aimed to correlate genotype-phenotype with humans, it is likely that the severe Hesx1 phenotype observed in mice is embryonic lethal in humans and thus, the population of patients harbouring the known Hesx1 mutations likely represents the milder spectrum of this phenotype.

3.2.2.1 Terminal differentiation of magnocellular neurons is affected in the Hesx1 mouse models

As previously explained, the neuroendocrine hypothalamus consists of two distinct neuronal populations, namely the magnocellular and parvocellular neuro-secretory cells, which regulate two separate entities of the pituitary gland, the PP and AP, respectively. In situ hybridisation against magnocellular terminal differentiation markers, Avp and Ot, revealed a severe reduction of positive transcripts within the PVN and SON of Hesx1<sup>-/-</sup> embryos at 18.5 dpc, when compared to wild-type controls (Figure 3.3e-f, k-l; n=3). The severity in reduction of Avp and Ot transcripts varied between Hesx1<sup>-/-</sup> mutant embryos, with the first Hesx1<sup>-/-</sup> mutant
displaying unilateral expression of these two genes (data not shown), the second showing no expression (Figure 3.3f, l), and the last one revealing a substantial reduction with bilateral expression of Avp and Ot transcripts (Figure 3.3e, k). Likewise, analyses of Avp and Ot expression in Hesx1I26T/− embryos at 18.5 dpc revealed a vast reduction of these transcripts compared to controls, similar to that observed in Hesx1I−/− mutants (Figure 3.3d, j; n=3). In contrast, analysis of Hesx1I26T/I26T embryos at this stage, revealed a moderate reduction in the expression of Avp and Ot transcripts when compared to stage-matched controls (Figure 3.3c, i; n=3). Subsequent quantification confirmed the evident reduction observed in the in situ experiments. This analysis suggests that the expression of Avp and Ot within the hypothalamus of the Hesx1 allelic series is significantly reduced in a graded manner corresponding to the levels of Hesx1 activity of the specific allele (Figure 3.3g, m; one-way ANOVA: Hesx1I26T/I26T; Hesx1I26T; Hesx1I−/−; p <0.01).
Figure 3.3 - Terminal differentiation of magnocellular neurons is affected in the *Hesx1* allelic series, corresponding to the severity of the postnatal phenotype (b-f; h-l) *In situ* hybridisation against the terminal differentiation markers of the magnocellular neurons, *Avp* (b-f) and *Ot* (h-l) on coronal sections of *Hesx1*^+/+, *Hesx1*^I26T/I26T, *Hesx1*^I26T/- and *Hesx1*-/- at 18.5 dpc. (a) Schematic representation of an 18.5 dpc embryo indicating the plane of section. (a’) illustrates the approximate location of the supraoptic region of the hypothalamus, in which the PVN and SON nuclei reside. Both *Avp* and *Ot* transcripts showed a similar expression domain within genotypes (b, h) *Avp* and *Ot* transcripts are clearly identifiable within the PVN and SON of control embryos at 18.5 dpc. In contrast, expression of *Avp* and *Ot* is drastically altered in *Hesx1*-/-, which ranged from (e, k) bilateral sparse expression in both the PVN and SON through to (f, l) an entire absence of these transcripts. (d, j) Analysis of the *Hesx1*^I26T/- embryos revealed a similar pattern to the *Hesx1*-null embryos with only sparse detection of *Avp* and *Ot* transcripts whereas (c, i) *Hesx1*^I26T/I26T embryos displayed a moderate reduction of expression. Arrowheads delimit single cells whereas arrows show groups of positive cells. Quantification of (g) *Avp*- and (m) *Ot*-mRNA transcripts in *Hesx1*^+/+, *Hesx1*^I26T/I26T, *Hesx1*^I26T/- and *Hesx1*-/- embryos at 18.5 dpc (n=3 per genotype). Error bars represent the mean ± SEM. One-way ANOVA revealed a significant reduction in positive transcripts in *Hesx1*^I26T/I26T, *Hesx1*^I26T/- and *Hesx1*-/- embryos when compared to controls. Abbreviations: 3V, third ventricle; V, trigeminal ganglion; WT, wild-type. Scale bar, 100 µm.
3.2.2.2 Terminal differentiation of parvocellular neurons is moderately affected in the *Hesx1* mouse model

To further dissect the role of *Hesx1* within the developing neuroendocrine hypothalamus, terminal differentiation markers of parvocellular neurons, which govern the AP, were analysed by *in situ* hybridisation (*Ss, Ghrh*) and immunohistochemistry (GnRH) at 18.5 dpc. Contrary to the expression of magnocellular neuronal markers, *Ss*+ve cells were present within the aPV in the *Hesx1*−/− mutants; however, these appeared reduced in numbers in all embryos analysed. Additionally, no signs of unilateral expression were evident in any of these mutants (Figure 3.4e; *n*=3). A similar reduction was identified in the *Hesx1*126T/− mutants (Figure 3.4d; *n*=3). Interestingly, little differences in *Ss* expression were observed in the *Hesx1*126T/126T when compared to controls (Figure 3.4c; *n*=3). Analysis of *Ghrh* at 18.5 dpc did not reveal any obvious differences in the expression of this marker between genotypes (Figure 3.4f-i; *n*=3 per genotype). *Ghrh*+ve signal was correctly located at the level of the median eminence in all mouse lines analysed. This result was verified by quantification of *Ghrh*+ve neurons (Figure 3.4j; one-way ANOVA: *Hesx1*126T/126T; *Hesx1*126T/−; *Hesx1*−−; *p*>0.05). Unfortunately, owing to the wide expression of *Ss* within the entire brain and the difficulty in morphologically distinguishing the aPV, *Ss*+ve cells were not quantified, as it proved challenging to determine *Ss*-expressing neurons solely belonging to the aPV nuclei (Michaud et al., 1998; Morales-Delgado et al., 2011).

To summarise, these data suggest that terminal differentiation of both neuroendocrine neuronal types is affected by the loss of *Hesx1*. Interestingly, the magnocellular neuro-secretory system appeared to be more sensitive to diminishing levels of *Hesx1* activity. In mice homozygous for the hypomorphic *Hesx1*-I26T allele there was approximately a 50% reduction in numbers of AVP and OT neurons, whereas a further reduction in *Hesx1* activity, as in the *Hesx1*126T/− and *Hesx1*−− mouse lines, essentially led to an entire loss of these cells. With regards to the parvocellular neurons, the work presented here demonstrates that the expression of *Ss*
mRNA is more sensitive to the loss of Hesx1 activity when compared to Ghrh-expressing neurons, which remained unaltered. However, the effect on Ss expression was still not as pronounced as the defects observed within the magnocellular neurons. Taken together, these findings suggest that a defect within the neuroendocrine hypothalamus may contribute to the Hesx1-null phenotype. As previous studies have shown that the combined loss of OT, AVP and SS-expressing neurons results in perinatal death, these findings may provide an explanation for the observed post-natal phenotype in the various Hesx1 mouse models (Acampora et al., 1999; Goshu et al., 2004; Hosoya et al., 2001; Michaud et al., 1998; Nakai et al., 1995; Schonemann et al., 1995; Wang and Lufkin, 2000). As the Hesx1I26T/− mouse provides a similar phenotype to the Hesx1/− mouse, this mouse line was omitted for further analyses and thus, the two most interesting mouse lines – the hypomorph Hesx1I26T/I26T and null Hesx1/− – were selected for further analysis and became the subject of this chapter hereon.

Previous analyses of Hesx1/− embryos have elucidated a role for Hesx1 in the development of anterior forebrain structures, such as telencephalic and optic vesicles (reviewed in detail in the general introduction, Chapter 1). Of interest to this work, Hesx1-deficient mutants also display abnormalities in the olfactory bulbs, and regions of the ventral hypothalamus often appear abnormal in morphology, mainly due to the severe pituitary dysplasia (Andoniadou et al., 2007; Dattani et al., 1998). Detailed analysis of GnRH neuron migration has demonstrated that these cells migrate through the nasal cavity, where they enter the forebrain through the olfactory bulbs and migrate caudally, projecting their axons to the median eminence (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989). Interestingly, our analyses of these neurons revealed that despite disruptions in anterior forebrain tissue, GnRH-expressing neurons withhold the ability to migrate through to the level of the median eminence, as clearly evidenced by the presence of GnRH+ve neuronal fibres within the hypothalamus at the level of the pituitary (Figure 3.4k-m, k’, l”, m’”; n=3 per genotype). Additionally, GnRH immunoreactivity was isolated within the pituitary gland of all mutants analysed (Figure 3.4k’,
1’, m’; \( n=3 \). Thus, it appears that these neurons appear to be less sensitive than the magnocellular neurons to altering levels of \( Hesx1 \).
Figure 3.4 - Terminal differentiation of SS-parvocellular neurons is altered in the Hesx1 allelic series whereas GHRH- and GnRH-parvocellular neurons remain unaffected

(b-i) In situ hybridisation and (k-m) immunolabelling against the terminal differentiation markers of parvocellular neurons on coronal sections of Hesx1+/+, Hesx1I26T/+, Hesx1I26T/I26T and Hesx1−/− at 18.5 dpc. (a) Schematic representation of an 18.5 dpc embryo indicating the plane of sections. (a’) illustrates the approximate location of the aPV within the supraoptic region of the hypothalamus, which house SS-expressing neurons, whereas (a’’) demonstrates the location of the Arc within the tuberal hypothalamus that contains GHRH-expressing neurons. (b) Ss-expressing neurons are present within the aPV of control embryos. (c) A similar pattern of Ss expression is observed in Hesx1I26T/embryos. (d-e) In contrast, expression of Ss appears reduced in Hesx1I26T and Hesx1−/− when compared to controls. Arrows delimit Ss expression in the aPV. (f-j) Little difference in Ghrh expression is observed between genotypes, which is confirmed through quantification of Ghrh mRNA in the hypothalamus of Hesx1+/+, Hesx1I26T/, Hesx1I26T/ and Hesx1−/− embryos at 18.5 dpc (n=3 per genotype). Error bars represent the mean ± SEM. One-way ANOVA revealed no significant differences in positive transcripts between all genotypes analysed. (k-m) GnRH protein is detectable in ventral hypothalamus and pituitary of control, Hesx1I26T/ and Hesx1−/− embryos. (k’, l’, l”, m’, m”’) High magnification images of the boxed regions in (k-m). (k’) GnRH+ve fibres are present at the base of the hypothalamus surrounding the median eminence. Additionally, GnRH+ve labelling is identified within the pituitary gland, in a similar pattern to the pattern of gonadotrophs’ distribution. (l’, l”’) GnRH+ve neuronal projections are present in the ventral hypothalamus adjacent to the median eminence of Hesx1I26T/embryos. (l’) GnRH+ve labelling is present within the pituitary gland of the Hesx1I26T/embryos despite aberrant pituitary morphology. (m’, m”’) Similarly, GnRH+ve fibres in the ventral hypothalamus and protein in the pituitary gland are observed in the Hesx1−/−mutants, again, despite the highly dysmorphic structures. Abbreviations: BS, basosphenoid tissue; ME, median eminence; OR, oral cavity. WT, wild-type. Scale bars, (b-i and k-m) 100 μm, (k’, l’, l”, m’, m’’) 200 μm.
3.2.3 Sf1 and Pomc1 hypothalamic expression in Hesx1-deficient embryos

To evaluate whether this defect was specific to the neuroendocrine hypothalamus or represented a global hypothalamic abnormality, terminal differentiation markers of neurons not directly involved in the neuroendocrine system were examined. Brief analysis of this was conducted through study of the Sf1- and Pomc1-expressing neurons within the hypothalamus of control, Hesx1\textsuperscript{I26T/I26T} and Hesx1\textsuperscript{−/−} embryos.

The ventro-medial nucleus (VMH) of the hypothalamus is a cell group localised above the Arc of the tuberal hypothalamus. Many studies have shown that the nuclear receptor Sf1 is crucial for VMH development and function. In close collaboration with the Arc, this hypothalamic nucleus functions to regulate food intake and body weight homeostasis (Jo and Chua S Jr, 2009; Kim et al., 2011; Szarek et al., 2010). In situ hybridisation against Sf1 at 18.5 dpc did not reveal any substantial differences in expression between genotypes (Figure 3.5a-c; \(n=3\) per genotype). Similar to the function of Sf1, Pomc1 expression can be observed in the Arc of the hypothalamus, where it plays a significant role in feeding behaviours (Jo and Chua S Jr, 2009; Szarek et al., 2010). In situ hybridisation of Pomc1 expression at 18.5 dpc showed comparable expression of Pomc1 transcripts within the Arc of control, Hesx1\textsuperscript{I26T/I26T} and Hesx1\textsuperscript{−/−} embryos (Figure 3.5d-f; \(n=3\) per genotype). This result came in disagreement with previous findings from our lab. Analyses of both Hesx1\textsuperscript{I26T} and Hesx1\textsuperscript{−R160C} (phenotypically indistinguishable from Hesx1\textsuperscript{−/−} mice) mouse models showed decreasing levels of Pomc1 expression in the ventral diencephalon at 12.5 dpc, with a more severe loss of signal in Hesx1\textsuperscript{R160C/R160C} embryos (Sajedi et al., 2008b). To investigate these findings further, in situ hybridisation with Pomc1 was analysed at 12.5 dpc in Hesx1\textsuperscript{I26T/I26T} and Hesx1\textsuperscript{−/−} mutants. Interestingly, this analysis failed to identify any obvious differences in Pomc1 transcript expression between genotypes (Figure 3.5g-i; \(n=3\) per genotype). The discrepancies reported here may be accounted by a delayed onset of Pomc1 expression in the former analysis, as previously reported by Dasen et al. (2001).
Figure 3.5 - Terminal differentiation of Sf1 and Pomc1 neurons in the neuroendocrine hypothalamus remain unaffected in the Hesx1<sup>126T/I26T</sup> and Hesx1<sup>-/-</sup> mouse models

(a-i) In situ hybridisation against the terminal differentiation markers, Sf1 and Pomc1, on coronal sections of Hesx1<sup>+/+</sup>, Hesx1<sup>126T/I26T</sup> and Hesx1<sup>-/-</sup> at 18.5 dpc (a-f) and 12.5 dpc (g-i). (a) Sf1 transcripts are clearly present within the VMH of a control embryo at 18.5 dpc. (b,c) Similar expression of these transcripts is observed in the hypothalamus of Hesx1<sup>126T/I26T</sup> and Hesx1<sup>-/-</sup> embryos at 18.5 dpc. (d-i) Comparable expression of Pomc1 is located within the Arc of (d) control, (e) Hesx1<sup>126T/I26T</sup> and (f) Hesx1<sup>-/-</sup> at 18.5 dpc, in a pattern that is traceable back to earlier stages of hypothalamic development (g-i). Abbreviations: BS, basosphenoid tissue; ME, median eminence; Pit, pituitary; VD, ventral diencephalon; VMH, ventro-medial nucleus. Scale bar, 100 µm.
3.2.4 Early marker analysis of the neuroendocrine hypothalamus in the Hesx1 allelic series

To determine whether the defects observed at late gestation in the Hesx1 mutants could be traced back to an abnormality during early specification of the neuroendocrine hypothalamus, embryos were examined at 12.5 dpc.

Analysis of the ventral diencephalon marker, Nkx2.1, on coronal sections through the ventral diencephalon revealed minimal differences in the expression pattern of this gene between genotypes (Figure 3.6b-d; n=3). As a result, it appears that the specification of the ventral diencephalon occurs as normal in these Hesx1-deficient mutants. Otp and Sim1 are two genes that are required for the coordinate development of the hypothalamic-pituitary axis. Specifically, the loss of Otp or Sim1 from the developing hypothalamus results in the absence of SS, AVP, OT, CRH and TRH expression, ultimately causing neonatal death of these mutant mice (Acampora et al., 2000; Michaud et al., 1998; Wang and Lufkin, 2000). To further examine the possibility that specific neuronal populations were affected in the developing hypothalamus of Hesx1 mutants, in situ hybridisation using specific Otp and Sim1 anti-sense riboprobes was conducted in the Hesx1 allelic series. Expression of these two markers in the Hesx1126T/126T embryos appeared comparable to controls, despite the moderate reduction of Avp- and Ot-expressing neurons observed at late gestation (Figure 3.6f, i; n=3). In contrast, both Otp and Sim1 expression domains were clearly reduced in the ventral diencephalon of Hesx1-/- mutants when compared to Hesx1126T/126T and control littermates (Figure 3.6g, j; n=3). Collectively these data further support the hypothesis that Hesx1 acts in a defined manner to control the specification of specific neuronal populations within the neuroendocrine hypothalamus.
Figure 3.6 - Expression of Otp and Sim1 is reduced in the Hesx1<sup>−/−</sup> mutants

(b-i) In situ hybridisation to detect early specification markers on coronal sections of Hesx1<sup>+/+</sup>, Hesx1<sup>I26T/I26T</sup> and Hesx1<sup>−/−</sup> at 12.5 dpc (a) Schematic of an 12.5 dpc embryo indicating the level of sections in b-j, as illustrated by a’. (a) Nkx2.1 transcripts are clearly present within the ventral area of the developing hypothalamus of control embryos. (c-d) Similar expression of these transcripts is observed in the ventral diencephalon of Hesx1<sup>I26T/I26T</sup> and Hesx1<sup>−/−</sup> embryos at 12.5 dpc. (e) Otp expression is present in the lateral regions of the ventral diencephalon of control embryos with few positive transcripts identified close to the region of the developing pituitary. (f) Comparable expression of Otp was present in Hesx1<sup>I26T/I26T</sup> embryos in lateral regions of the developing hypothalamus, but fewer transcripts are present within the ventral region adjacent to the pituitary when compared to controls. (g) In contrast, Otp transcripts are substantially reduced, both laterally and ventrally in the developing diencephalon of Hesx1<sup>−/−</sup> mutants. (h) Sim1 transcripts show a similar pattern of expression to Otp in the lateral regions of the ventral diencephalon. (i) Sim1 expression in Hesx1<sup>I26T/I26T</sup> mutants showed little deviation from control embryos, whereas (j) Sim1 expression in Hesx1<sup>−/−</sup> appears reduced. Abbreviations: VD, ventral diencephalon; Inf, infundibulum; Pit, pituitary. Scale bar, 100 µm.
3.2.5 Descendants of Hesx1-expressing cells colonise the ventral diencephalon and hypothalamus

The data described demonstrate a novel role for Hesx1 within the neuroendocrine hypothalamus, however, how Hesx1 exerts this role still remains unanswered. In mouse, Hesx1 is an early marker of the developing forebrain, with transcripts detectable within the ventral diencephalon and proximal regions of the optic stalk by 9.0 dpc. Subsequently, by 10.5 dpc, expression is localised only to the pituitary primordium, RP (Martinez Barbera et al., 2000; Martinez-Barbera et al., 2000). As Hesx1 is expressed within regions that give rise to the developing hypothalamus, albeit transiently, the possibility that Hesx1-expressing cell descendants contribute to the development of the mature hypothalamic nuclei still exists.

To explore this hypothesis, we employed two previously described mouse lines: (1) the Hesx1-Cre mouse model, in which the entire open reading frame is replaced by the Cre recombinase coding sequence. Accordingly, Cre expression recapitulates the endogenous Hesx1 expression pattern (Andoniadou et al., 2007); (2) R26R-YFP reporter mouse containing the YFP coding sequence preceded by a floxed STOP cassette in the R26R locus (Srinivas et al., 2001). Subsequent inter-crossing of these mouse lines generated double heterozygous Hesx1^{Cre+};R26R^{YFP/+} embryos and mice, in which YFP expression was permanently activated by Cre excision of the loxP-flanked transcriptional stop sequence. As a result, Hesx1-expressing cells and their descendants could be traced through immunofluorescence labelling.

Immunofluorescence against YFP was performed on Hesx1^{Cre+};R26R^{YFP/+} embryos between 9.5-18.5 dpc. YFP staining on sagittal sections through 9.5 dpc embryos demonstrated that a substantial number of cells within the ventral diencephalon labelled positive for YFP. At this stage, virtually all cells within the developing RP were also YFP^{+ve} (Figure 3.7a; n=3 embryos). Consecutive analysis of YFP expression at 11.5 dpc in Hesx1^{Cre+};R26R^{YFP/+} embryos revealed that in contrast to the pituitary in which all cells were YFP labelled, the ventral diencephalon displayed a mosaic pattern of YFP staining (Figure 3.7b; n=3). Therefore at this
stage, *Hesx1*-expressing cell descendants populate the ventral diencephalon. The striated pattern observed here further reiterates that these descendants are unlikely to influence the development of the entire hypothalamus, but rather specific neuronal populations. Further analysis of YFP expression at 18.5 dpc in these embryos demonstrated much less occupation of YFP⁺ve cells in the hypothalamus. These cells were very few in number, but tended to inhabit regions of the hypothalamus in close proximity to regions of the third ventricle (Figure 3.7; n=3). When compared to the known distribution and cell numbers of the magnocellular and parvocellular neurons, these YFP positive cells appeared substantially sparse, not appearing to occupy any particular nuclei. Collectively, these data suggest that the terminal differentiation defects observed in the *Hesx1* mutants are unlikely due to the cell-autonomous effects of *Hesx1* loss.
Figure 3.7 - Descendants of *Hesx1*-expressing cells colonise regions of the ventral diencephalon and hypothalamus during late stages of gestation

(a-c) Immunolabelling against YFP on (a) sagittal and (b-c) coronal sections of *Hesx1*^{Cre/++;R26R^{YFP/+}} embryos at (a) 9.5, (b) 11.5 and (c) 18.5 dpc. (a) At 9.5 dpc, YFP^{+ve} labelling is present in a number of cells of the developing ventral diencephalon. At this stage, the vast majority of cells within RP are stained positive for YFP. (b) By 11.5 dpc, YFP^{+ve} cells are still identified within the developing ventral diencephalon, but are mosaic in distribution. In contrast, most RP cells are YFP^{+ve}. (c) At 18.5 dpc, much fewer YFP^{+ve} cells are detectable within the hypothalamus of these embryos. YFP^{+ve} cells appear to occupy regions close to the ventricular zone. Similar to early stages of development, practically all cells within the AP are YFP^{+ve}. Abbreviations: FB; forebrain; Hypo, hypothalamus; ME, median eminence; VD, ventral diencephalon. Scale bars, 100 µm.
The exact mechanism to why the loss of *Hesx1* in the developing hypothalamus results in the absence of particular neuro-secretory cells remains unknown. Therefore, it was reasoned that comparison of the hypothalamic regions in *Hesx1*\textsuperscript{Cre/+};*R26R\textsuperscript{YFP/+} and *Hesx1*\textsuperscript{Cre/-};*R26R\textsuperscript{YFP/+} would provide insight into the fate of cells that were *Hesx1* deficient, as the *Cre* and *YFP* dosage remained same in both compound embryos. Interestingly, negligible differences in YFP expression were observed between genotypes at 12.5 and 18.5 dpc (Figure 3.8; \(n=3\) embryos per genotype/stage). At 12.5 dpc, YFP labelled cells colonised intermittent regions of the ventral diencephalon, in a similar pattern of labelling to that previously observed at 11.5 dpc. Again most, if not all, cells of RP equally expressed YFP in *Hesx1*\textsuperscript{Cre/+};*R26R\textsuperscript{YFP/+} and *Hesx1*\textsuperscript{Cre/-};*R26R\textsuperscript{YFP/+} embryos at this stage (Figure 3.8a-b). At 18.5 dpc, YFP\textsuperscript{+ve} cells were identifiable within the hypothalamus of mutant embryos alike to the numbers of YFP-expressing cells observed in wild-type embryos, although the pattern of these cells appeared to be less focal (Figure 3.8c-d). This is believed to be mainly due to the dysmorphology of the ventral hypothalamus, induced by the abnormally-fused anterior pituitary tissue. While this part of the study is only brief in analysis, this data suggests that the loss of *Hesx1* from ventral diencephalon precursors does not result in premature death or failure of these cells to proliferate, although this remains to be fully clarified. Additionally, it still remains unclear whether *Hesx1*-expressing cells adopt different fates, as observed in anterior forebrain structures (as described in detail in Section 1.5.2.1; (Andoniadou et al., 2007)) or simply remain undifferentiated, failing to express markers of terminal differentiation.
Figure 3.8 - Descendants of Hesx1-expressing cells colonise regions of the ventral diencephalon and hypothalamus equally in Hesx1\textsuperscript{Cre/++;R26R\textsuperscript{YFP/+}} and Hesx1\textsuperscript{Cre/--;R26R\textsuperscript{YFP/+}} embryos.

(a-d) Immunolabelling against YFP on coronal sections of Hesx1\textsuperscript{Cre/++;R26R\textsuperscript{YFP/+}} and Hesx1\textsuperscript{Cre/--;R26R\textsuperscript{YFP/+}} embryos at (a-b) 12.5 and (c-d) 18.5 dpc. (a) At 9.5 dpc, YFP\textsuperscript{+ve} labelling are present in a number of cells of the developing ventral diencephalon. At this stage, the vast majority of cells within RP stain positive for YFP. (b) At 12.5 dpc, YFP\textsuperscript{+ve} cells are present within the ventral diencephalon and RP in a comparable pattern between Hesx1\textsuperscript{Cre/++;R26R\textsuperscript{YFP/+}} and Hesx1\textsuperscript{Cre/--;R26R\textsuperscript{YFP/+}} embryos. (c-d) Equally, no differences in YFP labelling are observed within the hypothalamus at 18.5 dpc between genotypes. Similar YFP staining is detected within the AP of Hesx1\textsuperscript{Cre/++;R26R\textsuperscript{YFP/+}} and Hesx1\textsuperscript{Cre/--;R26R\textsuperscript{YFP/+}} embryos, despite the highly dysmorphic appearance of AP tissue in mutant embryos. Red staining represents autofluorescent cells. Abbreviations: Hypo, hypothalamus; OR, oral cavity; VD, ventral diencephalon. Scale bars, 100 µm.
3.3 CONCLUSIONS

To summarise, the results presented in this chapter illustrate a novel role for *Hesx1* in the development of the neuroendocrine hypothalamus. Within this structure, it appears that *Hesx1* may act in a restricted manner, to coordinate the development of specific neuronal subtypes. For instance, I have shown that the terminal differentiation of the magnocellular neurons that govern the PP, seem to be more sensitive to the loss of *Hesx1* expression than the parvocellular neurons, with only an alteration of SS-expressing neurons. Furthermore, I show that these defects are not due to an overall abnormality in the specification and/or patterning of the hypothalamus, as indicated by the normal expression of the hypothalamic marker *Nkx2.1*. Instead, *Hesx1* functions, most likely indirectly, in the specification of particular neuroendocrine nuclei, a result that can be traced back to earlier hypothalamic development, as evidenced by the reduced expression of *Otp* and *Sim1*. Lastly, fate mapping of *Hesx1* descendant cells revealed that although these cells colonise the majority of ventral diencephalon during early development, a minority of these cells occupy the hypothalamus at late gestation. This may be accounted for by the arrival of cells from other locations, or alternatively, the scattered appearance of these YFP+ve cells could be due to the transient expression of Cre within this structure. The use of a better Cre reporter line, such as the *Rosa<sup>Tomato</sup>*, may help to label more cells, thereby providing a more accurate picture (Madisen et al., 2010). However as it stands, given the distribution of these cell descendants, the results presented in this chapter allude to the possibility that the defects observed in the *Hesx1*-deficient mutants occur largely in a non-cell autonomous manner.
CHAPTER 4: FUNCTIONAL
CHARACTERISATION OF NOVEL \textit{HESX1}
MUTATIONS
4.1 INTRODUCTION

Since the initial association of HESX1 and SOD in humans, a number of mutations in HESX1 have been identified in patients manifesting with isolated hypopituitarism and/or SOD (discussed in the general introduction; Chapter 1). It is the functional characterisation of these mutations \textit{in vitro} that has increased our understanding of HESX1 at a molecular level. Utilising a similar approach, this chapter aims to assess the functional consequences of three novel HESX1 variants, further contributing to previous studies in delineating functional domains of HESX1. The screening and clinical evaluation of patients was performed in collaboration with Prof. Thierry Brue (University of Marseille, France). My contribution to this manuscript was the generation of plasmids containing the identified mutations (as detailed in Chapter 2) and the functional analysis \textit{in vitro} of the mutant proteins, which is described herein (Reynaud et al., 2012).

4.1.1 Mutational screen

Consistent with the PP defects in $Hesx1^{-/-}$ mice presented in the previous chapter, HESX1 was one of the first genes to be associated with pituitary stalk interruption syndrome (PSIS), a syndrome characterised by the phenotypic presentation of abnormal stalk morphology or ectopic PP, forming a unique subset of congenital hypopituitarism (di et al., 2009; McCabe et al., 2011; Simmons et al., 1992). To date, only four genes - HESX1, LHX4, OTX2 and SOX3 - are known to be involved with the underlying pathology in no more than 3% of overall cases (Reynaud et al., 2011).

In a recent screening for HESX1 variants in cases of PSIS-associated hypopituitarism within the GENPITHYPO network - a multicentre study for the screening of genetic determinants of congenital hypopituitarism in paediatric and adult centres - three allelic variants of HESX1 were identified. These variations include a previously described nonsense homozygous p.R109X mutation, and two novel allelic variants p.S67T and p.F156S (Figure 4.1) (Reynaud et al., 2011). In addition, this cohort was screened for variations in the aforementioned known genes associated with PSIS. Lastly, as a hypothesis based approach,
screening for two members of the prokineticin 2 pathway, the ligand PROK2 and receptor PROKR2, was conducted, as former studies have identified that these genes mediate angiogenesis and neuronal migration, and furthermore they are both expressed in pituitary and hypothalamic tissue (Lin et al., 2002; Martin et al., 2011; Monnier and Samson, 2010). Of interest, the propositus harbouring the S67T mutation also carries an unrelated genetic defect affecting PROKR2 p.R85H. No other alterations in the open reading frame of all other genes tested, including LHX4, OTX2, PROK2 and SOX3, were found.

Sequencing of parental DNA of each of these patients revealed that R109X mutation was present in a heterozygous state in the father, mother, and one sister, all of whom were asymptomatic. Interestingly, the same heterozygous F156S allelic variant was identified in the father, but not the mother of the proband. Conversely, both father and mother were phenotypically normal. Lastly, the allelic variant S67T was found in the same heterozygous form in the father, however without the PROKR2 allelic variant. The mother of this patient did not carry any other allelic variant in any of the genes analysed. Thus, the PROKR2 mutation occurred de novo. The phenotypic presentation of each patient is summarised in Table 4.1.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Mutation</th>
<th>MRI</th>
<th>Endocrine deficit</th>
<th>Additional information</th>
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<tr>
<td>1</td>
<td>R109X</td>
<td>Hypoplastic AP gland; Ectopic PP at median eminence; interrupted pituitary stalk</td>
<td>GH and ACTH deficiencies at 1 year. Panhypopituitarism at 14 years.</td>
<td>SOD, midline brain defects, or other malformations were absent.</td>
</tr>
<tr>
<td>2</td>
<td>F156S</td>
<td>Pituitary aplasia; interrupted pituitary stalk</td>
<td>GH and TSH deficiencies at 1 year. ACTH reduction at 4 years. Panhypopituitarism at 14 years.</td>
<td>SOD or midline brain defects were absent. Abnormal aspect of white matter (possibly the result of infantile hypoglycaemic seizures).</td>
</tr>
<tr>
<td>3</td>
<td>S67T</td>
<td>Hypoplastic AP gland; Ectopic PP at median eminence; interrupted pituitary stalk</td>
<td>GH and TSH deficiencies at 18 months. Panhypopituitarism at 14 years.</td>
<td>SOD, midline brain defects, or other malformations were absent. Olfactory lobes were normal.</td>
</tr>
</tbody>
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AP, anterior pituitary; PP, posterior pituitary; SOD, septo-optic dysplasia. Information collated from (Reynaud et al., 2012).
4.1.2 Functional domains of *HESX1* protein

*Hesx1/HESX1* encodes for a 185 amino acid protein. To date, previous studies have deduced that this protein contains two functional domains. The homeodomain located at the C-terminus facilitates the DNA-binding properties of this transcription factor. Structural analyses of the homeodomain have revealed that this region consists of three α-helices and a fourth helix, and it is the second and third helices that form a helix-turn-helix motif that recognises and binds to the major groove of DNA [reviewed in (Wigle and Eisenstat, 2008)]. Belonging to the paired-like class of homeodomain proteins, HESX1 binds as homodimers to the palindromic DNA sequence **TAATYNRATTA**, which is termed ‘PIII’ due to the two inverted TAAT core sequences separated by 3 bp (Wilson et al., 1995). In addition its DNA-binding properties, the homeodomain is also able to confer repressor ability; however, it is the eh1 domain located within the N-terminal that is sufficient and necessary for HESX1-mediated repression (Carvalho et al., 2003). This function is largely mediated through the recruitment of the mammalian Groucho orthologues, TLE1 and TLE3 to the eh1 domain (Carvalho et al., 2010; Dasen et al., 2001).

Comparative sequence homology demonstrates that residues R109 and F156 are both invariant between species, occurring within the homeodomain, whereas S67 is located in a region linking the N-terminal repression domain with the homeobox and retains very little homology between species (Figure 4.1). As R109X yields a premature stop codon occurring within the first few residues of the homeodomain and F156 occurs within the third helix of the homeodomain, it is predicted that the mutations R109X and F156S will impede the DNA-binding properties of HESX1. The functional consequences of the S67T substitution are more difficult to anticipate as this mutation is non-conserved between species and occurs in a region of unknown function.
Figure 4.1 - Mutational analysis of HESX1

(a) Schematic diagram of HESX1 protein structure showing the position of the three identified mutations discussed in this study, in relation to known functional domains of HESX1. The eh1 domain resides between 21-27 amino acids, whereas the homeodomain encompasses amino acids 108-167. (b) Heterozygous missense mutation c.200G>C leads to the substitution of a serine residue (codon 67) by a threonine (S67T). Likewise, the heterozygous missense mutation c.467T>C results in an amino-acid change from a phenylalanine (codon 156) to a serine (F156S). In contrast, the nonsense mutation c.325C>T (p.R109X) is predicted to result in a severely truncated protein (loss of 74 amino-acids, including the homeodomain). (c) Snapshots of the predicted reading frame of human HESX1 compared with the mouse HESX1 and *Xenopus* orthologue XANF1. The boxed regions indicate the homeodomain. Mutations in this study highlighted in yellow. Both mutations within the homeodomain - R109X and F156S – are highly conserved between species. Conversely, mutation S67T is located within a less defined region of HESX1 and shows divergence between the named species. Schematic in (c) adapted from (Dattani et al., 1998).
4.2 RESULTS

To investigate the effect of these mutations on known HESX1 molecular properties, mutant proteins were generated through site directed mutagenesis and were expressed either as HA-tagged proteins, or fused to the GAL4 DNA-binding domain (Chapter 2).

4.2.1 Subcellular localisation of HESX1 mutations

As HESX1 is a transcription factor, nuclear localisation of the protein is essential for its physiological function. To evaluate the effect of each mutation on the subcellular localisation of the protein, HEK-293T cells were transfected with plasmids expressing the mutant and wild-type proteins fused to the HA epitope. Indirect immunofluorescence labelling using an anti-HA antibody clearly demonstrated that wild-type HESX1 localised to the nucleus of the cell, with little or no cytoplasmic staining (Figure 4.2a). Analyses of F156S and S67T constructs revealed a similar pattern within the nuclei of transfected cells (Figure 4.2c, d). In contrast, the truncated R109X protein could not be detected within the transfected cells (Figure 4.2b), a result that was replicated after several independent transfections and by using multiple HA-R109X plasmids originating from E. coli clones (data not shown).

The failure of this protein to accumulate in transfected cells was further demonstrated by western blot analysis. Whole cell lysates from HEK-293T cells transfected with HA-wild-type-HESX1, HA-F156S and HA-S67T contained a protein of approximately 25 kDa – a size corresponding to the predicted molecular weight of the HA and HESX1 fusion protein – which was expressed at comparable levels (Figure 4.2e). However, the R109X protein (estimated at ~15 kDa in molecular weight) was very weakly expressed (Figure 4.2e). To exclude the possibility of aberrant protein translation, HA-wild-type-HESX1, HA-F156S, HA-S67T and two independent clones of HA-R109X were in vitro transcribed/translated and protein extracts immunoblotted for HA. Using this technique, HA-wild-type-HESX1, HA-F156S and HA-S67T proteins were all detectable through the presence of a 25 kDa band (Figure 4.2f). In contrast to the immunoblot obtained from whole cell lysates, in vitro translation of R109X mutant plasmids
clearly revealed a protein of approximately 15 kDa, with comparable intensity to all other loaded proteins.

Taken together, this analysis suggests that the effects of the F156S and S67T substitutions are solely due to the impairment of the molecular activities of the mutant proteins. However, the premature termination of translation at R109 yields a truncated protein, which lacks the whole of the homeodomain and is expressed at very low levels under normal physiological conditions, failing to accumulate in the nucleus.
Figure 4.2 - Subcellular localisation of wild-type and F156S, R109X, S67T mutant human HESX1 proteins

Indirect immunofluorescence of HEK-293T cells transfected with plasmids expressing either (a) wild-type HESX1 or (b-d) mutant HESX1 fused to HA and counterstained with DAPI. Note that (a) wild-type HESX1, (c) F156S and (d) S67T mutant proteins localise in the nuclei of transfected cells (pink), but the truncated protein (b) R109X is not detectable. Scale bar, 100 µm. (e) Western blot analysis of transfected cells immunoblotted with either an anti-HA antibody (top panel) or an anti-β-actin antibody (bottom panel). Wild-type, F156S and S67T HESX1 proteins are expressed at comparable levels, but the truncated protein R109X is very weakly expressed. (f) In contrast, in vitro transcription/translation of wild-type and mutant HESX1 reveals the presence of the 15 kDa HA-R109X protein (c1 and c2 represent two independent clones), which is expressed at equal levels to HA-wild-type, HA-F156S and HA-S67T proteins.
4.2.2 Molecular analysis of the F156S, S67T and R109X HESX1 mutant proteins

The work described in this section is organised into three subsections. The first subsection explores the repressor ability of wild-type and mutant HESX1, dependent on the protein’s ability to bind DNA. The second subsection evaluates the repressor ability of wild-type HESX1 and mutant proteins independently of DNA-binding activity. And finally, the third subsection elaborates on the ability of wild-type and mutant HESX1 to interact with known corepressors, such as TLE1. For these sets of experiments, wild-type and mutant HESX1 proteins were expressed fused to the GAL4 DNA-binding domain. This protein fragment, consisting of N-terminal region of GAL4 (1-147 amino acids), allows for the strong and specific binding to a specific 17 bp nucleotide sequence, but is incapable of conferring any transcriptional activity unless fused to a protein that can contribute to transcriptional function (Sadowski et al., 1992).

4.2.2.1 Mutations R109X and F156S result in the inability of HESX1 to bind DNA

Analysis of the repressing activity of wild-type HESX1 and mutant proteins was initially assessed in a mammalian one-hybrid system. This system utilises the interaction between a specific DNA-binding motif and its cis-element providing a measure for DNA-protein interactions, and has previously been used to determine the functional effects of formerly identified human HESX1 mutations (Brickman et al., 2001; Carvalho et al., 2003; Cohen et al., 2003; McNay et al., 2007). As HESX1 functions as a repressor, the luciferase-reporter vector was chosen to contain a minimal SV40 promoter downstream of a DNA-binding motif. Under these conditions, if HESX1 is able to bind DNA, either directly (Figure 4.4a-b) or indirectly (Figure 4.5a-b), luciferase activity would be repressed, a change that can be quantitatively measured by light emission.

Initial experiments were conducted in order to determine the optimum conditions for detection of HESX1 repression. CHO cells have previously been used by many groups, including our group, for the assessment of HESX1’s repressor ability, and therefore, they were
employed for this evaluation (Carvalho et al., 2003; McNay et al., 2007). To this end, the luciferase reporter containing the SV40 promoter and six copies of the paired-like binding site PIII, which wild-type HESX1 binds to with high affinity, was used (6PIII-SV40-Luciferase reporter) (Brickman et al., 2001; Dattani et al., 1998). Under basal conditions, luciferase is constitutively active under the SV40 promoter. Subsequently, if HESX1 is able to bind to the PIII motif, the SV40 promoter should be repressed resulting in a reduction in luciferase activity (Figure 4.4a-b). Transfection with 10 ng and 100 ng of wild-type HESX1 plasmid resulted in a dose-dependent repression of the SV40 promoter, from 22% to 52% respectively, of basal levels of luciferase activity (Figure 4.3b). As a consequence, the optimum amount of 100 ng for each plasmid was transfected into CHO cells for the subsequent mammalian one-hybrid experiments described.
Figure 4.3 - Optimisation of conditions for HESX1-mediated repression

(a) Schematic of reporter plasmid containing the six dimeric paired-class binding sites (PIII), which HESX1 binds to, upstream of the SV40 promoter (6PIII-SV40-Luciferase reporter). This reporter plasmid was used in the subsequent experiments to detect levels of HESX1-mediated repression. (b) Wild-type HESX1 is able to repress the SV40 promoter in CHO cells in a dose-dependent manner. Data shown represent one experiment consisting of three samples per experimental group.
Similarly to the previous experiment, the 6PIII-SV40-Luciferase reporter vector was utilised to evaluate the repressor ability of the mutant proteins, dependent on their ability to bind DNA (Brickman et al., 2001). In this situation, wild-type HESX1 and S67T-mutant HESX1 were able to repress the basal levels of SV40-mediated transcriptional activation (approximately 29% and 31%, respectively; Figure 4.4c). In contrast, R109X and F156S showed no repressing activity and in fact, basal levels of luciferase reporter activity appeared increased relative to the control transfections (Figure 4.4c). From these results alone, it appears that the DNA-binding ability of the S67T mutation is conserved and indistinguishable from wild-type HESX1, whereas alterations occurring in the homeodomain, either through the F to S substitution (F156S) or the lack of the entire homeodomain (R109X), results in disrupted DNA-binding.
Figure 4.4 - Mutant proteins R109X and F156S are unable to bind to the PIII consensus sequence
(a-b) Schematic of the employed mammalian one-hybrid system, utilising a reporter plasmid with six
copies of the HESX1-binding motif, PIII, upstream of the SV40 promoter. (a) Under normal conditions,
luciferase activity is constitutively active due to presence of the SV40 promoter. (b) Upon HESX1
binding to the PIII consensus, repression of the SV40 promoter occurs, resulting in reduced luciferase
activity. (c) Analysis of the repressing activity of the R109X, F156S and S67T mutant proteins in an
assay that is dependent on their DNA-binding properties. A total of 100 ng of plasmids expressing GAL4-
HESX1 fusion proteins (either wild-type or mutant) were co-transfected with 100 ng of a reporter plasmid
containing six dimeric paired-class binding sites (PIII), which HESX1 binds to, upstream of the SV40
promoter (6PIII-SV40-Luciferase reporter). Wild-type HESX1 and S67T mutant proteins are able to bind
to DNA and repress transcription to equivalent levels (29% and 31% of the basal level, respectively). In
contrast, both HESX1-R109X, which lacks the entire homeobox, and HESX1-F156S, which contains a
substitution in the highly conserved F156 of the third helix of the homeobox, cannot repress transcription
mediated by the SV40 promoter, and there is an apparent increase of the basal luciferase activity (t-test; \( p < 0.001 \)).
Strategy: Mammalian one-hybrid system
4.2.2.2 Mutant proteins are able to repress transcription

To further clarify the effect of the mutations, a series of experiments were conducted using a reporter plasmid containing GAL4 binding sites, rather than the PIII sequences (Gal4BS-SV40-Luciferase reporter) (Brickman et al., 2001). Under these conditions, the repressor activity of the mutant proteins could be evaluated independently of their DNA binding properties (Figure 4.5a-b). In these experiments, all three mutant proteins were consistently able to repress the SV40-mediated transcriptional activation of luciferase expression (Figure 4.5c). This finding was anticipated as the repressor ability of HESX1 is mainly facilitated through its interaction with the corepressors, TLE1 and TLE3, which is mediated through the N-terminal eh1 domain (Brickman et al., 2001; Carvalho et al., 2003; Carvalho et al., 2010; Dasen et al., 2001). Therefore, the lack of repressing activity of the R109X and F156T mutant proteins observed when using the PIII reporter plasmid was indicative of the lack of DNA-binding to its target sequence. This is not unexpected considering that the R109X lacks the entire homeodomain and the F156T substitution occurs in an entirely conserved F residue in the third helix of the homeodomain which is known to play an essential role in mediating binding to DNA sequences (Wilson et al., 1995).
Figure 4.5 - R109X, F156S and S67T mutant proteins can repress transcription in an assay that is independent of their DNA-binding capabilities

(a-b) Schematic of mammalian one-hybrid system utilising a reporter plasmid containing GAL4-binding motifs, upstream of the SV40 promoter. (a) Under normal conditions, luciferase activity is constitutively active due to the SV40 promoter. (b) Upon GAL4 binding to the GAL4 cis element, HESX1 is able to mediate repression of the SV40 promoter, resulting in reduced luciferase activity. Thus, the repressor ability of wild-type and mutant HESX1 can be analysed independently of their ability to bind DNA. (c) A total of 100 ng of plasmid expressing GAL4-HESX1 fusion proteins (either wild-type or mutant) were co-transfected with 100 ng of a reporter plasmid containing GAL4-binding sites upstream of the SV40 promoter (Gal4BS-SV40-Luciferase reporter). Wild-type HESX1 caused a 32% repression of the basal luciferase activity, while R109X, F156S and S67T mutant proteins lead to a 51%, 41% and 47% repression, respectively. Asterisks indicate significant differences of mutant proteins compared with wild-type HESX1 (t-test; p <0.05).
Strategy: Mammalian one-hybrid system
4.2.2.3 Assessment of the ability of mutant proteins to bind the corepressor TLE1

As previously described, the repressing activity of HESX1 is largely mediated by its ability to bind TLE1, which is mainly facilitated through the eh1 domain at the N-terminus of HESX1. To investigate the effect of the mutations on protein-protein interactions, a mammalian two-hybrid system was employed. This method is a genetic, in vivo assay based on the reconstitution of the function of a transcriptional activator through the interaction of two proteins: one containing a DNA binding domain and another one with a transcriptional activator function (Luo et al., 1997). Subsequent interaction between these two chimeric proteins in a mammalian cell line will result in the activation of the reporter gene.

Our assay utilised a promoter-less luciferase reporter plasmid containing the PIII binding sites, and the Gal4-HESX1 and Tle1-VP16 effector plasmids. As a result, binding to the reporter is mediated through the HESX1-moiety of the GAL4-HESX1 fusion protein (Figure 4.6a-b). Interaction of wild-type HESX1 with TLE1 was demonstrated by the significant elevation of luciferase expression in the co-transfected cells relative to control transfections (approximately 17 fold-increase; Figure 4.6c). Similarly, the S67T mutant protein was able to interact with TLE1 and luciferase expression levels were essentially identical to those obtained with wild-type HESX1 (Figure 4.6c). In contrast, expression of either F156S or R109X together with TLE1-VP16 resulted in activation of the reporter to levels comparable to those in the control transfections (Figure 4.6c).

In an attempt to assess the ability of the HESX1 wild-type and mutant proteins to bind TLE1 independent on DNA binding, a similar mammalian two-hybrid system was adopted, but using a reporter containing GAL4-binding sites instead. Unfortunately, neither wild-type HESX1 nor the mutant proteins analysed were able to demonstrate any interaction with TLE1, upon multiple attempts (data not shown). The reason for this result remains unclear; however, it may suggest that the HESX1/TLE1 interaction requires HESX1 DNA binding.
Figure 4.6 - Analysis of the interaction between the corepressor TLE1 and the HESX1 mutant proteins

(a-b) The TLE1/HESX1 interaction was analysed using a reporter plasmid containing six dimeric paired class binding sites, which HESX1 binds to, and is therefore dependent of the HESX1-DNA binding capabilities (6PIII-SV40-Luciferase reporter). (a) Under basal condition, luciferase activity is minimal due to lack of the SV40 promoter. (b) Subsequently, HESX1 ability to bind the VP16-fused TLE1 through the eh1 domain results in a dramatic increase in luciferase activity. (c) GAL4-HESX1 expression constructs (50 ng) were transfected with plasmids encoding the TLE1 fused to the activation domain of the VP16 viral protein (200 ng) and the reporter plasmid (100 ng). Wild-type HESX1 and S67T mutant protein showed a 17-fold increase in basal luciferase activity. In contrast, both R109X and F156S showed levels of luciferase activity comparable to those obtained with empty expression vectors, suggesting a failure to interact with TLE1-VP16. Asterisks indicate significant differences of mutant proteins compared with wild-type HESX1 (t-test; p <0.001).
Strategy: Mammalian two-hybrid system
4.3 CONCLUSIONS

Together, these *in vitro* studies demonstrate that the R109X- and F156S-HESX1 mutant proteins have the capability to repress transcription, possibly through interaction with TLE1, as the eh1 domain remains unaffected in both mutant proteins. However, their DNA-binding properties are disrupted by the F to S substitution or the lack of the entire homeodomain. Of interest, the finding that the R109X protein was weakly expressed when expressed as a HA-tagged protein implies that this protein may be unstable *in vivo* and susceptible to degradation. Finally, the S67T mutant protein is indistinguishable from wild-type HESX1 in the tested assays, suggesting that this could be an example of a rare polymorphism.
CHAPTER 5: ANALYSIS OF HYPOTHALAMIC PITUITARY AXIS
DEFECTS IN $HESX1^{CRE/+};SOX2^{FL/FL}$ MICE
5.1 INTRODUCTION

As *Hesx1* is one of the earliest markers to be expressed within the developing RP, the *Hesx1<sup>Cro/+</sup>* mouse model has provided a useful tool in conditionally activating/depleting factors for the investigation of their role during physiological and pathological pituitary development (Andoniadou et al., 2007; Hermesz et al., 1996).

Recently, the transcription factor SOX2, a member of the B1 subfamily of HMG proteins, has been shown to play a crucial role in pituitary, forebrain and eye development in humans (Kelberman et al., 2006; Kelberman et al., 2008). Furthermore, Sox2 expression is conserved within the hypothalamus and pituitary in mouse, making this developmental model an ideal avenue to explore the role of this transcription factor during hypothalamic-pituitary organogenesis (Fauquier et al., 2008; Kelberman et al., 2006). However, consistent with the requirement of Sox2 during early embryogenesis, Sox2<sup>−/−</sup> embryos do not survive implantation, hampering the investigation of its function during later development (Avilion et al., 2003). To circumvent this problem, many groups have applied the Cre/LoxP system to conditionally remove Sox2 from their tissue of interest. Formerly, studies have shown an essential role of Sox2 in the development of the CNS, in maintaining neuronal progenitor identity (Bylund et al., 2003; Graham et al., 2003). Moreover, elegant studies using various combinations of hypomorphic Sox2 mouse models have identified that Sox2 is required during normal retinal development to maintain proliferative and differentiation capacity in neural progenitor cells, in a manner that is dose-dependent (Taranova et al., 2006).

In this chapter, the function of Sox2 was investigated by deleting Sox2 using the *Hesx1<sup>Cro/+</sup>* and Sox2<sup>fl/fl</sup> mouse lines with the aim to provide further insights into the function of this important transcription factor within the developing mammalian pituitary. In addition, this conditional approach was utilised to reveal insights into the pathogenesis of the hypothalamic-pituitary abnormalities in humans with SOX2 haploinsufficiency (Section 1.6.4).
5.2 RESULTS

5.2.1 Genotypic analysis of Hesx1\textsuperscript{Cre+};Sox2\textsuperscript{fl/fl} embryos and mice

Pre-implantation lethality of Sox2-null embryos (Avilion et al., 2003) impedes investigation of the role of Sox2 within the developing pituitary gland. Therefore, in order to assess the primary function of Sox2 in this tissue, we employed the Hesx1\textsuperscript{Cre+} animals (Andoniadou et al., 2007) to be crossed with Sox2\textsuperscript{ββ} mice (Taranova et al., 2006). The latter animals carry a loxP-Sox2-loxP conditional allele that can be excised in Cre-expressing cells, such as the developing pituitary gland in the case of the Hesx1\textsuperscript{Cre+} animals. Previous studies from our laboratory have shown that the Hesx1 promoter drives Cre expression from the Hesx1\textsuperscript{Cre} allele in RP progenitors fated to become hormone-producing cells in the AP later in gestation (Andoniadou et al., 2007; Gaston-Massuet et al., 2011).

Genotypic analysis of embryos between 9.5-18.5 dpc derived from crosses between Hesx1\textsuperscript{Cre+};Sox2\textsuperscript{fl/+} animals and either Sox2\textsuperscript{fl/+} or Sox2\textsuperscript{fl/fl} animals revealed no statistical difference from expected numbers (Tables 5.1 and 5.2; Sox2\textsuperscript{fl/+}: n=136, Chi-square test, \( p = 0.780 \); Sox2\textsuperscript{fl/fl}: n=165, Chi-square test, \( p = 0.230 \)). In contrast, genotyping of animals from birth to weaning failed to identify any viable Hesx1\textsuperscript{Cre+};Sox2\textsuperscript{fl/fl} animals (Tables 5.1 and 5.2; n=0/88; Sox2\textsuperscript{fl/+}: Chi-square test, \( p = 0.010 \); Sox2\textsuperscript{fl/fl}: n=0/48; Chi-square test, \( p <0.001 \)). Collectively, these data suggest that Sox2 deletion from the Hesx1 expression domain results in neonatal lethality.
Table 5.1 - Genotypes obtained from *Hesx1*<sub>Cre/+<sub>;<Sox2<sup>fl/+</sup> x *Sox2<sup>fl/fl</sup> intercrosses</p>

<table>
<thead>
<tr>
<th>Stage</th>
<th>*Sox2&lt;sup&gt;+&lt;/sup&gt;/; *Hesx1&lt;sup&gt;+&lt;/sup&gt;/</th>
<th>*Sox2&lt;sup&gt;+&lt;/sup&gt;/; *Hesx1&lt;sub&gt;Cre/+&lt;/sub&gt;/</th>
<th>*Sox2&lt;sup&gt;fl/+&lt;/sup&gt;; *Hesx1&lt;sup&gt;+&lt;/sup&gt;/</th>
<th>*Sox2&lt;sup&gt;fl/+&lt;/sup&gt;; *Hesx1&lt;sub&gt;Cre/+&lt;/sub&gt;/</th>
<th>*Sox2&lt;sup&gt;fl/fl&lt;/sup&gt;; *Hesx1&lt;sup&gt;+&lt;/sup&gt;/</th>
<th>*Sox2&lt;sup&gt;fl/fl&lt;/sup&gt;; *Hesx1&lt;sub&gt;Cre/+&lt;/sub&gt;/</th>
<th>Total</th>
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<tbody>
<tr>
<td></td>
<td>(12.5%)</td>
<td>(12.5%)</td>
<td>(25%)</td>
<td>(25%)</td>
<td>(12.5%)</td>
<td>(12.5%)</td>
<td></td>
</tr>
<tr>
<td>10.5 dpc</td>
<td>5</td>
<td>6</td>
<td>8</td>
<td>3</td>
<td>8</td>
<td>4</td>
<td>34</td>
</tr>
<tr>
<td>12.5 dpc</td>
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<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
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<td>4</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>20</td>
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<tr>
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<td>8</td>
<td>6</td>
<td>4</td>
<td>6</td>
<td>29</td>
</tr>
<tr>
<td>17.5 dpc</td>
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<td>3</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>18</td>
</tr>
<tr>
<td>18.5 dpc</td>
<td>1</td>
<td>2</td>
<td>8</td>
<td>7</td>
<td>3</td>
<td>5</td>
<td>26</td>
</tr>
</tbody>
</table>

Embryos† (% observed) (12.5%) (13%) (24%) (18%) (16%) (15.5%)

Pups (% observed) (21.5%) (17%) (18%) (29.5%) (13.5%)

a Derived from expected Mendelian ratios.

*Chi-square test showed a significant deviation from the expected 12.5% ratio (P=0.010).

†Chi-square test showed no significant deviation from the expected Mendelian ratio.

Table 5.2 - Genotypes obtained from *Hesx1*<sub>Cre/+<sub>;<Sox2<sup>fl/+</sup> x *Sox2<sup>fl/fl</sup> intercrosses</p>

<table>
<thead>
<tr>
<th>Stage</th>
<th>*Hesx1&lt;sup&gt;+&lt;/sup&gt;/; *Sox2&lt;sup&gt;fl/+&lt;/sup&gt; (25%)</th>
<th>*Hesx1&lt;sup&gt;+&lt;/sup&gt;/; *Sox2&lt;sup&gt;fl/fl&lt;/sup&gt; (25%)</th>
<th>*Hesx1&lt;sub&gt;Cre/+&lt;/sub&gt;/; *Sox2&lt;sup&gt;fl/+&lt;/sup&gt; (25%)</th>
<th>*Hesx1&lt;sub&gt;Cre/+&lt;/sub&gt;/; *Sox2&lt;sup&gt;fl/fl&lt;/sup&gt; (25%)</th>
<th>Total</th>
</tr>
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<td>4</td>
<td>3</td>
<td>10</td>
<td>9</td>
<td>26</td>
</tr>
<tr>
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<td>6</td>
<td>6</td>
<td>4</td>
<td>21</td>
</tr>
<tr>
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<td>2</td>
<td>1</td>
<td>6</td>
</tr>
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<td>15</td>
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<td>41</td>
</tr>
<tr>
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<td>3</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
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<td>5</td>
<td>8</td>
<td>16</td>
<td>6</td>
<td>35</td>
</tr>
<tr>
<td>18.5 dpc</td>
<td>7</td>
<td>7</td>
<td>6</td>
<td>8</td>
<td>28</td>
</tr>
</tbody>
</table>

Embryos† (% observed) (20%) (21%) (35%) (24%)

Pups (% observed) (54%) (14.5%) (31%) (0*)

a Derived from expected Mendelian ratios.

*Chi-square test showed a significant deviation from the expected 25% ratio (P<0.001).

†Chi-square test showed no significant deviation from the expected Mendelian ratio.
5.2.2 Histological analysis of the developing pituitary gland in \textit{Hesx1}^{Cre/+};\textit{Sox2}^{fl/fl} embryos

To assess the consequences of \textit{Sox2} loss in embryonic pituitary organogenesis, embryos were analysed histologically from 9.5 dpc, when RP induction is proceeding, to 18.5 dpc immediately prior to birth, in \textit{Hesx1}^{Cre/+};\textit{Sox2}^{fl/fl} and control (\textit{Hesx1}^{Cre/+};\textit{Sox2}^{fl/+} and all remaining genotypes) littermates. To ensure that similar levels of the pituitary were used for comparisons, the presence of the infundibulum in each section was used as a landmark.

Haematoxylin and eosin staining showed that the developing RP at 9.5-10.5 dpc in \textit{Hesx1}^{Cre/+};\textit{Sox2}^{fl/fl} embryos appeared morphologically comparable to control littermates (Figure 5.1a-b; \(n=81\)). The first apparent evidence of a morphological defect was observable by 12.5 dpc (Figure 5.1c-d; \(n=50\)). Typically it presented as small AP hypoplasia, which was further pronounced by 14.5 dpc (Figure 5.1e-f; \(n=35\)). By 18.5 dpc, mutant pituitaries displayed a fully penetrant phenotype of severe AP hypoplasia with remnants of pituitary tissue often embedded within the oral ectoderm (Figure 5.1g-i; \(n=54\)). As a consequence, normal development of the basosphenoid cartilage was disrupted in these mutant embryos, remaining as two separate entities surrounding the ectopically located AP tissue. In contrast, the PP and IL were grossly normal in \textit{Hesx1}^{Cre/+};\textit{Sox2}^{fl/fl} mutants (Figure 5.1g-h; \(n=54\)). To summarise, this analysis suggests that the initial induction of the RP occurs as normal in the \textit{Sox2}-deficient pituitaries, but subsequently, absence of \textit{Sox2} results in the developmental failure of the AP to thrive, ultimately resulting in a small and partially ectopic AP at late gestation.
Figure 5.1 - Conditional deletion of Sox2 from the developing RP results in a hypoplastic pituitary at late gestation

Haematoxylin and eosin staining on (a-f) sagittal and (g-i) frontal sections of control and Hesx1^Cre/++;Sox2^fl/fl pituitaries throughout organogenesis. (a-b) At 9.5 dpc, invagination of RP from the oral ectoderm is comparable between genotypes. (c-d) By 12.5 dpc, mutant pituitaries appear dysmorphic demonstrating early signs of AP hypoplasia when compared to control sections, a phenotype that is exacerbated by (e-f) 14.5 dpc. (g-h) By 18.5 dpc, severe AP hypoplasia is observed in mutant pituitaries. (h) In addition, disruption of basosphenoid tissue can be detected, where the AP remains attached to the oral cavity inhibiting the basosphenoid bone to fuse correctly. (i) Note, development of the PP remains largely unaffected. Abbreviations: AP, anterior pituitary; BS, basosphenoid tissue; Hypo, hypothalamus; Inf, infundibulum; OR, oral cavity; PP, posterior pituitary; RP, Rathke’s pouch; VD, ventral diencephalon. Scale bars, 100 µm.
5.2.3 Assessment of the levels of recombination of the Sox2 locus in 

$Hesx1^{Cre/+};Sox2^{fl/fl}$

To analyse the effectiveness of the Cre-mediated excision of Sox2 within the developing pituitary and hypothalamus in the $Hesx1^{Cre/+};Sox2^{fl/fl}$ mouse model, immunofluorescence using a specific SOX2 antibody was performed on control and mutant sections. Sections from 10.5 dpc control embryos demonstrated that SOX2 expression is detectable uniformly throughout RP. In contrast, SOX2 labelling revealed a dorso-ventral gradient within the ventral diencephalon, with SOX2 protein being highly expressed dorsally (Figure 5.2a; $n=3$). Analysis of mutant sections at this stage revealed a comparable staining of SOX2, both within RP and ventral diencephalon (Figure 5.2b; $n=3$). This equivalent expression of SOX2 between mutant and controls at these early stages is likely due to SOX2 protein persistence rather than inefficiency of the Cre protein, as it has been previously demonstrated that $Hesx1$-Cre mediated excision using the Rosa26-loxP-Stop-loxP-YFP mouse line results in extensive YFP staining within RP at 9.5-10.5 dpc (Chapter 3). By 12.5 dpc, little or no SOX2 label was detectable in mutant pituitaries, in contrast to control pituitaries where SOX2 protein was localised dorsally within RP. Of relevance to this study, SOX2 expression within the prospective hypothalamus and infundibulum was comparable between genotypes at this stage (Figure 5.2c-d, c’, d’, $n=6$ embryos per genotype). Absence of SOX2 from the periluminal region of the AP was also confirmed in 18.5 dpc mutant embryos (Figure 5.2f, f’; $n=4$).
Figure 5.2 - SOX2 is excised from RP by 12.5 dpc

Immunofluorescence against SOX2 on (a-d) sagittal and (e-f) frontal sections through control and Hesx1\textsuperscript{Cre\textasciitilde};Sox2\textsuperscript{fl/fl} pituitaries during organogenesis. (a-b) At 10.5 dpc, SOX2 is detectable throughout the developing RP in a similar pattern between control and mutant embryos. Comparable labelling between genotypes is also observed in the ventral diencephalon, which shows a dorsal-ventral gradient within this tissue. (c-d) By 12.5 dpc, equal staining of SOX2 can be seen within the developing infundibulum in control and mutant sections. (c’) In control sections, SOX2 staining within the developing pituitary is confined to RP. (d’) In contrast, mutant pituitaries show little SOX2 staining throughout the developing pituitary at this stage. This finding is later confirmed at 18.5 dpc. (e) In control pituitaries, SOX2 is mainly restricted to the periluminal regions of the AP. Few SOX2\textsuperscript{+ve} cells are also dispersed within the AP. (f) In contrast, little SOX2 staining is noted within the entire AP in mutant embryos. Abbreviations: AL, anterior lobe; AP, anterior pituitary; IL, intermediate lobe; Inf, infundibulum; PL, posterior lobe; RP, Rathke’s pouch; VD, ventral diencephalon. Scale bars, 100 µm.
5.2.4 Early pituitary patterning in \textit{Hesx1}^{Cre/+};\textit{Sox2}^{0/0} embryos

As in many other organs, the development of the AP relies on the presence of morphogenic gradients that are determined by the concerted actions of multiple signalling molecules emanating from both the overlying ventral diencephalon/infundibulum and surrounding mesenchyme. \textit{Bmp4}, a member of the Bmp family, is one of the earliest signalling molecules known to be expressed in the presumptive infundibulum and is necessary for the invagination and initial specification of RP progenitors within the oral ectoderm (Davis and Camper, 2007; Ericson et al., 1998; Treier et al., 1998). Similarly, members of the Fgf family, \textit{Fgf8} and \textit{Fgf10}, are expressed within this region of the developing ventral diencephalon/infundibulum and are required for the maintenance and proliferation of RP precursors through the induction of LIM homeodomain proteins, \textit{Lhx3} and \textit{Lhx4} (Sheng et al., 1996; Sheng et al., 1997). At 9.5-10.5 dpc, the expression domains of \textit{Bmp4}, \textit{Fgf8} and \textit{Fgf10} in the ventral diencephalon were comparable between genotypes, as was the expression domain of the pituitary-specific transcription factor, \textit{Lhx3} within RP (Figure 5.3a-f, i-j; \textit{n}=3 embryos each). Consistent with these data, p-ERK, a downstream effector of the Fgf signalling pathway, appeared unaltered in \textit{Hesx1}^{Cre/+};\textit{Sox2}^{0/0} embryos when compared to stage-matched control embryos (Figure 5.3g-h, \textit{n}=4 each).
Figure 5.3 - Early patterning of the ventral diencephalon and pituitary is unaltered in $Hesx1^{Cre+};Sox2^{bop}$ embryos

(a-f, i-l) *In situ* hybridisation and (g-h) immunofluorescence on control and mutant sagittal sections between 9.5 and 10.5 dpc. The expression patterns and levels for (a-b) *Bmp4*, (c-d) *Fgf8* and (e-f) *Fgf10* transcripts and (g-h) p-ERK protein within the prospective hypothalamus are comparable between genotypes. Furthermore, similar expression of *Lhx3* mRNA is detectable within the RP at this stage. Abbreviations: RP, Rathke’s pouch; VD, ventral diencephalon. Scale bars, 100 µm; (a-f; g-h; i-l).
In addition to the early roles that these signalling molecules play during induction and specification of RP, maintenance of these signals through to mid-gestation is required for the proper proliferation of pituitary progenitors (Ericson et al., 1998; Treier et al., 1998). Given this, premature attenuation of these signals could equally contribute to the hypoplastic phenotype observed in the \textit{Hesx1}^{Cre/\ast};\textit{Sox2}^{0/0} pituitaries. \textit{In situ} hybridisation against \textit{Bmp4} (Figure 5.4a-b; \( n=3 \)) and \textit{Fgf8} (Figure 5.4c-d; \( n=3 \)) failed to reveal any differences within the ventral diencephalon in control and mutant embryos at 12.5 dpc. In contrast, the expression domain of \textit{Lhx3} in \textit{Hesx1}^{Cre/\ast};\textit{Sox2}^{0/0} pituitaries was clearly reduced in a pattern corresponding to the overall size of pituitary tissue at this stage, when compared to controls (Figure 5.10a-b; \( n=3 \)). Taken together, it is likely that the hypoplastic pituitary described is due to an intrinsic effect of the depleted \textit{Sox2} within the developing pituitary, rather than the consequence of aberrant hypothalamic signalling.
Figure 5.4 - Patterning of the ventral diencephalon remains unaltered in $Hesx1^{Cre/+};Sox2^{fl/fl}$ embryos

*In situ* hybridisation on sagittal sections through the ventral diencephalon and pituitary of control and mutant embryos at 12.5 dpc. The expression of (a-b) *Bmp4* and (c-d) *Fgf8* transcripts persists within the developing hypothalamus to a similar degree in control and mutant embryos. Abbreviations: Inf, infundibulum; RP, Rathke’s pouch; VD, ventral diencephalon. Scale bar, 100 µm.
5.2.5 Terminal differentiation of somatotrophs and mature thyrotrophs is disrupted in Hesx1\textsuperscript{Cre/+};Sox2\textsuperscript{fl/fl} pituitaries

Immunohistochemistry against pituitary hormones at 18.5 dpc revealed a substantial reduction of somatotrophs (GH\textsuperscript{+ve}) and thyrotrophs (TSH\textsuperscript{+ve}) in Hesx1\textsuperscript{Cre/+};Sox2\textsuperscript{fl/fl} mutant pituitaries, with only a few scattered positive cells identified within serial sections of the pituitary (Figure 5.5a-d; \(n=3\) pituitaries each). In contrast to this, the staining pattern of corticotrophs/melanotrophs (ACTH\textsuperscript{+ve}) and thyrotrophs/gonadotrophs (αGSU\textsuperscript{+ve}) cells appeared more comparable between genotypes (Figure 5.5i-l; \(n=3\)). Immunoreactivity against FSH and LH further confirmed the presence of the gonadotrophs within Hesx1\textsuperscript{Cre/+};Sox2\textsuperscript{fl/fl} pituitaries (Figure 5.5m-p; \(n=3\) ). Finally, lactotrophs (PRL\textsuperscript{+ve}) were detectable in mutant pituitaries, as shown by immunolabelling against PRL (Figure 5.5e-f; \(n=3\)) and further confirmed by \textit{in situ} analysis using a specific Prl anti-sense riboprobe (Figure 5.6a-b; \(n=2\)).
**Figure 5.5 - Sox2 depletion from the developing pituitary gland results in a reduction of somatotrophs and thyrotrophs at late gestation**

Immunohistochemistry against terminal differentiation markers on frontal sections through control and *Hesx1* 
^cre/+/;Sox2*fl/fl* pituitaries at 18.5 dpc. Numbers of (a-b) somatotrophs (GH^+ve^) and (c-d) thyrotrophs (TSH^+ve^) are severely reduced in *Hesx1* 
^cre/+/;Sox2*fl/fl* pituitaries when compared to controls. The few positive somatotrophs and thyrotrhps detected in mutant sections are indicated by arrowheads in b and d, respectively. Similarly, (g-h) immunoreactivity against PIT1 is greatly diminished in mutant pituitaries when compared to controls. In contrast, (e-f) lactotrophs (PRL^+ve^), (i-j) corticotrophs and melanotrophs (ACTH^+ve^), and (k-p) gonadotrophs (αGSU^+ve^, FSH^+ve^ and LH^+ve^) are present within mutant pituitaries, despite the reduction in AP tissue. Abbreviations: AL, anterior lobe; IL, intermediate lobe, PL, posterior lobe. Scale bar, 100 µm.
Figure 5.6 - *Prl* mRNA transcripts are present within *Hesx1*<sup>Cre/+;Sox2</sup><sup>fl/fl</sup> pituitaries

(a-b) *In situ* hybridisation using a specific *Prl* anti-sense riboprobe on control and mutant pituitary frontal sections at 18.5 dpc. *Prl*-expressing cells are scattered throughout the AP in both control and mutant sections. Abbreviations: AL, anterior lobe; IL, intermediate lobe; PL, posterior lobe. Scale bar, 100 µm.
Ontogenic analyses have previously illustrated that the initial expression of Pit1 within the caudo-medial pituitary at 14.5 dpc is required for the subsequent activation of Gh, Prl and Tshβ genes. Furthermore, throughout development and into adulthood, Pit1 gene expression is known to persist within somatotrophs, lactotrophs and thyrotrophs (Dolle et al., 1990; Li et al., 1990; Simmons et al., 1990). Our terminal differentiation data thereby imply a defect within the differentiation of the Pit1 cell lineage. To explore this hypothesis, immunostaining against PIT1 was conducted between 12.5 dpc, prior to normal PIT1 induction, through to 18.5 dpc. At all stages analysed from 14.5 dpc, when PIT1 expression commences, it was apparent that there was a severe reduction in the number of PIT1+ve cells (Figures 5.7a-d, 5.8a-b, 5.5g-h; n=3 embryos per genotype at each stage). As observed at late gestation, this finding appeared to be restricted solely to the Pit1 cell lineage, as immunostaining against ACTH and αGSU revealed little difference in expression between control and mutant pituitaries at 15.5 dpc (Figure 5.8c-f; n=4).

Immunofluorescence against GH and TSH at 15.5 dpc complements our hypothesis of defective cell lineage commitment in the Hesx1Cre+/+;Sox2ββ mutant pituitaries. At 15.5 dpc, few somatotrophs were identifiable in control pituitaries as determined by the presence of GH+ve cells (Figure 5.9a; n=3). Similar to the terminal differentiation data at 18.5 dpc, no somatotrophs were detectable in Hesx1Cre+/+;Sox2ββ pituitaries (Figure 5.9b; n=3). In contrast, immunofluorescence against TSH clearly revealed the loss of the Pit1-dependent thyrotrophs from the caudo-medial region of the developing anterior lobe in Hesx1Cre+/+;Sox2ββ mutants, whereas the Pit1-independent population of rostral tip thyrotrophs were comparable between genotypes (Figure 5.9c-d; n=4).
Figure 5.7 - Pit1 cell lineage specification is not initiated in \textit{Hesx1}^{Cre/+},\textit{Sox2}^{fl/fl} pituitaries

Immunofluorescence against PIT1 on sagittal control and mutant pituitary sections at (a-b) 12.5 and (c-d) 14.5 dpc. (a-b) At 12.5 dpc, no PIT1$^{\text{+ve}}$ cells are detectable within the developing AP of control and mutant embryos. (c-d) By 14.5 dpc, PIT1$^{\text{+ve}}$ cells occupy a large proportion of the developing AP in control pituitaries, however, no PIT1$^{\text{+ve}}$ cells are detected in mutant pituitaries at this stage. Inserts show high-magnification images of boxed regions. Abbreviations: AP, anterior pituitary; Inf, infundibulum. Scale bar, 100 µm.
Figure 5.8 - Removal of Sox2 from the developing pituitary gland results in a reduction of PIT1\textsuperscript{+ve} cells

Immunofluorescence on frontal sections through pituitaries from control and $Hesx1^{Cre/+};Sox2^{fl/fl}$ embryos at 15.5 dpc. (a) PIT1\textsuperscript{+ve} cells appear to occupy a large proportion of the AP in control sections. (b) In contrast, very few or no PIT1\textsuperscript{+ve} cells are detectable in $Hesx1^{Cre/+};Sox2^{fl/fl}$ pituitaries. (c-d) ACTH\textsuperscript{+ve} cells are dispersed throughout the AP and IL of control and mutant pituitaries. (e-f) Likewise, comparable distribution of αGSU\textsuperscript{+ve} cells is located within the AP between genotypes. Inserts show high-magnification images of boxed regions. Yellow signal represents autofluorescent blood cells. Abbreviations: AL, anterior lobe; IL, intermediate lobe; PL, posterior lobe. Scale bar, 100 µm.
Figure 5.9 - Pit1-dependent somatotrophs and thyrotrophs are absent in \(Hesx1^{Cre/+};Sox2^{ff/+}\) pituitaries

Immunofluorescence labelling against GH and TSH on sagittal sections through control and mutant pituitaries at 15.5 dpc. (a) Emergence of somatotrophs, as labelled by GH antibody, commences as a positive cell, present within the caudo-medial region of the AP of control embryos (depicted by arrowhead in a’’). (b) No somatotrophs are detectable throughout \(Hesx1^{Cre/+};Sox2^{ff/+}\) pituitaries. TSH\(^{+ve}\) cells represent two separate populations within the developing pituitary gland; Pit1-independent TSH\(^{+ve}\) cells localised to the rostral-tip of the AP, and a succeeding Pit1-dependent population of TSH\(^{+ve}\) found within the caudo-medial AP. (c) The presence of TSH\(^{+ve}\) cells is clearly detectable within both the (c’) rostral tip and (c’’) caudo-medial region of the developing AP in control embryos. (d) Thyrotrophs are present within the (d’) rostral tip of the developing AP, however they are not detectable within the (d’’) caudo-medial AP of \(Hesx1^{Cre/+};Sox2^{ff/+}\) embryos. High-magnification images of boxed regions shown below each picture. Yellow signal represents autofluorescent blood cells. Abbreviations: Inf, infundibulum. Scale bar, 100 \(\mu\)m.
Analysis of two Prop1 mouse models, the spontaneous Ames dwarf (Prop1\textsuperscript{df}) and the genetically engineered Prop1\textsuperscript{null} mouse has highlighted a positive role of Prop1 in the determination of the Pit1 lineages (Andersen et al., 1995; Sornson et al., 1996). \textit{In situ} hybridisation against Prop1 demonstrated a clear reduction of Prop1 expression in mutant pituitaries compared with control genotypes at 12.5 dpc (Figure 5.10c-f; \textit{n}=4). NeuroD1, a member of the bHLH family of transcription factors, is required for the differentiation of the corticotroph cell population through the activation of Pomc1 transcription (Lamolet et al., 2004; Poulin et al., 1997). Analysis of NeuroD1 at 12.5 dpc revealed a complementary pattern of expression within the ventral region to that of Prop1 in both control and mutant pituitaries. Specifically, NeuroD1 appeared to expand dorsally in Hesx1\textsuperscript{Cre/+};Sox2\textsuperscript{fl/fl} mutant pituitaries (Figure 5.10g-h; \textit{n}=4). Despite this, little difference in the expression of Pomc1 at 13.5 dpc was identified between genotypes (Figure 5.10i-j; \textit{n}=3).
Figure 5.10 - Altered expression of intermediate lineage commitment markers in $Hesx1^{Cre+};Sox2^{fl/fl}$ pituitaries

In situ hybridisation on (a-h) sagittal and (i-j) frontal sections through pituitaries of control and mutant 12.5 dpc embryos. (a-b) The $Lhx3$ expression domain is reduced in mutant pituitaries when compared to control pituitaries at 12.5 dpc, reflecting an overall reduction in pituitary size. (c-f) The expression levels of $Prop1$ mRNA transcripts are greatly reduced in mutant pituitaries when compared to controls, as demonstrated at two different axial levels. (g-h) Conversely, the expression of $NeuroD1$ transcripts appear dorsally expanded in $Hesx1^{Cre+};Sox2^{fl/fl}$ pituitaries in a complementary pattern to $Prop1$ expression. (i-j) However, $Pomc1$ mRNA levels appear comparable between genotypes. AP, anterior pituitary; Abbreviations: AP, anterior pituitary; Inf, infundibulum. Scale bar, 100 µm.
To conclude, the data presented so far suggest that the intrinsic loss of Sox2 from the developing pituitary gland results in a severe reduction of Pit1-dependent somatotrophs and thyrotrophs, which could in-part be explained by an initial reduction in Prop1 expression. Surprisingly, lactotrophs fully differentiate and express PRL despite the lack of PIT1+ve cells. Lastly, corticotrophs and gonadotrophs are able to fully differentiate within the Sox2-deficient pituitaries irrespective of the overall reduction in AP tissue.

5.2.6 Reduced proliferation of RP progenitors in Hesx1(Cre);Sox2(fl/fl) mutant pituitaries

The observed hypoplasia of the AP in the Hesx1(Cre);Sox2(fl/fl) mutants could be due to increased apoptosis of RP progenitors, decreased proliferation, premature differentiation, and/or a combination of these factors.

Cell death is thought to have an early and transitory role in normal pituitary organogenesis. Although little cell death occurs within RP and the developing AP, apoptotic cells are restricted to the border between the invaginating pouch and the surrounding oral ectoderm tissue between 10.5 and 12.5 dpc, where they are believed to play an important role in the separation of the pouch from the rest of the oral ectoderm (Charles et al., 2005). Immunolabelling against cleaved caspase-3, an indicator of apoptosis, at 12.5 dpc clearly identified apoptotic cells within the oral ectoderm adjacent to the developing pouch in both control and mutant sections (Figure 5.11a-b, a’’, b’’; n=3). However, no apoptotic cells were detected within control and mutant pituitaries at both 12.5 and 14.5 dpc (Figure 5.11a’, b’, c-d; n=3 pituitaries per genotype per timepoint). Analyses at these two timepoints were undertaken, as these are the stages at which the morphological defects first appear and evolve.
Figure 5.11 - Increased apoptotic cell death is not observed in Hesx1<sup>Cre/+;Sox2<sup>fl/fl</sup></sup> pituitaries

Immunofluorescence against activated caspase-3 on sagittal sections through the pituitary glands of control and mutant embryos at (a-b) 12.5 and (c-d) 14.5 dpc. (a-b) At 12.5 dpc, control sections reveal cells undergoing apoptosis within the oral ectoderm immediately adjacent to the developing pituitary (between arrowheads in a, magnified region insert a’’). A similar expression pattern of cleaved caspase-3 is also evident in mutant embryos (between arrowheads in b, magnified region insert b’’). In both control and mutant pituitaries, no activated caspase-3 positive cells are detected within RP and developing AP, at (a-b, magnified region inserts a’-b’) 12.5 and at (c-d) 14.5 dpc. Yellow signal represents autofluorescent blood cells. Abbreviations: AP, anterior pituitary; Inf, infundibulum. Scale bar, 100 µm.
Recent studies have demonstrated a role for the Cip/Kip family of negative cell cycle regulators during a crucial time for pituitary development, namely when proliferating cells exit the cell cycle, and prepare to undergo differentiation. \( p57^{Kip2} \), in conjunction with Cyclin E, appears to control the cell cycle exit of proliferating precursors generating a population of non-cycling progenitor cells. Induction of \( p57^{Kip2} \) occurs in non-cycling cells progressing into differentiation. Subsequently, \( p27^{Kip1} \), another cell cycle inhibitor, is expressed in differentiated cells and acts to prevent these cells from re-entering the cell cycle (Bilodeau et al., 2009). To investigate whether premature cell cycle exit and differentiation could contribute to the hypoplastic AP, expression of these two cell cycle regulators was analysed in control and mutant pituitaries at 12.5 and 14.5 dpc. At all stages, negligible differences in the expression of \( p57^{Kip2} \) were observed between genotypes (Figure 5.12a-d; \( n=3 \) embryos each per timepoint). However at both stages, a modest increase in \( p27^{Kip1} \) was noted in mutant pituitaries when compared to control sections (Figure 5.12e-f; \( n=3 \) embryos per genotype per timepoint). To investigate this finding further, subsequent immunofluorescence labelling against ACTH, \( \alpha \)GSU and TPIT, all early lineage commitment markers, was performed at developmental stages immediately prior to when their normal gene expression first commences. These analyses did not show any temporal alterations in the onset of expression of these markers in the developing RP and AP in \( Hesx1^{Cre+};Sox2^{fl/fl} \) pituitaries when compared to control genotypes (Figure 5.13a-f; \( n=4 \)). Furthermore, analysis of the TPIT immunolabel at 14.5 dpc in control and mutant pituitaries did not identify any overt abnormalities (Figure 5.13g-h; \( n=3 \)).
Figure 5.12 - Mild increased expression of $p27^{kip1}$, but not $p57^{kip2}$, in $Hesx1^{Cre/+};Sox2^{fl/fl}$ mutant pituitaries

Immunostaining on sagittal sections through pituitaries of control and mutant embryos at 12.5 and 14.5 dpc. (a-d) At both stages, expression of $p57^{kip2}$, a marker of cell cycle exit, is widely distributed within RP progenitors and the developing pituitary gland. Negligible differences between genotypes are apparent. (e-h) In contrast, the expression domain of $p27^{kip1}$, an indicator of differentiating cells, appears to be increased in mutant pituitaries at both (e-f) 12.5 and (g-h) 14.5 dpc. Inserts show high-magnification images of boxed regions in c-d and g-h. Yellow signal represents autofluorescent blood cells. Abbreviations: BS, basosphenoid tissue; Inf, infundibulum. Scale bar, 100 µm.
Figure 5.13 - Premature differentiation does not contribute to the hypoplasia of Hesx1*Cre/+;Sox2<sup>fl/fl</sup> pituitaries

Immunofluorescence on sagittal pituitary sections from control and mutant embryos at (a-f) 12.5 and (g-h) 14.5 dpc. At 12.5 dpc, no (a-b) ACTH<sup>+</sup>, (c-d) αGSU<sup>+</sup>, (e-f) TPIT<sup>+</sup> cells were detected within RP and developing AP in both control and mutant pituitaries. At this stage similar expression of ACTH<sup>+</sup> and TPIT<sup>+</sup> cells within the developing ventral diencephalon was observed between genotypes (as indicated by arrowheads in a-b and e-f, respectively). (g-h) At 14.5 dpc, comparable expression of TPIT was present within the developing AP of control and Hesx1*Cre/+;Sox2<sup>fl/fl</sup> pituitaries. Inserts show high-magnification images of boxed regions. Abbreviations: AP, anterior pituitary; Inf, infundibulum; VD, ventral diencephalon. Scale bar, 100 µm.
Thus, despite the moderate increase in expression of p27<sup>Kip1</sup>, neither cell death nor premature differentiation appear to be important mediators of the pituitary hypoplasia in the Sox2-deficient pituitaries, and thereby implicate that proliferation rates are likely to be altered in the Hesx1<sup>Cre/+;Sox2<sup>0/0</sup></sup> pituitaries.

Cell proliferation was initially assessed through intraperitoneal injections of BrdU into 12.5 dpc timed pregnant dams, 120 minutes prior to harvest. BrdU, a thymidine analogue, is incorporated into replicating DNA strands during the S phase of the cell cycle. During normal pituitary organogenesis, injected BrdU is mainly incorporated within the RP periluminal progenitors at 12.5 dpc, but not in the developing AP, which represents an early population of differentiating cells (mainly rostral tip thyrotrophs) (Davis et al., 2011). During the course of pituitary organogenesis, the proportion of BrdU<sup>+</sup> cells is at its maximum between 11.5-12.5 dpc, subsequently declining through pituitary development. At later stages of gestation, BrdU<sup>+</sup> cells become a small population of scattered cells within the periluminal region of the AP (Davis et al., 2011; Ward et al., 2005). At 12.5 dpc, mutant pituitaries contained fewer BrdU<sup>+</sup> cells in the periluminal epithelium when compared to stage-matched controls (Figure 5.14a-b; t-test; \(p = 0.0252; n = 3\) pituitaries each). Similarly, further studies at 14.5 dpc using pregnant dams that were injected with BrdU 90 minutes prior to being sacrificed, revealed fewer BrdU<sup>+</sup> cells within the periluminal epithelium of mutant pituitaries compared with controls (Figure 5.14c-d; t-test; \(p = 0.0001; n = 3\) each).
Figure 5.14 - BrdU incorporation is reduced in $\text{Hesx}^\text{Cre}^\text{+};\text{Sox}^{2\beta\gamma}$ pituitaries at 12.5 and 14.5 dpc

Double-immunolabelling for BrdU (green) and PITX1 (red) on sagittal pituitary sections from control and mutant embryos exposed to BrdU at 12.5 and 14.5 dpc. PITX1 was used to highlight pituitary cells from surrounding tissue. (a) 120 minute pulse of BrdU results in a large proportion of BrdU stained cells within RP of control pituitaries at 12.5 dpc. (b) A similar pattern can be seen in mutant pituitaries at this stage, however the number of BrdU$^{+}$ cells is smaller. (c) 90-minute pulse of BrdU at 14.5 dpc also results in BrdU$^{+}$ cells (although less in number) localised to the periluminal region, with the AP largely devoid of this label in controls. (d) Under the same experimental conditions, fewer BrdU$^{+}$ cells can be detected within the periluminal region of mutant embryos. Note that white cells represent auto-fluorescent blood cells. (e) Quantification of BrdU$^{+}$ cells in control and mutant pituitaries, represented as a percentage of total periluminal PITX1$^{+}$ and DAPI-counterstained nuclei ($n=3$ each). Error bars represent the mean ± SEM. Abbreviations: AP, anterior pituitary; Inf, infundibulum. Scale bar, 100 µm.
Cyclin D2 is expressed during the G1 phase of the cell cycle, and is essential for the progression to S phase (Ward et al., 2006). To complement our BrdU experiments, immunostaining against Cyclin D2 was conducted at 12.5 and 14.5 dpc, and the proportion of Cyclin D2\(^{+ve}\) cells within the periluminal epithelium was calculated in control and mutant pituitaries. At both stages analysed, fewer Cyclin D2\(^{+ve}\) cells were observed in mutant pituitaries when compared to littermate controls (Figure 5.15a-d; t-test; 12.5 dpc, \(p = 0.0011\) and 14.5 dpc, \(p < 0.0001\) respectively; \(n=3\) pituitaries per genotype per stage). Collectively, these data strongly support the hypothesis that the capacity of RP progenitors to proliferate is compromised in the \(Hesx1^{Cre+};Sox2^{loxp}\) pituitaries, subsequently accounting for the smaller AP observed at late gestation.
Figure 5.15 - Numbers of Cyclin D2<sup>+</sup> cells are reduced in \textit{Hesx1<sup>Cre<sup>+</sup>\textsubscript{;Sox2<sup>R<sup>fl/fl</sup>}} pituitaries}

Immunolabelling for Cyclin D2 on sagittal sections through pituitaries of control and mutant embryos at 12.5 and 14.5 dpc. (a) At 12.5 dpc, a large proportion of RP progenitors are positive for Cyclin D2 in control embryos. (c) By 14.5 dpc, Cyclin D2<sup>+</sup> cells are still evident within RP of control pituitaries, but are reduced in number when compared to 12.5 dpc. (c‘’) At this stage, the developing AP is completely devoid of Cyclin D2<sup>+</sup> cells. (b-d) At both stages, the numbers of Cyclin D2<sup>+</sup> cells within the periluminal region are significantly reduced in mutant pituitaries when compared to (a-c) controls, despite normal distribution and location of these cells. (c-d) Note that green cells represent auto-fluorescent blood cells. Inserts show high-magnification images of boxed regions. (e) Quantification of Cyclin D2<sup>+</sup> cells in control and mutant pituitaries, represented as a percentage of total periluminal PITX1<sup>+</sup> and DAPI-counterstained nuclei (\(n=3\) each). Error bars represent the mean ± SEM. Abbreviations: AP, anterior pituitary; Inf, infundibulum. Scale bar, 100 µm.
$Hesx1^{Cre/+}, Sox2^{fl/fl}$ $Hesx1^{Cre/+}, Sox2^{fl/fl}$

(a) 12.5 dpc

(b) 12.5 dpc

(c) 14.5 dpc

(d) 14.5 dpc

(e) 

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$Hesx1^{Cre/+}, Sox2^{fl/fl}$  
$Hesx1^{Cre/+}, Sox2^{fl/fl}$

% Cyclin D2 +ve cells

12.5 dpc  | 14.5 dpc

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5.2.7 Absence of SOX2 leads to depletion of embryonic precursors in the developing RP

The onset of pituitary hormone expression is well described; at 12.5 dpc, the developing AP contains no differentiated cells (Bilodeau et al., 2009; Seuntjens and Denef, 1999). Subsequently, as early as 13.5 dpc, the first appearance of TSHβ^ve cells (thyrotrophs) is detectable within the developing anterior lobe. At the same time, few ACTH^ve cells (corticotrophs) are present however this number strongly increases by 14.5 dpc, when a large proportion of the AP is occupied by this cell type. At this stage, few weakly-stained-PRL cells (lactotrophs) are detectable, along with the emergence of TSHβ cells within the caudo-medial region of the anterior lobe. At 15.5 dpc, the first few LH/FSH^ve cells (gonadotrophs) appear, closely followed by GH^ve cells (somatotrophs), the last cell type to be born (Seuntjens and Denef, 1999). Interestingly, although gonadotrophs terminally differentiate late during pituitary organogenesis, it has been noted that their precursors commit to their cell fate very close in time as the ‘early’-born corticotrophs and rostral tip thyrotrophs. As the data herein shows that corticotrophs, rostral tip thyrotrophs and gonadotrophs are the least affected lineages and that there is a significant decrease in proliferation of RP precursors in the Hesx1^Cre/+;Sox2^fl/fl mutants, it was hypothesised that premature depletion of precursors, required for the generation of ‘late’-born cell lineages (e.g. Pit1-dependent thyrotrophs and somatotrophs), may underlie the observed defects.

To address this, pulse-chase experiments were performed by injecting 10.5 dpc pregnant dams with a single dose of BrdU, and subsequently harvesting the embryos at 14.5 dpc. This technique relies on the principles that unincorporated BrdU is cleared within 2 hours post-injection and that incorporated BrdU is diluted with every cycle of cell division (Ward et al., 2005). Consequently, embryos collected several days after injection would reveal strong BrdU-retained label mostly in descendants of cells that were in S phase within the 2-hour window post-injection and exited cell cycle soon after. With this rationale in mind, late-‘born’
cells, such as the precursors of Pit1-dependent thyrotrophs and somatotrophs would incorporate the BrdU label at 10.5 dpc and go through several rounds of divisions (thereby diluting the BrdU label), prior to exiting the cell cycle and differentiating. In contrast, early-'born' cells, such as corticotrophs, rostral tip thyrotrophs and gonadotrophs, would exit the cell cycle shortly after BrdU incorporation, thus retaining a constant level of BrdU label.

In agreement with this notion, BrdU immunoreactivity was highest at the most rostral and ventral tip of the developing AP, corresponding to the 'early'-born population of thyrotrophs and few corticotrophs in 14.5 dpc control pituitaries (Davis et al., 2011). Conversely, the periluminal epithelium containing RP progenitors was completely devoid of any BrdU staining. An intermediate area located within the caudo-medial region of the developing gland, a region where PIT1 cells typically reside, displayed weaker, more diffused BrdU staining (Figure 5.16a, c; n=3 pituitaries). This BrdU staining pattern created a distinct visual gradient (high rostral to low caudal) in control pituitaries that was not so well-defined in Hesx1\textsuperscript{Cre/+};Sox2\textsuperscript{fl/fl} pituitaries. Rather, a more uniform intense staining of BrdU\textsuperscript{+ve} cells was found within the highly dysmorphic mutant pituitaries, most likely representing the 'early'-born AP cell types. Additionally, BrdU staining could be detected within the ventral part of the periluminal epithelium, indicating that these progenitors had retained the label for longer (Figure 5.16b-c; n=3). Quantification of BrdU\textsuperscript{+ve} cells within the entire pituitary gland revealed a substantial increase in BrdU\textsuperscript{+ve} cells within Hesx1\textsuperscript{Cre/+};Sox2\textsuperscript{fl/fl} mutant pituitaries when compared to controls (Figure 5.16c; t-test; p = 0.0158; n=3 each). This was not unforeseen, as not only are the mutant RP progenitors dividing slower and therefore diluting the incorporated BrdU at the time of injection to a lesser extent, but also, the size of the entire gland, as determined by the number of DAPI- and PITX1-positive cells, was greater in control pituitaries than mutants. Collectively, the pattern of BrdU staining observed in the Hesx1\textsuperscript{Cre/+};Sox2\textsuperscript{fl/fl} mutants is consistent with the terminal differentiation data presented earlier, in which corticotrophs, rostral tip thyrotrophs and gonadotrophs remain less affected, however terminal differentiation of the Pit1 lineage is severely altered. In addition, the persistence of BrdU-
labelled cells in RP periluminal progenitors is compatible with the lower proliferation capacity observed in the mutant pituitary at 12.5 and 14.5 dpc (Figures 5.14 and 5.15).

In summary, these data demonstrate the absolute requirement of SOX2 during normal pituitary organogenesis, to maintain RP progenitors in a proliferative state during a defined temporal window, in order to attain a correctly-sized and fully differentiated gland.
Figure 5.16 - Late-‘born’ cell types are absent in Hesx1Cre⁺;Sox2⁻²⁰⁻ pituitaries

Immunostaining for BrdU (green) and PITX1 (red) on 14.5 dpc sagittal pituitary sections obtained from control and mutant embryos exposed to BrdU at 10.5 dpc. PITX1 was used to highlight pituitary cells from surrounding tissue. (a) In control pituitaries, retention of BrdU labelling reveals a distinct pattern of BrdU incorporation, which was lost within (b) mutant pituitaries (summarised in schematic). (a) Strong BrdU staining is detected within the rostral tip of the developing AP, representing a population of cells that exited the cell cycle shortly after BrdU incorporation. Weaker BrdU staining was observable within the caudo-medial region of the AP, marking cells that underwent numerous cell cycle divisions, following BrdU retention. Finally, little or no staining of BrdU was present within RP progenitors lining the pituitary lumen. (b) In contrast, strong and weak BrdU labelled cells were intercalated in a disorganised manner throughout the AP of mutant pituitaries. (c) Quantification of BrdU⁺⁺ cells in control and mutant pituitaries, represented as a percentage of total DAPI/PITX1-stained nuclei (n=3 each). Error bars represent the mean ± SEM. Abbreviations: AP, anterior pituitary; Inf, infundibulum; RP, Rathke’s pouch. Scale bar, 50 µm.
5.2.8 SOX2 expression is not required for SOX9 expression

Sox9, a member of the SoxE subfamily, has previously been shown to represent a population of differentiating progenitors in the embryonic pituitary, as well as co-localising with most SOX2$^{+}$ cells in the adult (Fauquier et al., 2008). *In vitro* studies have postulated that SOX2$^{+}$SOX9$^{-}$ cells represent a population of multipotent, uncommitted progenitors, whilst SOX2$^{-}$SOX9$^{+}$ label a transient, more committed progenitor cell type in the pituitary. To investigate whether SOX9 cells were present in the Sox2-deficient pituitaries, immunolabelling for SOX9 was performed at 18.5 dpc. SOX9$^{+}$ cells were detectable within the cleft of *Hesx1*<sup>Cre/+;Sox2<sup>fl/fl</sup> pituitaries as well as scattered through the highly dysmorphic AP tissue, similar to that observed in control pituitary sections (Figure 5.17a-b; n=4). Thus it appears that SOX9$^{+}$ cells are independent of SOX2 expression and may represent a precursor niche within these Sox2-deficient pituitaries.

*Figure 5.17 - SOX9$^{+}$ cells are present within *Hesx1*<sup>Cre/+;Sox2<sup>fl/fl</sup> mutant pituitaries*

Immunostaining for SOX9 on control and mutant frontal pituitary sections at 18.5 dpc. (a) Intense SOX9 staining is observed within the brain and basosphenoid tissue of control sections. In addition, SOX9$^{+}$ cells are mainly localised to cells within the IL and the lining of the lumen in the AP. Few positive cells are present scattered throughout the AP. (b) A similar pattern of SOX9 staining was detectable in mutant sections, despite the dysmorphology. Abbreviations: AL, anterior lobe; BS, basosphenoid tissue; IL, intermediate lobe. Scale bar, 100 µm.
5.2.9 Lack of GnRH neurons in the hypothalamus of \textit{Hesx1}^{\text{Cre/+};Sox2^{fl/fl}} mutant embryos

As previously explained in the general introduction (Chapter 1), all patients with hypopituitarism due to mutations in \textit{SOX2} present with AP hypoplasia on MRI image, in conjunction with the unusual phenotype of isolated gonadotrophin deficiency (HH) [reviewed in (Tziaferi et al., 2008)]. Intriguingly, our analyses of terminal differentiation in \textit{Hesx1}^{\text{Cre/+};Sox2^{fl/fl}} pituitaries revealed the presence of both FSH- and LH-expressing cell types, despite the highly dysmorphic and hypoplastic AP (Figure 5.5m-p; \( n=3 \) each). Moreover, \textit{Sf1}, a gonadotroph-specific transcription factor expressed prior to terminal differentiation, appeared normal or slightly increased in mutant pituitaries relative to controls (Figure 5.18c-d; \( n=3 \)). Of relevance to this, \textit{Sf1} mRNA expression within the overlying hypothalamus appeared comparable between genotypes (Figure 5.18a-b; \( n=3 \)).

Given these results, I sought to investigate the reason underlying the HH consistently observed in humans with \textit{SOX2} mutations. Previous analysis of both \textit{Sox2} heterozygous mice and human subjects has indirectly alluded to the theory that \textit{SOX2} may act on multiple levels, implicating the hypothalamus as a possible contributor to the pathophysiological phenotype observed (Kelberman et al., 2006; Kelberman et al., 2008).
Figure 5.18 - Sf1 expression within the hypothalamus is similar between genotypes, however, is increased in pituitary glands of Hesx1\textsuperscript{Cre/+}\(;\)Sox2\textsuperscript{fl/fl} embryos

*In situ* hybridisation using a specific anti-sense Sf1 riboprobe on control and mutant frontal sections at 18.5 dpc. (a-b) Little differences in expression of Sf1 transcripts were observed within the ventro-medial hypothalamus of control and mutant embryos at late gestation. (c) In control pituitaries, Sf1 mRNA is restricted to ventral regions. In contrast, Sf1 expression appeared expanded in mutant pituitaries, scattered throughout the hypoplastic AP with few positive cells ectopically located within the IL (as indicated by arrowheads in d). Abbreviations: AL, anterior lobe; IL, intermediate lobe. Scale bar, 100 µm.
The mature neuroendocrine hypothalamus consists of two distinct neuro-secretory systems controlled by the magnocellular and parvocellular neurons [reviewed in (Acampora et al., 2000; Andersen and Rosenfeld, 2001; Szarek et al., 2010)]. The magnocellular neurons, grouped within the PVN and SON, project their axons to the PP, where they release AVP and OT directly into general circulation. In situ hybridisation using specific Avp and Ot riboprobe revealed comparable expression of these two genes between genotypes at 18.5 dpc (Figure 5.19a-d; n=3 brains each). Parvocellular neurons project to the median eminence where they release hypophysiotrophic hormones into the hypophyseal-portal system, conveying these signals to the AP. GHRH-expressing parvocellular neurons are located within the Arc of the hypothalamus, and act to stimulate the release of GH from the somatotrophs. Expression domains of Ghrh mRNA transcripts within the Arc of controls and mutant sections were similar at 18.5 dpc (Figure 5.19i-j; n=3). Together, these data further confirm that the deletion of Sox2 induced by the Hesx1-Cre mouse line exerts little or no effect on the development of the hypothalamus, as previously suggested.

Finally, I sought to analyse the presence of GnRH neurons within the brains of Hesx1^{Cre/+};Sox2^{fl/fl} 18.5 dpc embryos. GnRH neurons are very sparse in number and are dispersed throughout the rostro-caudal continuum of the hypothalamus, failing to accrue within particular nuclei. However, their projections into the median eminence can be easily detected by immunolabelling with specific anti-GnRH antibody, as this releasing factor accumulates in the axon terminals. Accordingly, immunolabelling against GnRH confirmed the abundency of these neuronal projections within the median eminence of control embryos. In contrast, these projections were completely absent in Hesx1^{Cre/+};Sox2^{fl/fl} embryos (Figure 5.19k-l; n=5 brains per genotype), suggesting that either these neurons were reduced in numbers or created erratic projections. To resolve these possibilities, quantitative analysis of GnRH neurons was performed, which revealed a substantial reduction of these neurons in mutant brains when compared to controls (Figure 5.20a-b, i; control 583.5 ± 60.50, mutant 269.0 ± 44.00; n=2 brains per genotype). This finding was further confirmed through an independent analysis of
these mutant brains at 18.5 dpc by Dr Cariboni (a collaborator in this project (UCL, Division of Cell and Developmental Biology)). Collectively, these data suggest that the underlying cause of the HH observed in patients harbouring SOX2 mutations may be the result of a primary defect within the hypothalamus, rather than the pituitary.

GnRH neurons are a unique population of parvocellular neurons, unlike any other releasing factor neuron; these neurons are born outside of the brain within the nasal placode beginning at 10.5 dpc, where they migrate along olfactory/vomeronasal neurons initially to the olfactory bulbs and subsequently, to the medial basal hypothalamus by 18.5 dpc. Thus, failure in the specification of these neurons from within the developing olfactory epithelium, and/or a defect in the migration of these neurons to the hypothalamus, leads to insufficient pituitary gonadotrophin release, and is one of the causes of HH in humans (Cariboni et al., 2007). To investigate the origin of the GnRH phenotype in our Hesx1^{Cre+};Sox2^{fl/fl} embryos, immunohistochemistry against GnRH was analysed at 13.5 dpc, a timepoint when migrating neurons are present within the nasal mesenchyme and forebrain (Cariboni et al., 2007). In agreement with the data at late gestation, I confirmed a vast reduction of GnRH^+ve cells at 13.5 dpc. Here, GnRH neurons appeared to migrate and enter into the brain, however, the absolute numbers of these neurons were significantly diminished (Figure 5.20c-d, j; t-test \( p = 0.0111; n=3 \) heads each). This finding was traceable back to earlier stages when fewer GnRH^+ve cells were identified within the nasal epithelium at 12.5 dpc (Figure 5.20e-h; \( n=2 \) per genotype). Thus, it appears that the depletion of SOX2 from the Hesx1-expression domain results in the reduced genesis of GnRH neurons, rather than aberrant migration of these cells into the brain.
Figure 5.19 - GnRH neurons are absent within the hypothalamus of *Hesx1^{Cre/+};Sox2^{fl/fl}* embryos
(a-j) *In situ* hybridisation and (l-k) immunohistochemistry on frontal sections through the hypothalamus of control and mutant embryos at 18.5 dpc. The expression domains of (a-d) *Avp* and (e-h) *Ot* mRNA transcripts within the (a-b, e-f) PVN and (c-d, g-h) SON were comparable between control and mutant embryos. (i-j) Similarly, little or no differences in expression of *Ghrh* transcripts within the Arc were detected between genotypes. (k) Immunolabelling against GnRH in control embryos highlighted expression of GnRH protein within the terminal projections of parvoceullar neurons located at the median eminence. (l) In contrast, no GnRH protein was found within the median eminence of mutant embryos. Abbreviations: 3V, third ventricle, ME, median eminence. Scale bars, 100 µm.
Figure 5.20 - GnRH neurons are reduced in $Hesx1^{Cre/+};Sox2^{fl/fl}$ embryos at all stages analysed

(a-h) Immunohistochemistry on (a-b) frontal and (c-h) sagittal sections through the brain and nasal cavity of control and mutant embryos at (a-b) 18.5, (c-d) 13.5 and (e-h) 12.5 dpc. (a) Immunostaining against GnRH in control embryos revealed the expression of GnRH neurons within the medial preoptic region of the hypothalamus. (b) In contrast, fewer GnRH$^{+ve}$ cells were identified within this region. (a-b) Inserts show high-magnification images of boxed regions. (c-h) This finding was also confirmed at earlier stages. (d, f, h) $Hesx1^{Cre/+};Sox2^{fl/fl}$ embryos displayed fewer GnRH$^{+ve}$ throughout the nasal cavity and within regions of the brain at both 13.5 and 12.5 dpc, when compared to (c, e, g) wild-type controls. (c-d) Inserts show high-magnification images of boxed regions. Arrowheads depict GnRH$^{+ve}$ neurons within the nasal area, whereas arrows highlight these neurons within the forebrain. (i) Quantification of GnRH$^{+ve}$ cells within the entire brains of control and mutant embryos at 18.5 dpc ($n=2$ each). (j) Quantification at 13.5 dpc of GnRH$^{+ve}$ neurons within the entire heads of control and mutant embryos ($n=3$ each). Error bars represent the mean ± SEM. Data presented in a-h was collected by Dr. Cariboni. Abbreviations: FB, forebrain; NA, nasal area. Scale bars, 100 µm.
5.2.10 Hesx1-expressing cell descendants do not co-localise with GnRH neurons within the olfactory epithelium

To investigate whether Hesx1-expressing cells, or their descendants, occupy regions of the olfactory epithelium where GnRH neurons are specified, Hesx1\textsuperscript{Cre/\textast}\textsuperscript{+};R26\textsuperscript{YFP/+} mice were re-utilised (Chapter 3). Previous studies have shown that before commencing their journey towards the brain, GnRH neurons can be identified within the ventro-medial region of the olfactory epithelium at 11.5 dpc (Tucker et al., 2010). Immunostaining against YFP at this timepoint revealed YFP\textsuperscript{+ve} cells within the olfactory epithelium; however, no apparent pattern, both in location and number, could be identified within this structure (Figure 5.21a-c; n=3 embryos). Interestingly, double-immunolabelling against YFP and GnRH at this stage demonstrated minimal co-localisation implying that only a small minority of Hesx1-descendant cells are fated to become GnRH neurons (Figure 5.21b-e; n=3). Instead, YFP staining was often detected in close proximity to these nascent neurons. Collectively, these data illustrate that SOX2 removal from the Hesx1-expression domain likely impacts GnRH neurogenesis in a non-cell autonomous manner.
Figure 5.21 - *Hesx1*-expressing cell descendants colonise regions of the olfactory epithelium where GnRH are specified, but rarely co-localise with these cells

Double-immunolabelling for YFP (green) and GnRH (red) on (a-e) coronal and (f-g) sagittal sections from *Hesx1*<sup>Cre/+, R26R<sup>YFP/+</sup></sub> embryos at 11.5 dpc. YFP was used to mark *Hesx1* cell descendants. (a) *Hesx1*-expressing cell descendants are present within the olfactory epithelium at 11.5 dpc. Vast expression of YFP is also detectable within the telencephalon at this stage. Yellow cells represent auto-fluorescent cells. (b-e) GnRH neurons can be detected within the ventro-medial region of the olfactory epithelium at 11.5 dpc. In contrast, *Hesx1*-expressing cell descendants show no obvious pattern of distribution within this structure. As exemplified in (b-e), the greater majority of *Hesx1* descendants do not co-localise with GnRH neurons. (e) In fact, only two co-localised cells are highlighted by an *. Abbreviations: OE, olfactory epithelium; Te, Telencephalon; VM, ventro-medial. Scale bars, 100 µm.
Recently, Tucker et al. demonstrated that the graded expression of several transcription factors defines distinct domains of the olfactory epithelium at 11.5 dpc. Of relevance to this study, they revealed that SOX2 is expressed at low levels within the ventro-lateral olfactory epithelium and progressively increases to higher levels located dorso-medially (Tucker et al., 2010). To determine the effects of the Cre-mediated excision of SOX2 within the developing olfactory epithelium in the $Hesx1^{Cre/+};Sox2^{flo/d}$ mutant mouse, immunolabelling against SOX2 was conducted on control and mutant sections at 11.5 dpc. Despite the mutant olfactory epithelium showing signs of hypoplasia, a similar high medial, low lateral pattern of SOX2 expression was observed in both control and mutant olfactory epithelia (Figure 5.22a-b; $n=1$ each). By 12.5 dpc, negligible differences, both in the size of the nasal epithelia and expression pattern of SOX2, were detected (Figure 5.22c-d; $n=2$ per genotype). Thus, the removal of SOX2 from the $Hesx1$-expression domain results in minimal alterations within the nasal epithelium. This is consistent with the aforementioned mosaic pattern of $Hesx1$-descendants within the olfactory epithelium at earlier stages.
Figure 5.22 - Removal of SOX2 from the *Hesx1*-expression domain results in minimal alterations within the nasal epithelium

Immunofluorescence against SOX2 on (a-b) coronal and (c-d) sagittal sections through the nasal cavity of control and mutant embryos at (a-b) 11.5 and (c-d) 12.5 dpc. (a-b) Despite an overall reduction in size, SOX2 expression is present within the medial region of the nascent nasal epithelium in both control and mutant embryos at 11.5 dpc. (c-d) By 12.5 dpc, a similar expression pattern of SOX2 can be identified within the nasal epithelium of control and mutant embryos. No differences in the size of this structure are observed between control and mutant sections at this stage. Green signal represents autofluorescent cells.

Abbreviations: NC, nasal cavity; NE, nasal epithelium; NP, nasal pit. Scale bars, 100 µm.
5.2.11 Increased apoptosis does not contribute to the loss of GnRH neurons

To determine whether increased apoptosis could account for the GnRH phenotype observed in the \( Hesx1^{Cre/+};Sox2^{flo/flo} \) mouse model, immunostaining against cleaved caspase-3 was performed on control and mutant sections at 12.5 dpc, a timepoint coinciding with early stages of GnRH neurogenesis. In both control and mutant sections, sparse cleaved caspase-3 staining was observed within the entire nasal cavity at this stage (Figure 5.23a-b; \( n=2 \) embryos per genotype). Therefore, at least at this timepoint, apoptosis does not appear to be an important contributor to the diminished levels of GnRH neurons detected in \( Hesx1^{Cre/+};Sox2^{flo/flo} \) mouse.

Figure 5.23 - Little apoptotic cell death is observed within the nasal region of \( Hesx1^{Cre/+};Sox2^{flo/flo} \) at 12.5 dpc.

Immunostaining against cleaved caspase-3, an indicator of apoptosis, on sagittal sections through the nasal cavity of control and mutant embryos at 12.5 dpc. (a-b) Few apoptotic cells are present within the nasal regions of both control and mutant embryos at 12.5 dpc. Arrows highlight the few apoptotic cells present. Green staining represents autofluorescent signal. Abbreviations: NC, nasal cavity; NE, nasal epithelium. Scale bar, 100 µm.
5.3 CONCLUSIONS

To conclude, these data reveal that SOX2 acts on multiple levels to control the formation of the hypothalamic-pituitary axis. As demonstrated by this work, the absence of SOX2 in the developing RP of \textit{Hesx1}^{Cre+};\textit{Sox2}^0/0 embryos leads to severe anterior lobe hypoplasia at late gestation with a drastic reduction of PIT1 expression and severe disruption of somatotroph and thyrotroph differentiation. In contrast, other hormone-producing cell types such as corticotrophs, rostral tip \textit{Pit1}-independent thyrotrophs and, interestingly, lactotrophs and gonadotrophs are less affected. It appears that this hypoplasia is a consequence of reduced proliferation of RP progenitors and, as a result, a failure to yield a sufficiently large pool of precursors for all cell lineages of the anterior pituitary. Accordingly, differentiated cells derived from precursors exiting cell cycle at early stages (i.e. corticotrophs, rostral-tip thyrotrophs and gonadotrophs) are generated, whilst hormone-producing cells originating from late-born precursors (i.e. somatotrophs and \textit{Pit1}-dependent thyrotrophs) are severely impaired. Finally, I demonstrate that \textit{Hesx1}^{Cre+};\textit{Sox2}^0/0 embryos exhibit reduced neurogenesis of the GnRH neurons, which accounts for the consistent HH observed in patients with hypopituitarism due to SOX2 haploinsufficiency. Therefore, in addition to demonstrating an essential function of SOX2 for normal expansion of cell lineage precursors of differentiated pituitary cell types, these data provide insights into the mechanisms underlying the characteristic pituitary hypoplasia and HH observed in patients harbouring SOX2 mutations.
CHAPTER 6: DISCUSSION
6.1 SUMMARY OF MAIN THESIS FINDINGS

Extensive studies have previously shown that *Hesx1* is essential for normal forebrain and pituitary formation in mouse and humans [(Acampora et al., 2000; Andoniadou et al., 2011; Durmaz et al., 2011) and reviewed in (Kelberman et al., 2009)]. In this thesis, I illustrate a novel role for *Hesx1* in the development of the neuroendocrine hypothalamus and correlate these findings towards the differences in perinatal lethality observed in the mouse lines analysed, which, in turn, provides major implications for the understanding of human SOD, as discussed herein (Chapter 3; Section 6.2). In addition, these novel results provide an explanation of why subjects harbouring mutations in *Hesx1* are commonly associated with the unusual phenotype of PSIS or an ectopically located PP (summarised in Chapter 1; (Reynaud et al., 2011)). Finally, the functional analysis of three novel *Hesx1* variants associated with PSIS, was assessed *in vitro* [Chapter 4; (Reynaud et al., 2012)]. Consistent with previous studies, mutations that occur within the homeodomain of *Hesx1* result in the compromised ability of HESX1 to bind DNA, whilst repressor function is maintained. Conversely, the S67T variation, which occurs outside of any known functional domain, results in little deviation from wild-type *Hesx1* function. Whilst it could be that this variant represents a rare polymorphism, this allelic change also occurs in combination with the PROKR2-R85H mutation, suggesting that these genes may act synergistically in the pathogenesis of PSIS. Together, the work presented here further our basic understanding on HESX1 function during normal embryogenesis and its contribution to disease states.

More recently, mutations of *SOX2* have been implicated in the aetiology of SOD and associated conditions (Kelberman et al., 2006; Kelberman et al., 2008; Sato et al., 2007; Tziaferi et al., 2008). Regardless of this, the function of SOX2 within the hypothalamic-pituitary axis is unknown. In Chapter 5, I demonstrate a pituitary-autonomous role for SOX2. Removal of SOX2 during early stages of pituitary organogenesis allows for the initial development of RP, however, the successive expansion of the pouch is impaired, which ultimately leads to severe hypoplasia of the AP. Despite the severe hypoplasia reported at late gestation, the *Pit1* lineage
appears to be most sensitive to depleting levels of SOX2. Interestingly, in the absence of PIT1, lactotrophs still form, suggesting a Pit1-independent source for this cohort. Furthermore, in an attempt to reconcile the phenotype-genotype relationship, I show that the reduction of SOX2 leads to abnormal GnRH neurogenesis, which provides an explanation for the HH consistently observed in patients with hypopituitarism as a consequence of SOX2 haploinsufficiency (Chapter 5).

In the course of this discussion, these findings are addressed in accordance to the existing knowledge of Hesx1 and Sox2 and normal hypothalamic-pituitary development, providing a solid basis for future work. Each chapter will be discussed in turn.

6.2 HESX1 IS REQUIRED FOR NEUROENDOCRINE TERMINAL DIFFERENTIATION

Since the generation of the Hesx1−/− mouse mutants, the reasons underlying their perinatal lethality have been elusive. Here, I propose that defects originating within the hypothalamus primarily account for this (Chapter 3). In our mouse model, the entire magnocellular neuro-secretory system housed within the PVN and SON failed to develop (Figure 3.3). In addition, at least one of the main types of parvocellular neurons, namely the Ss-expressing cells located in the aPV, demonstrated reduced expression (Figure 3.4). Unlike the severe reduction of the magnocellular neurons, only a partial reduction of these neurons was observed. Absolute determination of this observed reduction has proved challenging to assess for two main reasons. Firstly, the aPV is less defined than the other neuroendocrine nuclei, and SS-expressing neurons also reside in other regions of the hypothalamus, including the suprachiasmatic area and VMH (Michaud et al., 1998; Morales-Delgado et al., 2011). Secondly, it is believed that SS-expressing neurons within the aPV are not fully represented embryonically and that this population is only fully visible at around P28 (Morales-Delgado et al., 2011). Thus, the deficit in this neuronal subtype remains to be fully determined and may possibly be
amplified during postnatal stages. Although not assessed in this study, CRH- and TRH-
expressing parvocellular neurons which function to regulate the synthesis and secretion of
ACTH and TSH respectively, from the AP also reside in the PVN. Previous mutagenesis
screenings in mice have revealed that these five neuro-secretory peptides (AVP, OT, SS, CRH
and TRH) are delineated through the same genetic pathway, in which Otp and Sim1/Arnt2 act in
parallel as originators in this cascade. In our studies, reduced expression of both Otp and Sim1
was detected in the Hesx1−/− mouse (Figure 3.6). From this, it is reasonable to suggest that Hesx1
acts upstream of these two genes to regulate terminal differentiation of AVP-, OT-, SS- (and
most likely CRH- and TRH-) expressing neuronal subtypes (Figure 6.1). It is the combined loss
of these neuronal subtypes that leads to the perinatal death of these mutants, as reported
previously in the Sim1−/−, Arnt2−/−, Otp−/−, Brn2−/− and Sim2−/− (Acampora et al., 1999; Goshu et al.,
2004; Hosoya et al., 2001; Michaud et al., 1998; Nakai et al., 1995; Schonemann et al., 1995;
Wang and Lufkin, 2000).

The findings that GHRH- and GnRH-expressing parvocellular neurons remained
unaltered in the Hesx1−/− mouse are not completely unforeseen (Figure 3.4), as these two
neuronal subpopulations are determined by completely independent genetic pathways to the
neuronal subtypes described above. The homeobox genes, Gsh1 and Hmx2/Hmx3 are required
for the expression of GHRH within the Arc (Li et al., 1996; Valerius et al., 1995; Wang et al.,
2004). Analysis of Otp−/− mouse mutants by two independent studies has shown that both GHRH
expression and its upstream determinant, Hmx2 remains unperturbed in this mouse model
(Acampora et al., 1999; Wang and Lufkin, 2000). Furthermore, Otp expression in Hmx2−/−;
Hmx3−/− double mutants is unchanged (Wang et al., 2004). Taken together, this is consistent
with our proposal that Hesx1 functions in a discrete manner to coordinate the terminal
differentiation of particular neuronal subtypes.

Although the genetic factors underlying the specification of GnRH neurons remain to be
resolved, these neurons are born within the olfactory placode where they migrate through the
nasal cavity and olfactory bulbs to reach areas of the preoptic hypothalamus (Schwanzel-Fukuda
Consequently, they are unlikely to be regulated by the same genetic determinants that define the rest of the neuroendocrine subpopulations that originate within the ventral diencephalon. Investigation of these neurons in the $Hesx1^{-/-}$ revealed the presence of these neuronal projections within the median eminence in addition to within the AP, comparable to wild-type controls (Figure 3.4). This is potentially of great interest for two reasons. Firstly, the finding that GnRH neurons are able to reach regions of the hypothalamus overlying pituitary tissue suggests that although the olfactory bulbs are defective in the $Hesx1$-null mutants, sufficient migratory cues remain intact in these mutants to direct these neurons to their ultimate locations within the ventral hypothalamus (Dattani et al., 1998; Sajedi et al., 2008b). Secondly, it has been well described that the parvocellular neurons secrete their neurohormone products into the portal vasculature, which ultimately functions to transport these signals to the AP. Consequently, a comparable GnRH$^{+ve}$ immunoreactivity within the AP of all $Hesx1$ mouse mutants to wild-type controls could imply that the portal system in these animals remains unchallenged. However, in many cases, including the $Hesx1^{I26T/I26T}$ mouse mutants, AP tissue is abnormally located, remaining attached to the ventral brain structures. As a clear distinction between AP tissue and the brain no longer exists in these mutants, the possibility that these factors may incorporate into an AP devoid of a portal system still exists. Indeed, Navratil and co-workers demonstrated that GnRH promotes the movement of gonadotrophs, both in vitro and in vivo, resulting in the active and directed movement of these cell types towards their releasing factor (Navratil et al., 2007). From this, additional analysis of vascularisation markers, such as platelet endothelial cell adhesion molecule (PECAM) and vascular endothelial growth factor A (VEGFA), would be of value to determine whether the hypophyseal portal system remains unperturbed in these mutants.
Figure 6.1 - *Hesx1* functions upstream of Otp and Sim1/Arnt2 to coordinate the development of the neuroendocrine hypothalamus

Taken together, previous proposals have suggested that the aPV and PVN may originate from a large contiguous area of similar molecular identity as early as 12.5 dpc (Goshu et al., 2004). Here I show, through the analysis of *Hesx1*-deficient mutant mice, that this region may initiate its specification process, as early as 9.0-9.5 dpc, when *Hesx1* is expressed within the presumptive ventral diencephalon (Figure 6.1).

**6.2.1 *Hesx1* is required in a dosage-dependent manner within the neuroendocrine hypothalamus**

The neuroendocrine phenotype observed in the *Hesx1*<sup>126T/126T</sup> mouse model further confirms that this mutation results in a hypomorphic allele, as previously shown *in vitro* and *in vivo* (Carvalho et al., 2003; Sajedi et al., 2008b). In this study, *Avp* and *Ot* expression was
reduced to 57.2% and 35.9% respectively, whereas negligible differences in Ss expression were observed (Figures 3.3 and 3.4). Despite these neuroendocrine deficits, these mice are largely viable and fertile (Sajedi et al., 2008b). A 50% reduction in AVP and OT levels has previously been reported in Brn2 heterozygous mice that express half the levels of Brn2. Likewise, these mice are also viable and reproductively active (Nakai et al., 1995). Interestingly, despite abnormal terminal differentiation of the magnocellular neurons at late gestation in Hesx1I26T/I26T mutant mice, little differences in Otp and Sim1 expression was noted when examined at 12.5 dpc (Figure 3.6). As these two factors are known to participate in the proliferation, as well as migration of these neuronal precursors, it remains to be determined whether a subtle difference in expression levels could account for this discrepancy (Acampora et al., 1999; Michaud et al., 1998; Wang and Lufkin, 2000). Closer investigation of these two markers at additional timepoints in the Hesx1I26T/I26T would help to clarify this possibility.

Moreover, the terminal differentiation defect described here appears to be dosage sensitive as Hesx1I26T/- embryos showed an intermediate phenotype on the development of these neuronal subtypes abovementioned (Figures 3.3 and 3.4). This is consistent with previous analysis of these embryos, which demonstrated that the frequency and severity of forebrain defects are increased when compared to homozygous embryos for the Hesx1-I26T mutation (Sajedi et al., 2008b). Furthermore, similar to Hesx1-deficient mice, Hesx1I26T/- mice are vastly perinatally lethal. Only a single viable mouse was generated from a total population of 100 mice. Interestingly, this mouse exhibited bilateral microphthalmia and stunted growth, indicative of hypopituitarism. This mouse was sacrificed at 2 months of age (data not shown; unpublished results).

As described in detail in the general introduction (Chapter 1), Hesx1 functions as a transcriptional repressor, which is mainly mediated through the eh1 domain. In vitro studies have clearly shown that mutations within this domain, as in the Hesx1-I26T mutation, result in reduced repressing activity, but uncompromised DNA-binding. It has previously been shown that one of the reasons underlying the reduced repressor activity of HESX1-I26T is that it is no
longer able to bind TLE1. This Groucho-related protein functions as a co-repressor and aids the repression activity mediated by HESX1. Given that the pituitary defects in these mice are phenotypically identical to Hesx1<sup>-/-</sup> mutant mice, it has been predicted that this HESX1/TLE1 interaction is essential for pituitary function (Dasen et al., 2001; Sajedi et al., 2008b). Conversely, forebrain development in these mice remains largely unaffected and is mainly restricted to the eyes (Sajedi et al., 2008b). Collectively, it is believed that in the mouse, the HESX1/TLE1 interaction is primarily required within RP, secondarily in eye precursors and finally within cells destined to become telencephalon. Here, analysis of the neuroendocrine system in the Hesx1<sup>II6T/II6T</sup> mouse has further elaborated on this theory, as in these mutants, a partial terminal differentiation and fully-penetrant abnormality was found. Therefore, it is now proposed that the HESX1/TLE1 interaction is required more importantly within the pituitary, followed by the ventral diencephalon, the eyes and ultimately, by the telencephalon.

This theory is consistent with prior investigation of TLE function during CNS development in the Xenopus. In this study, Tsuji and colleagues (2005) showed that in the absence of TLE, the prospective dorsal diencephalon territory is expanded at the expense of ventral diencephalic fates. Additionally, eye defects are observed in these mutants, and are believed to be consequential of this initial enlargement of dorsal diencephalon, as the optic vesicle is displaced proximally to the neural tube failing to associate with the lens placode. Similar to the Hesx1 mouse data, the development of the telencephalon in these mutants remains unaltered (Dattani et al., 1998; Sajedi et al., 2008b; Tsuji and Hashimoto, 2005).

### 6.2.2 Molecular mechanisms underlying the neuroendocrine defects in Hesx1-deficient mice

The finding that Hesx1 acts within the hypothalamus is intriguing, as Hesx1 transcripts are only briefly expressed within the developing hypothalamus (Hermesz et al., 1996; Thomas and Beddington, 1996). Therefore, the question remains: how does Hesx1 exert its effect on these neuronal populations?
Cell fate analyses of Hesx1 descendant cells showed that subsequent to endogenous Hesx1 expression, Hesx1-expressing cell descendants continued to colonise regions of the ventral diencephalon and subsequently the hypothalamus. However, these cells remained few and showed no evident signs of occupying regions of the aPV, PVN and SON (Figures 3.7 and 3.8). From this, it seems unlikely that Hesx1 is required cell-autonomously to direct fate decisions. Moreover, the lack of Hesx1 resulted in negligible effects in the gross morphology of this structure, as Hesx1 descendant cells appear to be similar in number and distribution within the hypothalamus at late gestation (Figure 3.8). Therefore, it is improbable that Hesx1 is required for the survival or proliferation of these neurons. However, it is still plausible that Hesx1-expressing cells adopt different fates, as observed in anterior forebrain structures or simply remain undifferentiated, failing to express markers of terminal differentiation (Andoniadou et al., 2007).

Taken together, this data implies that Hesx1 must function indirectly to coordinate the terminal differentiation of AVP-, OT- and SS-expressing neuronal subtypes. Although direct targets of HESX1 still remain unidentified, there is compelling evidence that Hesx1 is required during forebrain and pituitary development to antagonise members of the Wnt/β-catenin signalling pathway (Andoniadou et al., 2007; Andoniadou et al., 2011; Gaston-Massuet et al., 2008; Gaston-Massuet et al., 2011). Given that this function is conserved within these two tissues, the possibility that Hesx1 functions to repress Wnt/β-catenin within the presumptive hypothalamus exists. AXIN1 functions as a negative modulator of the Wnt/β-catenin pathway by cooperating with protein kinases to degrade β-catenin. Analysis of the Axin1-mutant in zebrafish has shown that inhibition of Wnt signals is required for the specification of the hypothalamus anlage, as well as later for its compartmentalisation (Kapsimali et al., 2004). Although specification of the hypothalamus, as determined by the presence of Nkx2.1, appeared unaffected in the Hesx1 mouse mutants (Figure 3.6), the restriction of Hesx1 to the ventral diencephalon between 9.0-9.5 dpc may function to locally modulate Wnt levels within the presumptive hypothalamus to confer identity to individual domains and define cell-fate
decisions. This could explain why the more caudally-located Arc, which normally receives higher levels of Wnt signals, remains unchallenged in the Hesx1-deficient mouse, whereas the more rostrally-located nuclei, such as the aPV and PVN, are altered.

Alternatively, although much emphasis has been placed on inductive signals from the ventral diencephalon to the pituitary primordium, studies have also highlighted that signals emanating from the pituitary may function in the development of the ventral diencephalon. For example, co-culture experiments have shown that the close association of pituitary tissue is required for the differentiation/proliferation of ACTH/MSH cells within the hypothalamus (Daikoku et al., 1983). Additionally, Hermesz and colleagues described a Hesx1 regulatory element that is sufficient to drive expression within the neuroectoderm when exposed to RP tissue. Furthermore, this finding was shown to be stage-dependent, as co-culture experiments with 13.5-14 dpc pituitary explants failed to induce transgene expression (Hermesz et al., 2003). Thus, the expression of Hesx1 within RP during early stages of organogenesis could additionally function to pattern the developing hypothalamus. Great insight into this theory could be provided by the analysis of the neuroendocrine hypothalamus in Lhx3−/−;Lhx4−/− double mutants, in which the pituitary primordium fails to develop beyond primitive rudimentary pouch stages (Sheng et al., 1997).

6.2.3 Implications/translational impact

The main focus of this investigation was to study the possible relationship between hypothalamic dysfunction in patients presenting with SOD. Given that HESX1 was the first and remains one of the few genes associated with this pathology, Hesx1 mouse models were utilised as an in vivo approach to study this hypothesis (Dattani et al., 1998). Our results support a hypothalamic contribution to this disease.

In this study, a crucial finding is that terminal differentiation of the magnocellular AVP- and OT-expressing neurons failed in the absence of Hesx1. As described in the general introduction (Chapter 1), it is the axonal projections of these magnocellular neurons that
constitute the PP and therefore, it is not unexpected that loss of these neuronal subtypes results in abnormalities of the PP. Consistently, previous analyses of $Otp^{-/-}$, $Sim1^{-/-}$, $Arnt2^{-/-}$ and $Brn2^{-/-}$ mutants report hypoplasia of the PP without any morphological abnormalities of the AP and IL (Acampora et al., 1999; Hosoya et al., 2001; Michaud et al., 1998; Nakai et al., 1995; Schonemann et al., 1995; Wang and Lufkin, 2000). In this analysis, the presence of a highly dysmorphic AP fused to the hypothalamus in the $Hesx1^{-/-}$ mutants concealed the identification of a definite PP structure. However, endorsing our findings of magnocellular anomalies in $Hesx1$-deficient mice, reports of abnormally positioned PP have been documented in patients harbouring $HESX1$ mutations (Chapters 1 and 4). In fact, $HESX1$ was one of the first genes to be associated with this trait and, along with $SOX3$, $LHX4$ and $OTX2$, remains one of the few known underlying pathological factors (Mitchell et al., 2002; Reynaud et al., 2011). Of importance, it has been noted that the presence of these structural abnormalities are highly indicative of the severity of the endocrinopathy outcome (Mehta et al., 2009). Taken together, our mouse models data correlates with the clinical findings derived from human observations. Structural abnormalities of the PP in patients with $HESX1$ mutations could be indicative of hypothalamic defects that may affect a range of physiological and behavioural functions, in addition to hypopituitarism, as discussed below.

Detection of structural abnormalities fails to determine whether the neurons of the PP are functional. AVP is a key regulator of fluid balance and acts to stimulate water uptake by the kidney tubules, whereas OT has been classically associated with lactation and parturition. Although loss of these two peptides is not essential for survival, they both act to regulate vital physiological processes.

Reports in rats have demonstrated that the loss of AVP results in diabetes insipidus which is characterised by high water intake accompanied by excretion of an excessive amount of dilute urine (Schmale and Richter, 1984). However, although patients with forms of SOD have been reported to manifest diabetes insipidus (Masera et al., 1994), patients with $HESX1$ mutations are never diagnosed with this symptom (personal communication, Prof. M. Dattani).
In an attempt to closely match our data with human patients, the more severely affected *Hesx1*<sup>−/−</sup> embryos were excluded from this analysis. Yet, embryos exhibiting milder phenotypes still die perinatally and therefore, they still represent a more severe phenotype to patients diagnosed with *HESX1* mutations, as these patients do not die, but do require medical attention. It could be that surviving human patients are more alike to the *Hesx1<sup>I26T/I26T</sup>* hypomorph, which is also viable.

As abnormal PP function can range from subtle defects including reduced AVP release following osmotic challenge, through to persistent nocturnal enuresis or symptomatic diabetes insipidus, a possibility that mild water and electrolyte disturbances exist in *HESX1*-deficient patients, but remains undiagnosed (Secco et al., 2011).

Although OT is not required for normal parturition or reproductive behaviour, studies in mice have shown that it is essential for normal nursing. Pups are born and retain the ability to suckle normally, but failed milk injection by OT-deficient mothers results in the neonatal death of the pups (Nishimori et al., 1996; Young, III et al., 1996). In addition to this function, over the past years OT has also been implicated in the mediation of social behaviours and social memory in rodents [reviewed in (Heinrichs et al., 2009; Heinrichs and Domes, 2008; Meyer-Lindenberg et al., 2011)]. Loss of OT and its receptors result in deficits in social interactions that are characteristic of a spectrum of autistic disorders (Pobbe et al., 2011). Indeed, clinical studies have shown that the administration of OT to subjects diagnosed with autistic traits significantly reduces repetitive behaviours, as well as improves social interactions, such as communication and facial recognition (Guastella et al., 2010; Hollander et al., 2003; Hollander et al., 2007). Interestingly, in a recent study of 83 patients diagnosed either with SOD or optic nerve hypoplasia, 31% of this cohort were clinically diagnosed with an autistic-spectrum disorder (Parr et al., 2010). In light of the mouse data obtained, patients harbouring mutations in *HESX1* are currently being assessed for OT deficits (in collaboration with Prof. M. Dattani; unpublished results).

In addition to housing the cell bodies of the magnocellular neurons, the PVN has also been implicated in the regulation of appetite. Classical lesion experiments of the PVN results in
excessive food intake and consequently, obesity (Leibowitz et al., 1981). Corroborating this, Sim1 heterozygotes have been reported to develop early onset obesity believed to be due to PVN hypocellularity (Michaud et al., 2001). Moreover, Sim1 haploinsufficiency in humans is one of the few monogenic causes of obesity (Holder, Jr. et al., 2000). By contrast to the hypocellular PVN phenotype described by Michaud et al. (2001), Kublaoui and colleagues (2006) observed no difference in the PVN neuronal number using a Sim1-enhanced green fluorescent protein (GFP) transgenic reporter, but subsequently reported that Ot mRNA and peptides are diminished by 80% in Sim1+/− mice, whereas the expression of Trh, Crh and Avp are only reduced by 20-40% (Kublaoui et al., 2006; Kublaoui et al., 2008). From this, they proposed that the reduced levels of OT were responsible for the hyperphagia of the Sim1+/− mice. In support of this theory, patients with Prader-Willi syndrome, a human genetic disorder that is associated with severe obesity, have a 42% reduction in PVN OT neurons (Swaab et al., 1995). In our study, both Sim1 and Ot expression levels were vastly reduced in the Hesx1−/− mutant (Figures 3.3 and 3.6). As obesity remains a common trait in patients with SOD, reduced SIM1 and OT levels could be an underlying cause for this clinical presentation. In addition to the PVN, the Arc and adjacent VMH are two other nuclei well known for regulating food intake and body weight homeostasis. Pomc1 neurons housed in the Arc and Sf1 neurons located within the adjacent VMH represent two of the main neuronal subtypes for controlling these actions (Jo and Chua S Jr, 2009; Kim et al., 2011). Intriguingly, the present analysis revealed minimal alterations in the expression of both Pomc1 and Sf1 (Figure 3.5). Thus, it appears that the obesity trait is more likely due to a defect within the neuroendocrine PVN, rather than in one of the additional hypothalamic populations implicated in this behaviour.

Finally, hypopituitarism has been intimately associated with patients harbouring mutations in HESX1, which can range from the mildest form of isolated GH deficiency through to CPHD (summarised in the general introduction, Chapter 1). The work presented in this chapter suggests that a neuroendocrine deficit in the Hesx1 mouse model could account for the hypopituitarism observed in human subjects, but reveals that only particular neuroendocrine
subtypes are affected in these mutants. Intriguingly, the expression of GHRH remained unaltered in these mutants (Figure 3.4), yet GH deficiency is the most common clinical presentation in HESX1 patients [reviewed in (Kelberman et al., 2009)]. Thus, the question remains: what is the reason behind the GH deficiency frequently reported in HESX1 patients with forms of hypopituitarism? As mentioned, the portal system remains a vital component to the neuroendocrine-pituitary axis, and therefore, perturbations of this structure could compromise the function of the AP. Indeed, the analysis of GnRH neurons demonstrates these peptides are able to reach the AP in all the Hesx1 mutants analysed. However, as described, this could be due to the ectopically-located AP fused to the ventral hypothalamus, which may allow for this function, independent of an intact portal vasculature. In patients with Hesx1 mutations, fusion of AP tissue to the brain has never been reported. Therefore, it exists that an intact portal system is vital for AP function in humans.

Furthermore in Chapter 3, I reported deficits in OT levels. Previous observations have shown that OT stimulates the migration and sprouting of human endothelial cells in vitro and that endothelial cells can express the OT receptor in situ (Cassoni et al., 2006; Thibonnier et al., 1999). Prompted by these findings, recent work in zebrafish has established that OT is crucial for the formation of the vascularisation to the PP. Morpholinos-induced knock-down of OT or its receptor results in an aberrant vascular phenotype and therefore, it has been hypothesised that OT-release is required focally for endothelial morphogenesis of this structure (Gutnick et al., 2011). Deductively, the loss of OT in our mouse model could additionally affect the formation of the portal system, in turn, deregulating AP function. Collectively, the hypopituitarism phenotype in HESX1-deficient patients may be a consequence of both perturbed neuroendocrine function coupled with a defective portal vasculature.
6.3 MUTATIONS OCCURRING WITHIN THE HOMEODOMAIN OF HESX1 RESULTS IN IMPAIRED DNA BINDING, WHILST REPRESSOR FUNCTION IS MAINTAINED

Chapter 4 reports the functional consequences of three recently identified HESX1 variations: R109X and F156S that both occur within the C-terminal homeodomain, and S67T which occurs in a region of unknown function. In addition, S67T was found to occur in combination with a PROKR2 mutation, R85H. These allelic variants were identified in patients diagnosed within the subgroup of congenital hypopituitarism, PSIS. Clinical evaluation of these patients demonstrated that all subjects displayed signs of severe hypopituitarism, which commenced during infancy and later presented as panhypopituitarism at puberty. The increased severity of the clinical outcome noted in these patients is consistent with all the data presented above, suggesting that additional defects within the neuroendocrine hypothalamus may be in play (Chapter 3; Section 6.2).

By the nature and location of the allelic variants R109X and F156S, impaired DNA binding is expected, and the in vitro experiments documented here confirm this. When HESX1 repressor ability was assessed dependent on its ability to bind to the PIII sequence, both mutant proteins showed no repressor ability, and in fact, revealed an increase in luciferase activity relative to controls (Figure 4.3). It is proposed that the increased luciferase activity observed when R109X or F156S mutant proteins are transfected may be the result of sequestration by these two mutant proteins of essential cofactors required to mediate repression of the SV40 promoter, if DNA binding to the PIII site is disrupted (Figure 4.3). However, both R109X and F156S HESX1 mutant proteins retained their ability to repress transcription when DNA binding was facilitated by the GAL4 DNA domain (Figure 4.4). These data imply that the DNA binding of HESX1 is essential for its repressor action and corroborates previous mouse data from our group which demonstrates that the loss of DNA-binding, by the presence of a point mutation within the third α-helix of homeodomain (R160C), results in a null phenotype (Dattani et al.,
1998; Sajedi et al., 2008b). Taken together, it is likely that these two mutations play a causative role in the observed hypopituitarism.

The finding that little or no protein expression was observed when the R109X mutant protein was expressed as a HA-tagged protein is perplexing, as when the mutant R109X was expressed as a GAL4 fusion protein, this mutant protein appeared to be stable, retaining the ability to induce an effect on luciferase activity, identical to the F156S mutation (Figures 4.1, 4.3, 4.4 and 4.5). It could be that the presence of the GAL4 binding domain (147 amino acids in length) helps to stabilise this protein, whereas the HA tag (approximately 54 amino acids) is insufficient to protect this protein from degradation. From this, it is highly probable that the R109X-HESX1 protein is degraded under in vivo conditions. Indeed, a former study has shown that a similar nonsense HESX1 mutation, which is predicted to lack the entire homeodomain, leads to degradation mediated by the nonsense-mediated decay pathway, a surveillance mechanism of protein synthesis that is employed by cells to rapidly degrade mRNA when a premature stop codon is present more than 50-55 nucleotides upstream of the 3’ exon-exon junction (Vivenza et al., 2011).

In contrast to the two mutations described above, a S67T substitution resulted in minimal effects on the functional ability of HESX1 (Figures 4.1, 4.3, 4.4 and 4.5). Consistent with this data, the father of this patient also carried the same mutation in a heterozygous state, and was asymptomatic. Combined, it is likely that this mutation represents a rare HESX1 polymorphism and is not a primary cause of hypopituitarism observed in this patient. Interestingly, this patient was found to carry a mutation (R85H) in the open reading frame of the G-protein coupled receptor, PROKR2. When functionally analysed, the R85H mutation was found to have deleterious effects on the protein action, thus supporting a causative role in the phenotype (Reynaud et al., 2012). However, this mutation has previously been associated with HH/Kallmann syndrome devoid of additional endocrine deficits, suggesting that although the S67T mutation exerts minimal consequences on the function of HESX1, oligogenic interactions could have a combinatory effect in vivo in the pituitary (Cole et al., 2008; Dode et al., 2006;
Indeed, digenic mutations have been previously associated with HH and Kallmann syndrome, where mutations in two independent genes synergistically produce a more severe phenotype in HH families than alone (Pitteloud et al., 2007). Analysis of a double heterozygous mouse model, Prokr2<sup>++</sup>;Hesx1<sup>++</sup> would help to clarify this possibility.

As evidenced from the characterisation of Hesx1 mouse models, the pituitary, followed by the neuroendocrine hypothalamus, and then the eyes and forebrain appear to be most sensitive to levels of HESX1 function (Dattani et al., 1998; Sajedi et al., 2008b) (Chapter 3; Section 6.2.1). The finding that all the patients here present with hypopituitarism without any reported abnormalities within the eyes and forebrain further support this hypothesis that the pituitary and neuroendocrine hypothalamus remain the most sensitive structures to diminishing levels of HESX1 (Table 4.1). Additionally, whilst previous literature has shown that HESX1 homozygous mutations are most often associated with a more severe clinical outcome [reviewed in (McCabe et al., 2011)], the observation that the R109X homozygous mutation only results in severe hypopituitarism without any additional forebrain malformations is noteworthy, but further reiterates the highly variable nature of this condition (Table 1.2).

Of further interest, an intriguing finding is that the father of the propositus possessing the F156S in a heterozygous state, also contained the exact mutation, but remained asymptomatic. Two possible explanations could account for this inconsistency. Similar to the homozygous R109X mutation, the father of this proband may represent a milder case within the spectrum, either devoid of any abnormalities or possibly harbouring a mild form of hypopituitarism, which would most likely present as isolated GHD. Alternatively, the hypopituitarism reported in this patient may be oligogenic in basis, to which this alteration in HESX1 could contribute. Although this patient was not shown to possess any additional mutations within the open reading frame of the additional genes screened in this study, mutations in unknown genes may exist. This is in accordance with the reporting that only 3% of patients displaying PSIS phenotypes are resultant from mutations in known genes (Reynaud et al., 2011), strongly implying that other pathogenic genes are yet to be identified. Indeed, despite
the association between SOD and HESX1 for just over a decade, mutations in SOX2 have only been recently associated with related pathologies.

6.4 SOX2 IS REQUIRED WITHIN RP FOR THE CORRECT EXPANSION OF THE PROGENITOR POOL

In mouse, Sox2 is expressed in the multipotent stem cells in the inner cell mass of the blastocyst. Its ablation causes early embryo lethality at pre-implantation stages, and it is believed that the persistence of maternal SOX2 in these Sox2-null embryos allows for survival to this stage (Avilion et al., 2003). Within the nervous system, Sox2 is expressed in stem cells and early precursors, and in a minority of mature neurons. In neural progenitors, its downregulation is associated with progression from an undifferentiated state towards a more committed cell type (Graham et al., 2003). Consistently, previous characterisation of SOX2 expression within the developing pituitary shows that SOX2 is normally expressed within the progenitor cells of RP and its levels wane as differentiation proceeds, persisting only in a minority of cells localised to the periluminal area at late gestation (Figure 5.2) (Fauquier et al., 2008). Expression of SOX2 within the adult pituitary is maintained within this periluminal region and these SOX2\(^{+ve}\) cells exhibit many properties of progenitor cells; \textit{in vitro} culture of these cells demonstrate that they have the ability to self-renew and differentiate into all hormone-producing cell types (Fauquier et al., 2008). Collectively, it could be hypothesised that SOX2 is required within the developing pituitary gland for the correct proliferation and/or survival of these precursors prior to the onset of cellular differentiation. Indeed, the pituitary phenotype observed in the \textit{Hesx1}^{Cre/}\textit{;Sox2}\(^{0/0}\) mouse model supports this notion, as in the absence of SOX2, the pituitary failed to progress beyond a primitive structure, which resulted in a highly dysmorphic and hypocellular gland at late gestation (Figure 5.1).

Loss-of-function mutations in other early pituitary transcription factors, such as \textit{Pitx2} and \textit{Lhx3}, also result in a similar hypoplastic phenotype (Sheng et al., 1996; Zhao et al., 2006).
In these cases, excessive apoptotic cell death in combination with reduced proliferation is believed to contribute to the phenotype observed. In contrast, in the \textit{Hesx1^{Cre+};Sox2^{fl/fl}} mutants, little or no ectopic apoptosis was noted at all stages analysed during pituitary organogenesis (Figure 5.11). Instead, the removal of SOX2 from the developing gland resulted in decreased numbers of proliferating RP progenitors, as evidenced by the analysis of the proliferation markers, Cyclin D2 and BrdU (Figures 5.14 and 5.15). It is proposed that this reduced proliferation ultimately results in an early depletion of progenitor cells required for the appropriate number of precursor cells in the pituitary gland, preventing the generation of all cell types. Our BrdU label-retaining experiments are in agreement with this notion, as the weaker labelled BrdU cells, that represent cells that have undergone more ‘rounds’ of cell division appeared less evident in the Sox2-deficient pituitaries when compared to controls (Figure 5.16).

Moreover, a modest increase in the expression of the cell cycle inhibitor, p27\textsuperscript{Kip1}, was noted in mutants compared to wild-types, implying that increased cell cycle exit occurs in the Sox2-deficient pituitaries (Figure 5.12). Despite this, no precocious differentiation was observed in these mutants (Figure 5.13). This has previously been shown in mice deficient for the Notch downstream target gene, \textit{Hes1}. These mice display a concomitant increase in both cell cycle inhibitors, p57\textsuperscript{Kip2} and p27\textsuperscript{Kip1}, however, little differences in the expression of intermediate differentiation markers are observed (Monahan et al., 2009). From this data, it is predicted that an increase of cells exiting the cell cycle is not indicative of accelerated cellular differentiation. Our findings are consistent with this.

Combined, these data suggest that reduced proliferation, and perhaps premature cell cycle exit, results in the pituitary hypoplasia observed in the Sox2-null pituitaries. It would be of interest to overexpress SOX2 within differentiated cell types, such as under the control of the \textit{aGSU} or \textit{Pit1} promoter, to determine whether the absolute downregulation of SOX2 is required for later differentiation.
6.4.1 SOX2 is required for terminal differentiation of Pit1 precursors

Despite the vast reduction of AP tissue observed in the Hesx1\textsuperscript{Cre/}\textsuperscript{+};Sox2\textsuperscript{fl/fl} embryos, only cellular differentiation of Pit1 lineage appeared to be affected in these mutants. To a certain degree, this can be explained by the diminished levels of Prop1 expression at earlier stages during development in these Sox2-deficient pituitaries. I suggest that the observed differentiation phenotype is related to the timing of cell fate specification, as detailed below.

The mature pituitary gland contains six distinct hormone-producing cell types that all arise from a common progenitor. Two independent birthdating studies have highlighted that the vast majority of AP cell types exit the cell cycle concurrently between 11.5-13.5 dpc (Davis et al., 2011; Seuntjens and Denef, 1999). At 13.5 dpc, a transitional zone composed of non-cycling, undifferentiated progenitors appears immediately ventral to the periluminal area and is marked by the expression of p57\textsuperscript{Kip2} and Cyclin E. These cells do not express any markers of cellular differentiation (Bilodeau et al., 2009). It is now believed that this transitional zone is likely to be the correct time and place where cell fate commitment into the AP cell types occurs (Davis et al., 2011). Consequently, under normal conditions, extensive proliferation occurs during early stages of pituitary organogenesis, which then leads to a finite pool of progenitors sufficient for the correct generation of all pituitary cell types.

Several lines of evidence have shown that although gonadotrophs fully differentiate late in development (>16.5 dpc), progenitors of this cell lineage commit to this cell fate at much earlier stages, as determined by the expression of αGSU (11.5-12.5 dpc) (Barnhart and Mellon, 1994; Holley et al., 2002; Japon et al., 1994). Thus, rostral tip thyrotrophs, corticotrophs and gonadotrophs that are specified early, represent the first populations to deplete this progenitor pool. This is closely followed by the Pit1-dependent lineage, comprising of thyrotrophs, lactotrophs and somatotrophs. Lastly, melanotrophs that compose the IL have been shown to be delayed in the cell cycle until after all the AP cell types (Davis et al., 2011). Therefore, the terminal differentiation defect that is observed in the Hesx1\textsuperscript{Cre/};Sox2\textsuperscript{fl/fl} mouse line may just be consequential of a reduced progenitor pool, which would allow for the generation of the earlier
born cell types (rostral tip thyrotrophs, corticotrophs and gonadotrophs) at the expense of the later-born Pit1-dependent cell types. Indeed, the pulse-chase data supports this hypothesis (Figure 5.16). As the generation of the Pit1 lineage is dependent upon the prior expression of Prop1 (Andersen et al., 1995; Sornson et al., 1996), a reduction in Prop1 expression and not an absence, as observed in the Sox2-deficient pituitaries, may be a reflection of less cells present to commit to this fate, due to this reduced progenitor pool. The inverse expression pattern of NeuroD1 in relation to Prop1 further endorses this proposal (Figure 5.10).

This finding of cell fate conversion has also been demonstrated in Hes1-deficient mutants. As previously mentioned, in the absence of Hes1, cells of the AP prematurely exit the cell cycle, which ultimately leads to a hypoplastic AP gland. In addition, somatotrophs are ectopically located within the IL at the expense of melanotrophs (Kita et al., 2007; Raetzman et al., 2006). In this case, the hypoplasia observed does not appear to be as dramatic as the phenotype reported here. Thus, a larger AP progenitor pool may exist in these mutants, only short for the ultimate melanotrophs. This finding is also of relevance to my work, as it could imply that within the Pit1 lineage, somatotrophs are the last cell type to commit to their fate. In fact, previous analysis of terminal differentiation, as assessed by the onset of hormone expression, demonstrated that lactotrophs are found as early as 14 dpc concurrently with caudo-medial thyrotrophs, which are then followed by somatotrophs at 16 dpc (Seuntjens and Denef, 1999). This could in part provide an explanation for the PRL+ve cells observed in the Hesx1Cre+;Sox2f/f mutants.

However, it cannot be ruled out that PRL expression could evolve independently of Pit1 expression. Analysis of the Snell mouse model, which harbours a mutation in the Pit1 gene, has led to the identification of an independent thyrotroph population, not reliant on the former expression of Pit1. In this particular study, PRL expression was not examined (Lin et al., 1994). Moreover, detailed analysis of the somatotrophs population has unravelled the presence of two sub-populations; one dependent upon GHRH for proliferation, and the other independent of this signalling factor (Godfrey et al., 1993; Lin et al., 1992). Thus, the possibility remains that two
distinct lactotroph cohorts exist within this seemingly homogeneous population. Prl transcriptional activation in the absence of Pit1 has previously been reported in a cell line, and our data somehow provides support to the concept that there may be a Pit1-independent lactotroph cell population in the pituitary at late gestation (Gellersen et al., 1995). Further studies of Prl expression in other mouse models deficient for either Prop1 or Pit1 would help to conclude this finding.

6.4.2 How does SOX2 mediate this effect?

The data presented herein demonstrates an essential role for Sox2 within the developing pituitary gland; however, the molecular mechanisms underlying SOX2 actions remain elusive. Several lines of evidence suggest that Sox2 may function, at least in part, in coordinating the effects of several signalling pathways, including Notch and Wnt, as discussed below. Expression analysis of both Notch and Wnt signalling components in our Sox2-deficient mouse model would prove vital to further our understanding of Sox2 within this gland.

Former studies have shown that Notch signalling is necessary for maintaining the progenitor-like state in the developing pituitary gland. For example, loss of the Notch intracellular mediator, RBP-J, as well as Notch downstream targets Hes1 and Hes5, results in AP hypoplasia due to reduced proliferative capacity of RP progenitors (Kita et al., 2007; Zhu et al., 2006). Of interest, Notch signalling has been found to function downstream of Sox2 within neural progenitor cell populations, such as the developing neural tube and retina (Bani-Yaghoub et al., 2006; Taranova et al., 2006). Thus, it seems likely that SOX2 may mediate its effects through the control of Notch signalling within the pituitary. In support of this, complete genetic ablation of Notch signalling in Rbp-J-deficient embryos during pituitary organogenesis phenocopies the loss of SOX2 during early stages of pituitary development as observed in this study; these Pitx1Crea/;Rbp-JΔflo mice demonstrate severe AP hypoplasia with the concomitant loss of Pit1 lineage. Furthermore, it was demonstrated that, in addition to the aforementioned Notch targets, Prop1 is transcriptionally regulated by Notch signalling. Similar to our findings,
Notch signalling was not required for the initial expression of Prop1, but is necessary for its maintained expression. Thus, it has been proposed that Notch signalling maintains the expression of Prop1, providing molecular information essential for the subsequent generation of the Pit1 lineage (Zhu et al., 2006).

In addition to regulating Notch signalling, emerging evidence suggests that SOX proteins may have widespread roles in modulating Wnt signalling in development and disease [reviewed in (Hayward et al., 2008)]. Previous studies have shown that murine SOX2 is capable of associating with β-catenin to inhibit β-catenin-mediated activation of target genes. It appears that this function of Sox2 is independent of its HMG DNA binding domain, and in fact, is mediated by the C-terminal region (Mansukhani et al., 2005). Furthermore, it has been shown that SOX2 is able to transcriptionally activate Hesx1, a Wnt/β-catenin antagonist (Kelberman et al., 2008; Okuda et al., 2010). Together, this suggests that SOX2 may function within the pituitary to antagonise Wnt/β-catenin signalling. It is of interest that in many instances of vertebrate development, Wnt and Notch signalling are often associated with the alternative fate decisions of bi-potential precursors, and are usually antagonistic in nature, such that low Wnt signalling tends to be associated with high Notch levels, and vice versa [reviewed in (Hayward et al., 2008)]. Thus, Sox2 may act bi-directionally to maintain low levels of Wnt in addition to promoting Notch signalling.

From this, it could be predicted that loss of Sox2 will result in an overactivation of β-catenin signalling. Consistent with this, premature activation of the β-catenin pathway under the control of the Pitx1-Cre, which drives Cre expression from 9 dpc within regions of the oral ectoderm and developing RP, results in the complete loss of the gland by 13.5 dpc. Lhx3 expression is initially expressed, indicating that specification occurs as normal, but fails to be maintained (Olson et al., 2006).

However, contrary to this one finding, many studies have shown that the overactivation of Wnt signalling during earlier stages of pituitary development, results in an overproliferative gland, a completely opposite phenotype to that observed in the Hesx1Cre+/Sox2fl/fl mouse model.
Additionally, in the work presented here, little differences in \textit{Hesx1} expression were noted during early pituitary organogenesis in the \textit{Sox2}-deficient pituitaries (data not shown). Given the high expression levels of \textit{Sox2} during early stages of pituitary organogenesis, it may be that \textit{Sox2} functions at early stages to regulate the proliferative effects of Wnt/β-catenin signalling, and during later stages, this function is no longer required. Indeed, overproliferation of the pituitary gland is only observed when β-catenin-mediated actions are overactivated during early stages of pituitary organogenesis, under the control of \textit{Hesx1}-Cre, rather than at later stages, when directed by \textit{Pit1-Cre} or \textit{GH-Cre} lines (Gaston-Massuet et al., 2011). The prolonged expression of SOX2 protein following \textit{Cre}-mediated excision in \textit{Hesx1}^{Cre+/+};\textit{Sox2}^{fl/fl} pituitaries may be sufficient to repress the activation of the Wnt/β-catenin signalling pathway during these early stages.

Interestingly, work from our laboratory has recently demonstrated that the overactivation of β-catenin function also results in the blockage of the \textit{Pit1} lineage (Gaston-Massuet et al., 2011). Therefore, the loss of the \textit{Pit1} lineage in the \textit{Hesx1}^{Cre+/+};\textit{Sox2}^{fl/fl} pituitaries could be a combined effect of the loss of Notch at these stages as described above, as well as the overactivation of the Wnt/β-catenin pathway during later stages.

### 6.4.3 Reasons underlying the neonatal lethality reported in \textit{Hesx1}^{Cre+/+};\textit{Sox2}^{fl/fl} mice

An intriguing finding was that \textit{Hesx1}^{Cre+/+};\textit{Sox2}^{fl/fl} embryos die perinatally, thus the question remains: what is the underlying cause of death? It seems reasonable to suggest that irregular morphogenesis of the oropharyngeal cavity induced by pituitary phenotype in the \textit{Hesx1}^{Cre+/+};\textit{Sox2}^{fl/fl} embryos, could create both breathing and suckling problems in these neonatal animals, accounting for their early lethality. Indeed, feeding difficulties in these animals may also exist as oesophageal atresia has been documented in some \textit{SOX2} human patients (Williamson et al., 2006). As it has been noted that \textit{Hesx1} descendant cells populate regions of
the gut endoderm, excision of SOX2 under the regulatory elements of Hesx1 may ablate SOX2 from these regions, in addition to the developing pituitary gland (Andoniadou et al., 2007).

Furthermore, analysis of the Prop1-null mutant mice has previously shown that approximately half of these mice die at birth, whereas the remaining pups die within the first week of life, failing to thrive. Findings from this particular study revealed that lung anomalies, secondary to the loss of thyrotrophs in the developing pituitary, were the primary cause of death in these animals (Nasonkin et al., 2004). Thus, the loss of functional thyrotrophs in the Hesx1\textsuperscript{Cre/+};Sox2\textsuperscript{fl/fl} mice could also contribute to the perinatal lethality reported here.

6.4.4 Aberrant GnRH neurogenesis accounts for the hypogonadotrophic hypogonadism phenotype associated with human SOX2 mutations

Human patients carrying SOX2 mutations invariably display a phenotype characterised by AP hypoplasia and HH. However, there is no overall endocrine compromise, with most hormones detected at normal levels except for the gonadotropins FSH and LH that are abnormally low, causing delayed or absence of puberty. Even one patient without pituitary hypoplasia, shows HH, suggesting that there may be a specific requirement of SOX2 for normal development of the hypothalamic-gonadotrophic axis, as summarised in the general introduction (Chapter 1). To date, the reasons underlying this phenotype are intriguing and remain unexplained.

Analysis of the Hesx1\textsuperscript{Cre+};Sox2\textsuperscript{fl/fl} mouse model has proved fruitful in the investigation of this complex condition. As mentioned, minimal abnormalities in the terminal differentiation of gonadotrophs were noted in Hesx1\textsuperscript{Cre+};Sox2\textsuperscript{fl/fl} embryos, strongly implying that aberrant pituitary function is not the primary source of this defect (Figures 5.5 and 5.18). As it is well established that gonadotroph function is reliant upon the inducing actions of hypothalamic GnRH neurons, either a loss of these neurons, or their misdirected migration from their origin of the olfactory placode, could impair reproductive capacity. Of value, there is evidence to suggest that GABAergic mediated signalling is crucial for the migration and organisation of GnRH
neurons from the nasal compartment to the forebrain. Moreover, *in vitro* and *in vivo* experiments have shown that SOX2 is required for the number and connectivity of GABAergic neurons, and that the olfactory bulbs are hypocellular in Sox2 hypomorphs (Cavallaro et al., 2008). Thus, it is hypothesised that diminished migration of these neurons could account for the compromised reproduction in patients with SOX2 mutations.

In this work, I demonstrate that impairment of the hypothalamic-gonadotrophic axis is due to reduced neurogenesis of GnRH neurons originating from the olfactory placodes. A substantial reduction of GnRH neurons was observed at all stages analysed in the Hesx1 Cre/+;Sox2 fl/fl embryos when compared to controls (Figures 5.19 and 5.20). Although superficial in analysis, the finding of minimal ectopic caspase-3 staining combined with reduced GnRH neuronal numbers at 12.5 dpc, suggests that reduced neurogenesis rather than excessive cell death may account for the reduced GnRH cohort in Sox2-deficient conditions (Figure 5.23). This finding is consistent with previous work, as excess apoptosis was not shown to be a major contributor to the early embryo lethality reported in the Sox2/- mouse, as well as in this analysis of the Hesx1 Cre/+;Sox2 fl/fl pituitaries, as described above (Avilion et al., 2003). However, in opposition to the previous hypothesis, despite the overall reduction in numbers, migration of GnRH neurons did not appear totally impaired in the Sox2-conditional mouse model, as evidenced by migratory GnRH cells at 12.5 and 13.5 dpc and a few cells within the hypothalamus at late gestation (Figure 5.20). In addition, the overall morphology of the olfactory bulbs in the Hesx1 Cre/+;Sox2 fl/fl mutants appeared comparable to controls (data not shown). Together, although abnormalities in neuronal migration may contribute to a weakened hypothalamic-gonadotrophic axis, it appears that reduced genesis of these neurons is the primary cause for this condition in humans with SOX2 mutations. Moreover, unaltered expression of other hypothalamic releasing factors, namely Avp, Ot and Ghrh (as well as Sf1), is coherent with the sole invariable HH observed in patients with SOX2 mutations (Figures 5.18, 5.19 and 5.20).
Although this finding provides an explanation for the human phenotype observed, the mechanism as to why this defect arises remains unclear. Within the olfactory epithelium at 11.5 dpc, $Hesx1$ descendant cells demonstrated a mosaic pattern of expression within this structure (Figure 5.21). Consistent with this, excision of SOX2 from these $Hesx1$ cell descendants appeared to have an initial effect on the development of this structure, as evidenced by a hypocellular olfactory epithelium at 11.5 dpc (Figure 5.22). However later in development, minimal effects on both the morphology and expression of SOX2 were found within the nasal cavity (Figure 5.22). Moreover, $Hesx1$ descendants infrequently co-localised with GnRH neurons (Figure 5.21). Together, the effects of SOX2 excision from the $Hesx1$-expression domain are likely to impact the neurogenesis of GnRH neurons in a non-cell autonomous manner.

Several lines of evidence have suggested that fate specification of the murine GnRH neuronal system is dependent upon the presence of FGF8 signalling acting through FGFR1 receptor (Gill et al., 2004). For instance, $Fgf8$ hypomorphs, that retain half the wild-type levels of $Fgf8$ mRNA, result in an entire loss of the GnRH neuronal system (Falardeau et al., 2008). This is also supported by clinical studies that have identified that patients harbouring $Fgf8$ or $FGFR1$ mutations manifest delayed puberty [as reviewed in (Miraoui et al., 2011)]. Recently, it has been shown that FGF8 promotes neurogenesis within the olfactory epithelium through enhancing SOX2 expression. In the medial olfactory epithelium region of 11.5 dpc $Fgf8$ hypomorph embryos, SOX2 expression is attenuated and wild-type pattern of SOX2 expression is difficult to discern (Tucker et al., 2010). Moreover, reduced dose of SOX2, as in the $Sox2^{hpic}$, results in diminished neurogenesis, however, the specification of all olfactory epithelium neuronal cell types occurs as normal, albeit reduced in numbers. In further support of our findings, these neurons were detected within the olfactory epithelium, nasal cavity and forebrain of these mutants, implying normal migration. Collectively, it appears that the ablation of SOX2 expression within the developing olfactory epithelium of the $Hesx1^{Cre+},Sox2^{fl/fl}$ mouse results in a partial reduction of neurogenesis, which ultimately accounts for the reduced GnRH neurons
seen at late gestation. Given that diminished SOX2 levels results in the reduced genesis of all olfactory-derived neurons (Tucker et al., 2010), it is highly likely that anosmia cannot be excluded from patients harbouring SOX2 mutations, a symptom that has not been investigated to date (Prof. Dattani, personal communication).

6.5 SUMMARY AND FUTURE DIRECTIONS

From all the data combined, the results presented in this thesis have provided important insights into the contribution of *Hesx1* and *Sox2* during normal and pathological hypothalamic-pituitary development, posing new avenues for further research. Future directions should include: investigating the mechanism of action of these two proteins within these structures, as well as identifying *Hesx1* and *Sox2* target genes. Identifying downstream targets will not only increase our understanding of the molecular mechanisms of action that these two protein have, but will also provide future leads for genes that may be mutated in hypopituitary phenotypes with unknown aetiology.

During this study, I have shown that *Hesx1* plays a role in the development of the neuroendocrine hypothalamus, only affecting a particular subset of hypothalamic neurons, demonstrating for the first time that SOD could be primarily a hypothalamic disease, rather than a pituitary defect (Chapter 3). This is in line with a recent study conducted by Zhao and colleagues (Zhao et al., 2012). Using an elegant transgenic approach, the authors achieved a specific ventral-diencephalic deletion of *Shh* by crossing a *Shh<sub>loxP/loxP</sub>* with the SBE-Cre line, where Cre is expressed under the *Shh*-brain enhancer-2, *SBE2*. Using this technique, it was shown that Shh signalling from within the ventral diencephalon is required for the correct patterning of the prospective hypothalamus, which ultimately results in hypoplasia of the AP. Furthermore, loss of Shh signalling from this region affects the correct patterning of the retina as well as for the formation of the optic disc. Of value, these authors were able to show that *Shh* signalling within the ventral diencephalon is directly regulated by members of the SOXB1
family, Sox2 and Sox3, implying that SOD defects identified in patients with mutations in both Sox2 and Sox3 could be due to a loss of Shh signalling from within the ventral diencephalon.

In support of a hypothalamic origin for the SOD characteristics observed in the Hesx1\textsuperscript{−/−} mice, the pituitary phenotype in these mutants is alike to the pituitary defects secondary to faulty hypothalamic development, as in the Sox3\textsuperscript{−/−}, Tcf4\textsuperscript{−/−} and Wnt5a\textsuperscript{−/−} mutants (Brinkmeier et al., 2007; Cha et al., 2004; Rizzoti et al., 2004; Woods et al., 2005). Specifically, using classical two-dimensional techniques, previous research have shown that the absence of Hesx1, the developing pituitary gland becomes highly dysmorphic, but terminal differentiation of all pituitary cell types occurs as normal (Section 1.5.2.2; (Dasen et al., 2001; Dattani et al., 1998; Sajedi et al., 2008b)). Despite this however, it cannot be ruled out that a pituitary-specific defect could additionally contribute to the hypopituitarism symptoms observed. For example, recent pituitary studies have highlighted the importance of cell networks within the gland to promote optimal pituitary function [reviewed in (Hodson and Mollard, 2012; Mollard et al., 2012)]. Thus, whether these networks fail to form in Hesx1-deficient pituitaries, thereby contributing to the hypopituitarism phenotypes reported, remains to be evaluated. Together, SOD may result from pituitary malformations, hypothalamic defects and/or a combination of both.

To resolve this issue a little further, it would be of value to study the effects of Hesx1 deletion separately from within the developing pituitary and hypothalamus. One approach to tackle this question would be to create a conditional Hesx1 mouse line (Hesx1\textsuperscript{fl/fl}) which could be crossed with the Nkx2.1-Cre mouse to remove Hesx1 expression solely from the developing ventral diencephalon, whereas the use of a Lhx3-Cre line could help to distinguish the pituitary-specific role of Hesx1 (Fogarty et al., 2005; Kessaris et al., 2006; Sharma et al., 1998; Xu et al., 2008). This would not only help to differentiate and decipher the requirement of Hesx1 within these two tissues, but would also improve our understanding of the developmental origins of SOD.

As discussed in detail in Section 6.2, the close resemblance between Hesx1\textsuperscript{−/−} mouse model and SOD validates the use of the Hesx1 mouse lines in modelling the human conditions.
of SOD and hypopituitarism. Whilst the analysis conducted in Chapter 3 identifies a role for
*Hesx1* in the development of particular neurons, many nuclei remain to be assessed in these
mutants. For instance, are neuronal components that form the suprachiasmatic nuclei intact in
these mutants? Here the *Hesx1* mouse models could be used to delve further into understanding
SOD by performing detailed analysis of other vital nuclei important for normal homeostasis as
well as examining the vasculature which nourishes this structure. Furthermore, the I26T mouse
model is a particularly useful resource, as these mice are viable and fertile postnatally, providing
a closer mouse model to the human *HESX1* patient phenotype. It would be of interest to perform
physiological experiments, such as feeding the *Hesx1*\(^{I26Ti26T}\) on high-fat chow diets to assess
whether these mice are more susceptible to develop obesity than their wild-type counterpart, or
even challenging the mice by subjecting them to glucose tolerance tests and monitoring their
blood insulin levels, to assess whether these mice may have an increased sensitivity to develop
diabetic traits. If these findings appear to be true, patients identified with *HESX1* mutations
could be closely monitored to help prevent or improve diagnosis of these symptoms.
Collectively, it is evident that continued work on these mouse models will undoubtedly provide
valuable clinical insights that would otherwise be unobtainable.

To clarify the role of *Hesx1* within both the developing ventral diencephalon and
pituitary gland, it is essential to identify direct HESX1 targets. This could be achieved by using
a combination of techniques including microarray analysis, promoter binding assay and ChIP
analyses. Indeed, previous studies by our group have taken advantage of the *Hesx1*\(^{eGFP-DTA}\)
mouse line to profile anterior forebrain *Hesx1*-expressing precursors, which has confirmed
previous research by our lab demonstrating that *Hesx1* acts as a direct repressor of the canonical
Wnt/\(\beta\)-catenin pathway within this structure (Andoniadou et al., 2007; Andoniadou et al., 2011).
Using a similar methodology, microarray analysis of flowed sorted cells derived from
*Hesx1*\(^{eGFP/+}\) and *Hesx1*\(^{eGFP/eGFP}\) 12.5 dpc pituitaries could help to identify potential HESX1
targets within the pituitary gland, allowing better understanding of HESX1 within this tissue.
Common targets between the forebrain and pituitary could then facilitate the identification of downstream targets of *Hesx1* within the ventral diencephalon.

In Chapter 5, I demonstrated that mutations occurring within the homeodomain, such as the HESX1-F156S, results in impaired DNA (Figure 4.4). This construct could provide as a useful tool to complement the microarray analysis into deciphering direct HESX1 transcriptional targets by ChIP-on-chip analysis. Here, given the lack of a specific HESX1 antibody, indirect ChIP analyses could be performed using a robust anti-HA antibody. Consequently, expression of the HESX1-F156S-HA construct into *Hesx1*-deficient ES cells (available in our laboratory) could pose as an appropriate negative control to the wild-type HESX1-HA construct, facilitating the identification of any false positives obtained by this technique. This experiment, taken in combination with the previous forebrain analysis as well as the pituitary microarray, could help to identify common HESX1 targets within these discrete tissues.

In Chapter 6, I revealed the requirement for *Sox2* within the developing pituitary gland in maintaining the correct progenitor pool to allow for the production of all anterior pituitary cell types. Despite these findings, the molecular actions of *Sox2* still remain to be identified. To understand better how *Sox2* mediates its effects within the developing pituitary gland, the elucidation of direct SOX2 transcriptional targets as well as SOX2 partner proteins are essential future studies.

In a similar approach to studies suggested for generating HESX1 transcriptional targets, use of the *Sox2*<sup>2GFP</sup> mouse line could be used to flow-sort SOX2<sup>+</sup> cells for subsequent microarray analysis, to identify pathways regulated by SOX2 within the developing pituitary gland (Ellis et al., 2004). To complement this approach, ChIP-on-chip analysis could be performed on wild-type pituitaries at 11.5 dpc, when SOX2 expression is observed in the vast majority of pituitary cells. Previous work conducted by Taranova and colleagues have successfully performed ChIP assays using an anti-SOX2 antibody to identify DNA regulatory
sequences in the eye, demonstrating that SOX2 functions upstream of Notch signalling within this structure (Taranova et al., 2006).

In line with this research, the loss of SOX2 from the developing pituitary phenotypically recapitulates the loss of Notch signalling within this structure (Section 6.4.2; (Zhu et al., 2006)). To confirm whether Sox2 lies upstream of Notch signalling within the pituitary gland, Hesx1\textsuperscript{Cre\(+\)};Sox2\textsuperscript{fl/fl} mice could be crossed with Rosa\textsuperscript{Notch}, where the intracellular domain of the mouse Notch1 gene is expressed from the Rosa locus when exposed to a Cre line, resulting in constitutive signalling activity, to determine whether the phenotype could be rescued within this structure through the overactivation of the Notch pathway (Murtaugh et al., 2003).

As highlighted in the General Introduction (Section 1.6), SOX proteins require the assistance of partner proteins to facilitate their DNA-binding properties. To investigate SOX2 partner proteins specifically within the pituitary, a one-hybrid yeast system can be used, which would allow for the rapid identification of heterologous transcription factors that can interact with the newly identified DNA sequences derived from the ChIP-chip screening. Proteins can then be assessed for their co-localising potential with SOX2 by testing their ability to interact in vitro, both in the presence and absence of DNA, as well as seeing whether they would be able to synergistically activate transcription using luciferase reporter assays. Together these studies will increase our understanding of the transcriptional cascade in which Sox2 participates.

An interesting finding was that the removal of Sox2 from the Hesx1 domain resulted in impaired neurogenesis of GnRH neurons from the olfactory pits, providing an explanation for the invariable HH associated with SOX2 haploinsufficient patients. Fate-mapping analyses of these cells at 11.5 dpc failed to identify much co-localisation of GnRH with YFP, suggesting that Hesx1 descendant cells do not give rise to GnRH neurons. However, as GnRH expression commences at this stage, the possibly remains that insufficient GnRH expression had occurred by the stage. A closer, in-depth analysis of this fate-mapping at later stages is required to fully conclude this statement.
Lastly, the loss of GnRH staining at the level of the median eminence of $Hesx1^{Cre/+};Sox2^{fl/fl}$ embryos (Figure 5.19) suggests that axonal projection of these neurons may be defective in these mutants. To investigate this possibility further, olfactory pits from $Hesx1^{Cre/+};Sox2^{fl/fl}$ 11.5/12.5 dpc embryos could be co-cultured with wild-type ventral diencephalon tissue. Subsequent whole-mount antibody staining against GnRH could then help to distinguish whether these neurons have migrated and formed axonal projections effectively. This will help to assess whether defects in axonal projection are intrinsic to the GnRH neurons or dependent on guidance factors secreted by the ventral diencephalon itself. Conversely, as Sox2 is expressed within the ventral diencephalon, along with Sox3, Sox2 ablation from regions of the ventral diencephalon could alter guidance cues crucial for effective GnRH axonal projection. Co-culture of wild-type olfactory pits along with the ventral diencephalon tissue of $Hesx1^{Cre/+};Sox2^{fl/fl}$ embryos could help to resolve this possibility.

On a final note, the results presented in this thesis have provided important insights into the contributions of $Hesx1$ and Sox2 during hypothalamic-pituitary development, demonstrating that these two genes are both involved during various levels of hypothalamic and pituitary organogenesis. It is clear that the past decades have witnessed great advances in our understanding of the molecular components that govern pituitary organogenesis, and thus, have improved our knowledge of pituitary related conditions. Conversely, it is evident that our comprehension of hypothalamic development remains rudimentary. Given its physiological importance, it is necessary to focus on the identification of novel cellular molecules and pathways that are involved in hypothalamic morphogenesis. This will be facilitated by advances in molecular and cellular biology, including the generation of hypothalamic transcription factor downstream targets by the microarray and ChIP analyses, as proposed above for both $Hesx1$ and Sox2, as well as the characterisation of novel mouse models generated by N-ethyl-N-nitrosourea (ENU) mutagenesis. These approaches will help to address vital questions regarding the role of morphogens in patterning the hypothalamic primordium, the timings and mechanisms of hypothalamic neuronal specification, and unravelling the complete transcriptional cascades.
encoding terminal differentiation. Thus, subsequent studies proposed will not only build upon
the data presented in this thesis in further establishing the molecular mechanisms by which
*Hesx1* and *Sox2* regulate hypothalamic and pituitary development, but ultimately assist with
genetic counselling, diagnosis and with early commencement of treatment.
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