THE ROLE OF SONIC HEDGEHOG IN THE HYPOTHALAMUS

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Abstract

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Solsire E. Zevallos
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Sonic hedgehog (Shh) is a morphogen secreted during early development that is required for the formation of the ventral neural tube, including the ventral forebrain. The prechordal source of Shh underlying the forebrain is required early to bifurcate the cerebral hemispheres and eye vesicles as well as to specify a rostroventral forebrain region, the hypothalamus. We hypothesized that Shh expressed later in the hypothalamus may be required for other known functions of Shh, such as patterning within a region, cell proliferation, cell specification, and axon guidance. To evaluate this hypothesis, Shh was genetically excised from the hypothalamus using a cre/loxp strategy: Shh brain enhancer 2 (SBE2), with specific activity in the hypothalamus, drove cre recombination of the Shh conditional allele. SBE2-cre;Shh/loxp mice (or ShhDhyp) exhibited patterning abnormalities that resulted in morphologically abnormal hypothalamic ventral midline, pituitary, and optic nerve. The constellation of patterning defects and phenotypic abnormalities reflect the human disorder known as septo-optic dysplasia (SOD). These findings contribute to our understanding of the developmental mechanisms involved in proper formation of the hypothalamic-pituitary axis and the optic nerve, the two main aspects of the etiology of SOD.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Neuroscience

First Advisor
Douglas Epstein

Keywords
Sonic hedgehog, hypothalamus, pituitary, optic nerve, optic disc, sept-optic dysplasia

Subject Categories
Congenital, Hereditary, and Neonatal Diseases and Abnormalities | Developmental Biology | Developmental Neuroscience | Disease Modeling | Embryonic Structures | Endocrine System | Endocrine System Diseases |

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THE REQUIREMENT OF SONIC HEDGEHOG IN THE HYPOTHALAMUS

Solsire E. Zevallos

A DISSERTATION

in

Neuroscience

Presented to the Faculties of the University of Pennsylvania in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

2011

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ABSTRACT

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CHAPTER 1
INTRODUCTION

Sonic hedgehog (Shh) is required during vertebrate embryonic development to mediate the formation of the brain and spinal cord (Chiang et al., 1996). The Shh mutant and a portion of patients with holoprosencephaly (HPE) have deficiencies in Shh that result in the severe malformations of the brain and face that are salient and characteristic (Chiang et al., 1996; Solomon et al., 2010). The cerebral hemispheres and optic vesicles fail to separate into bilateral structures and instead appear fused. Ventral subcortical structures such as the hypothalamus are particularly affected, appearing fused across the midline in many HPE patients or even absent in Shh−/− mice (Hahn and Barnes, 2010). Shh is not only required to attain the overall end result of proper morphology, but more importantly, to establish the comprising pattern of distinct neural regions and cell fates within the CNS (Ribes and Briscoe, 2009). Shh has a critical role as a morphogen, and in this capacity, Shh patterns the ventral neural tube, including the hypothalamus and adjacent optic vesicles and pituitary (Szabo et al., 2009; Shimogori et al., 2010; Wang et al., 2010; Zhao et al., 2010).

**Sonic hedgehog: A secreted signal activates a transcriptional cascade**

Shh is a secreted protein that activates a transcriptional cascade in receptive cells. Transcription factor activity in turn dictates the expression profile and character of cells. In this manner, Shh promotes cells to adopt a specific identity and developmental program. The concentration of Shh ligand and the timing of exposure influence a cell’s response. This process has been well characterized in the developing spinal cord.

The initial source of Shh ligand in the developing spinal cord is the notochord, a mesodermal tissue that underlies the neural tube. This ventral source of Shh induces a second source at the ventral midline of the neural tube, the floorplate. These two sources establish a concentration gradient of Shh ligand that is high ventral and low dorsal.
Neural progenitor cells along the dorsoventral axis of the neural tube respond to the graded concentration of Shh ligand (Ribes and Briscoe, 2009). Neural progenitors detect Shh via the transmembrane receptor Patched1 (Ptc1). The binding of Shh and Ptc1 results in the activation of another transmembrane protein, smoothened (Smo), and its translocation to the tip of the cell’s primary cilium. Smo regulates Gli transcription factors: Gli1, -2, and -3, which in turn mediate changes in the expression of target genes. Shh target genes are transcription factors that regulate the expression profile and identity of progenitors. For example, in the developing spinal cord, the Shh target genes Nkx2.2, Olig2, and Nkx6.1 are transcription factors that characterize the three most ventral neural progenitor cell groups: p3, pMN, and p2, and determine their fates: V3 interneurons, motor neurons, and V2 interneurons, respectively. Shh signaling also influences the more dorsal p1 and p0 neural progenitor domains, which give rise to V1 and V0 interneurons, respectively.

Dorsal neural progenitors are farther from the ventral source of Shh and receive little or no Shh ligand. In the absence of Shh, Ptc1 maintains Smo inactive, and protein kinase A (PKA) promotes proteolytic degradation of Gli2 and also partial processing of Gli2 and Gli3 to truncated transcriptional repressor forms, Gli2R and Gli3R. GliRs repress Shh target gene expression; hence Shh target genes are not expressed in dorsal regions of the neural tube. Loss of Gli3 in mice leads to a dorsal expansion of Shh target genes Nkx6.1 and Nkx6.2 in intermediate regions of the dorsoventral axis of the neural tube (Persson et al., 2002). This result revealed that Gli2R function cannot compensate for Gli3R. Moreover, transgenic introduction of the Gli3R form, specifically, rescued the phenotype, showing that Gli3R is sufficient.

In more ventral cells that do receive Shh, Smo is active and inhibits the proteolytic processing of Gli2 and Gli3, reducing the levels of Gli3R. This removal of Gli3R is sufficient for the specification of p1, p2, and pMN neural progenitors, as shown by the rescue of these progenitors when Gli3 is inactivated in Shh mutants (Persson et al., 2002). To specify the most ventral neural progenitor population, p3, and the floor plate, higher levels of Shh and activated Smo mediate an accumulation of the full length transcriptional activator form of Gli2, Gli2A.
these cells, transcription of Gli3 is repressed, while transcription of Gli1, a target gene itself, is
activated. Gli1 has only been shown to act as a transcriptional activator of Shh target genes and
works in tandem with Gli2A. Loss of Gli2 or loss of both Gli1 and Gli2 results in the absence of
the floor plate marked by the Shh target gene Hnf-3β and a reduction in the p3 domain marked by
the target gene Nkx2.2 (Matise et al., 1998).

In addition to the levels of Shh ligand and Gli activity, the duration of signaling also plays
an important role in assigning character to neural progenitors. Fate-mapping experiments and
transgenic manipulations of chick neural tube explants have shown that neural progenitor
domains are induced in a temporally sequential stepwise fashion, from dorsal to ventral. More
ventral progenitors require higher concentrations of Shh ligand and longer maintenance of Shh
pathway activation in a mechanism called “temporal adaptation” (Dessaud et al., 2007).

For example, in response to a high ventral concentration of Shh ligand, the Olig2 motor
neuron progenitor domain is induced before Nkx2.2 is expressed ventrally adjacent. Over time,
the population of Olig2+ cells expands dorsally, and the most ventral Olig2+ neural progenitors
begin to express Nkx2.2 and downregulate Olig2. Although both Olig2+ and Nkx2.2+ progenitors
are first initiated adjacent to the ventral source of Shh with the same high concentrations of
ligand, Nkx2.2 requires sustained intracellular Shh pathway activation to be upregulated
(Dessaud et al., 2007). Shh pathway activation in Olig2+ progenitors, on the other hand, is
dampened over time by negative feedback in the form of the upregulation of Ptc1 and its capture
of Smo in an inactive state. As the Nkx2.2 progenitor domain expands, the more dorsal Olig2
domain becomes further removed from higher concentrations of Shh ligand required to compete
with negative feedback and maintain the highest levels of intracellular pathway activation.

The duration of Shh signaling is also important in the specification of the three progenitor
domains dorsal to the Olig2 domain, V0-V2. For these progenitors, lower, non-saturating
concentrations of Shh ligand yield proportional Gli activation and more ventral progenitor
identities with increased duration of exposure (Dessaud et al., 2010).
A sufficient interval of Shh signaling is required not only to specify, but also to maintain identity. Premature removal of Shh signaling can cause progenitors to revert to a more dorsal identity (Dessaud et al., 2010). This effect was observed in vivo in transgenic mouse mutants that lose the floor plate source of Shh by embryonic day 10.5 (E10.5). Even though the dorsoventral pattern of neural progenitor markers was normal at E9.5, by E10.5, a reduction in ventral identities and a concomitant expansion of dorsal markers was observed.

In summary, Shh provides positional identity to neural progenitors along the dorsoventral axis through (1) the code of its concentration gradient arising from a ventral source, and (2) the code of time of maintained intracellular pathway activation. Thus, the positional identity of progenitors is assigned in a progressive and dynamic manner by “the time integral—the cumulative level and duration—of Shh signaling” (Dessaud et al., 2010).

Shh is not the only variable in neural tube patterning, however. Other inductive signals also regulate transcription factor expression to pattern and determine cell fate in the neural tube. Retinoic acid (RA), Wnts, and members of the TGF-β superfamily can antagonize or synergize with Shh signaling. Wnt ligands and bone morphogenic proteins (Bmps) of the TGF-β superfamily are expressed in the dorsal neural tube and induce dorsal transcription factor domains. RA is expressed in the somites flanking the neural tube and induces intermediate dorsoventral fates. Both the Wnt and Bmp signaling pathways have interactions with the Shh signaling pathway so that the signaling of one pathway effects changes in the other (Nishi et al., 2009).

The output of these inductive signals along the dorsoventral axis of the neural tube is an array of transcription factor expression domains. Some of these expression domains overlap, so that a neural progenitor group giving rise to a specific cell fate is defined by the expression of a combination of transcription factors. This combinatorial code of transcription factor expression is different for distinct progenitor groups along the dorsoventral axis. Furthermore, the borders between adjacent progenitor groups are sharpened by transcriptional repression between transcription factors in adjacent domains. These cross-repressive interactions become evident
when a transcription factor is reduced or forcibly overexpressed, and the bordering transcription factor concomitantly expands or is downregulated, respectively.

A clear relationship has been observed between Shh signaling and the transcription factors that form cross-repressive pairs in the dorsoventral axis of the neural tube (Briscoe et al., 2000). Transcription factors induced by Shh, such as the target genes Nkx2.2 and Nkx6.1, are termed Class II genes. Those that are repressed by Shh signaling are called Class I, and include Irx3 and Pax6. Class II transcription factors form cross-repressive, bordering pairs with Class I transcription factors. For example, Nkx2.2 and Pax6 cross-repress and border each other, although Pax6 is an activator that indirectly represses Nkx2.2 (Matsunaga et al., 2000). Forced expression of Pax6 within the Nkx2.2 domain leads to downregulation of Nkx2.2, and vice versa. For this reason, removal of Shh causes a loss of Nkx2.2 and also results in a ventral expansion of the Pax6 domain that is no longer under Nkx2.2 repression (Dessaued et al., 2010; Chapter 3, Fig. 1). Cell mixing of distinct neural progenitors in Gli3; Smo compound mutants (Wijgerde et al., 2002) raises the possibility that Shh may also be involved in sorting of similar progenitors via affinity or repulsion mechanisms (Nishi et al., 2009).

**Dynamic domains of Shh expression pattern the forebrain**

The morphogenic activity of Shh extends to the most anterior region of the neural tube, the forebrain. As in the spinal cord, Shh signals in the forebrain are also initially derived from a ventrally underlying mesendodermal source, the prechordal plate, which is anterior to the notochord. Shh signals from the prechordal plate induce a second source of Shh in the ventral midline of the forebrain, similar to the floor plate source of Shh in the developing spinal cord. The Shh concentration gradient arising from these ventral sources leads to Gli activity and the upregulation of Shh target genes *Nkx2.1* and *Nkx2.2* in ventral progenitors of the forebrain. Other inductive signals such as Wnts and Bmps are secreted from the roof plate and help to pattern the dorsal forebrain similar to their role in the spinal cord.
The expression pattern of Shh in the forebrain is more dynamic and complex than in the spinal cord, however, and this difference likely contributes to the greater morphological complexity of the forebrain. First, the prechordal source of Shh is transient, lasting only from about E7.75 to E8.75. Although short-lived, the prechordal plate is a critical source of signals that are necessary to establish the rostroventral forebrain, or the primordium of the hypothalamus. While prechordal plate Shh ventralizes the forebrain, prechordal plate-derived Bmps induce rostral character. In explants, both Shh and Bmps are required to upregulate Nkx2.1, a marker of the hypothalamus not expressed in more caudal CNS regions (Dale et al., 1997; Ohyama et al., 2005).

Second, Shh in the ventral forebrain is repressed from the midline of the caudal hypothalamus at E9.5, forming an expression pattern of two bilateral stripes flanking the midline (Chapter 2, Supplementary Fig. 1). Midline expression of Shh is maintained in the anterior hypothalamus and all neural tube regions caudal to the hypothalamus. In the caudal hypothalamus, Shh-responsive cells expressing Gli1 are found dorsal to the bilateral stripes of Shh, and not at the ventral midline (Ohyama et al., 2008; Chapter 3, Fig. 2). Instead, Bmp4 is expressed in the ventral midline in this region, along with another family of inductive signals, fibroblast growth factors (Fgfs) 8 and 10 (Chapter 2, Fig. 2; Chapter 3, Fig. 3). This Bmp4+ and Fgf8/10+ ventral midline region is critical for the formation of the pituitary gland, which is partially derived from this tissue and underlies the hypothalamus in the adult (Chapter 2, Fig.1, Supplementary Fig. 2).

Bmps appear to trigger the downregulation of Shh and the upregulation of Fgf10 in the caudal ventral midline of the hypothalamus (Manning et al., 2006). Specifically, Bmps upregulate Tbx2, a transcriptional repressor of Shh. In mice, our collaborators have shown that a closely related T-box transcription factor, Tbx3, is required to repress Shh in the hypothalamus (Andreas Kispert, unpublished). In vivo, Tbx2/3 transcription factors are expressed in the caudal ventral midline of the hypothalamus (Chapter 2, Fig.1; Pontecorvi et al., 2008). Their onset of expression is concomitant with the downregulation of Shh at the midline and its restriction to a mutually
exclusive domain that flanks Tbx2/3 midline expression (Manning et al., 2006; Chapter 3, Fig. 2). According to Manning et al. (2006), the downregulation of Shh in these midline cells is required for a temporary arrest in their cell cycle that gives them the capacity for a later wave of proliferative expansion.

What remains in question is the source of the signal that induces Tbx2/3. Bmp signaling is known to induce Tbx2/3, but the Bmp ligand may be (1) Bmp7/4/2 secreted from the prechordal plate, (2) Bmp4 from the ventral midline, or (3) Bmp7 in the ectoderm ventrally adjacent to the neural tube (Chapter 3, Fig. 3). Alternatively, Manning et al. (2006) found that a Wnt antagonist, WIF, is sufficient to rescue Tbx2 expression in explants with blocked Bmp signaling. The authors suggest that Bmp signaling may mediate the downregulation of Wnt activity in vivo in the hypothalamus. However, this interaction has yet to be characterized. Another possibility is that hypothalamic explants have to attain sufficient rostral character to express genes such as Nkx2.1 and Tbx2 of the hypothalamus, an anterior region of the neural tube. As mentioned previously, Bmps from the prechordal plate induce rostral hypothalamic character (Dale et al., 1997; Ohyama et al., 2005). However, since Bmps are blocked in the explants, the cells may attain sufficient rostral character to express Tbx2 by the inhibition of Wnt signaling. The inhibition of Wnts is a well-characterized mechanism that induces anterior character in the neural tube. When Wnt antagonists or transcriptional repressors are deficient in vivo, the specification of the forebrain, including the hypothalamus, does not occur and only more posterior neural tube regions are observed (Hoch et al., 2009).

Anterior-posterior patterning also plays an important role in specifying the domain of Shh expression in the forebrain. Six3 is a transcriptional repressor of Wnt7 and is thus critical in establishing the anterior forebrain domain. At the same time, Six3 also acts as a transcriptional activator of Shh in the anterior midline of the hypothalamus (Geng et al., 2008; Jeong et al., 2008). Six3 activates Shh in the hypothalamus by binding to Shh brain enhancer 2 (SBE2), a hypothalamic-specific enhancer of Shh (Jeong et al., 2006).
Bmp signaling must also be regulated to mediate proper anterior-posterior patterning and Shh expression in the forebrain. The Bmp antagonists noggin and chordin are expressed in the prechordal plate and the anterior neural ridge (ANR), an anterior source of rostralizing, forebrain-inducing signals such as Fgf8 (Anderson et al., 2002). Noggin and chordin from these sources are critical for limiting Bmps, excessive levels of which are known to downregulate Shh and Fgf8 (Ohkubo et al., 2002). Interestingly, although there is no Shh in the forebrain or prechordal plate of Chrd<sup>−/−</sup>;Nog<sup>−/−</sup> mutants at E8.5, by E9.5, the bilateral stripes of Shh in the caudal hypothalamus are present (Anderson et al., 2002). However, as in mutants haploinsufficient for Six3, Shh in the anterior midline of the hypothalamus is absent in Chrd<sup>−/−</sup>;Nog<sup>−/−</sup>. Similarly, when only noggin is deleted, the anterior midline domain of Shh is reduced (Davis and Camper, 2007). Thus, it appears that the expression of Shh in this anterior domain is particularly sensitive to imbalances in anterior-posterior patterning.

Nevertheless, aberrant anterior-posterior patterning in the hypothalamus does not always affect Shh expression, signifying that Shh can be in a separate pathway or upstream of other anterior-posterior patterning factors. For example, Hesx1 is another transcriptional repressor required to repress Wnt/β-catenin signaling and promote forebrain identity. In Hesx1 null embryos, Fgf8/10 expression in the caudal midline of the hypothalamus expands rostrally into the anterior midline where Shh is expressed (Dasen et al., 2001). Shh expression, however, is not affected in these animals (Dattani et al., 1998).

In other studies of mutants with aberrant anterior-posterior patterning in the hypothalamic midline, the pattern of Shh expression was not reported. These studies include analysis of Wnt pathway members expressed in the anterior midline of the hypothalamus: Wnt5a and Tcf4 (Chapter 2, Fig. 3), which are required to restrict the caudal Bmp4<sup>+</sup> and Fgf8/10<sup>+</sup> domain (Brinkmeier et al., 2007; Potok et al., 2008). In addition, Sox3, a transcription factor expressed in neural progenitors throughout the CNS (Chapter 2, Fig. 4), functions through an uncharacterized mechanism to prevent Bmp4 and Fgf8/10 expansion (Rizzoti et al., 2004).
When Bmp4 and Fgf8/10 expand rostrally, it can be at the expense of the domains of transcription factors such as Six6 that are co-expressed with Shh in the anterior midline of the hypothalamus (Chapter 2, Fig. 2). Six6 is rostrally restricted and reduced in noggin mutants (Davis and Camper, 2007).

Future experiments are required to incorporate all these factors into one mechanism of anterior-posterior patterning in the hypothalamic ventral midline. Regardless of the cause, the aberrant expansion of the domain of Bmp4 and Fgf8/10 signals in the caudal midline of the hypothalamus induces excessive pituitary tissue from the underlying ectoderm and results in pituitary dysmorphology (see "Specification and Function of the Pituitary" for more information). Although not yet characterized due to the focus in the pituitary in these experiments, such a defect in anterior-posterior patterning in the hypothalamus is likely to affect the identities of neural progenitors and their hypothalamic cell fates.

Lastly, at E10.0, two new domains of Shh expression are observed rostral and dorsal to the hypothalamus within the forebrain (Shimamura et al., 1995). First, rostral Shh expression is detected in the telencephalon, the region of the developing forebrain that will give rise to the cerebral cortex dorsally and the basal ganglia ventrally. Specifically, Shh is expressed in the medial ganglionic eminence (MGE) and the adjacent preoptic area (POA) at the ventral midline (Chapter 2, Fig. 1). The transcription factor Nkx2.1 is co-expressed with Shh in these regions (Chapter 2, Fig. 1). The MGE develops into the globus pallidus, a nucleus of the basal ganglia involved in motor control. In addition, the MGE gives rise to inhibitory interneurons that migrate dorsally and function in the cortex (Anderson et al., 2001). The POA, on the other hand, is involved in maintaining homeostasis and regulating endocrine function along with other nuclei, or subregions, of the hypothalamus. In fact, the POA is considered part of the hypothalamus in all disciplines except embryology. The POA considered separate from the hypothalamus due to its formation from a separate, later-induced domain of gene expression during embryonic development (Puelles and Rubenstein, 2003).
Second, Shh expression in the shape of a spike rising dorsally from the caudal ventral midline forms the zona limitans intrathalamica (ZLI; Chapter 2, Fig. 1). The ZLI is a critical source of signals for patterning the thalamus, the region of the brain dorsal to the hypothalamus. The thalamus is the central integrator of all the various sensory input the brain receives. As it develops, the thalamus expresses the Class I transcription factor Pax6, while the more ventral hypothalamus expresses Class II transcription factors Nkx2.2 and Nkx2.1 (see “Sonic hedgehog: A secreted signal activates a transcriptional cascade”; Chapter 3, Fig. 1). Together, the developing thalamus and hypothalamus comprise the diencephalon in the forebrain (reviewed in Blackshaw et al., 2010).

**Specification and function of the hypothalamus**

The hypothalamus regulates vital autonomic and endocrine functions and associated behaviors. Specifically, the hypothalamus mediates thermoregulation, fluid & electrolyte balance, stress response, circadian rhythms, feeding and metabolism, sexual behavior and reproduction, and arousal and sleep. The hypothalamus consists of about a dozen nuclei that regulate these functions and synchronize with each other. For example, through multiple connections between hypothalamic nuclei, daily patterns of sleep and wakefulness are intimately linked to the regulation of feeding and energy metabolism (Gooley et al., 2006).

Along the anterior-posterior axis of the hypothalamus, nuclei can be divided into anterior, medial, and posterior groups with general functions. Gene expression patterns, however, subdivide these broad categories even at early embryonic stages (Shimogori et al., 2010). The Shh expression domain along the anterior-posterior axis of the hypothalamus serves as a spatial and ontogenic guide with which to relate the expression of region-specific genes (Chapter 2, Fig. 1). In fact, most hypothalamic nuclei can be identified during embryonic development based on gene expression, even though the cells have not settled into their final positions and condensed into nuclei. Hypothalamic cells are born near the third ventricle and then move out.
laterally as they differentiate, so that lateral nuclei are born before medial nuclei flanking the ventricle.

At the most anterior level, the POA regulates fluid and electrolyte balance, body temperature, and sexual hormones (Saper, 2009). The ventrolateral preoptic area specifically promotes sleep. Cells of the anterior hypothalamus (AH), just caudal to the POA, have similar roles. In the embryo, the POA is actually of telencephalic origin and thus expresses the telencephalic marker Foxg1, differentiating it from other hypothalamic nuclei. As mentioned previously, the POA expresses Shh and Nkx2.1, but it does not express other Shh target genes such as Nkx2.2 and Nkx6.2 (Shimogori et al., 2010). The AH, on the other hand, develops within the Shh-responsive zone and does express Nkx2.2 and Nkx6.2 (Puelles and Rubenstein, 2003; Shimogori et al., 2010).

Within the anterior region of the hypothalamus, the suprachiasmatic nucleus (SCN) can be identified ventrally, sitting on top of the optic chiasm. The SCN is the “master clock” of circadian rhythms for the body. Shimogori et al. (2010) have found that the SCN is marked by the transcription factor Lhx1 by E12.5. By this time, Lhx1 can be found within a zone that is just caudal to Nkx6.2, but sits rostral to the more caudal domain of Shh expression. Although not confirmed, it is likely that neural progenitors that give rise to Lhx1+ cells express the target gene Nkx2.2 (Seth Blackshaw, personal communication).

In relation to the SCN, the paraventricular nucleus (PVN) lies dorsally while the related supraoptic nucleus (SO) lies laterally. Neurons in the PVN and SO derive from a common supraopto-paraventricular region (SPV) during embryonic development that can be marked by genes such as Otp and Sim1 that are expressed dorsal to the Shh-signaling domain (Shimogori et al., 2010; Puelles and Rubenstein, 2003). These nuclei contain the magnocellular neurons of the hypothalamus. As the name implies, these cells have large somas that synthesize one of two hormones, vasopressin or oxytocin, for direct release into the body’s circulatory system. The axons of these cells terminate in the posterior lobe of the pituitary gland, which is also called the neurohypophysis due to its content of these axons (Saper, 2004). Once released from the
posterior pituitary, the hormone oxytocin acts mostly in the brain to mediate responses related to labor, breastfeeding, and bonding behavior. Vasopressin, on the other hand, acts on the kidneys and promotes the body’s conservation of water.

In addition to magnocellular neuroendocrine neurons, the PVN also has smaller, parvocellular neuroendocrine neurons (Saper, 2004). Parvocellular neurons are also found in other nuclei, such as the POA and the arcuate (see below). These neurons synthesize hormone signals and release them at the median eminence, where the capillaries of the portal system absorb and carry the signals to cells in the anterior lobe of the pituitary. Anterior pituitary cells then respond by synthesizing and releasing hormones active in the body. For example, parvocellular neurons of the PVN synthesize corticotropin-releasing hormone that promotes anterior pituitary cells to release adrenocorticotropic hormon, or ACTH. ACTH acts on the adrenal gland and regulates the release of adrenal hormones in response to stress. In this manner, the hypothalamus controls the release of hormones from the pituitary, which is in turn “the master gland” of the body.

The middle third, or tuberal, hypothalamus contains nuclei that regulate feeding, energy metabolism, stress responses, and coordinate all these with wake-sleep cycles (Saper 2009). Although more caudal, the premamillary nucleus also fits in with these nuclei functionally, as it has been found to integrate energy signals with reproductive ability (Donato et al., 2009). Again, gene expression patterns subdivide this region even at early embryonic stages (Shimogori et al., 2010). From rostral to caudal, Pomc marks the arcuate nucleus (Arc), Nr5a1 (previously Sf-1) is expressed exclusively in the ventromedial nucleus (VM), Lhx8 marks the dorsomedial nucleus, and Lef1 marks the premamillary region (PM). All of these markers are found within the domain of Nkx2.1 (Shimogori et al., 2010), which is expressed in response to Shh signaling in the ventral region of the hypothalamus. The caudal bilateral stripes of Shh also run through this region and may partially overlap with these markers.

The caudal end of the hypothalamus contains nuclei that are critical for maintaining wakefulness, responding to emergencies, and establishing memories (Vann and Aggleton, 2004;
Saper, 2009). In the embryo, the caudal expression of Nkx2.1 and Shh continues into this domain. Like the PVN, the mamillary nucleus (MM) in this region also expresses Otp and Sim1, and in addition Foxb1 (Shimogori et al., 2010; Puelles and Rubenstein, 2003). Just caudal to the mamillary and at the rostral base of the ZLI, the supramamillary nucleus (SM) can be identified by expression of Irx5 (Shimogori et al., 2010).

The expression of Shh near or in the domains of many hypothalamic nuclei markers raises the question whether Shh is required in the hypothalamus for the specification of many of these subregions (Chapter 2, Fig. 1). Until recently, the role of the expression of Shh in the hypothalamus was unknown, since the hypothalamic region fails to form in Shh mutants without the prechordal plate source of Shh. Recently, two groups conditionally removed Shh expression from the hypothalamus, although adjacent areas outside the hypothalamus also underwent recombination in both experiments.

First, Szabó et al. (2009) genetically deleted Shh from the caudal hypothalamus and the ZLI, leaving Shh in the prechordal plate and the anterior ventral midline of the hypothalamus intact. In these Foxb1-Cre<sup>−/−;Shh<sup>loxP/loxP</sup> mutants, called Shh-c, Nkx2.1 was still expressed, highlighting that Shh is not required in this domain to specify hypothalamic character (Sussel et al., 1999). The intact prechordal source of Shh was likely sufficient to induce Nkx2.1, although the hypothalamic region was reduced and the third ventricle was expanded. The authors evaluated whether the hypothalamic neuroepithelium was reduced due to a requirement for Shh in proliferation or cell survival, as such a requirement for Shh had been identified in the diencephalon at E9.0 (Ishibashi and McMahon, 2002). However, cell death was normal as compared with controls in Shh-c, and only a 20% decrease in proliferation was visible at E12.5 but not E10.5. Rather, Shh from the caudal hypothalamus and ZLI appeared to be required for the proper specification of hypothalamic subregions and the prethalamus. The lateral regions of the hypothalamus and tuberal nuclei such as the VM were reduced, while terminal differentiation of the MM did not occur. Loss of Shh from the ZLI resulted in loss of the prethalamus, as
previously characterized (Kiecker and Lumsden, 2004). The anteroventral hypothalamic nuclei, on the other hand, were relatively unaffected, likely because Shh in the anterior ventral midline of the hypothalamus was maintained.

Second, Shimogori et al. (2010) deleted Shh from the hypothalamus, the POA, and the MGE using \( Nkx2.1-cre;Shh^{loxP/loxP} \) mice. Similar to Szabó et al. (2009), this group noticed an expansion of the third ventricle and thinning of the hypothalamus. Tuberal nuclei were lost. However, MM and SM nuclei were unaffected, as was the prethalamus with the preservation of Shh in the ZLI. Particularly, Shh at the base of the ZLI is within the SM, and this source of Shh was likely sufficient for the proper development of the SM and the adjacent MM in these animals. Since \( Nkx2.1-cre;Shh^{loxP/loxP} \) mice additionally lose Shh in the hypothalamic anterior midline, POA, and MGE, nuclei in the anterior hypothalamus are lost and the ventral telencephalon is reduced. The authors verified that the telencephalic marker Foxg1 was still present and had not expanded caudally into the hypothalamic domain. In fact, the markedly reduced hypothalamic region was still marked by an early marker, Rax. Thus, an early hypothalamic domain had been specified, but it seemed to have failed to differentiate and expand without the hypothalamic source of Shh.

It is not clear from these experiments whether the telencephalic or the hypothalamic source of Shh in the anterior midline of the hypothalamus is required for the development of anterior nuclei. Also, if loss of Shh did not lead to marked changes in proliferation or cell death (Szabó et al. 2009), why did the Rax domain fail to expand? One possible mechanism is altered patterning of factors downstream of Rax. In the developing spinal cord (see “Sonic hedgehog: A secreted signal activates a transcriptional cascade”), for example, when Shh target genes are downregulated due to a loss of Shh, more dorsal transcription factors expand into their domains. What genes expand into the hypothalamic domain to mediate its altered morphology in the absence of Shh? My experiments in Chapter 3 address these questions.
**Specification and function of the pituitary**

The pituitary gland sits just below the hypothalamus, and a stalk called the infundibulum connects both structures. Hypothalamic hormone signals modulate cell proliferation, hormone synthesis, and secretion of the adenohypophysis, which includes the anterior and intermediate lobes of the pituitary. The neurohypophysis, or posterior lobe of the pituitary, consists of the axon terminals of the hypothalamic magnocellular neurons, as discussed previously. Hormones secreted by the pituitary, or “the master gland,” act on several target organs in the body.

The adenohypophysis contains six different cell types characterized by the different hormones they produce and secrete (Zhu et al., 2007). Somatotropes secrete growth hormone (GH) and regulate linear growth and metabolism. Lactotropes produce prolactin (PRL), regulating milk production in mammalian females. Corticotropes secrete ACTH, which is a proteolytic product of proopiomelanocortin (POMC). Thyrotropes produce thyroid-stimulating hormone (TSH), which promotes thyroid hormone (TH) secretion and modulates skeletal remodeling. Gonadotropes produce luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which promote sexual maturation and maintain reproductive function. In the intermediate lobe, melanotropes secrete α-melanocyte stimulating hormone (α-MSH). α-MSH is a product of processed ACTH and regulates the production of the pigment melanin by melanocytes in the body.

The generation of these six different cell types is the result of pituitary patterning, proliferation, and cell differentiation during development. First, morphogenic movements beginning at around E9.0 lead to the formation of the pituitary primordium. In mammals, the anterior pituitary develops from an invagination of oral ectoderm called Rathke’s pouch. Rathke’s pouch has a fingerlike shape that points up towards the overlying hypothalamus. Simultaneously, a portion of the Bmp4⁺ caudal hypothalamus invaginates downwards, forming a small protrusion called the infundibulum which abuts Rathke’s pouch dorsocaudally. The apposition of the infundibulum to Rathke’s pouch is critical for pituitary development. These two vesicular
structures progressively grow and pinch off during embryonic development to form the anterior and posterior lobes of the pituitary gland.

The initial, most critical signals for the formation of the pituitary are Bmp4 and Fgf8/10 secreted by the hypothalamus (Chapter 2, Fig. 2). Thus, a hypothalamic domain must be specified before the pituitary can begin to form. This requirement is observed in Shh and Gli1;Gli2 mutants that lack the hypothalamus (Park et al., 2000; Treier et al., 2001). In Nkx2.1 and severe Gli2 mutants, the hypothalamus is barely present as is the expression of Bmp4 and Fgf8 (Takuma et al., 1998; Wang et al., 2010). Hence, a rudimentary pouch begins to form in these mutants but is not maintained. Bmp4 appears to be required for invagination of Rathke’s pouch, as this failed to occur in Bmp4 mutants on a mixed background (Takuma et al., 1998). Once induced, the pituitary continues to require Bmp signaling to specify all cell types except a few corticotropes (Treier et al., 1998). Fgf10 and its receptor are also required after initial induction for cell survival within Rathke’s pouch and to prevent agenesis of the anterior pituitary by E14.5 (Ohuchi et al., 2000). Bmp4 and Fgfs in the infundibulum appear to function by upregulating Lhx3, the definitive marker of the adenohypophysis from the E9.5 Rathke’s pouch until adulthood (Chapter 2, Fig. 1). Similar to the requirement for Bmp and Fgf signaling, the transcription factor Lhx3 is required for cell survival within the pouch and the specification of all cell types except a few corticotropes (Sheng et al., 1996). Specifically, in vitro studies have shown that Lhx3 synergizes with other transcription factors to upregulate markers of pituitary cell fates (Sloop et al., 2001).

In addition to stimulating proliferation and inducing Lhx3 expression in Rathke’s pouch explants, the infundibulum patterns the pouch (reviewed in Zhu et al., 2007). The infundibulum has been shown to promote dorsal progenitor identities by antagonizing ventral identities marked by Isl1 and αGSU. Isl1 and αGSU are upregulated by ventralizing Shh and Bmp2 signals. Shh is expressed just outside the base of Rathke’s pouch in the oral ectoderm, and it signals to the pouch to promote proliferation and upregulate BMP2 in the ventral pouch. BMP2, in turn, induces
expression of Isl1 and αGSU, while suppressing expression of dorsal ACTH fates (Treier et al., 1998).

As mentioned previously, expansion of the Bmp4+ and Fgf8/10+ infundibular domain can lead to dysmorphology of Rathke’s pouch. Forced overexpression of Fgf8 causes ectopic Lhx3 induction and pituitary hyperplasia with dramatic expansion of dorsal ACTH cell lineages (corticotropes and melanotropes) and inhibition of other fates (Treier et al., 1998; 2001). In addition, the pituitary appears dysmorphic with multiple lumens. Increased Bmp signaling in noggin mutants also leads to a similar defect (Davis and Camper, 2007). Rathke’s pouch appears hyperplastic and rostrally shifted with multiple invaginations. Similar phenotypes are observed in Hesx1, Wnt5a, Tcf4, and Sox3 mutants, in which expansion of the infundibular domain is regarded as the main cause for the dysmorphology (Dasen et al., 2001; Potok et al., 2008; Brinkmeier et al., 2007; Rizzoti et al., 2004).

Dorsal Fgf and ventral BMP2 gradients regulate the expression of transcription factors that determine the proliferation program, positional identity, and fate of pituitary progenitors (Zhu et al., 2007). In the most ventral region of the anterior pituitary, Tbx19 marks progenitors that differentiate early to give rise to corticotropes. Gonadotropes are also found in the ventral pituitary but they differentiate later from Sf-1+ cells. Tbx19 is also required in the most dorsal portion of the adenohypophysis to specify the melanotropes of the intermediate lobe. Pit1 marks progenitors in the caudomedial region of the pituitary gland at E13.5. Pit1+ cells will give rise to somatotropes, thyrotropes, and lactotropes. However, most lactotropes arise postnatally when Pit1 and the transcriptional co-repressor N-CoR repress the growth hormone gene in a population of somatotropes to specify lactotropes (Scully et al., 2000).

An important aspect of pituitary development that has not been addressed is the role of Shh in the hypothalamus. Shh in the anterior midline of the hypothalamus is just rostral to the domain of critical Bmp4 and Fgf10 signals for pituitary development. The requirement for Shh signaling to cells in Rathke’s pouch was evaluated, but this approach did not differentiate
between the oral ectoderm and the hypothalamic source (Treier et al., 2001). In addition to signaling to cells in the pituitary, Shh in the hypothalamus may play an important role in patterning the infundibular region so that it expresses Bmp4 and Fgf10 appropriately for proper pituitary development. In Chapter 2, I evaluate this requirement for Shh in the hypothalamus.

**The role of Shh in the development of the visual system**

Shh is required for proper development of the eyes, especially at the initial stages of their development. Early in embryonic development, the primordium of the two bilateral eyes in vertebrates is a single midline tissue in the anterior neural plate. Shh signals from the underlying prechordal plate are required for the eye field to split into discrete left and right eye primordia (reviewed in Roessler and Muenke, 2010). If the prechordal plate or Shh signals are absent, a single eye will develop at the midline: a condition known as cyclopia. As the neural tube closes, the bilateral eye primordia begin to evaginate to form the optic vesicles. At E9.5, these protrusions can be found on the sides of the forebrain at the anterior-posterior level between the preoptic area and the anterior hypothalamus, sites of Shh expression at the ventral midline.

Several morphogenetic events occur in the optic vesicle (Fuhrmann, 2010). First, as the optic vesicle grows outward, it makes contact with the surface ectoderm and induces the formation of the lens. Second, the optic vesicle undergoes a basal constriction proximal to the brain to form the optic stalk. Third, ventral-lateral optic vesicle tissue invaginates, resulting in the double-layered optic cup (Chapter 2, Fig. 3). The inner layer of the optic cup gives rise to the neural retina, while the outer layer differentiates into the retinal pigment epithelium (RPE).

Shh continues to be required for eye development during the morphogenetic events of the bilateral optic vesicles (Yang, 2004). Shh from the prechordal plate and/or the forebrain ventral midline regulates the transcription factors that specify optic stalk, retina, and RPE identity. As in other CNS regions, Bmp4 expressed in the dorsal retina antagonizes the effects of ventrally-derived Shh. Shh upregulates markers of ventral and proximal regions of the optic vesicle and cup, while downregulating the following dorsal and distal markers: (1) Tbx5 in the
dorsal retina promoted by Bmp4 (Koshiba-Takeuchi et al., 2000); and (2) Class I transcription factor Pax6 (Chapter 2, Fig. 3) of the retina and RPE (Walther and Gruss, 1991). Specifically, Shh induces Pax2, Vax1, and Vax2, which specify the optic stalk and ventral retina (Chapter 2, Fig. 3). Pax2, Vax1, and Vax2 then in turn repress Pax6 (Macdonald et al., 1995; Mui et al., 2005). Vax transcription factors have cross-repressive interactions with Tbx5 (Schulte et al., 1999), and Pax2 and Pax6 cross-repress each other (Schwarz et al., 2000).

Changing the expression domains of these factors causes a concomitant change in the morphology of the optic cup and stalk. Overexpression of Shh in early zebrafish embryos causes a reduction of Pax6 and an expansion of the Pax2 expression domain, resulting in fewer retina and RPE cells and expanded optic stalks (Macdonald et al., 1995). Loss of Vax1 and Vax2, on the other hand, causes loss of Pax2 and the expansion of Pax6+ retina into the optic stalk (Miu et al., 2005).

The timing and source of Shh signals for patterning of the optic vesicle and cup remain unconfirmed. Recently, results by Zhao et al. (2010) showed that Shh signaling was required after formation of the optic vesicles. Zhao et al. (2010) evaluated the requirement for Shh signaling in the optic vesicle and stalk from E9.5 by conditionally deleting Smo in the CNS. Although at first the optic vesicle appeared normal and expressed Vax1, Vax2, and Pax2, by E10.0, progressive failure of ventral optic cup development was detectable. Increased cell death in the optic vesicle likely contributed to the defect. Bmp4 upregulation and expansion as early as E9.5 may have caused the increase in cell death, as this is a previously characterized function of Bmp4 in the eye (Trousse et al., 2001). In addition, the authors observed a reduction in proliferation in the ventral optic cup and also a progressive loss of Vax1, Vax2, and Pax2 from this region, even at stages when this tissue was present. By E11.0, the optic stalk marked by Pax2 was shorter, and by P0 the animals were born without eyes. Although this study provided valuable information on the continued requirement for Shh signaling after optic vesicle formation, it did not address the source of Shh signals. In Chapter 2, I evaluate the requirement of Shh signals from the midline of the hypothalamus for optic cup patterning.
Towards the end of eye cup formation, the ventral retina and ventral optic stalk undergo morphogenetic movements to create a passage way for the exit of retinal axons with visual information and the entrance of the hyaloid artery with blood supply to the eye (Otteson et al., 1998). Along the ventral midline of the eye cup and ventral optic stalk, Pax2+ cells invaginate forming the optic fissure (Chapter 2, Fig. 3). Mesenchymal cells enter the eye cup through the optic fissure to form the hyaloid artery. As the fissure begins to seal closed, the first retinal ganglion cell (RGC) axons exit the eye just adjacent to the hyaloid artery through the fissure. These exiting pioneer axons will be joined by more axons as more RGCs differentiate, forming the thick axon fiber bundle that is the optic nerve. At the interface between the eye and optic stalk, invaginating Pax2+ cells of the optic fissure encircle the exiting RGC axons. These Pax2+ cells differentiate into glia that support the RGC axons and secrete the axon attractant Netrin1 (Deiner et al., 1997). If the eye is removed, the transection of the exiting RGC axon bundle has the appearance of a white disc. Hence, the site where RGC axons exit the eye is called the optic disc, and the Pax2+ and Netrin1+ glial cells that encircle the RGC axons as they exit are called optic disc cells (Chapter 2, Fig.3).

Proper patterning of the optic cup is critical for the formation and closure of the optic fissure and related optic disc. Mutations in Shh and the transcription factors it regulates during the morphogenesis of the optic cup: Pax6, Pax2, Vax1 and Vax2, can result in coloboma, or failure to close the optic fissure (Gregory-Evans et al., 2004). The optic disc is often affected in coloboma, and this can result in blindness. For example, when Vax1 and Vax2 are absent, expansion of Pax6+ retina into the optic stalk prevents closure of the optic fissure and formation of the optic disc (Miu et al., 2005).

A more subtle phenotype results from haploinsufficiency of Pax2 in the Krdr mouse (Otteson et al., 1998). In these mice, the invagination of the optic fissure does not extend to the optic stalk. Thus, Pax2+ cells fail to encircle exiting RGC axons and instead remain outside the eye in the optic stalk. Consequently, the optic disc is malformed, with RGC axons exiting the eye in fascicles through the retina before converging into the optic nerve. Interestingly, an expansion
of RPE was observed, which is consistent with a possible expansion of the Pax6 domain in the presence of insufficient Pax2 repressor.

Another Bmp, Bmp7, is also required for the formation of the optic fissure and disc (Morcillo et al., 2006). Bmp7 is expressed in the ventral midline, proximal optic vesicle and cup, and in the mesenchymal cells that form the hyaloid artery. In Bmp7 mutants, several patterning markers of the optic cup and stalk were maintained. Only the invaginating Pax2⁺ cells that form the optic fissure appeared reduced, in addition to a defect in proliferation and apoptosis in the ventral optic cup. Since Bmp7 may be downstream or in a separate signaling pathway from other patterning markers of the optic vesicle, it would be interesting to determine what factors regulate Bmp7. In Chapter 2, I evaluate whether Shh in the hypothalamus regulates Bmp7 expression in the midline and the optic vesicle.

As eye cup morphogenesis is completed, a new domain of Shh expression is initiated in RGCs (Wallace, 2008). Newly differentiated RGCs initially arise in the center of the eye cup and progressively differentiate towards the periphery, forming the RGC layer adjacent to the vitreous fluid of the eye. RGCs secrete Shh signals to the adjacent, inner neuroblast layer, where Shh is required to promote proliferation of retinal progenitor cells. At the same time, Shh from RGCs provides negative feedback to limit the differentiation of retinal progenitors to RGCs.

Retinal Shh was also found to be required to specify optic disc cells and prevent the expansion of RPE into the optic stalk (Dakubo, 2003). However, the Thy1-Cre mouse line (“703”) used in the study did show recombination in the hypothalamus (Campsall et al., 2002), so that a requirement for hypothalamic Shh for the specification of optic disc cells cannot be excluded. In Chapter 2, I evaluate this requirement for hypothalamic Shh.

Finally, Shh can be transported down the axons of RGCs, where a role for Shh in their guidance has been characterized (Bovolenta, 2005). In addition, Shh is critical for patterning the forebrain and the expression domains of axon guidance factors such as Slit chemorepellents (Barresi et al., 2005). Slits guide RGC axons on their trajectory once they exit the eye. Specifically, most RGC axons cross under the hypothalamus to form the optic chiasm, and the
expression of Slit repellents in the POA and hypothalamus are required to restrict the path of RGC axons and guide chiasm formation (Plump et al., 2002). After crossing at the chiasm, RGC axons form the optic tract and project to the thalamus and midbrain for initial stages of visual information processing.

In conclusion, Shh is required throughout most of the development of the eyes and for the proper development of the retinal projection. Shh signals from the hypothalamus may be required during certain time points, such as the morphogenesis of the optic vesicle. At other time points, hypothalamic Shh may not play a role and the retinal source of Shh, for example, may be critical for the development of the visual system. Thus, different aspects of visual system development may require different sources Shh, and deciphering these requirements may have implications for the etiology of blindness in patients with congenital brain malformations.

**Shh insufficiency results in holoprosencephaly (HPE) in humans**

Loss of Shh in humans is exhibited as a condition known as holoprosencephaly (HPE). HPE is characterized by incomplete separation of the bilateral cerebral hemispheres and dysmorphology of the facial midline (Solomon et al., 2010). Severely affected patients may have microcephaly and cyclopia below a proboscis (a non-functioning, nose-like appendage). Patients with severe craniofacial malformations are more likely to have alobar HPE, characterized by a single “monoventricle” resulting from a complete failure to separate the cerebral hemispheres. Less severe facial abnormalities include varying degrees of hypotelorism, cleft lip and/or palate, and flattened nasal bridge. Often, “the face predicts the brain,” so that the milder the facial phenotype, the less severe is the brain malformation. Partial separation of the cerebral hemispheres occurs in the following types of HPE, in order of decreasing severity: semilobar, lobar, and middle interhemispheric fusion variant (MIHF/MIHV). Subcortical structures, such as the basal ganglia, thalami, and hypothalami, may also fail to be separated.

These malformations are consistent with the early requirement for Shh signals from the prechordal plate in the separation of the eye field and cerebral hemispheres into bilateral
structures. The requirement for Shh in this process is further supported by mutations in Shh pathway genes, such as **GLI2** and **PTCH1**, which can also cause HPE (Solomon et al., 2010). Similarly, mutations in **SIX3** can lead to HPE, and this transcription factor is required for early forebrain formation and has been shown to activate Shh in the anterior midline of the hypothalamus (Geng et al., 2008; Jeong et al., 2008). Apart from activating Shh, formation of the prechordal plate mediated by the HPE genes **NODAL** and **ZIC2** is also required (Gritsman et al., 2000; Warr et al., 2008). Although necessary for prechordal plate formation, Nodal signals may also need to be regulated, as evidenced by the requirement for the HPE gene **TGIF1**, a transcriptional regulator of TGFβ signaling (El-Jaick et al., 2007).

In humans, a loss of function mutation in one allele of **SHH** is sufficient to generate the HPE phenotype, while mice heterozygous for Shh mutations are phenotypically normal. Nevertheless, large families have been identified in which certain members have SHH mutations but lack an overt phenotype, while the same deleterious mutation resulted in HPE in the proband (Solomon et al., 2010). Upon genetic identification, the seemingly unaffected family members with SHH mutations may be found to have an HPE microform, such as mild microcephaly, hypotelorism, or a single maxillary central incisor (SMCI). The variable penetrance of SHH mutations, like many other autosomal dominant disorders, is hypothesized to involve interactions with other gene loci and the environment. Alcohol, for example, is a teratogen that can cause HPE (Lipinski et al., 2010).

The structural brain anomalies in HPE often result in severe neurological impairment and seizures. Malformations of the hypothalamus can lead to autonomic deregulation of temperature control and electrolyte imbalance, which can also result in seizures (Solomon et al., 2010). Seventy percent of HPE patients suffer from central diabetes insipidus (DI), a deficit in the release of antidiuretic hormone, also called vasopressin (see “Specification and function of the hypothalamus”; Hahn et al., 2005). Vasopressin is synthesized in the hypothalamus and released at the posterior pituitary. Lack of vasopressin can result in dehydration and seizures due to
polyuria, especially if the other typical symptom of polydipsia is absent. A possibility exists that compromised hypothalamic osmoreception in HPE may be the root cause for failure to release vasopressin. Hahn et al. (2005) found that the severity of DI correlated with the grade of HPE and hypothalamic non-separation. Other endocrinopathies consistent with anterior pituitary dysfunction were found to be much less common.

In my experiments, I evaluate whether Shh in the hypothalamus is required for the morphogenesis of the forebrain and associated structures affected in HPE.

**Loss of Shh in the hypothalamus may result in septo-optic dysplasia (SOD)**

Loss of Shh in the hypothalamus may lead to milder congenital malformations than HPE, since Shh in the hypothalamus is a later source of Shh than the prechordal plate and a subdomain of Shh forebrain expression. Shh in the hypothalamus may be important for patterning within the hypothalamus, which is in turn critical for pituitary development (see “Dynamic domains of Shh expression pattern the forebrain” and “Specification and function of the pituitary”). In addition, Zhao et al. (2010) showed that Shh signaling was required for eye development after formation of the optic vesicles, at a time point when Shh signals from the hypothalamic midline, medial to the optic vesicles, may be necessary (see “The role of Shh in the development of the visual system”). As discussed previously, the final stages of optic cup development are particularly important for the morphogenesis of the optic disc and the exit of RGC axons from the eye, forming the optic nerve. A congenital condition that comprises these possible deficits in the hypothalamic-pituitary axis and the optic nerve is called septo-optic dysplasia (SOD).

Septo-optic dysplasia (SOD), also known as de Morsier Syndrome, is rare, affecting 1 in 10,000 live births (Webb and Dattani, 2010). The condition is highly heterogeneous and is currently defined by any combination of (1) optic nerve hypoplasia (75–80% of patients), including absence of the optic disc; (2) midline neuroradiological abnormalities such as agenesis of the corpus callosum and absence of the septum pellucidum; and (3) pituitary hypoplasia and consequent panhypopituitarism. Around 30% of SOD patients meet all three diagnostic criteria.
Most patients (62-80%) suffer from hypopituitarism, especially growth hormone deficiency, and 60% have an absent septum pellucidum. Malformation of the brain midline appears to be the central deficit resulting in SOD features.

SOD symptomatology can be partially predicted from the malformations detected by MRI (McCabe et al., 2011). Coincident with optic nerve hypoplasia on an MRI, neurological deficits are common, ranging from global retardation to focal deficits such as epilepsy. 23% of SOD patients have significant visual impairment. Anterior pituitary hypoplasia, an undescended posterior pituitary, and an absent pituitary stalk are predictors of hypopituitarism. An ectopic posterior pituitary can also be observed in SOD, although it is not as strong a predictor of hypopituitarism as an undescended posterior pituitary. The extent of pituitary dysfunction in SOD patients is highly variable, ranging from isolated growth hormone deficiency to panhypopituitarism.

Both environmental and genetic factors are thought to cause the disease (reviewed in Webb and Dattani). Currently, a genetic diagnosis is found in less than 1% of SOD patients. Environmental factors include increased prevalence of prenatal drug and alcohol abuse and younger maternal age in SOD cohorts.

Genetic mutations in $HESX1$, $SOX2$ and $SOX3$, and recently $OTX2$, have been found to be associated with SOD (reviewed in McCabe et al., 2011). All of these genes have an early role in specifying forebrain fate, and then also later roles in the development of the eye and/or pituitary.

$Hesx1$ is required for proper formation of the eyes and the hypothalamic-pituitary axis affected in SOD. Specifically, $Hesx1$ mouse mutants can have absent optic vesicles, microphthalmia and anophthalmia, and hypoplasia of the anterior pituitary, ventral diencephalon, and hypothalamus (Dasen et al., 2001). SOD patients with $HESX1$ mutations have variable hypopituitarism with an undescended posterior pituitary. Forebrain abnormalities are less frequent. The penetrance of $Hesx1$ mutations appears highly variable.
Sox2 and Sox3 are members of the SoxB1 [SRY-related high mobility group (HMG) box] family of transcription factors. Both proteins are early markers of neural progenitor cells (Chapter 2, Fig. 4). Heterozygous loss of Sox2 in mice causes pituitary hypoplasia and dysmorphology resulting in panhypopituitarism (Kelberman et al., 2006). Sox2 also has an important role in eye development, particularly lens induction (Kamachi et al., 2001). Consistent with findings in the mouse, SOX2 mutations in humans are associated with anophthalmia or microphthalmia, malformation of the corpus callosum, and anterior pituitary hypoplasia.

SOX3 is located on the X-chromosome (Xq27). The Sox3 null mutation in male mice on a mixed background yields hypopituitarism and midline central nervous system defects (Rizzoti et al., 2004). Similarly, male patients, with both under and over-dosage of the gene, exhibit variable hypopituitarism, infundibular hypoplasia, and an ectopic posterior pituitary. Abnormalities of the corpus callosum are also present, with or without mental retardation.

Otx2 (Orthodentic homeobox 2) is a transcription factor with characterized roles in eye and brain development (reviewed in McCabe et al., 2011). Heterozygous mouse mutants have a variable phenotype ranging from normal to severe eye and brain abnormalities (anophthalmia, holoprosencephaly or anencephaly). OTX2 accounts for 2–3% of anophthalmia/microphthalmia syndromes in humans, and has been recently implicated in the aetiology of hypopituitarism. SOD patients with heterozygous mutations in OTX2 have highly variable hypopituitarism, with or without an ectopic posterior pituitary, in addition to anophthalmia/microphthalmia.

Interestingly, both Hesx1 and Sox3 were shown to be required in mice to prevent anterior expansion of the infundibular domain marked by Bmp4 and Fgf8/10 (see “Dynamic domains of Shh expression pattern the forebrain”; Dasen et al., 2001; Rizotti et al., 2004). As mentioned previously, the expansion of this posterior domain of the hypothalamic midline results in pituitary dysmorphology (see “Specification and function of the pituitary”), consistent with pituitary abnormalities found in SOD.

In my experiments in Chapter 2, I found that Shh in the anterior midline of the hypothalamus appears to border the posterior midline expression of Bmp4 and Fgf8/10 and is
required to restrict their anterior expansion. As observed in Sox3 and Hesx1 mutants, the expansion of the infundibular domain results in hypothalamic and pituitary dysmorphology with a rostrally ectopic posterior pituitary. Although mice with loss of the hypothalamic source of Shh (Shh$^{\Delta hyp}$) die shortly after birth, a significant reduction in several pituitary cell types was identified at E18.5 and is consistent with symptoms of hypopituitarism in SOD. The characteristic optic nerve hypoplasia of SOD was also observed in Shh$^{\Delta hyp}$ mice, and uncovered a requirement for Shh signals from the hypothalamus in the morphogenesis of the optic disc. My colleague, Li Zhao, further identified that Sox2 and Sox3 mediate activation of hypothalamic Shh expression by binding to the SBE2, the hypothalamus-specific enhancer of Shh. Together, our results indicate that mutations in Sox genes may result in SOD due to a requirement for these transcription factors to regulate hypothalamic Shh, which in turn mediates morphogenesis of the hypothalamus, pituitary, and optic disc.

In Chapter 3, I delineated the role of SBE2-regulated Shh in patterning of the hypothalamus. Shh in the hypothalamus is required to limit the expansion of the Tbx3$^+$, non-proliferative ventral midline. Expansion of this midline domain results in loss of anteroventral and tuberal hypothalamic nuclei. This result serves as a mammalian in vivo validation of the interaction between Shh and Tbx2 in chick hypothalamus explants, although loss of Shh resulted in an overall decrease in proliferation in the hypothalamus (Manning et al., 2006; see “Dynamic domains of Shh expression pattern the forebrain”). Since Manning et al. (2006) also showed that Bmps can upregulate Tbx2, my results are consistent with antagonism between the Shh and Bmp signaling pathways, a pervasive patterning mechanism in the developing embryo. Li Zhao also collaborated with me in this study, and found that Tbx3 interferes with Sox2/3 activation of SBE2, thereby repressing Shh from the Tbx3$^+$ ventral midline.

In summary, my thesis work uncovers multiple requirements for Shh signals from the hypothalamus in the patterning of the hypothalamus, pituitary, and eye. These findings are important for understanding the etiology of SOD and HPE, congenital conditions that can now be
viewed within the same spectrum of Shh deficiency. Consistent with the literature, HPE results from a failure to properly execute the early morphogenetic events mediated by the prechordal source of Shh. SOD, on the other hand, reflects a deficiency in the later source of hypothalamic Shh and its function in the morphogenesis of the hypothalamus, pituitary, and optic disc. The hypothesis that SOD and HPE can result from a deficiency in Shh that is temporally and spatially different is further supported by (1) the specific regulation of Shh hypothalamic expression by transcription factors (Jeong et al., 2008; this work); and (2) the possibility of teratogens such as prenatal alcohol affecting Shh signaling at different developmental time points (Loucks et al., 2007).
REFERENCES


CHAPTER 2

Disruption of the SoxB1 dependent regulation of Sonic hedgehog transcription in the hypothalamus results in Septo-Optic Dysplasia

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Running title: Regulation of \textit{Shh} expression in the hypothalamus

Words: 2767
Acknowledgements: We thank Dr. Jean Richa and his staff at the University of Pennsylvania Transgenic and Mouse Chimeric Facility for their assistance in transgenic mouse production, as well as the staff in the Biological Services Section at NIMR. We are grateful to Drs. Dan Kelberman and Mehul Dattani (University College London, London, UK) for kindly providing the human Sox2 expression constructs. We also thank Drs. Steve Liebhaber and Kenny Campbell for constructive comments on the manuscript. This work was supported by NIH grant R01 NS039421 from NINDS (DJE), March of Dimes grant #1-FY08-421 (DJE), and the MRC (U117512772) (KR and RLB).

Key words: Septo-optic dysplasia, Sonic hedgehog, SoxB1, hypothalamus, pituitary, optic disc
ABSTRACT

Septo-optic dysplasia (SOD) is a congenital brain malformation syndrome resulting in pituitary, optic nerve, and midline forebrain defects (Kelberman et al., 2008). The etiology of SOD is poorly understood, with the majority of cases being sporadic. In rare instances, SOD is caused by mutations in Sox2, Sox3 or Hesx1, but how this manifests in disease is not entirely certain (Kelberman et al., 2006; Laumonnier et al., 2002; Woods et al., 2005; Dattani et al., 1998). We demonstrate here that mouse embryos lacking Sonic hedgehog (Shh) in the prospective hypothalamus exhibit key features of SOD, including pituitary hypoplasia and absence of the optic disc. The hypothalamic source of Shh is required to maintain gene expression boundaries along the anteroposterior and mediolateral neural axes that are important for proper pituitary and eye development, respectively. We further reveal that Sox2 and Sox3 are dose dependent regulators of Shh transcription, which directly bind and activate a long-range Shh forebrain enhancer. These data indicate that reduced levels of Shh in the prospective hypothalamus cause SOD.
INTRODUCTION

The temporal and spatial control of Shh expression within defined signaling centers is essential for its ability to impart patterns of growth and identity to neuronal progenitors throughout ventral regions of the vertebrate neural tube (Fuccillo et al., 2006). During early stages of brain development, Shh is expressed in the prechordal plate, an axial mesendodermal tissue that transiently underlies the anterior CNS (Echelard et al., 1993). Shh signaling from the prechordal plate is necessary for the separation of the cerebral hemispheres, eye fields and other craniofacial structures (Chiang et al., 1996). Shh−/− mouse embryos exhibit holoprosencephaly (HPE), a structural brain malformation resulting in the fusion of bilateral forebrain and craniofacial structures due to the loss of Shh signaling from the prechordal plate (Chiang et al, 1996). A similar HPE phenotype is observed in humans that are Shh haploinsufficient (Roessler et al., 1996). Shh also imparts regional identity within the ventral forebrain (Fuccillo et al., 2006). In chick embryos, the combination of Shh and Bmp7 from the prechordal plate induces the hypothalamus, which subsequently becomes a secondary site of Shh transcription (Dale et al., 1997). Unfortunately, due to the severity of the brain defects in Shh−/− mouse embryos, the full extent of Shh function in the hypothalamus has not been realized.
METHODS

Electromobility Shift Assays (EMSA)
Cos-1 cells were transfected with pcDNA3-Flag, pcDNA3-Flag-Sox2 or pcDNA3-Flag-muSox2 using FuGENE 6 (Roche Applied Science) according to the manufacturer's instructions. 48 hours after transfection, whole cell lysates were prepared in a RIPA buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, and 0.5% sodium deoxycholate and protease inhibitor cocktail (Roche Applied Science). EMSA was performed as described (Jeong et al., 2008). The sequence of the sense strand of oligonucleotide probes is as follows:

SBE2(WT): 5’- ACTAGTTAGCCCTATTGTGGCTTGAATTAC -3’;
SBE2(ΔSoxB1): 5’- ACTAGTTAGCCCTATAGCTCAGCTCGTTGAATTAC -3’;
MHP: 5’- CGCTTAAGGAGTTAATTGTGGTTTGAAG -3’.

Transient transfection and dual reporter assays
Cos-1 cells were cultured under standard conditions in DMEM (GIBCO BRL) supplemented with 10% fetal bovine serum, and transfected at 50–70% confluency using FuGENE 6 (Roche Applied Science). 1 μg of an SBE2-luciferase reporter construct pGL4.23[luc2/minP] (Promega) was mixed with 0.25-4μg of pcDNA-Flag-Sox2 expression plasmid or empty vector for compensation, and 5 ng of pRL-TK vector (Promega) as an internal control and applied to cells grown in a 3-cm dish. Cells were harvested 48 hours after transfection and assayed for firefly and renilla-luciferase activities (Dual Luciferase assay system, Promega). Enhancer activity was expressed as fold induction relative to that of cells transfected with the empty pcDNA3 vector. At least three independent experiments were performed for each construct in triplicate.

Chromatin Immunoprecipitation
Approximately, 10-15 embryos at E10.5 were harvested in DMEM (with 10% fetal bovine serum), pooled into head and trunk fractions, cut into small pieces and fixed with 1% PFA for 15 minutes with shaking. Crosslinking was blocked by treating samples with 100 mM glycine for 15 min.
Tissues were homogenized with a B dounce pestle in cell lysis buffer (5 mM Pipes, pH 8, 85 mM KCl, 0.5% NP-40) with protease inhibitor cocktail, then nuclei were spun down and resuspended in nuclei lysis buffer (50 mM Tris-HCl, pH8.1, 10 mM EDTA, 1% SDS). Chromatin was sonicated and diluted in buffer (0.5% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH8.1, 150 mM NaCl). After preclearing with protein A and G agarose beads (Upstate), the chromatin was incubated overnight with 4μg of Anti-Sox2 (Abcam) or Anti-IgG (Santa cruz) antibodies, followed by 2 hours of incubation with protein A and G agarose beads which were washed overnight in dilution buffer. Beads were washed twice with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH8.1, 150 mM NaCl), twice with high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH8.1, 500 mM NaCl), twice again with low salt buffer, once with Li buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH8.1), and finally once with TE. After elution with 100 mM NaHCO3 and 1% SDS and decrosslinking, DNA was purified with phenol/chloroform/isoamylalcohol and subjected to quantitative PCR as described (Jeong et al., 2008) with the following primer sets:

SBE2: (F) 5’-CATATTCTAACCAGTTGCGCTA-3’,
(R) 5’-TTGTCACATGGATCCATTCA-3’.

6.5 kb 3’ of SBE2: (F) 5’-CACATCGCTGCTGCTCCCTAC-3’,
(R) 5’-GGTACATTCTTGTAGCTTCG-3’.

Plasmid Construction

SBE2 reporter constructs were generated by cloning a 750 bp SBE2 sequence into a vector containing the Shh minimal promoter, lacZ gene and SV40 poly(A) signal. A construct containing a deletion of the SoxB1-binding site (TTGTTTG) was generated by ligating two PCR products immediately flanking this site that were amplified with the following primer sets:

E355 (5’-GCAGCTGGATCCACAAAGCGACTTAGAGAATCTT-3’) and
E360 (5’-GCAGCTTCTAGATAGGGCCACTGGTTAGAATATG-3’);
E361 (5’-GCAGCTTCTAGATAGGGCCACTGGTTAGAATATG-3’);
E343 (5’-GCAGCTGCGGCCGCACAGTCATTAGGCAAACAG-3’).
The cloning of full-length wild-type or mutant versions of human Sox2 into the pCMV/SV-Flag expression vector was described previously (Kelberman et al., 2006).

Mouse lines
Transient transgenic embryos were generated by pronuclear injection of SBE2 reporter constructs into fertilized eggs derived from the (BL6×SJL) F1 mouse strain (Jackson Labs, Bar Harbor ME). Sox2; Sox3 compound mutant embryos were produced by crossing Sox2<sup>−/−</sup> males with Sox3<sup>−/−</sup> females (Rizzoti et al., 2007). The SBE2-cre line was generated by pronuclear injection of a construct containing cre cloned downstream of SBE2 and a minimal Shh promoter. Stable lines were screened by in situ hybridization for cre expression in the SBE2 domain within the ventral diencephalon. The Shh<sup>-/+</sup> and Shh<sup>fl/+</sup> mouse strains were procured from the Jackson labs (Bar Harbor, ME).

Whole mount β-galactosidase staining and whole mount in situ hybridization
The activity of β-galactosidase was detected using X-gal (Sigma) as substrate. Whole-mount RNA in situ hybridization was performed using digoxigenin-UTP-labeled riboprobes according to a previously described protocol (Jeong et al., 2006). Stained embryos were dehydrated in methanol, cleared in benzyl alcohol:benzyl benzoate (1:1), and photographed. Representative embryos were rehydrated, sunk in 30% sucrose overnight, embedded in O.C.T (optimal cutting temperature embedding medium), quick frozen on dry ice and cryosectioned at 20 μm.

Immunohistochemistry and section in situ hybridization
Embryos were collected at specified developmental stages (plug day = 0.5) and fixed in 4% paraformaldehyde at 4°C for either 90 min, for immunohistochemistry, or overnight, for in situ hybridization. Embryos were washed extensively in PBS, sunk in 30% sucrose overnight at 4°C, embedded in OCT, quick frozen on dry ice and cryosectioned at 20 μm. Primary antibodies used for immunohistochemistry and dilutions are as follows: Shh (5E1; 1:100); Lhx3 (1:100); Pax6
Neurofilament (1:250) (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, Iowa); Nkx2.1 (1:1000; Dr. Carole Mendelson, UT Southwestern Medical School), Pax2 (1:250; Invitrogen), Tcf4 (1:200; Exalpha Biologicals, Inc.), Sox2 (1:250; Millipore), Sox3 (1:100; R&D Systems). Detection of primary antibodies was achieved using Cy3-(Jackson ImmunoResearch Laboratories) or Alexa 488- (Molecular Probes) conjugated secondary antibodies. Vector Labs' Vectastain Elite ABC Kit (Rabbit IgG) and ImmPACT DAB Peroxidase Substrate were used to detect Pax2 staining on sagittal sections through the eye. Section in situ hybridization was performed as described (Nissim et al., 2007). At least 3-5 control and mutant embryos were evaluated for each antibody or in situ probe.

**Pituitary hormone cell counts and antibody staining**

Images from every fourth coronal section through the pituitary were thresholded for positively stained cells in Fiji (an ImageJ package). The total number of positively stained cells detected per pituitary was used to derive the cell type number average per genotypic group (n = 3-4). The F-test was used to determine equal variance, and a twotailed, unpaired t-test was run to determine the statistical difference between genotypes for each pituitary cell type. To account for the five pituitary cell type comparisons between genotypes, the Bonferroni correction was applied and an alpha level of 0.01 was used, although each pituitary cell type was analyzed in different animals. The following rabbit antisera to rat antigens, used at 1:500, were obtained from Dr. Parlow at the National Hormone & Peptide Program, NIDDK: growth hormone (rGH), Beta thyroid stimulating hormone (rBetaTSH), Beta luteinizing hormone (rBetaLH), and adrenocorticotropic hormone (rACTH). Sheep anti-alpha-melanocyte stimulating hormone (aMSH) was obtained from Millipore and used at 1:20,000.
RESULTS

*Shh* expression in the ventral diencephalon (prospective hypothalamus) of E10.5 mouse embryos is detected in two bilateral stripes on either side of the ventral midline from the mammillary region posteriorly, to the level of the optic vesicles anteriorly, at which point *Shh* converges at the midline and extends into the preoptic area (POA) at the base of the telencephalon (Supplemental Fig. 1a). To address the function of this hypothalamic source of *Shh*, we used an *SBE2-cre* mouse line to conditionally inactivate a floxed allele of *Shh*, thus producing a hypothalamic specific knock-out of *Shh*. SBE2, an upstream regulatory element from *Shh*, was particularly advantageous for this purpose as it only activates *cre* transcription within *Shh* expressing cells of the hypothalamus (Jeong et al., 2006). Selective abrogation of *Shh* from the hypothalamus was observed as early as E9.5 in *SBE2-cre; Shh*^{loxP-}/embryos (referred to herein as *Shh*^{hyp}, for deletion of *Shh* in the hypothalamus) (Supplemental Fig.1b). At E12.5, *Shh* continued to be absent from the ventral hypothalamic domain of *Shh*^{hyp} embryos, but not other forebrain regions (Fig.1a,d). *Shh* signaling activity, as measured by the expression of *Gli1*, was greatly reduced in Shh responsive cells within the hypothalamus of *Shh*^{hyp} embryos (Fig. 1b,e).

This is consistent with recent results demonstrating a requirement for Shh in the formation of ventral hypothalamic nuclei (Shimogori et al., 2010). The loss of neuroendocrine and centrally projecting neurons in *Shh*^{hyp} embryos results, in part, from altered dorsoventral patterning in the diencephalon and will be described elsewhere. Instead, our analysis of *Shh*^{hyp} embryos focused on a unique aspect of Shh function, the regulation of anteroposterior hypothalamic identity.

*The hypothalamic source of Shh is required for proper pituitary morphology*

The Nkx2.1 homeoprotein is expressed in the ventral hypothalamus in response to Shh signaling from the prechordal plate (Fig. 1c; Dale et al., 1997). Nkx2.1 staining was maintained in *Shh*^{hyp} embryos (Fig. 1f). This result, along with the observation that the cerebral hemispheres and eyes were bifurcated in *Shh*^{hyp} embryos, suggests that Shh signaling activity from the prechordal plate was not compromised in these mutants (data not shown). However, we did
observe an irregularity in the development of the Nkx2.1+ infundibulum in Shh<sup>shyp</sup> embryos. The infundibulum evaginates from the ventral diencephalon to form the posterior lobe of the pituitary (Zhu et al., 2007). However, in Shh<sup>shyp</sup> embryos, the infundibulum was highly dysmorphic, failed to pinch off from the diencephalon, and was shifted anteriorly within the brain (Fig. 1c,f). Examination of Tbx2 confirmed the ectopic position of the infundibulum in Shh<sup>shyp</sup> embryos (Fig. 1g,j). The retrochiasmatic area of the hypothalamus (RCH) lies between the Tbx2+ infundibulum - posteriorly, and the Foxg1+ POA - anteriorly (Fig. 1h). The distance between Tbx2 and Foxg1 was greatly reduced in Shh<sup>shyp</sup> mutants, compared to control littermates, and the RCH took on a thinner cellular morphology, characteristic of the infundibulum (Fig. 1h,k). Thus, the anterior hypothalamus assumed a more posterior identity in Shh<sup>shyp</sup> embryos.

Anterior pituitary development was also affected in Shh<sup>shyp</sup> embryos. Rathke’s pouch, the primordium of the anterior and intermediate lobes of the pituitary, arises from a region of oral ectoderm that invaginates in response to inductive cues from the infundibulum (Zhu et al., 2007). Lhx3 staining, which marks Rathke’s pouch, revealed a duplicated invagination that failed to pinch off from the oral ectoderm and, like the infundibulum, was shifted anteriorly in the brain of Shh<sup>shyp</sup> embryos (Fig. 1i,l and Supplemental Fig. 2a).

**Shh is required for proper anteroposterior patterning in the hypothalamus**

To understand the molecular basis of the pituitary phenotype in Shh<sup>shyp</sup> embryos, we interrogated genes expressed along the anteroposterior axis of the hypothalamus at earlier stages of development (Fig. 2a). Previous studies implicated members of the Bmp and Fgf families in the induction and growth of Rathke’s pouch (Ericson et al., 1998; Treier et al., 1998; Takuma et al, 1998). Bmp4 and Fgf10 are expressed in the ventral midline of the posterior hypothalamus, including the infundibulum (Fig. 2a,b,d). This contrasts with the expression of Shh, Six6 and Tcf4, which mark the anterior hypothalamus, rostral to the infundibulum (Fig. 2a,f,h,j). In Shh<sup>shyp</sup> mutants, the anterior limit of Bmp4 and Fgf10 expression extended into the
RCH territory at the expense of Shh and Six6 (Fig. 2b-i). The anterior expansion of Bmp4 was observed as early as E9.5, suggesting that it occurred in direct response to the loss of Shh. The posteriorization of the anterior hypothalamus in Shh hyp embryos was not complete, as evidenced by the persistent expression of Tcf4 in the RCH (Fig. 2j,k). This finding argues that the partial loss of anterior hypothalamic identity in Shh hyp embryos results from a failure to suppress some posterior hypothalamic markers, rather than an ablation of anterior tissue.

Mouse models of SOD, including Hesx1−/−, Sox2−/− and Sox3−/− mutants, display hypothalamo-pituitary patterning defects of similar character to Shh hyp embryos (Kelberman et al., 2006; Dattani et al., 1998; Rizzoti et al., 2004; Dasen et al., 2001). The anterior expansion of Fgf and Bmp signals is a common occurrence in these mutants and is postulated to enhance the recruitment of oral ectoderm to Rathke’s pouch resulting in the formation of multiple and ectopic anterior pituitaries. SOD patients often present with deficiencies in one or more pituitary hormones (Kelberman et al., 2008). Likewise, Shh hyp mutants displayed significant reductions in the number of somatotropes, corticotropes, and thyrotropes in the anterior pituitary at E18.5, whereas gonadotropes and melanotropes were unaffected (Supplemental Fig. 2b,c). The hypopituitarism observed in Shh hyp embryos differs from that described in other Shh signaling mutants (Zhu et al., 2007), likely because the additional Shh expression domains impacting on pituitary development, including the prechordal plate and oral ectoderm, are intact in Shh hyp embryos.

Shh in the hypothalamus is required for optic disc formation

SOD patients also exhibit varying degrees of blindness due to optic nerve hypoplasia (Kelberman et al., 2008). To determine if Shh hyp embryos were afflicted with a similar eye defect, we stained retinal ganglion cell (RGC) axons for neurofilament. Unlike control embryos, which showed a tight bundle of RGC axons exiting the eye at E14.5, the majority of RGC axons remained trapped in the eye of Shh hyp embryos, resulting in a severely hypoplastic optic nerve (Fig. 3a,b). RGC axons exit the eye through the optic disc, which forms at the juncture of the optic
stalk and cup (Otteson et al., 1998). Pax2+, Netrin1+ optic disc cells were not detected in the eyes of Shh<sup>Δhyp</sup> mutants, although expression of these markers was detected in the optic stalk (Fig. 3c-f). The absence of optic disc cells was not due to altered Shh signaling in the eye, as previously shown (Dakubo et al., 2003), since the expression of Shh and Gli1, in RGCs and retinal progenitor cells, respectively, was unaffected in Shh<sup>Δhyp</sup> embryos (Fig. 3g-j).

To elucidate the molecular mechanism responsible for the optic disc defect in Shh<sup>Δhyp</sup> embryos, we assayed the expression of genes with known roles in eye development. At E10.5, Shh and Gli1 were missing from the ventral midline of the diencephalon and optic stalk, respectively, of Shh<sup>Δhyp</sup> embryos (Fig. 3k-n). Other markers, including Vax1, Pax2 and Netrin1 maintained their expression in the optic stalk, but were reduced from the ventral portion of the retina (Fig. 3o-t). Consequently, the dorsal retinal marker, Pax6, expanded ventrally in Shh<sup>Δhyp</sup> embryos. These data indicate that the role of the hypothalamic source of Shh in eye development is limited to patterning the retina, whereas the earlier source of Shh in the prechordal plate is required for the separation of the eye fields and formation of medial structures, such as the optic stalk (Chiang et al., 1996).

Subtle reductions in the ventral retina domain marked by Pax2 are known to affect the morphogenesis of the optic disc (Otteson et al., 1998). Pax2+ cells invaginate in the ventral retina and optic stalk to generate the optic fissure (Fig. 3w). The optic disc forms from the population of Pax2+ cells that invaginate at the juncture between the optic cup and optic stalk. We examined Pax2+ cells on sections cut parallel to the surface of the eye in control and Shh<sup>Δhyp</sup> embryos (Fig. 3x-cc). While Pax2+ cells successfully invaginated to form the optic fissure at lateral levels of Shh<sup>Δhyp</sup> embryos, they failed to invaginate at the medial extent of the optic cup. A similar phenotype was observed in Bmp7<sup>−/−</sup> mutants, which lack the optic disc as well as the optic fissure and show a downregulation in Pax2 expression (Morcillo et al., 2006). Interestingly, the faint domains of Bmp7 in the ventral retina, stalk and midline of the hypothalamus were absent in Shh<sup>Δhyp</sup> embryos (Fig. 3u-v). Therefore, the optic disc defect likely stems from the failure of the
hypothalamic source of Shh to properly maintain Bmp7 and/or Pax2 expression in the ventral region of the eye.

**SoxB1 proteins are dose dependent regulators of Shh expression in the anterior hypothalamus**

We next investigated how Shh, Sox2, Sox3 and Hesx1 might be integrated in a signaling network required for hypothalamo-pituitary development by assessing Shh expression in several mouse models of SOD. A previous study revealed that the hypothalamic expression of Shh was unaltered in Hesx1−/− mutants, ruling out a role for this homeodomain transcription factor in regulating Shh transcription (Dattani et al., 1998). We therefore focused our analysis on single and compound mutants in Sox2 and Sox3, two high-mobility group (HMG) containing DNA binding proteins that along with Sox1 comprise the SoxB1 subfamily (Pevny and Placzek, 2005).

Sox2 and Sox3 are expressed throughout the ventricular zone of the CNS where they function to maintain neuronal progenitor identity (Pevny and Placzek, 2005). Sox2 and Sox3 are coexpressed with Shh from the onset of its transcription in the ventral diencephalon and continue to share overlapping expression in the RCH at E10.5 (Fig. 4a–d and data not shown).

The intensity of Shh staining in the anterior hypothalamus of male hemizygous Sox3Y− mutants (Sox3 is X-linked) was subtly, albeit consistently, downregulated, especially at its posterior limit adjacent to the infundibulum (Fig. 4e,f). This area of reduced Shh transcription was coincident with an expansion in the anterior boundary of Fgf10 expression (Fig. 4i,j). A similar reduction in the anterior hypothalamic domain of Six6 was observed (Fig. 4m,n). Taken together with the results from our analysis of ShhΔhyp mutants, these data suggest that the reduced expression of Shh in the hypothalamus of Sox3Y− embryos may contribute to the pituitary hypoplasia described in these animals (Rizzoti et al., 2004).

SOD is typically less severe in Sox3Y− and Sox2+/− mice, compared to ShhΔhyp mutants (Rizzoti et al., 2004; Kelberman et al., 2006). To determine whether this is due to functional redundancy between SoxB1 family members, we examined the expression of hypothalamic markers in various compound mutants. A very similar pattern of misexpression was observed for
Shh, Fgf10 and Six6 in Sox3\textsuperscript{Y/-};Sox2\textsuperscript{Y/-} compared to Sox3\textsuperscript{Y/-} mutants, suggesting that the overall dose rather than the specific function of Sox2 and Sox3 is important for hypothalamic development (compare Fig. 4g,k,o to f,j,n). Remarkably, in embryos lacking Sox3 and heterozygous for Sox2 (Sox3\textsuperscript{Y/-}; Sox2\textsuperscript{Y/+}), the ventral midline of the anterior hypothalamus was completely devoid of Shh and Six6 expression and instead was replaced by an expanded domain of Fgf10 (Fig. 4h,l,p). The hypothalamic patterning defect in Sox3\textsuperscript{Y/-}; Sox2\textsuperscript{Y/-} embryos is highly reminiscent of Shh\textsuperscript{hyp} mutants and is consistent with the notion that SoxB1 family members function in a dose dependent manner to regulate the expression of Shh in the ventral midline of the anterior hypothalamus. Interestingly, Sox2 also regulates the postnatal expression of Shh in neuronal stem cell populations in the hippocampus and lateral ventricle (Favaro et al., 2009).

**Sox2 binds directly to SBE2 and is necessary for its activation**

The loss of Shh expression in compound SoxB1 mutant embryos was limited to the hypothalamus, implying a high degree of specificity to the SoxB1 dependent regulation of Shh transcription (Fig. 4e,h). One mechanism by which this might occur is through the direct binding of SoxB1 factors to SBE2. To explore this possibility, we scanned the 750 bp SBE2 sequence for SoxB1 binding sites using the rVista tool (Loots et al., 2002) and identified a single site closely matching the consensus sequence that was invariant across multiple phyla (Fig. 5a).

Since the SoxB1 binding site was not a perfect match with the consensus sequence, we evaluated its potential to be bound by a representative SoxB1 family member in an electrophoretic mobility shift assay (EMSA). A specific protein-DNA complex was observed when Cos-1 cell lysates transfected with Flag-tagged Sox2 were incubated with a radiolabeled probe overlapping the SoxB1 binding site in SBE2 (Fig. 5b, lane 2). Further evaluation of the specificity of the Sox2/SBE2 interaction was addressed in competition assays using unlabeled probes overlapping the SoxB1 motif in SBE2, or a previously characterized SoxB1 site in the mouse Hesx1 promoter (MHP) (Eroshkin et al., 2002). Both of these cold probes displaced Sox2 binding to the radiolabeled SBE2 (WT) probe with equivalent efficiency (Fig. 5b, lanes 3-8). In contrast,
an unlabeled mutant SBE2 (ΔSoxB1) probe was unable to compete efficiently for Sox2 binding (Fig. 5b, lanes 9-11).

To determine if SoxB1 factors are sufficient to stimulate transcription of an SBE2 driven luciferase reporter construct (pGL-SBE2 WT), we conducted co-transfection assays. Cos-1 cells transfected with increasing amounts of Sox2 showed a robust dose-dependent gain in luciferase activity, which was highly dependent on the presence of the SoxB1 binding site (Fig. 5c). Similar results were obtained for Sox3 (data not shown). Our finding that SoxB1 factors are potent and direct activators of SBE2-mediated transcription, afforded us with an assay to assess the mechanisms by which SOD causing mutations in these genes might affect Shh transcription. A previously described mutation in the HMG domain of Sox2 (L97P) impeded its binding to SBE2 (Fig. 5d; Ragge et al., 2005). Two other Sox2 variants carrying point mutations in the transactivation domain showed either no effect on DNA binding (A191T) (Kelberman et al., 2006), or enhanced binding to SBE2 (Q177X) (Fantes et al., 2003), the mechanism for which is unclear. Interestingly, none of the Sox2 variants activated SBE2 dependent luciferase expression to a level comparable to wild type Sox2 (Fig. 5d). These findings indicate that disease-causing variants in Sox2 failed to either bind and/or activate SBE2.

We next performed chromatin immunoprecipitation (ChIP) experiments to determine if SoxB1 binding to SBE2 occurred in vivo. A significant enrichment of SBE2 was detected in Sox2-bound chromatin isolated from embryonic brain extracts at E10.5 (Fig. 5e). A sequence 6.5 kb downstream of SBE2 was not enriched in Sox2-bound chromatin, suggesting that the recruitment of Sox2 to SBE2 was specific. ChIP experiments with an antibody against Sox3 produced similar results (data not shown). Unexpectedly, a slight enrichment in SBE2 sequence was also observed in Sox2 bound chromatin isolated from the trunk region of the embryo. The significance of this finding is unclear as SBE2 is not active in posterior regions of the embryo. Nonetheless, these data indicate that Sox2 is recruited to SBE2 in the embryonic forebrain at a stage when Shh is dependent on SoxB1 factors for its expression.
**SBE2 activity in the hypothalamus is dependent on a highly conserved SoxB1-binding site**

To address the in vivo significance of the SoxB1 binding site, we performed transgenic reporter assays with constructs containing either wild type (WT) or mutant (ΔSoxB1) versions of SBE2 lacking the SoxB1 motif. Embryos carrying the SBE2 (WT) lacZ construct showed a pattern of X-gal staining in the ventral diencephalon that is both typical for this enhancer and the endogenous expression of Shh (n = 3/4; Fig. 6a,c,e,g; Jeong et al., 2006). In contrast, embryos carrying the mutated SBE2 (ΔSoxB1) lacZ construct displayed a truncated pattern of X-gal staining that consistently failed to extend into the anterior hypothalamic domain, including the area showing reduced Shh expression in SoxB1 mutant embryos (n = 12/13; Fig. 5b,d,f,h). Thus, the putative SoxB1 binding site in SBE2 is essential for transcriptional activity in the hypothalamus.
DISCUSSION

In summary, our results identify unique roles for the hypothalamic source of Shh in regulating eye and pituitary morphogenesis. The phenotypes shared between \(Shh^{hyp}\) embryos and mouse models of SOD suggest that reduced Shh signaling from the hypothalamus underlies the pathogenesis of this disorder. Thus, SOD represents a later manifestation of a Shh dependent phenotype compared to HPE, the clinical features of which partially overlap with SOD but are often more severe due to the earlier perturbation of Shh signaling from the prechordal plate. Additional transcription factors are likely operating in combination, or in parallel, with SoxB1 proteins to ensure the proper temporal and spatial expression of \(Shh\) in the hypothalamus (Jeong et al., 2008). Identifying other trans determinants of \(Shh\) expression may add to the list of genes important for forebrain development that when disrupted cause SOD.
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Figure 1. The hypothalamic source of Shh is required for proper pituitary morphology at E12.5.

(a,b,d,e) Whole mount in situ hybridization for Shh and Gli1 on bisected brains from control (SBE2cre; Shh^+/loxp) and Shh^−hyp (SBE2cre; Shh^−/loxp) embryos. In the hypothalamus of control embryos, Shh is expressed in progenitors of the ventromedial nucleus (VM). Shh is missing from the VM domain of Shh^−hyp embryos, but other forebrain regions, including the zona limitans intrathalamica (ZLI) and
preoptic area (POA) maintain Shh expression. Gli1 marks Shh-responsive cells in ventral and anterior hypothalamic nuclei, including the mammillary (MM), premammillary (PM), arcuate (Arc), suprachiasmatic (SCN), anterior hypothalamus posterior region (AHP), and anterior hypothalamus anterior region (AHA). With the exception of the AHA and MM, all other hypothalamic regions show reduced Gli1 expression in Shh\(^{\Delta hyp}\) embryos. (c,f) Nkx2.1 immunostaining on midsagittal sections through control and Shh\(^{\Delta hyp}\) embryos counterstained with DAPI (blue). Nkx2.1 staining is present in the ventral hypothalamus and medial ganglionic eminence (MGE) of Shh\(^{\Delta hyp}\) embryos. However, the posterior pituitary (PP) is dysmorphic and ectopically positioned compared to control embryos. (g,j) In situ hybridization for Tbx2 on midsagittal sections confirms the ectopic PP in Shh\(^{\Delta hyp}\) versus control embryos. (h,k) Double labeling of the POA and PP with Foxg1 and Tbx2, respectively, shows that the intervening region corresponding to the retrochiasmatic hypothalamus (RCH) is reduced in Shh\(^{\Delta hyp}\) embryos. (i,l) Nkx2.1 and Lhx3 immunostaining reveals that the anterior pituitary (AP) is duplicated and shifted anteriorly in Shh\(^{\Delta hyp}\) mutants. D, dorsal; A, anterior.
Figure 2. Shh is required for proper anteroposterior patterning in the hypothalamus. (a) Diagram of a midsagittal section through a control E10.5 brain delineating the expression domains of anteroposterior patterning markers in the hypothalamus. Bmp4 and Fgf10 are co-expressed in the posterior ventral midline (blue), while Shh, Six6, and Tcf4 mark the anterior ventral midline (red). (b, c) At E9.5, Bmp4 expression is rostrally expanded (brackets) in Shh\textsuperscript{hyp} embryos. Bmp4 is also ectopically expressed in the oral ectoderm of mutants (arrow). (d, e) Fgf10 expression is expanded rostrally in Shh\textsuperscript{hyp} versus control embryos (brackets). (f, g) Shh transcription is repressed in the hypothalamus of Shh\textsuperscript{hyp} embryos. (h, i) Six6 expression is downregulated in the hypothalamus of Shh\textsuperscript{hyp} embryos. (j, k) Tcf4 expression is unaffected in the hypothalamus of Shh\textsuperscript{hyp} embryos. D, dorsal; A, anterior.
Figure 3. The optic disc fails to form in Shh<sup>Δhyp</sup> embryos. (a-j) Coronal sections through the eyes of control and Shh<sup>Δhyp</sup> embryos at E14.5. (a,b) RGC axons labeled with neurofilament (NF) bundle and exit the eye at the optic disc in control, but not Shh<sup>Δhyp</sup> embryos. (c-f) Pax2 and Netrin1 mark cells of the optic
disc (arrowheads) and optic stalk (arrows). The optic disc is absent in $Shh^{\Delta hyp}$ mutants. (g-j) $Shh$ and $Gli1$ expression is unaltered in the eyes of $Shh^{\Delta hyp}$ versus control embryos. (k-v) Coronal sections through the hypothalamus of E10.5 embryos at the level of the eye. (k-n) The expression of $Shh$ in the ventral midline of the hypothalamus and $Gli1$, in the optic stalk (OS) is lost in $Shh^{\Delta hyp}$ mutants. (o-t) The expression of $Vax1$, Pax2, and $Netrin 1$ is reduced in the ventral retina (below dotted line) of $Shh^{\Delta hyp}$ mutants, but is preserved in the optic stalk. Pax6 staining in the dorsal retina (above dotted line) expanded ventrally in $Shh^{\Delta hyp}$ embryos. (u,v) The weak $Bmp7$ expression in the ventral midline of the hypothalamus, optic stalk, and ventral retina in control embryos is absent in $Shh^{\Delta hyp}$ mutants. (w) Diagram of the eye and optic stalk in control and mutant, tilted to expose the ventral optic fissure (OF). The fissure and invagination of cells (curved arrows) does not extend to the stalk in mutants. The level of section in panels x-cc is shown.

Labels: dR, dorsal retina; L, lens; vR, ventral retina; dOS, dorsal optic stalk; vOS, ventral optic stalk. (x-cc) Sections cut parallel to the eye surface at E10.5. Pax2$^{+}$ cells invaginate at the lips of the optic fissure (arrows) in control and $Shh^{\Delta hyp}$ at surface and mid-cup levels. However, Pax2$^{+}$ optic disc precursors at the interface between the eye cup and stalk (arrowhead) fail to invaginate in $Shh^{\Delta hyp}$. D, dorsal; L, lateral; A, anterior.
Figure 4. SoxB1 proteins are dose dependent regulators of Shh expression in the anterior hypothalamus. (a-d) Coexpression of Shh, Sox2 and Sox3 on midsagittal sections through the hypothalamus of wild type embryos at E10.5. (e-p) In situ hybridization for Shh (e-h), Fgf10 (i-l) and Six6 (m-p) on midsagittal sections through the hypothalamus of control and SoxB1 mutant embryos at E10.5. The position of the infundibulum is marked with a dashed line, and the arrows point to the posterior boundaries of Shh and Six6 expression. A subtle reduction in the intensity and posterior demarcation of Shh and Six6 expression was detected in Sox3\(^{−/−}\) and Sox3\(^{−/−}\); Sox2\(^{−/−}\) mutants, concomitant with an anterior expansion in Fg10 expression. Sox3\(^{−/−}\); Sox2\(^{−/−}\) mutants showed a profound loss of Shh and Six6 expression in the anterior hypothalamus and corresponding gain in Fgf10.
Figure 5. Sox2 binds directly to SBE2 and is necessary for its activation.

(a) Schematic map showing the distribution of genes/exons (black rectangles) and regulatory elements (colored rectangles) within the 1Mb genomic region upstream of Shh on mouse chromosome 5. The
SoxB1 binding site (red) in SBE2 is highly conserved across species. (b) EMSAs performed with Cos-1 cell extracts transfected with pcDNA-Flag (lane 1) or pcDNA-Flag-Sox2 (lanes 2–11) expression vectors, incubated with a radiolabeled SBE2 wild type (WT) probe. The arrow points to the specific protein/DNA complex. Increasing concentrations of unlabeled wild type probes overlapping the SoxB1 site in SBE2 (lanes 3-5), or MHP (lanes 6-8) effectively competed for Sox2 binding, whereas the mutated SBE2 (ΔSoxB1) probe did not (lanes 9-11). (c) Sox2 regulates SBE2 activity in vitro. Cos-1 cells were co-transfected with either WT (blue) or ΔSoxB1 (magenta) versions of SBE2-luciferase constructs and increasing amounts of pcDNA3-Flag-Sox2 plasmids. Sox2 activated SBE2-luc (WT) in a dose-dependent manner, but not SBE2-luc (ΔSoxB1). Each point on the curve represents an average of three experiments performed in triplicate (*p<0.02, Student’s t-test). (d) Human mutations in Sox2 affect SBE2 binding and/or activation. Top: Schema of Sox2 protein showing amino acid substitutions arising from point mutations in either the HMG DNA-binding or transactivation domains. Middle: Cos-1 cell lysates transfected with pcDNA3-Flag (lane 1), pcDNA3-Flag-Sox2 (lane 2), or pcDNA3-Flag-muSox2 (lanes 3-5) were analyzed for binding to a radiolabeled SBE2(WT) probe. No complex formation was observed for the L97P mutant (lane 4), whereas, the A191T and Q177X mutants (lanes 3 and 5) showed normal or increased DNA binding, respectively. aFlag immunoblot demonstrates that wild type and mutant Sox2 proteins were expressed at equivalent levels, although the size of Q177X is smaller than WT. Bottom: Mutant Sox2 proteins showed significantly impaired transactivation of the SBE2 (WT) luciferase reporter construct. Each bar represents the average of three experiments performed in triplicate. Asterisk represents a statistically significant difference from wild type (p<0.05). (e) ChIP from embryos using anti-Sox2 or anti-IgG antibodies. QPCR results from three independent experiments revealed SBE2 enrichment in Sox2 bound chromatin isolated from forebrain and posterior trunk regions of E10.5 mouse embryos (*p<0.01, Student’s t-test). A control sequence, 6.5 kb downstream of SBE2, was not enriched in Sox2 bound chromatin.
Figure 6. SBE2 activity in the hypothalamus is dependent on a highly conserved SoxB1-binding site. X-gal staining of representative transgenic embryos carrying either wild type (WT; a,c,e,g) or SoxB1 deleted (ΔSoxB1; b,d,f,h) SBE2-lacZ reporter constructs at E10.5. The dashed lines in (a,b) show the plane of section in panels (c-h). The number of embryos showing representative reporter activity over the total number of transgenic embryos is indicated for each construct (a,b).
Figure S1. Shh expression in the hypothalamus is absent in SBE2-cre;Shh<sup>Δhyp</sup> (Shh<sup>Δhyp</sup>) embryos. (a) Shh expression in whole mount (top) and coronal brain sections through a control E10.5 embryo at two levels along the anteroposterior axis of the hypothalamus. In the posterior region, at the level of the presumptive ventromedial nucleus (VM), Shh is expressed in bilateral stripes adjacent to the ventral midline. In the anterior hypothalamus at the level of the optic stalk (OS), Shh is detected in the ventral midline. (b) Whole mount in situ hybridization of control and Shh<sup>Δhyp</sup> embryos at E9.5 with a probe against Shh exon 2, which is deleted in cells that have undergone cre-mediated recombination of the floxed Shh allele. The selective loss of Shh expression in the hypothalamus of Shh<sup>Δhyp</sup> embryos is indicated with an arrow (n = 4/5).
Figure S2. *Shh*\textsuperscript{Δhyp} embryos exhibit pituitary dysmorphology, ectopic positioning, and reduction in hormone-producing cell types at E18.5. (a) Near midsagittal sections of brains labeled with DAPI (blue).
and growth hormone (GH) (green). Lower panels show magnification of boxed areas in upper panels. Dashed lines indicate the rostrocaudal level of the hypothalamus corresponding to the anterior commissure (AC) or the mammillary (MM) nucleus. In control embryos, the pituitary is caudal to the MM, whereas in Shh<sup>△hyp</sup> mutants the dysmorphic pituitary is located rostral to the MM, with scattered GH<sup>+</sup> cells (arrows) even rostral to the AC. Note the islands of sphenoid bone (SP) in Shh<sup>△hyp</sup> mutants that have failed to fuse into a single plate underlying the brain. The third ventricle (3V) is expanded laterally in Shh<sup>△hyp</sup> embryos. Abbreviations: PP, posterior lobe; IL, intermediate layer; AP, anterior lobe. (b) Coronal sections through the pituitary labeled with pituitary hormone antibodies (green) and DAPI (blue). GH, growth hormone; TSH, thyroid stimulating hormone; LH, luteinizing hormone; ACTH, adrenocorticotropic hormone; αMSH, alpha-melanocyte stimulating hormone. Note that ACTH antibody also labels αMSH cells of the intermediate layer. (c) Quantification of data from (b). The number of cells expressing GH (<i>P</i> = 0.0009), ACTH (<i>P</i> = 0.0008), and TSH (<i>P</i> = 0.006) were significantly reduced in Shh<sup>△hyp</sup> mutants (asterisk, Student’s t-test).
CHAPTER 3

Shh restricts expansion of the non-proliferative ventral midline and specifies hypothalamic progenitor domains

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ABSTRACT

The hypothalamus is a small but critical brain region that regulates autonomic and endocrine functions necessary for life. The hypothalamus is organized into about a dozen interconnected nuclei that synchronize these vital processes in response to homeostatic information from the body and input from other brain regions. Proper formation of this intricate structure during embryonic development requires precise patterning by inductive signals. Sonic hedgehog (Shh) is a morphogen secreted from the prechordal plate initially required to specify the hypothalamic primordium. Subsequently, Shh is expressed within the hypothalamus, but the patterning role of this source of Shh is unclear. We observe that Shh in the hypothalamus is required to restrict expansion of Bmp4 and Tbx3 anteriorly and Tbx3 laterally in the ventral midline. Loss of Shh results in lateral expansion of the Tbx3+ ventral midline, a region that lacks proliferation at E10.5. Hence, proliferation within the ventral hypothalamus is reduced and the hypothalamus fails to expand in the absence of hypothalamic Shh. Bmp4 and Tbx3 also mark the infundibulum, an invaginating tissue in the posterior ventral midline that gives rise to the posterior pituitary. When the anterior boundary of these genes is not restricted due to loss of Shh, the infundibulum shifts rostrally at the expense of more anterior hypothalamic tissue at the ventral midline. In this manner, anterior and lateral expansion of the posterior midline leads to loss of anterior and ventral hypothalamic progenitors that give rise to anteroventral and tuberal hypothalamic nuclei.

In vitro assays further show that Tbx3 interferes with Sox2/3 activation of a Shh hypothalamic enhancer, SBE2. This interaction with TBX3 results in Shh reporter repression, revealing a mechanism for Shh repression from the Tbx3+ ventral midline domain. The mutual restriction between Shh and Tbx3 serves to properly specify the proliferative, neurogenic domain that will give rise to hypothalamic nuclei versus the non-proliferative ventral midline.
INTRODUCTION

The hypothalamus is a small brain region that regulates vital autonomic and endocrine functions and associated behaviors. Specifically, the hypothalamus mediates thermoregulation, fluid & electrolyte balance, stress response, circadian rhythms, feeding and metabolism, sexual behavior and reproduction, and arousal and sleep. The hypothalamus consists of about a dozen nuclei that synchronize with each other, send and receive projections from other brain regions, and send hormone signals to the pituitary gland, controlling its release of hormones into the body. The proper formation and organization of these hypothalamic nuclei is critical to sustain life.

During early embryonic development, the morphogen Sonic hedgehog (Shh) is required for the formation of the rostroventral forebrain region that will become the hypothalamus (Chiang et al., 1996). A mesendodermal tissue underlying the forebrain, the prechordal plate, first secretes Shh and ventralizes the forebrain. Shh from the prechordal plate is critical, for its deficiency can result in severe malformations of the brain and face midline known as the congenital disorder holoprosencephaly (HPE) in humans. The prechordal plate also secretes Bmps which serve to induce rostral character. In explants, both Shh and Bmps are required to upregulate Nkx2.1, a marker of the hypothalamus not expressed in more caudal CNS regions (Dale et al., 1997; Ohyama et al., 2005).

Shh expression is induced in the ventral midline of the neural tube including the hypothalamus. At E9.5, however, Shh is repressed from the midline of the caudal half of the hypothalamus, forming an expression pattern of two bilateral stripes flanking the midline. Bmp4, Fgf10, and T-box transcription factors Tbx2 and Tbx3 are upregulated in the caudal ventral midline where Shh was previously expressed. In chick explants, Bmps can upregulate Tbx2 and Fgf10, and Tbx2 in turn downregulates Shh (Manning et al., 2006). According to Manning et al. (2006), the downregulation of Shh in these midline cells is required for a temporary arrest in their cell cycle that gives them the capacity for a later wave of proliferative expansion. This hypothesis, however, has not been tested in vivo, and the role of Shh expression in the hypothalamus is still not clear.
Since the hypothalamic region fails to form in Shh\(^{-}\) mutants lacking prechordal plate, two groups have recently conditionally removed Shh expression from the hypothalamus and adjacent regions (Szabo et al., 2009; Shimogori et al., 2010). In both studies, Shh in the prechordal plate was sufficient to specify a hypothalamic domain, but the region was markedly reduced and failed to give rise to many ventral hypothalamic nuclei. Failure of the hypothalamic neuroepithelium to expand made the third ventricle appear enlarged. However, the brain regions outside the hypothalamus that also underwent recombination varied between studies and resulted in different sets of absent hypothalamic nuclei. Thus, the specific requirement of Shh in the hypothalamus for the formation of hypothalamic nuclei is not clear, and the molecular mechanisms responsible for the loss of nuclei in other studies remains to be elucidated.

Recently, we found that Sox2 and Sox3 mediate activation of hypothalamic Shh expression by binding to SBE2, the hypothalamus-specific enhancer of Shh (Chapter 2). Mutations in SOX2 and SOX3 can cause septo-optic dysplasia (SOD), a malformation of the brain that can affect the forebrain midline, the pituitary, and the optic disc (McCabe et al., 2011). The activation of hypothalamic Shh by Sox2 and Sox3 appears to be a critical factor in the etiology of SOD, since loss of Shh in this region was sufficient to result in a severely dysmorphic pituitary and the absence of the optic disc, consistent with the SOD phenotype (Chapter 2).

Here, we evaluated the role of SBE2-regulated Shh in patterning of the hypothalamus. Shh is required to limit the expansion of the posterior, non-proliferative ventral midline marked by Tbx3 and Bmp4. Anterior and lateral expansion of the non-proliferative midline results in the absence of anteroventral and tuberal hypothalamic nuclei in SBE2-cre; Shh\(^{lox\_}\) embryos (referred to as Shh\(^{\Delta hyp}\)). This is consistent with the expanded ventral midline observed previously in other SOD mouse models, specifically Hesx1 and Sox3 mutants (Dattani et al., 1998; Rizzoti et al., 2004). In vitro assays further show that Tbx3 interferes with Sox2/3 activation of SBE2, thereby repressing Shh from the ventral midline domain of the caudal hypothalamus (Zhou and Epstein, unpublished observations).
METHODS

Mouse lines

The SBE2-cre line was generated as previously described (Chapter 2). The Shh^+/+ and Shh^{lox/lox} mouse strains were procured from the Jackson labs (Bar Harbor, ME).

Immunohistochemistry and section in situ hybridization

Embryos were collected at specified developmental stages (plug day = 0.5) and fixed in 4% paraformaldehyde at 4°C for either 90 min, for immunohistochemistry, or overnight, for in situ hybridization. Embryos were washed extensively in PBS, sunk in 30% sucrose overnight at 4°C, embedded in OCT, quick frozen on dry ice and cryosectioned at 20 μm. Primary antibodies used for immunohistochemistry and dilutions are as follows: Pax6 (1:100); Nkx2.2 (1:100) (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, Iowa); Nkx2.1 (1:1000; Dr. Carole Mendelson, UT Southwestern Medical School); Ascl1/Mash1 (1:100; R&D Systems); Ki67 (1:1000; Novocastra); tyrosine hydroxylase (1:1000; Pel Freeze); Nr5a1 (1:1000; Ken Morohashi); Otp (1:250; Flora Vaccarino, Yale). Detection of primary antibodies was achieved using Cy3- (Jackson ImmunoResearch Laboratories) or Alexa 488- (Molecular Probes) conjugated secondary antibodies. Section in situ hybridization was performed as described (Nissim et al., 2007). At least 3-5 control and mutant embryos were evaluated for each antibody or in situ probe.

Cell Counting and Statistical Analysis

Sections at the approximate level of the ventromedial nucleus from E10.5 control and Shh^{+/-} mice were antibody-labeled and counterstained with DAPI. Cells positive for a given marker were counted individually using ImageJ. The total number of positive cells per representative section for each animal was determined, and then used to calculate the average number of positive cells per genotype group. Two-way Student’s t-tests and an alpha level of 0.05 were used for all statistical tests. Standard deviation values are reported in the text.
RESULTS

*Shh in the hypothalamus is required for ventral midline morphology and proper dorsoventral pattering*

Shh is a secreted morphogen that specifies distinct progenitor identities in a concentration and time-dependent manner (Ribes and Briscoe, 2009). Shh pathway activation modifies Gli transcription factor activity, which in turn mediates changes in Shh target gene expression. Shh-responsive cells upregulate *Gli1* which activates expression of Shh target genes, also known as Class II genes. In the hypothalamus, *Nkx2.1* and *Nkx2.2* (Fig. 1a, b) are Class II genes expressed ventrally (Puelles and Rubenstein, 2003). Just dorsal and marking the prethalamus, *Pax6* (Fig. 1a) is a Class I gene repressed by Shh signaling (Briscoe et al., 2000; Puelles and Rubenstein, 2003).

We sought to evaluate the requirement of the hypothalamic source of Shh for this morphogenetic activity. The prechodal plate is an early and transient source of Shh that could be sufficient to establish these progenitor domains in the forebrain. However, we hypothesized that Shh in the prechodal plate is not sufficient, and that the later, more enduring source Shh in the hypothalamus is required for aspects of patterning in the hypothalamus. To test the requirement of hypothalamic Shh, we evaluated *SBE2*-cre; *Shh*^loxP/loxP^ embryos (referred to as *Shh*^Δhyp^) in which Shh is genetically deleted specifically from the hypothalamus by E9.5 (Chapter 2). As previously described, SBE2 is a hypothalamus-specific enhancer of Shh (Jeong et al., 2006) that drives *cre* expression and recombination of the *Shh* conditional allele in the hypothalamus of these animals. Moreover, SBE2 is known to be regulated by *Six3* (Geng et al., 2008; Jeong et al., 2008) and Sox2/3 transcription factors (Chapter 2), whose coding sequences are targets of mutation in HPE and SOD, respectively (Wallis et al., 1999; Rizzoti et al., 2004; Kelberman et al., 2006). Thus, the SBE2-regulated domain of *Shh* in the hypothalamus is etiologically relevant.

To determine the morphogenetic requirement of SBE2-regulated *Shh*, the expression of Class II genes *Nkx2.1* and *Nkx2.2* and the Class I gene *Pax6* was evaluated in coronal sections through the hypothalamus at E10.5 in *Shh*^Δhyp^ animals versus control (Fig. 1). For this and
subsequent experiments, we chose to evaluate a medial level along the anterior-posterior axis likely to give rise to the ventromedial nucleus of the hypothalamus. At this level, rather than the narrowing “V” shape of the ventral midline in controls (bracket, Fig. 1a), the ventral midline in Shh$^{hyp}$ appeared expanded and flat (bracket, Fig. 1d). As previously described, Nkx2.1 still marked the hypothalamic region in the absence of hypothalamic Shh (Fig. 1a,d; Szabó et al. 2009). However, the number of Nkx2.1$^+$ cells in controls per representative section (704 ± 11[SD], n = 5) was reduced by more than half in Shh$^{hyp}$ (292 ± 24, n = 6, P < 0.01; Fig 1g). Pax6 was expanded ventrally and aberrantly abutted the Nkx2.1 domain in mutants (Fig. 1 a,d). In controls, the space between Nkx2.1 and Pax6 expression (arrow, Fig. 1 a,d) corresponds to the Nkx2.2 domain (arrow, Fig. 1 b), which is absent in mutants (arrow, Fig. 1 e). Nkx2.2 is known to repress Pax6, and Pax6 indirectly causes the repression of Nkx2.2 (Briscoe et al., 2000). Without Nkx2.2 repression in Shh$^{hyp}$, Pax6 expands ventrally.

To further validate the reduction in ventral progenitors marked by Nkx2.1 and Nkx2.2, we evaluated another marker, Mash1. Mash1 is required for neurogenesis in the ventral hypothalamus and also to promote certain ventral hypothalamic cell fates (McNay et al., 2006). We found that the number of Mash1 cells per representative section is almost ten-fold reduced in mutants (26 ± 17, n = 3) versus control (209 ± 53, n = 3, P < 0.05) and is particularly absent from the expanded ventral midline in mutants (Fig. 1c,f,g). These results show that Shh in the hypothalamus is required for proper dorsoventral patterning of progenitor domains and ventral midline morphology. Shh from the prechordal plate appears sufficient to induce expression of Nkx2.1, but is not sufficient to mediate proper expansion of the domain.

*Shh in the hypothalamus restricts lateral expansion of the ventral midline marked by Tbx3*

To determine whether patterning abnormalities in the ventral midline of Shh$^{hyp}$ correlate with its abnormal morphology, we first evaluated the pattern of Shh signaling in this region at E10.5. The absence of Shh adjacent to the midline in Shh$^{hyp}$ results in a loss of Shh-responsiveness marked by Gli1 (Fig. 2a-d). However, the Gli1 domain in controls is dorsal to the
Shh-expressing domain, indicating that the ventral midline is normally not Shh-responsive at E10.5. Ventral midline cells thus do not require Shh-signaling directly at this stage, but Shh is required to prevent dysmorphology of their domain.

We subsequently evaluated a gene that specifically marks the ventral midline of the hypothalamus: Tbx3 (Pontecorvi et al., 2008). In Shh\(^{\Delta hyp}\) mutants, Tbx3 is expanded versus control and marks the aberrantly wide and flat ventral midline tissue (Fig. 2e,f). In chick, a closely-related T-box transcription factor, Tbx2, mediates Shh downregulation in the hypothalamic midline (Manning et al., 2006). Our collaborators have shown that in mice, Tbx3 is required for this effect (Andreas Kispert, unpublished). Our result now shows that Shh in the hypothalamus is required to restrict Tbx3, indicating that Shh and Tbx3 delimit each other’s expression. Consistent with this mechanism, Shh and Tbx3 are observed in mutually exclusive domains (Fig. 2a,b,e,f).

**Shh in the hypothalamus is required for proper patterns of proliferation**

We hypothesized that a decrease in proliferation may account for the thin appearance of the ventral midline and the reduction in Nkx2.1\(^+\) progenitors in Shh\(^{\Delta hyp}\) mutants. In controls, immunofluorescence with the proliferation marker Ki67 revealed proliferating cells along the ventricle of the hypothalamus except for the narrow ventral midline (Fig. 2g, bracket). In mutants, the pattern of proliferation was similar to controls, except that the non-proliferative ventral midline domain was expanded (Fig. 2h, bracket). Shh\(^{\Delta hyp}\) animals had an increased number of non-proliferating ventral midline cells per section (51 ± 4, n = 4) versus control (15 ± 1, n = 3, \(P < 0.01\); Fig. 2i). This expansion of the non-proliferative ventral midline yielded a decrease in the overall number of proliferating cells within the Nkx2.1 domain in mutants (16 ± 2, n = 3) versus controls (44 ± 6, n = 3, \(P < 0.05\); Fig. 2i). In this manner, Shh promotes expansion of the Nkx2.1 domain by limiting the expansion of the non-proliferative ventral midline.

**Shh in the hypothalamus is required to restrict anterior expansion of Bmp4 and Tbx3**

In hypothalamus explants, Manning et al., (2006) showed that Bmps can upregulate Tbx2. We hypothesized that the expansion of the Tbx3 domain in Shh\(^{\Delta hyp}\) may be triggered by an
expansion in the expression domain of Bmps. We evaluated Bmp4 and Bmp7 expression at E9.5. First, however, we confirmed that Shh and Gli1 are absent by E9.5 in Shh<sup>Δhyp</sup> (Fig. 3a-d). In controls, Bmp4 is expressed in the caudal ventral midline of the hypothalamus (Chapter 2: Fig. 2b), and only weak expression can be observed at the more anterior level of the future ventromedial nucleus (Fig. 3e). In Shh<sup>Δhyp</sup> mutants, Bmp4 is expanded anteriorly (Chapter 2: Fig. 2c) and is thus strongly expressed at this level (Fig. 3f). Bmp7 in the ectoderm underlying the hypothalamus, on the other hand, appeared unaffected in Shh<sup>Δhyp</sup> as compared with control (Fig. 3g,h).

The changes in Bmp4 expression in Shh<sup>Δhyp</sup> correlated with lateral expansion of Tbx3 already present at E9.5 (Fig. 3i,j). Tbx3 is also detected more anteriorly in Shh<sup>Δhyp</sup> mutants versus controls (data not shown), and at the same anterior and medial levels, the Tbx3 domain appears wider or more expanded across the midline region in mutants than in controls (Fig. 2e,f; Fig. 3i,j). Interestingly, the morphology of the mutant ventral midline is not grossly different from control at E9.5, indicating that changes in Bmp4 and Tbx3 expression precede dysmorphism of the midline at E10.5 and may be causative. Shh in the hypothalamus is therefore required to restrict expansion of Bmp4 and Tbx3 anteriorly and Tbx3 laterally, patterning events likely required for proper formation of the ventral midline.

Shh in the hypothalamus is required for proper hypothalamic and posterior pituitary morphology

A portion of Bmp4/Tbx3<sup>+</sup> midline tissue invaginates and forms the infundibulum, which is specifically marked by Tbx2 (Chapter 2) and gives rise to the posterior lobe of the pituitary (Zhu et al., 2007; Pontecorvi et al., 2008). By E12.5, the posterior pituitary is visible in controls below the hypothalamus (Chapter 2, Fig. 1). In Shh<sup>Δhyp</sup>, however, the anterior expansion of Bmp4 is associated with an anterior shift in the Tbx2<sup>+</sup> infundibulum, which fails to adopt posterior pituitary morphology (Chapter 2, Fig. 1, 2, and Supplementary Fig. 2). Concomitantly, there is a severe loss of ventral hypothalamic tissue anterior to the infundibulum in Shh<sup>Δhyp</sup> (Chapter 2, Fig. 1, 2, and Supplementary Fig. 2).
We sought to more carefully characterize these abnormalities in hypothalamic morphology in Shh<sup>hyp</sup> on coronal sections at E12.5. At all anterior-posterior levels through the hypothalamus at this stage, the third ventricle appeared expanded versus control in Shh<sup>hyp</sup> animals (Fig. 4), likely a consequence of the expansion in the non-proliferating ventral midline observed at E10.5. Moreover, the midline at the level of the anterior hypothalamus had the appearance of the infundibulum in Shh<sup>hyp</sup> (Fig. 4a-f), consistent with the anterior position of the infundibulum in midsagittal sections evaluated previously (Chapter 2, Fig. 1).

*Shh in the hypothalamus is required for specifying anteroventral and tuberal nuclei identity, but not the identity of the dorsal supra-optoparaventricular and caudal mamillary areas*

We hypothesized that anterior and ventral hypothalamic nuclei, versus dorsal and caudal nuclei, would be more likely to be affected by the patterning and morphological abnormalities observed in the hypothalamus of Shh<sup>hyp</sup>. At E12.5, hypothalamic nuclei can be identified by gene expression (Shimogori et al., 2010), even though differentiating cells have yet to condense and form nuclei. To determine which nuclei are affected by the loss of Shh in the hypothalamus, we evaluated hypothalamic nuclei markers and dorsoventral patterning genes at E12.5 in Shh<sup>hyp</sup>.

In a coronal section through the anterior hypothalamus at E12.5, the transcription factors Nkx.2.2 and Otp can be used to mark several cell groups. Nkx2.2 is expressed ventrally in cells that will give rise to the suprachiasmatic nucleus and the posterior region of the anterior hypothalamus (Fig. 4a; Puelles and Rubenstein, 2003). Just dorsal to Nkx2.2, Otp marks the hypothalamic supra-optoparaventricular area (SPV) (Fig. 4b; Puelles and Rubenstein, 2003). The SPV gives rise to the paraventricular and supraoptic nuclei. Interestingly, a few dorsal-most Nkx2.2 cells co-label with Otp within the SPV (Fig. 4a,c, arrowheads). Otp also marks several cells at the ventral midline in the anterobasal nucleus (Fig. 4b, arrow), a region also marked by Nkx2.1 and Shh (Morales-Delgado et al., 2011). Cells in the anterobasal nucleus differentiate and express the peptide signal somatostatin. They subsequently disperse caudally to surround the ventromedial and arcuate nuclei (Morales-Delgado et al., 2011).
At an equivalent anterior level in \( Shh^{\Delta hyp} \), Nkx2.2\(^{+} \) cells were absent except for the few dorsal-most cells that co-label with Otp in the SPV (Fig. 4a,d,c,f, arrowheads). The Otp\(^{+} \) SPV was present in mutants, but Otp in the antero-basal nucleus was not, likely because the infundibulum was aberrantly present in its place at the midline (Fig. 4b,e, arrow).

At the more caudal level of the tuberal hypothalamus in controls, the edges of the Pax6\(^{+} \) prethalamus dorsally border the hypothalamic region (Fig. 4g, arrowheads; Puelles and Rubenstein, 2003). Just ventral to Pax6, a caudal and distinct domain of Otp expression marks the mammillary area (Fig. 4h; Puelles and Rubenstein, 2003). The tuberal hypothalamus is ventral to the rostral aspect of the Otp\(^{+} \) mammillary on a coronal section. Tuberal nuclei include the ventromedial and arcuate nuclei. Nr5a1 (previously Sf-1; Fig. 4i) specifically marks the ventromedial nucleus (Ikeda et al., 1995), and tyrosine hydroxylase (Fig. 4m) marks the A12 dopaminergic neurons of the arcuate nucleus (Baker et al., 1983). Otp is also observed in the arcuate nucleus (Fig. 4h, arrowheads) in cells that will synthesize somatostatin (Acampora et al., 1999).

In \( Shh^{\Delta hyp} \) mutants at an equivalent level, Pax6 marks the prethalamus as in controls, but this region is positioned much closer to the ventral midline (Fig. 4g,j, arrowheads). This revealed a reduction in the dorso-ventral extent of the hypothalamus below the Pax6 domain. In addition, Pax6 is found ectopically along the third ventricle in the ventral hypothalamic region where tuberal nuclei should develop in \( Shh^{\Delta hyp} \) (Fig. 4g,j). Shh in the hypothalamus is thus required to repress Pax6 from the tuberal region, consistent with the known function of Shh in promoting downregulation of Pax6 (Macdonald et al., 1995).

Between these two Pax6 domains in mutants, Otp marks the caudal mammillary area (Fig. 4h,k). However, the mammillary domain is also positioned abnormally close to the ventral midline for this anterior-posterior level. Together, the Pax6 and Otp expression domains mark most of the ventral neuroepithelium in \( Shh^{\Delta hyp} \) where tuberal nuclei should form. Neither Nr5a1\(^{+} \) cells of the ventromedial nucleus (Fig. 4i,l) nor cells of the arcuate nucleus marked by tyrosine hydroxylase or Otp (Fig. 4h,k,m,n) were present in mutants.
Tbx3 represses Shh from the ventral midline by interfering with SoxB1 activation of SBE2

Shh expression in the hypothalamus is regulated by the SBE2 enhancer, and transcription factors such as Six3 and SoxB1 factors Sox2 and Sox3 can bind SBE2 and activate hypothalamic Shh expression (Jeong et al., 2008; Geng et al., 2008; Chapter 2). To determine whether Tbx3 downregulates Shh through SBE2, Li Zhao in the lab carried out a series of experiments. She has found that Tbx3 interferes with Sox2/3 activation of SBE2, thereby repressing Shh from the ventral midline domain of the caudal hypothalamus (Zhao and Epstein, unpublished).
DISCUSSION

Shh in the hypothalamus is required to restrict expansion of Bmp4 and Tbx3 anteriorly and Tbx3 laterally. Expansion of ventral midline expression of Bmp4 and Tbx3 precedes and may cause the expanded, flat morphology of the ventral midline in animals that lose Shh in the hypothalamus. The ventral midline is a domain that normally lacks proliferation, and when it expands in the absence of Shh, the result is an overall decrease in proliferation within the ventral hypothalamus. Bmp4 and Tbx2/3 also mark the infundibulum in the posterior ventral midline (Chapter 2). When the anterior boundary of these genes is not restricted due to loss of Shh, the infundibulum shifts rostrally at the expense of more anterior hypothalamic tissue at the ventral midline. In this manner, anterior and lateral expansion of the posterior midline leads to loss of anterior and ventral hypothalamic progenitors and corresponding nuclei.

Shh in the hypothalamus is required for ventral midline patterning

Shh and Bmps from the prechordal plate at first cooperate to specify the rostroventral hypothalamic domain. At a later time point within the hypothalamus, however, they appear to antagonize each other as in the developing spinal cord and several other structures within the developing embryo (Patten and Placzek, 2002). In Chapter 2, we found that Shh is required to restrict the anterior border of Bmp4 expression in the caudal midline of the hypothalamus. This requirement for Shh may underlie Tbx3 anterior and lateral expansion in Shh\_hyp mutants (Fig. 5), since Manning et al., (2006) have shown that Bmps upregulate the closely related T-box transcription factor, Tbx2, in hypothalamic explants. Bmp7 in the prechordal plate and/or ectoderm may also mediate an expansion of Tbx3 in the absence of Shh in the hypothalamus, even though the domain of Bmp7 expression is not expanded. Future experiments are required to confirm and delineate Bmp-mediated upregulation of Tbx3 in vivo in mice. For example, an upregulation in Bmp signaling detected with pSMAD antibody can be evaluated in Shh\_hyp mutants and correlated with Tbx3 expansion and the timing and source of Bmp signals. If an
upregulation in Bmp signaling is confirmed, the expression of Bmp antagonists should also be evaluated, since Shh in the hypothalamus may also be required for their expression.

*Bmp4* expression in the ventral midline determines the domain of the infundibulum, which is a tissue that does not expand in thickness but rather stays thin and evaginates to form a pouch (Zhu et al., 2007). Anterior expansion of *Bmp4* may promote the anterior shift of the Tbx2+ midline infundibular domain Shh<sup>−</sup><sub>hyp</sub> animals (Chapter 2). The expanded Tbx3+ ventral midline of Shh<sup>−</sup><sub>hyp</sub> mutants appears to be representative of this infundibular tissue, since it is expanded anteriorly, thin, and lacks proliferation.

The role of Shh in restricting the expansion of the non-proliferative ventral midline may be related to its restriction of Tbx3, since Tbx2 and Tbx3 are known to mediate the transcriptional repression of cell cycle-related genes in other developing tissues (Stennard and Harvey, 2005). In the heart, for example, Tbx2 inhibits proliferation via the repression of *Nmyc*<sup>1</sup> (Cai et al., 2005). Interestingly, Manning et al., (2006) observed a decrease in proliferation due to forced expression of Shh in hypothalamic explants in vitro. In vivo, we observe a decrease in proliferation in the hypothalamus from loss of Shh (Fig. 2i). Our results highlight the value of in vivo experiments.

We propose a model for Bmp4 and Shh antagonism in the hypothalamus (Fig. 5). Bmp4 may upregulate Tbx3 to mediate repression of Shh and changes in proliferation necessary for midline and infundibular morphology. This is consistent with the role of Tbx2 in chick (Manning et al., 2006) and Tbx3 in mice (Andreas Kispert, unpublished) in downregulating Shh at the hypothalamic ventral midline. Moreover, our in vitro assays show that Tbx3 interferes with Sox2/Sox3 activation of Shh in the hypothalamus, thereby repressing Shh from the ventral midline of the caudal hypothalamus. Shh is required to restrict anterior expansion of Bmp4 and Tbx3. Since in controls the Tbx3 domain progressively widens across the midline at more caudal levels, its anterior expansion may result in its expanded appearance at anterior and medial levels in Shh<sup>−</sup><sub>hyp</sub> versus controls (Fig. 2e,f, Fig. 3i,j, Fig. 5, green lines). In this manner, Shh is also required to restrict the width of the Tbx3+ ventral midline domain. This antagonism between Bmp4 and Shh generates the unique pattern of Shh expression flanking the ventral midline specifically
in the hypothalamus, while in the rest of the anterior-posterior axis of the CNS, Shh is expressed in the ventral midline.

**Shh is required for the formation of hypothalamic nuclei**

Our results provide a molecular mechanism for the requirement of Shh in the hypothalamus in the development of hypothalamic nuclei. Expansion of Tbx3 in Shh\^{hyp} changes the fate of ventral progenitors anterior to the infundibulum to a non-proliferative ventral midline fate, including the infundibular fate. Hence, these hypothalamic cells do not expand and form ventral nuclei, including the anterobasal, suprachiasmatic, anterior hypothalamic, ventromedial, and arcuate nuclei. Dorsal hypothalamic progenitors that give rise to the SPV (paraventricular and supraoptic nuclei) are not affected by the expansion of Tbx3 in the ventral midline in the absence of Shh. Similarly, progenitors caudal to the Tbx3/Bmp4 domain that give rise to the mammillary area are not affected.

It is not clear whether progenitors marked by Nkx2.2, a Shh-target gene, fail to be specified in Shh\^{hyp} due to expansion of Tbx3 or a direct requirement for Shh in the hypothalamus to upregulate Nkx2.2. Experiments in the spinal cord show that maintenance of Shh expression is critical for Nkx2.2 expression, supporting a direct requirement for hypothalamic Shh (Dessaud et al., 2010). However, a few Nkx2.2\^{+} cells were identified at E12.5 in Shh\^{hyp} corresponding to the dorsal-most population of the Nkx2.2 domain at that level. This finding is consistent with Nkx2.2 upregulation in these cells by Shh in the prechordal plate, and the specification of more ventral cells to a Tbx3\^{+} identity due to Tbx3 expansion.

Our results also serve to clarify the requirement of Shh in the hypothalamus for the development of hypothalamic nuclei. Previous studies conditionally removed Shh from the hypothalamus but also adjacent regions (Szabó et al., 2009; Shimogori et al., 2010). By using SBE2 to drive conditional recombination, we genetically deleted Shh specifically from the hypothalamus. The regulation of SBE2 and the requirement of Shh in this domain have etiological relevance in HPE and SOD (Jeong et al., 2008; Geng et al., 2008; Chapter 2).
Supporting the results of previous studies, Shh in the hypothalamus is required for the development of ventromedial and arcuate nuclei (Szabó et al., 2009; Shimogori et al., 2010). We also determined that Shh in the hypothalamus is required for the specification of anteroventral hypothalamic nuclei such as the suprachiasmatic nucleus. This result corroborated findings by Shimogori et al. (2010), but not Szabó et al. (2009), who still detected the suprachiasmatic nucleus. This is likely because Szabó et al. (2009) removed Shh from the medial hypothalamus and more caudal regions but not from the anterior midline of the hypothalamus. Our results show that Shh in the anterior midline of the hypothalamus is likely required to restrict anterior expansion of Tbx3 and maintain the identity of anteroventral progenitors that will give rise to the suprachiasmatic nucleus.

We observed that Shh in the hypothalamus is not required for the specification of the SPV, which contrasts with the results of Shimogori et al. (2010). The SPV was likely absent in the Shimogori et al. (2010) study because Shh in the nearby telencephalic preoptic area is also deleted in addition to the hypothalamic source. These results indicate that Shh in the preoptic area may be required for the identity of progenitors of the SPV.

In agreement with Shimogori et al. (2010), we observed that Shh in the hypothalamus is not required for the formation of the mamillary area. However, our findings and those of Shimogori et al. (2010) disagree with those of Szabó et al. (2009), who observed a severely reduced mamillary area in the absence of hypothalamic Shh. The results of Szabó et al. (2009) can be explained by their genetic removal of Shh in more caudal regions than the Nkx2.1+ hypothalamus. In Shh<sup>hypl</sup> mutants, Shh is maintained at the base of the ZLI at the caudal end of the hypothalamus (Chapter 2: Fig. 1d). Near this domain of Shh expression, Gli1 is detected in the mamillary area in Shh<sup>hypl</sup> mutants (Chapter 2: Fig. 1e). These observations support a role for Shh outside the Nkx2.1+ SBE2-regulated hypothalamic domain in the development of the mamillary area.
The role of Shh in the hypothalamus in the etiology of SOD

Sox2 and Sox3 mutations are associated with SOD. In Chapter 2, I showed that Shh in the hypothalamus is required for proper formation of the pituitary and optic disc affected in SOD. Sox2 and Sox3 upregulate hypothalamic Shh, so that their requirement in SOD stems, at least in part, from their regulation of Shh (Chapter 2). The finding that Tbx3 plays a role in Sox2/3 regulation of hypothalamic Shh highlights Tbx3 as a possible candidate gene to study in humans in the etiology of SOD.

The deficits in hypothalamic development resulting from loss of hypothalamic Shh contribute to our understanding of the etiology and symptomatology of SOD. First, expansion of the hypothalamic midline may be a key event in the etiology of SOD, as other genes associated with the disorder, Sox3 and Hesx1, are also required to prevent this dysmorphology (Dattani et al., 1998; Rizotti et al., 2004). While Sox3 regulates Shh in the hypothalamus, Hesx1 does not appear to do this, as Shh is still expressed in Hesx1 null animals (Dattani et al., 1998). Second, characterization of hypothalamic malformations in the etiology of SOD provides an explanation for symptoms consistent with hypothalamic dysfunction, including obesity, sleep disorder, and temperature instability (Webb and Dattani, 2010). For these reasons, hypothalamic malformation and dysfunction may need to be investigated more carefully in patients, rather than just evaluation of pituitary abnormalities.

My current results together with my previous results in Chapter 2 show that Shh in the hypothalamus is required to prevent dysmorphology consistent with SOD, not HPE. The midline fusion of brain and craniofacial structures observed in HPE results from an early, known requirement for Shh in the prechorda plate (Roessler and Muenke, 2010). Shh in the hypothalamus, on the other hand, is a later source of Shh required for proper patterning of the ventral midline. Loss of Shh in the hypothalamus results in midline expansion, rather than fusion, and malformation of the hypothalamus and adjacent pituitary and optic disc structures affected in SOD (Chapter 2). Deficiency of Shh in humans may therefore generate a spectrum of brain malformations including HPE and SOD, depending on the timing and spatial loss of Shh.
Variability in Shh deficiency may be generated by mutations in transcriptional regulators of Shh, which can be time and spatially specific similar to SBE2, and environmental teratogens including prenatal alcohol (Loucks et al., 2007).
REFERENCES


Figure 1. Shh in the hypothalamus is required for ventral midline morphology and proper dorsoventral patterning. (a-f) Immunofluorescence on coronal sections through the hypothalamic ventral midline at E10.5 with markers of the dorsoventral axis of the hypothalamus. (a,d) Nkx2.1 (red) marks the ventral hypothalamic region that is reduced in Shh$^{\text{hyp}}$. Pax6 (green) marks the more dorsal thalamic region that is expanded ventrally in mutants.
Brackets demarcate the width of the ventral midline, which is expanded and flat in the mutant. Arrows point to a region that is negative for both Nkx2.1 and Pax6 in control (a), but where the two expression domains abut in $Shh^{hyp}$ (d), matching the site of the Nkx2.2 domain (arrows b,e). (b,e) Nkx2.2 expression is absent in $Shh^{hyp}$. Without repression by Nkx2.2, a Shh target gene, Pax6 expands ventrally (arrow, d). (c,f) Mash1 is markedly reduced in $Shh^{hyp}$ versus control, particularly in the ventral midline. (g) Quantification of the number of Nkx2.1$^+$ and Mash1$^+$ cells in control and $Shh^{hyp}$. The number of Nkx2.1$^+$ and Mash1$^+$ cells is significantly decreased in mutants versus control. (h) Diagram of sagittal section at E10.5 showing level of the future ventromedial nucleus evaluated in coronal sections.
Figure 2. Shh in the hypothalamus restricts lateral expansion of the ventral midline.
(a-h) Coronal sections through the hypothalamic ventral midline at E10.5. (a,b) Shh is adjacent to the ventral midline in controls and absent in Shh^{hyp}. (c,d) In controls, Gli1^{+} Shh-responsive cells are in a domain dorsally adjacent to Shh expression, not at the ventral midline. Gli1 is absent in mutants. (e,f) Tbx3 is expanded in the ventral midline of mutants versus controls. (g,h) The proliferation marker Ki67 marks cells along the ventricle, but not at the ventral midline in controls (bracket). In Shh^{hyp}, the non-proliferative ventral midline domain is expanded (bracket). (i) Quantification of changes in the pattern of proliferation. In Shh^{hyp}, the number of non-proliferative ventral midline cells is significantly increased versus controls. This expansion of the non-proliferative ventral midline yields a significant decrease in the number of proliferating cells within the ventral Nkx2.1 domain in mutants versus controls.
Figure 3. Shh in the hypothalamus is required to restrict anterior expansion of Bmp4 and Tbx3. (a-j) Coronal sections through the hypothalamic ventral midline at E9.5. (a,b) At this stage, Shh is in the midline in controls at the level that will give rise to the ventromedial nucleus. Shh is already genetically deleted in Shh<sup>hyp</sup> by this stage. (c,d) In controls, Gli1<sup>+</sup> Shh-responsive cells are in a domain dorsally adjacent to Shh expression, not at the ventral midline. Gli1 is absent in mutants. (e,f) Bmp4 in controls is only weakly expressed at this level, corresponding to the anterior edge of its domain. In Shh<sup>hyp</sup>, Bmp4 is strongly expressed at this level, consistent with the anterior expansion of its domain also observed in Chapter 2, Fig. 2. (g,h) Bmp7 is similarly expressed across controls and mutants in the oral ectoderm beneath the hypothalamus. (i,j) Tbx3 is expanded in the ventral midline of mutants versus controls already at this stage.
Figure 4. Shh in the hypothalamus is required for specifying anteroventral and tuberal nuclei identity, but not the identity of the dorsal supra-optoparaventricular and caudal mamillary areas. (a-n) Coronal sections through the hypothalamus at an anterior level (a-f) and
a tuberal level (g-n) at E12.5. At all levels in mutants versus controls, the third ventricle appears expanded. (a,d) Nkx2.2 marks cells in controls that will contribute to the suprachiasmatic nucleus and the posterior region of the anterior hypothalamus. In Shh<sup>Δhyp</sup>, most Nkx2.2<sup>+</sup> cells are absent, except for a few, dorsal-most cells (arrowheads). (b,e) Otp expression marks the SPV in controls and Shh<sup>Δhyp</sup>. Otp also marks cells of the anterobasal nucleus (arrow) in controls that are absent in mutants and apparently replaced by the infundibulum (arrow), more easily discernable in the overlay. (c,f) Overlay of Nkx2.2 and Otp expression. Arrowheads indicate a population of cells that are co-labeled with Nkx2.2 and Otp and maintained in Shh<sup>Δhyp</sup>. The dysmorphology of the midline in Shh<sup>Δhyp</sup> versus control can be more easily discerned (arrow). (g,j) Pax6 marks the prethalamus which dorsally borders the hypothalamus. In controls, the prethalamus is positioned dorsally out of view except for a few cells (arrowheads) at the ventral edge of the Pax6 domain. In Shh<sup>Δhyp</sup>, the Pax6<sup>+</sup> prethalamus is positioned much closer to the ventral midline, indicating a reduction in the dorsoventral extent of the hypothalamus. There are also ectopic Pax6 cells along the third ventricle in mutants. (h,k) Otp marks the mammillary area in controls and mutants. Otp also marks cells in the arcuate nucleus that are absent in Shh<sup>Δhyp</sup> (arrowheads). (i,l) Nr5a1 marks the ventromedial nucleus that is absent in mutants. (m,n) Tyrosine hydroxylase (TH) marks A12 dopaminergic neurons of the arcuate nucleus that are absent in Shh<sup>Δhyp</sup>.
Figure 5. Model: Antagonism between Shh and Bmps mediates ventral midline patterning.

Stylized representation of the ventral midline at E10.5 from a dorsal view, with anterior (A) to the left and posterior (P) to the right. Lateral sides are marked right (R) and left (L). In control mice, Shh (blue) in the anterior midline of the hypothalamus represses anterior expansion of Tbx3 (yellow) and Bmp4 (red) in the posterior midline. In \( Shh^{\Delta_{hyp}} \), Shh is lost from the hypothalamus, but maintained in the telencephalic preoptic area, where Shh may function similarly to repress Tbx3 and Bmp4 from expanding into this domain. Tbx3 is required to repress Shh from the posterior midline, and this repression involves inhibition of Sox2/3 upregulation of Shh. Bmps have been shown to upregulate a close homologue of Tbx3, Tbx2, in chick. In \( Shh^{\Delta_{hyp}} \), Bmp4 in the posterior midline is expanded rostrally and may mediate expansion of Tbx3. Bmp7 in the prechordal plate or underlying ectoderm may also upregulate Tbx3. Shh is required to restrict lateral expansion of Tbx3 in the midline observed in \( Shh^{\Delta_{hyp}} \) (compare green lines at the same A-P level of the ventromedial nucleus), although this may be secondary to anterior expansion.
CHAPTER 4
SUMMARY, DISCUSSION AND FUTURE DIRECTIONS

Shh is a morphogen secreted from ventral sources that promotes ventral character in the neural tube in a concentration and time-dependent manner. My thesis work evaluated the requirement of Shh expression in the hypothalamus during embryonic development. The ventralizing activity of Shh in the forebrain has been attributed to prechordal plate and/or hypothalamic sources, often without validation of the functional distinction between the two sources. Shh in the prechordal plate is an early and transient source of Shh that induces the rostroventral hypothalamus and morphogenetic splitting of the eye field and cerebral hemispheres. Failure of these processes results in HPE. Shh in the hypothalamus, on the other hand, is initiated later and is maintained longer during embryonic development. I have determined that Shh in the hypothalamus is required for patterning the hypothalamic midline, which affects the formation of both the hypothalamus and pituitary. I have also found that although Shh from the prechordal plate induces ventral markers of the optic vesicle and stalk, Shh in the hypothalamus is specifically required to maintain the proper boundaries of their expression and effect formation of the optic disc. Failure of these functions of Shh in the hypothalamus results in a phenotype consistent with SOD.

*Antagonism between Shh and Bmps patterns the ventral midline*

I found that Shh in the hypothalamus is required for proper patterning of the anterior-posterior axis of the hypothalamic midline. This role for Shh is novel since Shh is most well-known for patterning the dorsoventral axis of the developing CNS. Nevertheless, Shh antagonizes the effects of Bmps in dorsoventral patterning, and this theme repeats itself in patterning along the anterior-posterior axis of the hypothalamic midline.

According to Ericson et al. (1998), Bmp4 marks the posterior ventral midline as early as E8.5, preceding expression of Fgf8 in this domain. At this stage, Shh is expressed throughout the ventral midline of the hypothalamus, and Shh is also secreted from the underlying prechordal
plate (Shimamura et al., 1995). The prechordal plate also secrete Bmp7 (Anderson et al., 2002). Both Shh and Bmp7 are required to specify the Nkx2.1+ hypothalamic domain (Dale et al., 1997; Ohyama et al., 2005). The prechordal plate also expresses Bmp antagonists. Bmp antagonists are required to limit Bmp signaling and prevent loss of (1) Nkx2.1, (2) Shh in the prechordal plate and the anterior ventral midline, and (3) expression of Fgf10 at E10.5 (Anderson et al., 2002; Davis and Camper, 2007).

I found that in addition to Bmp antagonists, Shh in the hypothalamus is required to limit Bmps (Fig. 1). Shh restricts anterior expansion of Bmp4 in the ventral midline (Chapter 2, Fig. 2). Genes shown to be upregulated by Bmps: T-box transcription factors Tbx2 and Tbx3 also require Shh in the hypothalamus to properly specify their anterior limit (Chapter 2, Fig. 1). Hypothalamic Shh additionally restricts lateral expansion of Tbx3 (Chapter 3: Fig. 2, 3), and Tbx3 is required in mice to repress Shh from the posterior ventral midline at around E9.5 (Andreas Kispert, unpublished). In the absence of Shh in the hypothalamus, the loss of Nkx2.1 and Fgf10 that occurs with loss of Bmp antagonists is not observed. Rather, Nkx2.1 is maintained and Fgf10 is rostrally expanded (Chapter 2). This indicates that the requirement for hypothalamic Shh to limit Bmps arises once the hypothalamus has already gained competency to express Nkx2.1 and Fgf10, but before patterning in the ventral midline is determined.

**Shh in the hypothalamus may upregulate a Bmp antagonist in Shh-responsive cells**

Shh in the hypothalamus may restrict Bmp4 expansion by upregulating a Bmp antagonist in Shh-responsive cells (Fig. 1). Shimogori et al. (2010) show that the Bmp antagonist follistatin is expressed in the hypothalamus in a domain that is partially overlapping and dorsal to Shh expression. Such an expression domain is consistent with the domain of GlI1+ Shh-responsive cells found dorsally adjacent to Shh (Chapter 2, Fig.1). Follistatin may be secreted by Shh-responsive cells and inhibit Bmp signaling at the boundary of the Bmp4 expression domain. Heightened Bmp signaling in the absence of hypothalamic Shh is yet to be confirmed, but it may be the critical step leading to expansion of the Bmp4 and Tbx3 domains. To determine whether
Shh is required to upregulate follistatin, the expression of this Bmp antagonist can be evaluated in \( Shh^{hyp} \) mutants.

**Shh in the hypothalamus may upregulate a Bmp4 repressor**

Shh in the hypothalamus may also restrict Bmp4 expansion by upregulating a transcription factor that represses Bmp4. Six3 is a transcription factor that broadly marks the forebrain and is required for its formation. Six3 represses posteriorizing Wnt signals to specify the anterior forebrain (Lagutin et al., 2003). Six3 also upregulates Shh expression in the hypothalamus by binding to SBE2 (Geng et al., 2008; Jeong et al., 2008). Failure of this interaction is proposed as one mechanism by which mutations in Six3 cause the brain and face malformations observed in HPE. Mice with a human hypomorphic mutation in one allele of Six3 and heterozygous for Shh lose expression of Shh in the anterior ventral midline (Geng et al., 2008). This loss of Shh resulted in a progressive loss of Six3 from the anterior ventral midline of the hypothalamus in these animals (Geng et al., 2008). In this manner, Shh and Six3 upregulate each other in the anterior ventral midline.

Shh in the hypothalamus is likely to be required for Six3 expression in the anterior midline of the hypothalamus (Fig. 1). This hypothesis can be confirmed by evaluating Six3 in \( Shh^{hyp} \) mutants. Six3 may act as a transcriptional repressor of Bmp4, preventing its expansion into the anterior ventral midline (Fig. 1). Consistent with this second hypothesis, Six3 represses Bmp4 in neural plate specification in Xenopus and zebrafish (Gestri et al., 2005).

To determine whether a reduction in Six3 correlates with expansion of Bmp4 in the anterior ventral midline, Bmp4 can first be evaluated in embryos that lose Shh and Six3 in the anterior ventral midline (\( Six3^{+/-}; Shh^{+/+} \)). In these mutants, Bmp4 was maintained in the hypothalamus upon evaluation by whole mount in situ (Geng et al., 2008). However, a possible anterior expansion in Bmp4 was not evaluated carefully on midsagittal sections. Moreover, loss of anterior forebrain tissue in \( Six3^{+/-} \); \( Shh^{+/+} \) may be confounding for determining anterior expansion.
of Bmp4, and evaluation of an anterior, telencephalic marker like Foxg1 may be helpful as a landmark if present.

A reduction in Six3 may also correlate with expansion of Bmp4 in Shh<sup>hyp</sup> mutants. If a correlation exists, then it would be interesting to rescue Six3 expression in the hypothalamus of Shh<sup>hyp</sup> to determine whether it is sufficient to repress Bmp4 from the anterior ventral midline.

**SOD genes regulate midline patterning**

Six3 is an example of a gene that is required for specification of the forebrain region, and then subsequently mediates patterning of the ventral midline by upregulating Shh in this domain (Geng et al., 2008). Similarly, SoxB1 transcription factors, Sox2 and Sox3, are expressed in the initial stages of forebrain development in neural progenitors and upregulate several genes important for patterning the forebrain (Okuda et al., 2010). Li Zhao has found that Sox2/3 upregulate Shh in the hypothalamic ventral midline by binding to SBE2 (Chapter 2). Mutations in SIX3 cause HPE, while mutations in SOX2 or SOX3 cause SOD (Lacbawan et al., 2009; McCabe et al., 2011). The milder phenotype of mutations in SOX genes likely results from their redundancy in regulating Shh in the forebrain. Combined loss of three Sox2 and Sox3 alleles generates reductions in Shh and morphological abnormalities consistent with HPE (Chapter 2, Fig. 4).

Mutations in OTX2 and HESX1 can also cause SOD (McCabe et al., 2011). Otx2 is a transcription factor, which similarly to Six3, is required for specifying the forebrain region (Acampora et al., 1995). The transcription factor Hesx1 is regulated by Otx2 and Sox2/3 and is also required for development of the anterior forebrain (Dattani et al., 1998; Eroshkin et al., 2002). Similar to Six3, Hesx1 appears to repress Wnt signaling in the anterior forebrain.

One allele of any of these SOD genes may be sufficient to specify the forebrain region, but it may not be sufficient in humans to also antagonize posterior signals for proper placement of the anterior-posterior boundary in the ventral midline. Many of the genes involved in SOD appear to induce anterior character and repress posteriorizing signals such as Wnts. In the ventral
midline, Bmp4 also acts as a posterior signal that requires repression from the more anterior domain. SOD genes may therefore have a role in repressing Wnts or Bmp4 or both, emphasizing a need to evaluate both Wnt and Bmp pathway genes in mouse models of SOD.

Genes that pattern the ventral midline and are important for pituitary development also appear to repress Wnt-signaling and Bmp4. Tcf4, for example, acts mostly as a Wnt/β-catenin repressor and is required to restrict anterior expansion of Bmp4 (van Noort and Clevers, 2002; Arce et al., 2006; Brinkmeier et al., 2007; Hoppler and Kavanagh, 2007). Interestingly, Tcf4 expression in the ventral midline was not affected by loss of Shh and expansion of Bmp4 (Chapter 2, Fig. 2). One possibility is that Tcf4 and Shh act in parallel pathways to restrict Bmp4, and loss of either is sufficient to result in Bmp4 anterior expansion (Fig. 1). This hypothesis can be tested by evaluating a genetic interaction between Tcf4 and Shh in Tcf4<sup>−/−</sup>;Shh<sup>−/−</sup> mice.

The central deficit in SOD may therefore be mispatterning of the ventral midline of the hypothalamus. My results show that the midline of the hypothalamus is a critical source of signals that must be properly patterned for the development of hypothalamic nuclei, the pituitary, and the optic disc: structures affected in SOD. The alternative, that SOD genes are directly involved in the formation of all structures affected in SOD, is also possible. However, our results serve as at least one example of how a central deficit in mispatterning of the ventral midline can generate the SOD phenotype. Future study of candidate SOD genes should therefore evaluate both possible etiological mechanisms, and the expression of Shh in the hypothalamus should be considered a key factor to assess in SOD.

**Patterning of the hypothalamic midline affects pituitary development**

Shh and several other genes expressed in the hypothalamic midline are involved in pituitary development, including SOD genes. These genes are required to restrict anterior expansion of the infundibular domain marked by Bmp4 and Fgf8/10 (see Introduction: “Specification and function of the pituitary”). Expansion of Bmp4 and Fgf8/10 inductive signals for pituitary tissue recruitment and growth results in multiple Rathke’s pouch invaginations, and the infundibular domain itself adopts abnormal morphology, often failing to descend (Chapter 2;
(Rizzoti et al., 2004). The infundibulum also promotes dorsal ACTH pituitary cell lineages, including corticotropes and melanotropes (Treier et al., 1998; Treier et al., 2001). Consequently, pituitary dysmorphology is often coincident with an alteration in pituitary cell fates and abnormalities in the levels of hormones secreted by the pituitary gland.

Consistent with these mechanisms of pituitary patterning, loss of Shh in the hypothalamus resulted in pituitary dysmorphology and alterations in pituitary cell fates (Chapter 2). Interestingly, Shh<sup>Δhyp</sup> mutants had significantly decreased numbers of pituitary cell types (Chapter 2: Supplementary Fig. 2) expressing the hormones most often affected in SOD (in order of deficiency prevalence): growth hormone, thyroid stimulating hormone, and adrenocorticotropic hormone (Webb and Dattani, 2010). The numbers of melanotropes were not significantly decreased in Shh<sup>Δhyp</sup> likely because this is a dorsal cell fate promoted by infundibular signals. The numbers of gonadotropes were also not significantly affected; one possible explanation being that this ventral fate is specified by ventral Bmp2 and Shh in the oral ectoderm (Zhu et al., 2007). Shh in the ectoderm, particularly the posterior oral ectoderm near the pituitary, did not appear markedly affected in Shh<sup>Δhyp</sup> (data not shown).

Blood levels of pituitary hormones were not evaluated because Shh<sup>Δhyp</sup> animals die shortly after birth. This data would have determined whether synthesized hormones in pituitary cells are released into the bloodstream despite dysmorphology of the pituitary and surrounding tissues.

**Patterning of the hypothalamic midline affects the development of the optic disc**

The requirement for Shh in the hypothalamus for the development of the optic disc also highlights the importance of proper patterning in the ventral midline (Chapter 2). Upstream factors may be required to pattern the domain of Shh expression in the ventral midline, which in turn mediates proper development of the optic disc. In addition to known regulators of Shh through SBE2, namely Six3 (Geng et al., 2008; Jeong et al., 2008) and Sox2 and Sox3 (Chapter 2), other upstream factors may be required. Upstream factors such as Sox2 may also have direct roles in
eye development (Taranova et al., 2006), but it is nonetheless important to also explore their role in patterning the ventral midline and the effect of this patterning on the formation of the optic disc.

Integration of genes involved in the development of the hypothalamus, pituitary, and optic disc into one mechanism of midline patterning

A ventral midline hypothesis for SOD encourages future studies to integrate the functions of several factors involved in pituitary, hypothalamic, and optic disc development. For example, Wnt5a and Tcf4 are required to restrict the caudal Bmp4+ and Fgf8/10+ domain and prevent pituitary dysmorphology (Brinkmeier et al., 2007; Potok et al., 2008). Their role in the patterning of hypothalamic nuclei and the optic disc should also be assessed, since Shh in the midline may be lost with the expansion of Bmp4.

Genes important for the formation of the optic disc and nerve, such as Netrin1, Vax1, and Vax2, are expressed ventrally in the brain and may have roles in the formation of hypothalamic nuclei and the pituitary. Such a role was recently shown for Vax1, which was shown to be required to prevent anterior duplication of the pituitary and a second domain of Fgf10 expression in the ventral midline (Bharti et al., 2011).

The results of such studies can then be integrated to develop a more thorough etiological mechanism for SOD, outlining which genes are in the same or different pathways, and upstream or downstream of each other.

Loss of Shh at different time points yields distinct phenotypes

The finding that loss of Shh in the hypothalamus can cause an SOD phenotype, rather than the HPE phenotype typically associated with Shh deficiency, supports the hypothesis that differential loss of Shh in a time-dependent manner can result in different phenotypes (Cordero et al., 2004). The temporal loss of Shh in the generation of a phenotype is an important factor to consider, especially when evaluating the effects teratogen exposure. Prenatal alcohol, for example, is a teratogen that can affect Shh signaling (Loucks et al., 2007). Consistent with teratogen exposure resulting in SOD, an increased prevalence of prenatal drug and alcohol
abuse and younger maternal age has been identified in SOD cohorts (reviewed in Webb and Dattani, 2010). Future studies in model organisms may determine temporal correlations between teratogen exposure, Shh expression in the hypothalamic midline, and the SOD phenotype.
REFERENCES


Figure 1. Proposed model of ventral midline patterning.

Stylized representation of the ventral midline at E10.5 from a dorsal view, with anterior (A) to the left and posterior (P) to the right. Lateral sides are marked right (R) and left (L). Shh (blue) in the anterior midline of the hypothalamus represses anterior and lateral expansion of Tbx3 (yellow). Tbx3 represses Shh from the posterior midline through inhibition of Sox2/3 upregulation of Shh. Bmps can upregulate Tbx2 in chick hypothalamus explants, and likely its close homologue, Tbx3 in mice. I hypothesize (gold dashed lines) that Shh may upregulate the Bmp antagonist, follistatin (Fst), expressed in the hypothalamus. Through upregulation of Fst, Shh may restrict anterior expansion of Bmp4. Shh and Six3 upregulate each other in the anterior ventral midline. I hypothesize that Six3 may repress Bmp4 from the anterior midline, as this function for Six3 has been characterized in the earlier developmental context of the neural plate. In this manner, Shh restricts Bmp4 through upregulation of Six3. Like Shh, Tcf4 is also required to restrict anterior expansion of Bmp4. I hypothesize that Tcf4 and Shh may have parallel, synergistic roles in restricting Bmp4. As Tcf4 was not affected in Shh$^{shyp}$, it is not downstream of hypothalamic Shh.