# Role of Neural *Sonic hedgehog* in the Development of the Mouse Hypothalamus and Thalamus

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# Zusammenfassung

Der Hypothalamus ist eine wichtige Gehirnregion im rostralen Diencephalon und erforderlich für die Aufrechterhaltung der Homöostase und das Überleben. Es ist jedoch nur wenig über seine Entwicklung während der Embryogenese bekannt. Sonic hedgehog (Shh) ist ein sekretiertes Protein mit einem sehr dynamischen Expressionsmuster im Diencephalon. Die genauen Funktionen von Shh in diesen Bereichen sind allerdings noch unbekannt. Ziel dieser Arbeit war es daher, eine konditionelle Maus zu erstellen, in der die Expression von *Shh* spezifisch im Hypothalamus entfernt wird. Dies wurde erreicht mit der Kreuzung einer Mauslinie, bei der die Cre Rekombinase unter der Kontrolle des *Foxb1* Promoters steht, mit einer Mauslinie, bei der ein Teil des *Shh* Gens gefloxt ist. In diesen als *Shh-c* bezeichneten mutanten Mäusen wird die Cre Rekombinase bereits zu einem sehr frühen Zeitpunkt in der Neuralplatte exprimiert, so dass funktionelles *Shh* bereits ab E8.5 in der Neuralplatte entfernt wird, während die *Shh* Expression im Notochord intakt bleibt. Dies ermöglicht die Differenzierung der Funktion von neuralem gegenüber nicht-neuralem Shh. Die Analyse der *Shh-c* Embryonen ergab spezifische Veränderungen in allen Bereichen des Hypothalamus. Der laterale Hypothalamus war sehr reduziert und bildete keine Hypokretin/Orexin Neuronen aus. Der posterior-ventrale Hypothalamus war nicht vollständig spezifiziert und zeigte keine Expression des Forkhead Transkriptionsfaktors *Foxb1* bei E18.5, welcher für das Überleben der Neuronen in diesem Bereich erforderlich ist. Auch der Prethalamus war sehr reduziert und zu einem späteren Zeitpunkt der Entwicklung nicht detektierbar. Stattdessen wurde eine abnormale kortikale Struktur im Diencephalon nachgewiesen, welche den anterior-dosalen vom posterior-ventralen Hypothalamus trennte. Diese Ergebnisse verdeutlichen die Funktionen von neuralem Shh für die Entwicklung des Hypothalamus, unter anderem Stabilisierung von regionaler Bestimmung, Unterdrückung dorsaler Einflüsse und das Überleben der Neuronen.

Im zweiten Teil dieser Arbeit ergab die Untersuchung des Thalamus der *Shh-c* Mutante bei E18.5, dass alle Nuklei ausser den medialen und intralaminaren Nuklei nicht richtig ausgebildet waren. Dies verdeutlicht eine weitere wichtige Funktion von Shh für die Differenzierung der thalamischen Nuklei. Weiterhin konnte durch die Untersuchung der *Gbx2* defizienten Maus gezeigt werden, dass *Gbx2* für die richtige Differenzierung der medialen und intralaminaren thalamischen Nuklei in der *Shh-c* verantwortlich ist. Die Ergebnisse weisen ebenfalls darauf hin, dass Shh bei der Entwicklung des Thalamus nicht als klassisches Morphogen wirkt.

**Schlagwörter:** Hypothalamus, *Sonic hedgehog,* konditionelle Mutante, *Foxb1*, *Gbx2*, *Gli3*, Morphogen, Thalamus, Prethalamus.

# Abstract

The hypothalamus is an important brain region in the rostral diencephalon regulating homeostasis and survival but not much is known about its development. The secreted protein Sonic hedgehog (Shh) has a very dynamic expression pattern in the diencephalon but its functions in this part of the brain are not entirely clear. Therefore, the aim of this work was to generate a conditional mouse mutant termed *Shh-c* in which *Shh* is removed specifically in the hypothalamus. This was achieved by crossing a mouse line expressing Cre recombinase under the control of the *Foxb1* promoter with a floxed *Shh* mouse line. Since the Cre recombinase is expressed in a widespread pattern in the neural plate of the developing *Shh-c* embryos from E7.5 on all functional Shh could be ablated as early as E8.5 from the diencephalon while leaving *Shh* expression in the notochord intact. In this way it was possible to distinguish between the neural and non-neural sources of *Shh*. Specific alterations were observed in all subregions of the hypothalamus in the *Shh-c* mutant. The lateral hypothalamus was severely reduced and lacked hypocretin/orexin neurons. The posterior-ventral hypothalamus was incompletely specified and did not show expression of the forkhead transcription factor *Foxb1,* which is necessary for the survival of neurons in the posterior-ventral hypothalamus. Furthermore the prethalamus was very much reduced in size and disappeared later during development. Instead an abnormal cortical structure was detected in the diencephalon separating the anterior-dorsal from the posterior-ventral hypothalamus. This work uncovers essential roles of neural Shh in hypothalamic development, namely stabilization of regional fates, inhibition of dorsalizing influences and maintenance of hypothalamic survival pathways.

In the second part of this work a detailed study of the differentiated thalamus at E18.5 was performed to study the effect of the abolition of neural Shh on thalamic development. All nuclei except the medial and intralaminar thalamic nuclei were affected in the *Shh-c* mutant demonstrating the involvement of neural Shh in the differentiation of the thalamic nuclear groups. Surprisingly, the medial nuclei showed normal *Gbx2* expression, which was shown to be downstream of *Shh*. Therefore *Gbx2* is expressed by default in the absence of neural *Shh*. Analysis of the thalamus in *Gbx2* deficient mice revealed that *Gbx2* is specifically responsible for the development of the medial and intralaminar thalamic nuclear groups and therefore for the rescue of these nuclei in the *Shh-c* thalamus. Furthermore, Shh does not seem to act in a concentration-dependent manner in the development of the thalamus which is in contrast to the spinal cord where notochord-derived Shh acts as a classical morphogen.

**Keywords:** hypothalamus, *Sonic hedgehog,* conditional mutant, *Foxb1*, *Gbx2*, *Gli3*, morphogen, thalamus, prethalamus.

# 1 General Introduction

## 1.1 Patterning of the embryonic forebrain

The gastrulation of the developing embryo produces the three primitive germ layers: the outer layer, or ectoderm; the middle layer, or mesoderm; and the inner layer, or endoderm. The ectoderm gives rise to the major tissues of the central and peripheral nervous system and forms the neural plate, a columnar epithelium.

Underneath the neural plate lies the notochord, a distinct cylinder of mesodermal cells that extends along the midline of the embryo from mid-anterior to posterior. Its rostralmost portion is continued by the prechordal mesendoderm or prechordal plate.

As neurulation proceeds, the neural plate begins to fold at the midline, forming the neural groove. The lateral margins of the neural plate then meet in the midline, transforming the neural plate into a tube. This neural tube subsequently gives rise to the brain and spinal cord.

During embryogenesis anteroposterior (A/P), dorsoventral (D/V) and local patterning mechanisms specify regional identities already in the neural plate. Transient signaling centers produce diffusible molecules, which leads to the induction of certain transcription factors in the recipient cells. As a result, these cells acquire specific cellular identities.

The notochord and prechordal plate are two non-neural signaling centers that influence dorsoventral patterning of the neural tube by secreting the signaling protein Sonic hedgehog (Shh) (Echelard et al., 1993), which is necessary for ventral patterning throughout the neuraxis (Chiang et al., 1996) (Fig.1.1). Shh from the prechordal plate is also required for the bilateral subdivision of the eye field, and for the development of the optic stalks and the hypothalamus (Chiang et al., 1996).

Patterning of the lateral neural plate or dorsal neural tube is regulated by bone morphogenetic proteins (BMPs), that are expressed in the non-neural ectoderm, anterior neural ridge and roof of the forebrain and specify dorsal neural tube cell types (Liem et al., 1995).

A signaling molecule that influences A/P patterning is Fibroblast growth factor 8 (Fgf8). It is expressed in the anterior neural ridge (ANR), the cells at the junction between the

anterior neural and non-neural ectoderm, where it promotes telencephalic development by inducing *Foxg1* expression. *Fgf8* is also expressed in the isthmus, the region at the transition between midbrain and hindbrain, where it induces *En2* (engrailed 2) expression in the midbrain. Therefore, Fgf8 has different effects on forebrain and midbrain tissues thereby leading to different developmental fates in A/P patterning (Shimamura and Rubenstein, 1997). This demonstrates the important role of regionally distinct competence for the same signaling molecule in generating further complexities. The same is true for Shh, which induces motor neurons at the level of the spinal cord, whereas at the forebrain and midbrain levels it induces the hypothalamic neurons and tyrosine hydroxylase-positive neurons, respectively. The way cells respond to an organizing signal therefore depends on their intrinsic properties.

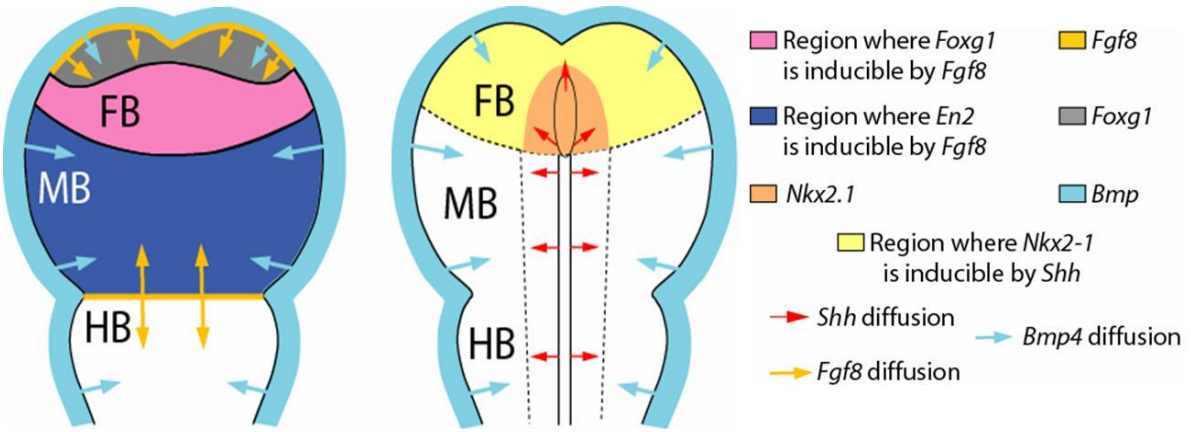


**Figure 1.1: Patterning in the neural plate.** Neural plate scheme showing anteroposterior (on the left) and dorsoventral (on the right) patterning mechanisms. Fgf8 signals (yellow arrows) from the anterior neural ridge induce *Foxg1* expression in the telencephalon, whereas Fgf8 signals from the isthmus lead to *En2* expression. Fgf8 applied to the forebrain induces *Foxg1* and Fgf8 applied to the midbrain, induces *En2*, showing the different effects of Fgf8 on different tissue types. Shh, a ventral patterning signal, is secreted by the prechordal plate and notochord (red arrows) and induces *Nkx2.1* expression in the forebrain. Bmp signals (blue arrows) from the non-neural ectoderm dorsalize the neural tube (Rubenstein and Beachy, 1998). Abbreviations: FB: forebrain; HB: hindbrain; MB: midbrain.

## 1.2 Sonic hedgehog (Shh)

Sonic hedgehog belongs to the hedgehog protein familiy and has very diverse functions during embryonic development. Shh signaling has been shown to be involved in

patterning of the nervous system, control of the proliferation of neural progenitors, axonal pathfinding and it may act as a survival factor.

In mouse embryonic development expression of *Shh* starts at late streak stages of gastrulation (E7.5) in the midline mesoderm of the head process. As somites form, the notochord and the node also express *Shh*. The prechordal plate and the anterior mesendoderm also come to express *Shh* as neurulation progresses. Shh from the notochord and prechordal plate is secreted and induces a second center of Shh production in the ventral midline cells of the neural plate at E8.5 (Roelink et al., 1995). The expression of *Shh* in the neuroectoderm is ventrally restricted and extends rostrally in the forebrain and caudally in the spinal cord (Echelard et al., 1993).

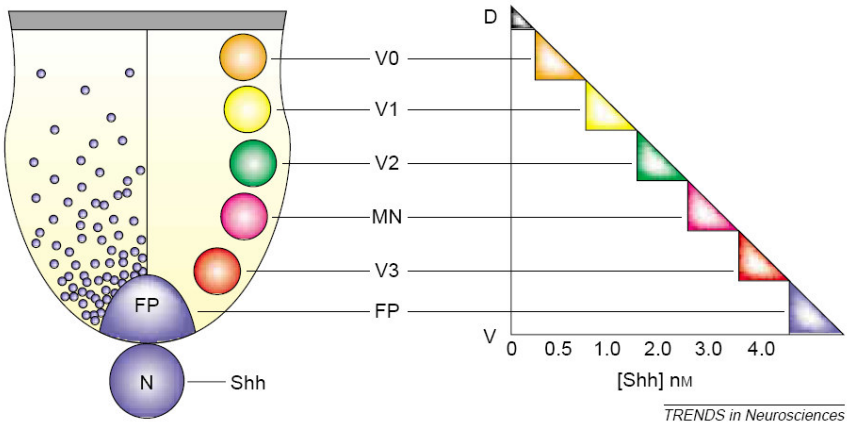


**Figure 1.2: Shh induces ventral spinal cord cell types in a concentration-dependent manner.** Schematic showing a transverse section of the spinal cord. Shh is secreted from the notochord (N) and floor plate (FP) and establishes a gradient of activity within the ventral neural tube. This leads to the formation of different progenitor domains in a concentration-dependent manner. Each progenitor domain generates different ventral interneuron subtypes (V0-V3) or motor neurons (MN).
Abbreviations: D: dorsal; FP: floor plate; MN: motor neuron; N: notochord; V: ventral (Machold and Fishell, 2002).

Before Shh can be secreted by the producing cells it has to undergo a series of post-translational modifications. Shh is produced as a large precursor protein that is auto-catalytically cleaved upon entry into the secretory pathway. The cleaved fragment is then cholesterol-modified at the C-terminus and palmitoylated near the N-terminus (Chen et al., 2004; Porter et al., 1996). These lipid attachments are necessary to produce a biologically active Shh, termed ShhNp, that is subsequently released from the cell by the multi-pass transmembrane protein dispatched 1 (Disp1).

The secreted ShhNp proteins are then assembled to soluble, high molecular weight complexes that mediate long-range signaling (Zeng et al., 2001).

In the spinal cord secreted Shh from the ventral regions establishes a gradient of activity that provides spatial information and controls cell identity in a concentration-dependent manner (Jessell, 2000) (Fig.1.2). In this way Shh secreted from the notochord acts as a long range morphogen in the spinal cord by first, acting on cells at a distance and second, inducing differential gene expression in a concentration-dependent manner (Chamberlain et al., 2008; Wolpert, 1969).

The Shh gradient is regulated by extracellular and transmembrane proteins. By binding their ligand they limit the range of Shh movement or alter the rate of Shh degradation. On the basis of their effect on Shh signaling, these proteins can be grouped into two classes. The first class of Shh binding cell surface proteins positively regulates Shh signaling through the binding and sequestration of Shh.

Ptch1, the Shh receptor, belongs to the second class of Shh binding proteins which inhibit Shh signal transduction. This twelve-pass transmembrane protein binds Shh with a sterol sensing domain. This leads to sequestration and subsequent endocytosis and degradation of Shh (Incardona et al., 2002).

For the intracellular transduction of Shh signaling the activation of the seven-pass transmembrane protein Smoothened (Smo) is necessary. In the absence of Shh, Ptch1 represses the activity of Smo. This inhibition is released upon binding of Shh to Ptch1. The signaling pathway downstream of Smo remains unresolved but leads to the regulation of the three Gli transcription factors (Bai et al., 2004). Whereas Gli1 and Gli2 function as transcriptional activators, Gli3 seems to act mainly as a transcriptional repressor. In the absence of Shh the full length form of Gli3 is proteolytically cleaved to generate a transcriptional repressor (GliR). Positive Shh signaling blocks the formation of GliR by inhibiting this post-translational cleavage. Furthermore, it prevents the degradation of Gli2 and induces the expression of the activator Gli1 (Jacob and Briscoe, 2003). The level of activity of each Gli protein is thereby proportional to the level of Shh signal transduction, which leads to the formation of a gradient of Gli activity.

Downstream of the Gli proteins, graded Shh signaling regulates the expression of a specific set of transcription factors (TF) in the ventral half of the neural tube. Class II TFs, like *Dbx1*, *Dbx2*, and *Nkx2.2* are induced by Shh, while class I genes, like *Pax6* and *Irx3*, are repressed. This leads to the combinatorial expression of specific TFs according to the Shh concentration that defines progenitor domains along the dorsoventral axis of the

neural tube, termed FP (floor plate), p3, pMN and p2-p0. Each progenitor domain has its own transcription factor code and generates different ventral interneuron subtypes (V0-V3) or motor neurons (MN) (Fig.1.2).

In this way the extracellular concentration gradient of Shh is translated into the intracellular specification of cell identities.

The set of transcription factors regulated by Shh signaling comprise a genetic network, termed neural tube gene regulatory network (GRN) (Dessaud et al., 2008). The class I and II transcription factors are members of the homeodomain (HD) and basic-helix-loop-helix (bHLH) families and function mainly as transcriptional repressors. Pairs of class I and class II proteins repress each other selectively and delineate thereby boundaries of progenitor domains. This elaborate network of crossregulation between members of the GRN is crucial for dorsoventral pattern formation in the neural tube.

During neural tube development genes of the GRN show dynamic changes in their expression pattern. But as development progresses the cells acquire a narrower potential with defined and restricted gene expression programs. Positive feedback loops stabilize and lock cells into a specification state, which is accompanied by epigenetic modifications, such as methylation of DNA, modification of histone tails and the presence of non-nucleosomal chromatin-associated proteins (Reik, 2007).

### 1.2.1 Shh in the forebrain

*Shh* shows a very dynamic expression pattern in the mouse forebrain, in contrast to the spinal cord, where *Shh* is restricted to the floor plate. Expression of *Shh* starts at E8.5 in the ventral forebrain and by E9.0 is continuously expressed in the ventral midline of the central nervous system (CNS) from the optic stalks to the presumptive spinal cord. At E9.5 *Shh* expression in the rostral diencephalon disappears from the ventral midline and starts expression in two ventrolateral bands that merge again in the floor of the forebrain at its rostral limit. The zona limitans interthalamica (ZLI) appears at E10.0 and also expresses *Shh* along its entire dorsoventral extent. This structure in the diencephalon is positioned at the boundary between the prethalamus anteriorly and the thalamus posteriorly and is the only region of the neural tube where *Shh* protrudes dorsally (Fig.1.3).

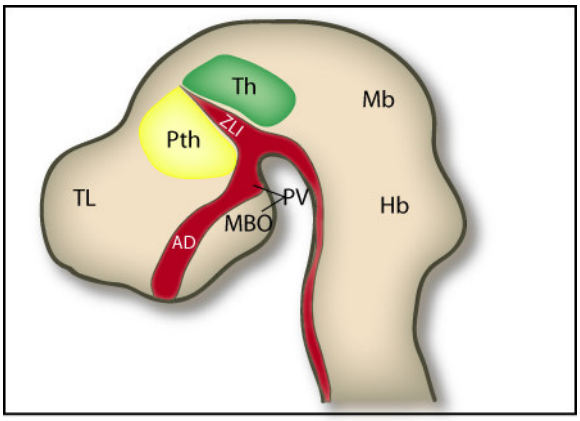


**Figure 1.3: Schematic representation of the dynamic expression pattern of *Shh* in the forebrain.** In the hypothalamus *Shh* (in red) is expressed in the anterior-dorsal (AD) region in a ventrolateral band and in the posterior-ventral (PV) region excluding the Mammillary body (MBO). The zona limitans interthalamica (ZLI) at the boundary between thalamus (Th) and prethalamus (Pth) in the caudal diencephalon also expresses *Shh*. Abreviations: MB: Midbrain; Hb: Hindbrain; TL: Telencephalon.

Shh signaling from the ZLI is necessary for region-specific gene expression in both thalamus and prethalamus and therefore regulates regional identity in the diencephalon (Kiecker and Lumsden, 2004). It has been shown in chicken that *Gbx2* expression in the thalamus and *Dlx2* expression in the prethalamus require functional hedgehog signaling. Furthermore, Shh downregulates *Pax6* in the thalamus and maintains *Pax6* expression in the prethalamus. These two diencephalic regions therefore have a differential competence in their response to Shh. All three *Gli* genes are expressed in the diencephalon complementary to the *Shh* expression pattern and seem to be the mediators of patterning in this region. The function of *Shh* expression in the PV and the AD in the hypothalamus is still elusive. It was reported in the chicken (Manning et al., 2006) that *Shh* downregulation in the ventral midline cells of the hypothalamus is required for their progression to hypothalamic progenitors and their further proliferation. But the mechanism that leads to the subsequent upregulation of *Shh* expression in the adjacent lateral hypothalamic band and its function in that region is still not known.

### 1.2.2 The Shh knockout mouse

The *Shh* gene has been disrupted by homologous recombination in embryonic stem (ES) cells to generate *Shh* knockout mice (Chiang et al., 1996). For this purpose the exon 2, which encodes part of the N-terminal domain crucial for Shh signaling, was replaced by a neomycin resistance gene driven by the mouse phosphoglycerate kinase promoter (*PGK-neo* cassette) leading to the production of a non functional truncated Shh protein. Mice heterozygous for the mutation are normal while *Shh* deficient embryos die at or just before birth and show severe morphological defects. The most striking and most obvious

abnormality in *Shh* deficient mutants is the presence of a cyclopic eye. In normal animals, a single and continuous eye field is formed at the midline of the early rostral neural plate. Secreted Shh from the prechordal plate mesoderm just underneath the rostral neural plate is necessary for the establishment of the ventral midline and the subdivision of the eye field into left and right. In *Shh* deficient mice a single fused optic vesicle protrudes at the ventral midline instead of bilateral eye structures. The prechordal plate is present during early embryogenesis in these mutants showing the requirement for Shh signaling. Additionally, *Shh* mutant embryos exhibit severe growth retardation through most of the embryo and lack forelimb and hindlimb structures. The forebrain, midbrain, hindbrain and spinal cord are reduced in size and have no basal plate or floor plate characteristics showing the major requirement for Shh for ventral cell fate specification. Also most of the diencephalon as an identifiable structure is missing in these embryos. While the hypothalamus seems to be absent completely, the dorsal diencephalon is reduced in size at E9.5 because of a reduced rate of proliferation of diencephalic precursors and an increased cell death (Fig.1.4) (Ishibashi and McMahon, 2002). Therefore, unlike other regions of the neural tube, Shh signaling is required for the normal development of both dorsal and ventral regions of the diencephalon.

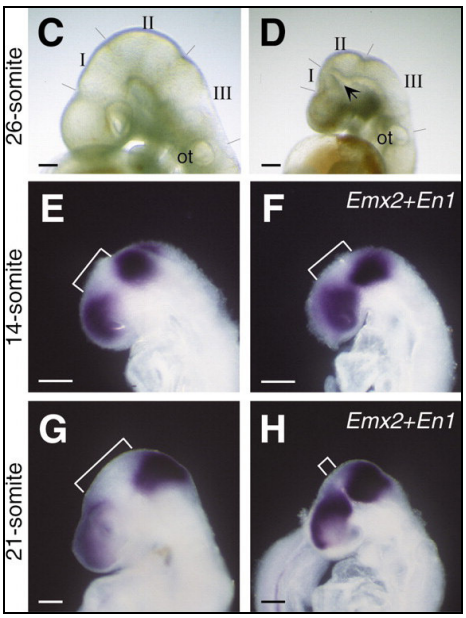


**Figure 1.4: The diencephalic and midbrain primordia in *Shh* deficient mutants fail to grow.** The normal morphology in wild type (wt) (C) and in the mutant (D) E9.5 (26 somites) embryo is shown. Region I corresponds to the presumptive diencephalon, region II to the midbrain and region III to the anterior hindbrain. Region I and II are reduced in size in the *Shh* mutant compared to wt. The arrow in D is pointing at the ventral constriction between diencephalon and midbrain which is much deeper in the mutant showing the lack of ventral structures. (E-H) Whole mount *in situ* hybridization for *Emx2* and *En1* at E9.0 (14 somites) and E9.5 (21 somites) in wt (E,G) and *Shh* mutant (F,H). The gap (represented by the bracket) between the *Emx2* and *En1* expression domains marks part of the dorsal diencephalon and the anterior midbrain. No difference is observed at 14 somites in these dorsal structures, but by 21 somites this gap is greatly reduced (Ishibashi and McMahon, 2002).

## 1.3 The hypothalamus

The hypothalamus is located in the ventral half of the diencephalon on both sides of the third ventricle and lies directly above the pituitary gland. It links the nervous system to the endocrine system via the pituitary gland by synthesizing and secreting neurohormones which in turn stimulate or inhibit the secretion of pituitary hormones. Neurons of the paraventricular nucleus for instance secrete the corticotropin-releasing hormone, which regulates adrenocorticotropic hormone (ACTH) secretion from the anterior pituitary gland. The hypothalamus consists of several nuclei which are involved in many different functions. It is responsible for the regulation of metabolism, control of body temperature, eating, drinking, fatigue, anger and circadian rhythms. Since it has to respond to many signals it is richly connected to many parts of the CNS.

Crosby and Woodburne (1940) divided the hypothalamus into three distinct longitudinal zones: (i) the periventricular, which is next to the third ventricle and contains most of the neurons that project to the pituitary, (ii) the medial zone, which has a series of rostro-caudally arranged neuronal aggregates and (iii) the lateral zone, which contains scattered neurons not forming aggregates. These three zones were further subdivided by LeGros Clark (1938) into four rostrocaudal levels based on the cell groups in the medial zone: the preoptic, anterior, tuberal and mammillary regions. These subdivisions divide the hypothalamus into twelve compartments (Fig.1.5) (Simerly, 2004).

On the basis of expression pattern, the hypothalamus can also be divided into an anterior-dorsal (AD), which comprises the preoptic, anterior and tuberal hypothalamus, and a posterior-ventral (PV) region, which generates the mammillary region (Mathieu et al., 2002).

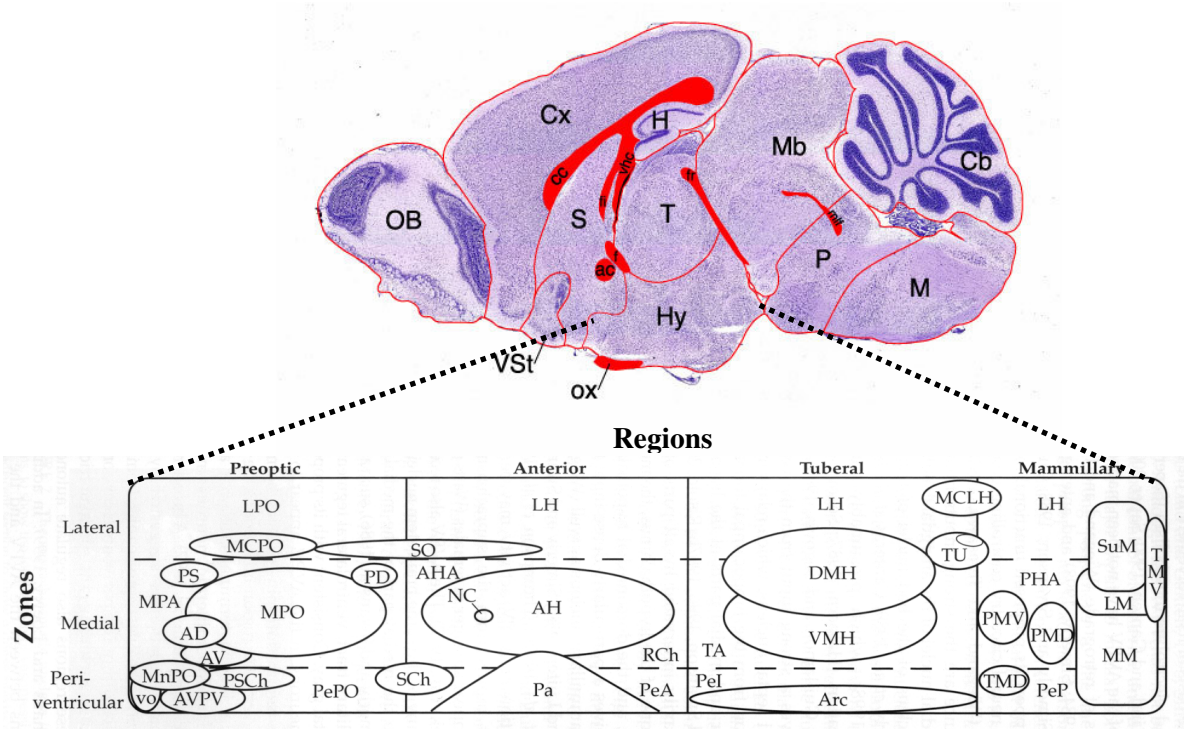


**Figure 1.5: The subdivisions of the hypothalamus.** On top, the sagittal view of an adult mouse brain is shown. The hypothalamus is located in the ventral part of the vertebrate brain. The schematic representation underneath is showing the three zones (Periventricular, Medial and Lateral) and the four regions (Preoptic, Anterior, Tuberal and Mammillary) of the hypothalamus with their nuclei (Simerly, 2004) (genepaint.org, modified by Dr. Alvarez-Bolado). Abbreviations: ac: anterior commissure; AD: Anterodorsal preoptic nucleus; AHA: Anterior hypothalamic area; AH: Anterior hypothalamic nucleus; Arc: Arcuate n.; AV: Anteroventral preoptic n.; AVPV: Anteroventral periventricular n.; Cb: Cerebellum; cc: corpus callosum; Cx: Cortex; DMH: Dorsomedial n.; f: fornix; fi: fimbria; fr: retroflex fascicle; H: Hippocampus; Hy: Hypothalamus; LH: Lateral hypothalamic area; LM: lateral mammillary nucleus; M: Medulla; Mb: Midbrain; LPO: Lateral preoptic area; MCLH: Magnocellular n.; MCPO: Magnocellular preoptic n.; MM: medial mammillary n.; MnPO: Median preoptic n.; MPA: Medial preoptic area; MPO: Medial preoptic n.; mtt: mammillotegmental tract; NC: Nucleus circularis; OB: Olfactory bulb; ox: optic chiasm; P: Pons; Pa: Paraventricular n.; PD: Posterodorsal preoptic n.; PeA: Anterior periventricular n.; PeI: Tuberal periventricular n.; PeP: Posterior periventricular n.; PePO: Preoptic periventricular n.; PHA: Posterior hypothalamic area; PMD: Dorsal premammillary n.; PMV: Ventral premammillary n.; PS: Parastrial n.; PSCh: Suprachiasmatic preoptic n.; RCh: Retrochiasmatic area; S: Septum; SCh: Suprachiasmatic n.; SO: Supraoptic n.; SuM: supramammillary n.; T: Thalamus; TA: Tuberal area; TMV: Ventral tuberomammillary n.; TMD: Dorsal tuberomammillary n.; TU: Tuberal n.; vhc: ventral hippocampal commissure; VMH: Ventromedial n.; vo: Vascular organ of the lamina terminalis; VSt: Ventral Striatum.

### 1.3.1 The mammillary body

The mammillary body is a large neuronal group in the medial zone of the mammillary region of the hypothalamus. It is formed by the larger medial mammillary nucleus (MM), the smaller lateral mammillary nucleus (LM), that consists of large, darkly stained neurons (Altman and Bayer, 1986), and the dorsal premammillary nucleus (PMd) (Fig.1.6). The medial nucleus can be further subdivided using cytological criteria into the pars medianus, pars medialis, pars lateralis and pars posterior (Paxinos, 2004). The mammillary body receives major afferents from several regions of the brain, for example from the subiculum by way of the fornix (Swanson and Cowan, 1977) and from the tegmentum by way of the mammillary peduncle (Cowan, 1964). It also sends major efferents to different brain regions (Fig.1.6). Fibers originating from the LM, MM and PMd form the principal mammillary tract that separates into the mammillotectal (mtc) and mammillotegmental tract (mtg). These axonal tracts project to Gudden´s tegmental nuclei, regions of the pons and to the superior colliculus (Morin, 1950). The axons of both, the mtc and mtg, branch interstitially by E17.5 and form the mammillothalamic tract (mtt) that innervates the anterior thalamic complex by E19.5 (Hayakawa and Zyo, 1989). The mammillary nuclei and its projections play therefore an important role in bridging the forebrain and midbrain. The mammillary body and the mammillothalamic tract are also part of the circuit of Papez (Papez, 1937), one of the major pathways of the limbic system, that connects the cingulate gyrus, the hippocampal formation and the hypothalamus. The mammillary body was also shown to be required for spatial learning and memory (Radyushkin et al., 2005; Sziklas and Petrides, 1998).

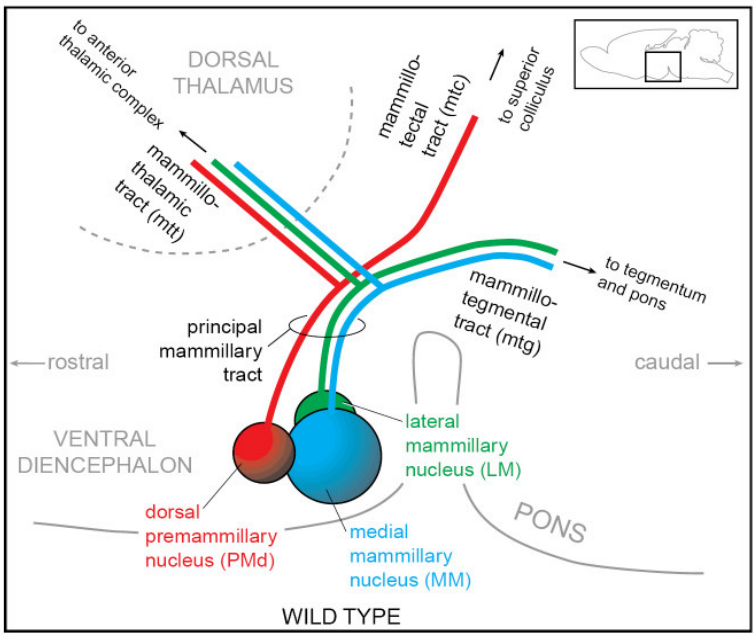


**Figure 1.6: The mammillary body and its projections.** A sagittal view of an adult mouse brain is shown. The inset in the upper right corner indicates the orientation of the brain. The mammillary body consists of three nuclei that send axons to several regions of the brain. The axons of the mammillotegmental (mtg) and mammillotectal (mtc) tract branch to form the mammillothalamic (mtt) tract that innervates the dorsal thalamus (Alvarez-Bolado et al., 2000b).

## 1.4 The thalamus (dorsal thalamus)

The thalamus is an oval shaped structure in the caudal diencephalon. It receives all sensory information except olfaction and is responsible for the initial processing of this input. Therefore, all sensory pathways relay in the thalamus that acts as a gatekeeper for information to the cerebral cortex, preventing or enhancing the passage of it. The ventral posterior lateral nucleus for example receives somatosensory information from the dorsal root ganglia and relays it via thalamocortical axons (TCA) to the primary somatosensory cortex. Visual information from the retina reaches the lateral geniculate nucleus which projects it to the primary visual cortex.

The thalamus is made up of several well-defined nuclei that are classified into anterior, medial, ventrolateral, and posterior groups with respect to the internal medullary lamina, a fiber bundle that crosses the thalamus rostrocaudally (Fig.1.7). The anterior group consists of the anterior thalamic nucleus (ATN), which is innervated by the mammillothalamic tract of the mammillary body and therefore is thought to participate in memory and emotion.

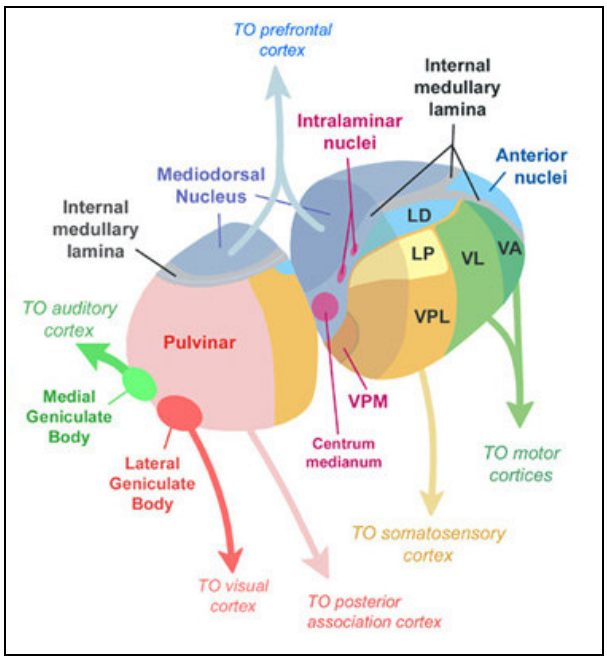


**Figure 1.7: The major subdivisions of the thalamus.** The thalamus is a relay for sensory information, which is processed and then conveyed to specific cortical areas. Visual information for example coming from the retina reaches the lateral geniculate nucleus, which conveys it to the primary visual cortex. The thalamus consists of several nuclei that are classified with respect to the internal medullary lamina.
Abbreviations: LD: laterodorsal nucleus; LP: lateral posterior; VA: ventral anterior; VL: ventral lateral; VPL: ventral posterior lateral nucleus.

The mediodorsal nucleus (MD) is the largest nucleus of the medial group. Its subdivisions are connected to the prefrontal cortex and have functions in memory.

The ventral anterior (VA) and ventral lateral (VL) nuclei are part of the ventral nuclear group. They receive input from the basal ganglia and cerebellum and relay it to the motor cortex. Therefore they have important functions in motor control. Another nucleus of that group is the ventral posterior lateral (VPL) nucleus that sends somatosensory information to the somatosensory cortex.

The posterior group consists of the medial geniculate (MG) nucleus, that sends auditory information to the cortex, the lateral geniculate (LG) nucleus, that conveys visual information to the cortex, the lateral posterior (LP) nucleus, and the pulvinar.

The thalamocortical axons originating from thalamic nuclei terminate in specific layers of the neocortex. They have to cross the diencephalic-telencephalic and the pallial-subpallial boundaries during embryonic development to reach their target area. Cortical neurons send at the same time corticofugal projections back to the corresponding thalamic nuclei and other additional nuclei (Lopez-Bendito and Molnar, 2003). Thalamocortical and

corticofugal axons together form the internal capsule (IC), a large axon bundle reciprocally connecting the cortex with the subcortical structures of the brain.
Some thalamic nuclei project instead of just to one specific cortical area to several cortical and subcortical regions. The paraventricular, parataenial, and reunions nuclei, which are located at the midline of the thalamus, belong to these nonspecific nuclei as well as the intralaminar nuclei within the internal medullary lamina. The intralaminar nuclei innervate for example the amygdala, the hippocampus, and the basal ganglia.
Most of the neurons in the thalamic nuclei utilize glutamate, the main excitatory transmitter in the brain and spinal cord (Kandel et al., 2000).
The prethalamus (formerly described as ventral thalamus) lies underneath the thalamus and is separated from it by the ZLI. Nuclei of the prethalamus are thalamic reticular nucleus, zona incerta and ventral lateral geniculate. In contrast to the thalamus it does not send efferents to the cortex but e.g. to the thalamus and striatum. Different domains of the prethalamus were shown to be required for TCA pathfinding (Tuttle et al., 1999).

## 1.5 The transcription factor Foxb1

Foxb1 (for *Forkhead box*), also known as Mf3, Fkh5 and TWH, belongs to the forkhead family of transcription factors. It has a 110 amino acid long, conserved DNA-binding domain, termed forkhead/HNF-3 domain (Kaufmann and Knochel, 1996). The three-dimensional structure of a forkhead domain bound to DNA was studied by X-ray crystallography (Clark et al., 1993). The structure was termed “winged helix” since it has a helix-turn-helix core of three α-helices and two loops or wings, which give it the appearance of a butterfly.
*Foxb1* expression during mouse development is very widespread in early ages but becomes very restricted in the adult (Alvarez-Bolado et al., 1999; Alvarez-Bolado et al., 2000a; Alvarez-Bolado et al., 2000b; Theil et al., 1999). It is first detected at E7.5 in the neural ectoderm and posterior primitive streak mesoderm (Kaestner et al., 1996). By E8.0 expression can be found in the ventricular layer of the diencephalon, midbrain, hindbrain and spinal cord. It then progressively recedes so that by E14.0 it is restricted to the mantle layer of the dorsal thalamus, the mammillary region and rostral midbrain and with fading intensity in regions of the hindbrain and caudal midbrain. In the adult, expression is only

found in the medial and lateral mammillary nucleus and the dorsal premammillary nucleus of the mammillary body and the superior colliculus in the rostral midbrain.
To assess its in vivo function, *Foxb1* has been knocked out (Alvarez-Bolado et al., 1999). *Foxb1* deficient mice are viable but show growth retardation and a hindlimb motility problem (Dou et al., 1997). The female knockout mice fail to generate the milk rejection reflex (Labosky et al., 1997). The mutant phenotype also includes impaired differentiation in the midbrain and diencephalon. In the diencephalon the mammillothalamic axons of the mammillary body do not reach the thalamus, which appears to be a non cell autonomous defect and the cells of the medial mammillary nucleus die by apoptosis perinatally (Alvarez-Bolado et al., 2000b). Consequently, adult *Foxb1* deficient mice have no medial mammillary nuclei, reduced lateral mammillary nuclei and no mammillothalamic tract. Since these structures have a crucial role in memory processes, these mice show impairment in spatial working memory (Radyushkin et al., 2005).

## 1.6 The Cre-loxP recombination system

The inactivation of a specific gene by deletion or modification and the study of the consequences in the mutant organism constitute a very useful approach to understand the *in vivo* functions of a given gene. This can be achieved by replacing a predetermined gene segment with a mutant version of this segment by homologous recombination in embryonic stem (ES) cells. If the mutated ES cells are transferred into mouse embryos the mutation can be transmitted into the mouse germline. This leads to the generation of classical (general) knockout mice in which the gene of interest is inactivated in all cells of the organism from the beginning of ontogeny. This approach has several disadvantages that make it difficult to study a gene's function. Some germline mutations may be lethal so that no knockout mouse can be generated. Some genes may exert their function at several stages of ontogeny, so that the initial stage of its function can be identified but not necessarily later stages. Other genes have important functions in different cell types which lead to complex phenotypes difficult to analyze. By using the Cre-loxP recombination system these problems can be avoided. With this method a cell-type-specific and/or inducible gene targeting, also termed conditional gene targeting is achieved (Fig.1.8).

For this purpose *loxP* sites, sequence motives of 34bp, are inserted by classical gene targeting into the genome of ES cells so that the target gene or a segment of that gene is flanked by *loxP* sites. This results in mutant mice that carry a *loxP*-flanked but functional gene in their genome. These animals are healthy and indistinguishable from wild type animals. By crossing these mutant mice with a Cre line, in which the Cre recombinase is expressed under the control of a tissue-specific or inducible promoter, conditional targeting of the *loxP*-flanked gene or gene segment can be achieved. The Cre enzyme, a P1 bacteriophage enzyme, recognizes the *loxP* sites and excises the flanked segment from the DNA leaving a single *loxP* site behind. In this way specific tissue types or cells can be genetically modified while other tissues of the organism remain unchanged (Fig.1.8).

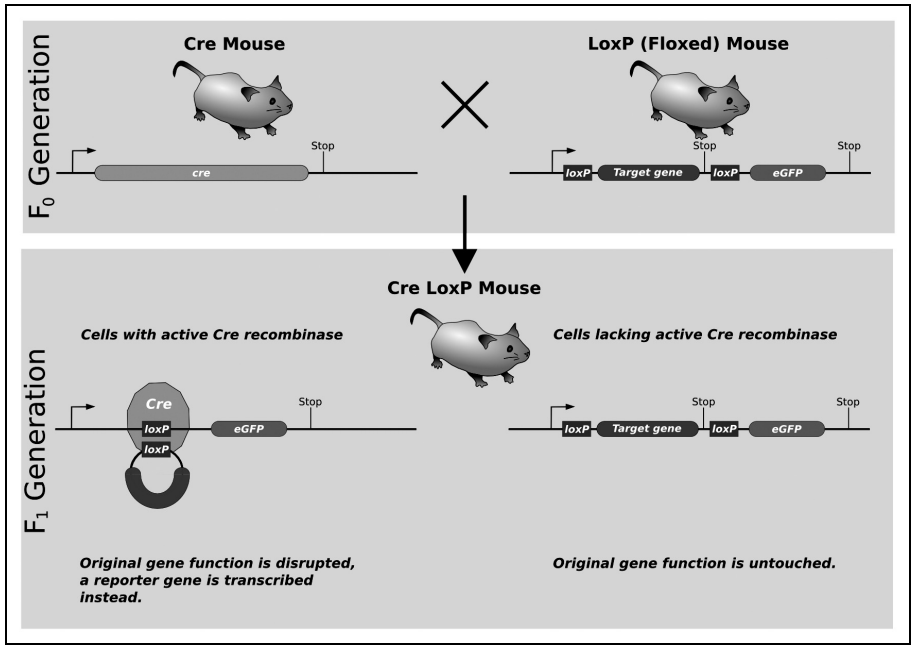


**Figure 1.8: The Cre-loxP recombination system.** A Cre mouse in which the Cre recombinase is controlled by a tissue-specific promoter is crossed with a loxP mouse in which the target gene is flanked by *loxP* sites. In the resulting Cre loxP mouse the flanked target gene is excised in the cells with active Cre recombinase while in cells that lack the activation of the Cre recombinase, gene function is untouched (wikipedia.org).

### 1.6.1 The ROSA26 reporter line

The ROSA26 (reverse orientation splice acceptor) mouse strain was produced by random retroviral gene trapping in embryonic stem cells (Friedrich and Soriano, 1991). With this method the reporter gene ß-galactosidase was integrated randomly into the genome leading to its expression from a tagged endogenous promoter. The integration site of the

reporter in the ROSA26 mouse gene trap line has been identified (Zambrowicz et al., 1997). The tagged promoter was shown to have constitutive activity and common features of a housekeeping gene promoter. The ROSA26 line therefore displays ubiquitous expression of the reporter gene during development starting at the morula-blastocyst stage. Homozygotes of the mutated tagged gene do not show an overt phenotype and are fertile.

This ROSA26 mouse line was used for the derivation of a reporter line for monitoring Cre expression (ROSA26R) (Soriano, 1999). For this purpose a neomycin (neo) expression cassette flanked by *loxP* sites, a *lacZ* gene and a polyadenylation (bpA) sequence was inserted close to the original gene-trap integration site. The neo expression cassette was followed by a triple polyadenylation site to prevent transcriptional readthrough. By Cre-mediated recombination the neo cassette and the bpA site are removed and ß-galactosidase is produced. Therefore, the ROSA26R mouse strain is very useful for monitoring Cre expression when crossed with a specific Cre line. The reporter is at the same time also a lineage marker, since all cells expressing the Cre recombinase and any daughter cells they generate by mitosis will permanently express ß-galactosidase.

### 1.6.2 The Foxb1-Cre mouse line

In our group we generated a mouse line expressing Cre recombinase under the control of the *Foxb1* promoter (Zhao et al., 2007). An iCre-IRES-EGFP cassette was knocked in the *Foxb1* locus by homologous recombination, so that not only Cre recombinase activity but also green fluorescent protein (GFP) reproduced *Foxb1* expression. To characterize Cre recombinase activity under the transcriptional control of *Foxb1*, we crossed the generated mouse line with the ROSA26R reporter mouse line. Staining for ß-gal in these crossings revealed that Cre-mediated recombination events took place already at E8.5 in the neural plate, the somites and the presomitic mesoderm of the tail. The staining was detected in places where *Foxb1* expression is expected showing the reliability of this marker. E9.5 embryos showed strong reporter expression in the somites and in all of the neural tube starting at diencephalic levels. At E12.5 ß-gal expression was found in the entire dorsal thalamus, part of the prethalamus and the PV of the hypothalamus. Since *Foxb1* expression is much more restricted at this age, the staining shows all the cells that derive from *Foxb1* expressing cells in which Cre-mediated recombination has taken place.

To further characterize the Foxb1-Cre line, it was crossed with the Z/AP reporter line expressing human placental alkaline phosphatase (hPLAP) after recombination (Lobe et al., 1999). In these crossings the Cre recombinase builds up slower than in the ROSA26R crossings but since hPLAP is a cell surface protein, the axonal processes of the cells in which Cre recombinase has been expressed are also stained. Therefore it is possible to label all thalamocortical axons by alkaline phosphatase (AP) staining since all thalamic cells are derived from *Foxb1* expressing cells.

### 1.6.3 The floxed Shh mouse line

In the *Shh* conditional allele exon 2 is flanked by *loxP* sites. Mice that are homozygous for this targeted mutation, also termed $Shh^{tm2Amc}$ are viable, fertile and display no abnormalities. Cre-mediated recombination excises exon 2 and some surrounding intronic sequences, generating a null allele. Exon 2 encodes part of the N-terminus of Shh, which has been shown to be essential for Shh function (Chiang et al., 1996). $Shh^{tm2Amc}$ mice can be used for temporal and tissue-specific ablation of Shh function when crossed with a strain expressing Cre recombinase in a tissue-specific manner (Dassule et al., 2000; Komada et al., 2008). When the floxed mutant is mated with a strain in which Cre recombinase is under the control of a CMV promoter and therefore expressed ubiquitously, the resulting phenotype resembles that of *Shh* knockout mice.

## 1.7 Aim of the work

*Shh* has a very dynamic expression pattern in the diencephalon with expression in the ZLI at the boundary between thalamus and prethalamus and in the lateral hypothalamic band in the hypothalamus. This suggests that Shh has important functions in diencephalic development. In the *Shh* knockout mouse the entire hypothalamus is abolished and the caudal diencephalon is reduced in size. Since all Shh is removed in these mice it is not possible to gain insight into the functions of the *Shh* expression domains in the diencephalon. Therefore, aim of this work was to specifically remove *Shh* expression in the diencephalon using the Cre-loxP recombination system. The Foxb1-Cre line expresses the Cre recombinase in parts of the rostral and caudal diencephalon, very much overlapping the *Shh* expression domains in the forebrain. This Cre line was crossed with the floxed *Shh* mouse line to generate a new mouse line that carries both mutations in its genome. For the analysis mice which were heterozygous for the Cre recombinase and homozygous for the floxed *Shh* allele were used and were referred to as *Shh-c*, for Shh conditional mutant. The first goal was to analyze the exact time point and region of Shh ablation in these mice by whole mount *in situ* hybridizations (ISH). Furthermore, the time point of the Shh pathway inactivation needed to be determined. Next the consequences of this region-specific removal should be characterized by ISH of fate markers. Specifically, the development of the hypothalamus and the thalamus of the Shh-c mutant was planned to be analyzed to gain more insight about the specific functions of neural Shh in these regions. Using explant cultures of wild type embryos and adding various substances to the culture medium to alter signaling pathways should be another approach to elucidate signaling cascades involved in early brain development.

# 2 Materials and Methods

## 2.1 Animal work

All work with animals was accomplished according to the regulations of the federal animal law for protection (BGB1 S.1105, ber. S. 181, Abschnitt 2 (§2+3), 5 (§7-9) und 8 (§119)). The Foxb1-Cre mouse line was generated in the 129Sv background and maintained in the 129Sv/C57BL/6 background (Zhao et al., 2007). For characterization of the Cre recombinase activity, this mutant was crossed with ROSA26R (Soriano, 1999) and with Z/AP (Lobe et al., 1999) reporter mice. The floxed Shh mouse line with the strain name 129-$Shh^{tm2Amc}$/J was made available by the Jackson Laboratory, Bar Harbor, donated by A. McMahon. Dr. A. Joyner provided the Gbx2 mutant mouse line.
Male and female mice were either crossed overnight and female mice were checked for vaginal plugs next morning. Alternatively, mice were crossed in the morning and separated at noon to receive timed mating plugs. For overnight plugs noon next day was considered as E0.5.

## 2.2 Genotyping

A short piece of tail (0.5-0.6 cm) from an adult mouse or leg tissue from a mouse embryo was incubated in 200µl of lysis buffer (10mM Tris-HCl, pH8.3; 2.5mM $MgCl_2$; 0.45% Tween20; 0.45% NP40, 10% gelatin; 25mM KCl) containing 0.2mg/ml proteinase K (Merck) in a thermomixer at 55°C with 850rpm until complete lysis was achieved (3-16 hours). The crude lysate was then incubated at 85°C for 45min on the thermomixer to inactivate the proteinase K. The sample was centrifuged for 3sec and stored at 4°C. For genotyping usually 1µl of this lysate was added to the PCR reaction.

### 2.2.1 Primers for genotyping

| Mouse strain | Primer name | Sequence |
|---|---|---|
| CMV-Cre | Cre-F | 5´ CGATGCAACGAGTGATGAGGTTCG 3´ |
| | Cre-R | 5´ AGCATTGCTGTCACTTGGTCGTGG 3´ |
| Z/AP | AP-1_for | 5´ GGAAGTCAGTGGGAGTGGTAAC 3´ |
| | AP-2_rev | 5´ GTCTCGGTGGATCTCGTATTTC 3´ |
| Gbx2 | Gbx2-3F | 5´ TCTCGGAACCCCAAGATTGTCGTC 3´ |
| | Gbx2-3R | 5´ TGGATGTCCACATCTAGGAGGTGC 3´ |
| | Gbx-3P1 | 5´ CACGAGACTAGTGAGACGTGCTAC 3´ |
| Shh | 1930 | 5´ ATGCTGGCTCGCCTGGCTGTGGAA 3´ |
| | 1931 | 5´ GAAGAGATCAAGGCAAGCTCTGGC 3´ |
| | 1840 | 5´ GGACACCATTCTATGCAGGG 3´ |
| Foxb1-Cre | GAB20 | 5′ CACTGGGATGGCGGGCAACGTCTG 3′ |
| | GAB22 | 5′ CATCGCTAGGGAGTACAAGATGCC 3′ |
| | EGFP | 5´ CTCGGCATGGACGAGCTGTACAAG 3´ |

### 2.2.2 Genotyping of the mouse lines Foxb1-Cre, CMV-Cre, and Z/AP

For the PCR reaction the PCR kit from GeneCraft was used with its own polymerase.

Each 20µl reaction:

1,0µl lysate
2,0µl 10x PCR buffer with 15mM $MgCl_2$
4,0µl Q-Solution
0,2µl dNTPs (20mM)
1,6µl $MgCl_2$ (25mM)
0,1µl primer 1 (100pmol/µl)
0,1µl primer 2 (100pmol/µl)
0,1µl primer 3 (100pmol/µl)
0,15µl polymerase (1U/µl)

Add $H_2O$ to a final volume of 20µl.

PCR program:

94ºC, 4min+ 30 x (94ºC, 20sec + 64ºC, 30sec + 72ºC, 40sec) + 72ºC, 5min

10µl of each PCR reaction were run on a 1% agarose gel. The sizes of the resulting fragments for Foxb1-Cre were 500bp (ko) and 258bp (wt), for CMV-Cre one band of 260bp and for Z/AP one band of 500bp.

### 2.2.3 Genotyping of the Gbx2 mouse line

PCR reaction:

1,0µl lysate
2,0µl 10x PCR buffer with 15mM $MgCl_2$
0,2µl dNTPs (20mM)
0,6µl $MgCl_2$ (25mM)
0,1µl primer 1 (100pmol/µl)
0,1µl primer 2 (100pmol/µl)
0,1µl primer 3 (100pmol/µl)
0,15µl polymerase (1U/µl)

Add $H_2O$ to a final volume of 20µl.

PCR program:

94ºC, 4min+ 30 x (94ºC, 20sec + 64ºC, 30sec + 72ºC, 40sec) + 72ºC, 5min

The resulting fragments of 500bp (ko) and 300bp (wt) were separated on a 1% agarose gel.

### 2.2.4 Genotyping of the Shh mouse line

PCR reaction:

1,0µl lysate
2,0µl 10x PCR buffer with 15mM $MgCl_2$
0,2µl dNTPs (20mM)
0,1µl primer 1 (100pmol/µl)
0,1µl primer 2 (100pmol/µl)
0,1µl primer 3 (100pmol/µl)
0,15µl polymerase (1U/µl)

Add $H_2O$ to a final volume of 20µl.

Gradient PCR program:

94ºC, 1:30min+ 35 x (94ºC, 30sec + 67ºC, 45sec – 0.5°C per cycle + 72ºC, 1min) + 72ºC, 2min

The PCR products were separated on a 1.5% gel. The wt band had a length of 449bp, the floxed mutant band 483bp, the band after Cre-mediated recombination was 115bp long.

## 2.3 *In situ* hybridization

### 2.3.1 Probe preparation

#### 2.3.1.1 RNA isolation

Total RNA from mouse brain (e.g. P0, P7) and mouse embryos (e.g. E10.5, E14.5) was isolated with the Qiagen RNeasy Mini Kit. The isolated RNA was mixed in a 1:1 ratio (on a mass basis) for subsequent cDNA synthesis.

#### 2.3.1.2 Reverse transcription

The cDNA was synthesized with the ThermoScript™RT-PCR System (Invitrogen). The following reagents were mixed for Mix1:

| | |
|---|---|
| Oligo(dT)-Primer | 1,0µl |
| RNA | 2,0µl (1µg/µl) |
| DEPC-treated water | 7,0µl |

To denature the RNA and anneal primer the mixture was incubated for 5min at 65°C and then placed on ice. Next Mix2 was prepared:

Mix2 (for one aliquot of cDNA):

| | |
|---|---|
| 5x cDNA Synthesis Buffer (vortex) | 4,0µl |
| 0.1M DTT | 1,0µl |
| RnaseOut™ (40 U/µl) | 1,0µl |
| DEPC-treated water | 1,0µl |
| 10 mM dNTP-Mix | 2,0µl |
| ThermoScript™RT (15 U/µl) | 1,0µl |

Mix2 was gently vortexed and added to Mix1. This mixture was then incubated for 60min at 50°C in a thermocycler and subsequently heated up to 85°C for 5min to stop the reaction. 1µl RNaseH was added and incubated for 20min at 37°C. In this form, the cDNA produced was frozen and kept at -80°C for long-term storage (>1 year).

#### 2.3.1.3 Primer design

A gene-specific oligonucleotide was designed by using a primer selection program or from own experience/strategy. In general, the GC content should be in the range of 40 to 60%, the 3´end of the primer, from which the polymerase will extend the sequence, should have a GC or CG, repeats should be avoided, no homology to other genes in the genome (BLAST search) should be present, no long hairpin structures or dimers should be formed. The length of the primers was in the range of 24 bases that bordered the desired sequence. Once the specific primer pair was selected, an RNA polymerase promoter sequence was added to the 5´end of the forward primer and the reverse primer. The sequence of the T7 promoter was GCGTAATACGACTCACTATAGGG and of the SP6 promoter GCGATTTAGG TGACACTATAG. The three underlined extra bases were attached to each of the promoters to allow better binding of the RNA polymerase. The primers were usually synthesized by Eurofins MWG Operon (Martinsried).

Primer sequences without the added RNA polymerase promoter sequence:

| Gene | GeneBank | Primer | Sequence |
|---|---|---|---|
| Foxb1 | U90538 | Forward | ATCGCTAGGGAGTACAAGATGCC |
| | | Reverse | GATCAGTGAGTTGGTCTTGTGGC |
| Wnt8b | NM_011720 | Forward | ATCGCTTACACACCAAG |
| | | Reverse | CAGGCAGTAGTCTGGGGAGT |
| Shh long probe | NM_009170 | Forward | ATGCTGCTGCTGCTGGCCAG |
| | | Reverse | GGGCCCCGAGTCGTTGTGCGG |
| Shh exon2 | NM_009170 | Forward | GACCGGCTGATGACTCAGAGGTGC |
| | | Reverse | TTTGGCCGCCACGGAGTTCTCTGC |
| Gli1 | NM_010296 | Forward | GCATGAGCTCTGCTTACACA |
| | | Reverse | CATTTGGGTTGTATCCCGGG |
| Ptch1 | NM_008957 | Forward | ACACGAACAATGGGTCTGATTCCT |
| | | Reverse | TGACATGAAAAGGCACATGAGGAT |
| Pitx2 | U80036 | Forward | AGAGCTTCCCCTTCTTCAACTCC |
| | | Reverse | ACAGGATGGGTCGTACATAGCAG |
| Gbx2 | NM_010262 | Forward | AGGTTCGCTATTCGAAGTCAAC |
| | | Reverse | AATTAAACACAACGGAGACGTG |
| Pax6 | NM_013627 | Forward | AGTTCTTCGCAACCTGGCTA |
| | | Reverse | ACTTGGACGGGAACTGACAC |
| Nurr1 | NM_013613 | Forward | ATGCCTTGTGTTCAGGCGCA |

| | | | |
|---|---|---|---|
| | | Reverse | CGTGCAGAGGCCCGTCGAAG |
| Nkx2-1 | NM_009385 | Forward | CAACAACTGCAGCAGGACAG |
| | | Reverse | GTCCGACCATAAAGCAAGGTAG |
| Lim1(Lhx1) | NM_008498 | Forward | ATCTCTCCAAGCGATCTGGTTCGC |
| | | Reverse | TCTCTGTACAACCACGAGCTCC |
| Calb1 | NM_009788 | Forward | AACTAGCAGAGTACACAGACC |
| | | Reverse | TCTCTGGCAGTAGACTTCTTGG |
| Avp | NM_009732 | Forward | ATGCTCGCCAGGATGCTCAACAC |
| | | Reverse | CTTGGCAGAATCCACGGACTCC |
| Pmch | BC048534 | Forward | GCACTCTTGTTTGGCTTTATGC |
| | | Reverse | GAGGTTTAATGCACACGTCAAGC |
| Hcrt | NM_010410 | Forward | AAGACGACGGCCTCAGACTTC |
| | | Reverse | CCATTTACCAAGAGACTGAC |
| Dlx2 | NM_010054 | Forward | CGGGGACGATTTTCTAACCT |
| | | Reverse | TACGTCGCAGCTTTCACAAC |
| Dbx1 | NM_001005232 | Forward | GGCCTCGAGCCAGATTCGGC |
| | | Reverse | TAGACAGGGAGACGGTGCCC |
| Emx1 | XM_132640 | Forward | AAGGGTTCCCACCATATCAACCG |
| | | Reverse | ACTAAGAACTACAGCAGGACCTGG |
| Irx1 | AF165984 | Forward | ACCTCGGTGCTAGGCATGTATGCC |
| | | Reverse | AGCCCTGTGCTAGGAACGCGCCGT |
| Nkx2-2 | NM_010919 | Forward | ACAGCCTACATTTCTGCGTGCTTT |
| | | Reverse | CTCCTTGTCATTGTCCGGTGACT |
| Lhx2 | NM_010710 | Forward | AGCAGCTAAGAGTGCAGGATTGGG |
| | | Reverse | CGTGGCAGTCTTTGAAAATACGG |
| Lhx5 | U61155 | Forward | GACCGACAATTCGACCTCATCGG |
| | | Reverse | TAGTTCAAGGAGGTGATGATGGG |
| Neurog2 | NM_009718 | Forward | TGATGCACGAGTGCAAGCGTCG |
| | | Reverse | ACAGCCTGCAGACAGCAATGGG |
| Cdh6 | NM_007666 | Forward | ACGGAGATTAAAAACCCAAAG |
| | | Reverse | TTAAGAGTCTTTGTCACTGTC |

#### 2.3.1.4 PCR I

The annealing temperature depends on the sequence of the gene specific part of the primer because the T7 or SP6 sequences do not bind to the cDNA. To amplify the sequence a touchdown (TD) PCR was performed first, usually in the range of 65°C-55°C. For the PCR reaction a Qiagen Kit with the Taq polymerase was used.

PCR reaction:

1,0µl cDNA
2,0µl 10x PCR buffer with 15mM $MgCl_2$
4,0µl Q-Solution (5x)
2,0µl dNTPs (2mM)
2,0µl forward primer (5pmol/µl)
2,0µl reverse primer (5pmol/µl)
0,1µl polymerase (1U/µl)

Add $H_2O$ to a final volume of 20µl.

TD PCR program:
94ºC, 2min + 20x (94ºC, 30sec + 65ºC, 30sec – 0.5°C per cycle + 72ºC, 1min) + 25x (94ºC, 30sec + 55ºC, 30sec + 72ºC, 1min) + 72ºC, 9min

The 20µl reaction was run on a 1% agarose gel to check for the right size of the PCR product and to separate the right band from possible additional bands. The right band was subsequently excised from the gel and purified with a gel extraction kit (QIAquick Gel Extraction Kit from Qiagen). The DNA was eluted with 22µl TE-buffer and was used for the next large scale PCR.

#### 2.3.1.5 PCR II (large scale PCR)

In order to prepare template for the riboprobe synthesis, a 100µl PCR reaction was prepared using the gel extracted DNA as template. The amount of template added depended on the yield of the first PCR. The gene specific primers that were used to generate the first PCR product from the cDNA or the T7/Sp6 primers were added to the PCR reaction. Amplification with the T7/Sp6 primers usually gave good results. Two PCR tubes were used to carry out the 100µl preparative PCR.

PCR reaction:

2-10µl DNA (20-50ng)
10µl 10x PCR buffer with 15mM $MgCl_2$
20µl Q-Solution (5x)
10µl dNTPs (2mM)
10µl forward primer (5pmol/µl)
10µl reverse primer (5pmol/µl)
0.5µl polymerase (1U/µl)

Add $H_2O$ to a final volume of 100µl.

If gene-specific primers were used, the same TD PCR program was run as in the first PCR. If the T7/Sp6 primers were added the following PCR program was used:

94ºC, 2min+ 35 x (94ºC, 20sec + 47.5ºC, 30sec + 72ºC, 1:10min) + 72ºC, 9min

5µl of the PCR reaction was subsequently analyzed on an agarose gel. The remaining 95µl were purified with a Qiagen PCR purification kit. The DNA was eluted with 22µl of Elution buffer (10mM Tris, pH 8.5). 1µl was used to measure the concentration and purity of the DNA. Typically the yield was 200-400ng/µl. 200ng of the DNA were sent for sequencing (Eurofin MWG Operon), for which the T7 primer was used to validate that the right sequence was amplified.

#### 2.3.1.6 RNA transcription

The Roche RNA transcription kit (DIG RNA Labeling Mix) was used. As template 500ng of the DNA obtained from the large scale PCR were added. The RNA polymerases were from Stratagene (T3) or from NEB (T7 and SP6).

| | |
|---|---|
| xµl | DNA template (500ng) |
| 2,0µl | 10x transcription buffer |
| 2,0µl | DIG RNA labeling mix |
| 1,0µl | RNase inhibitor |
| 1,0µl | RNA polymerase (SP6, T7 or T3) (in case of T7 only 0,7µl) |

Add DEPC-treated $H_2O$ to a final volume of 100µl.

The in vitro transcription reaction was incubated at 37°C for 2.5 hours. Following incubation 1µl of Stop solution was added per reaction. This stops the transcription and removes the DNA template.

Stop solution:

| | |
|---|---|
| 16,4µl | $H_2O$ DEPC-treated |
| 1,6µl | $MgCl_2$ (0.3M) |
| 2,0µl | DNase (10U/µl) |

This reaction was incubated for 15min at 37°C. To precipitate the RNA, 100µl of DEPC-$H_2O$, 72µl ice-cold $NH_4Ac$ (4M) and 470µl ice-cold 100% EtOH were added. The samples were briefly vortexed and placed at -80°C for 20min. After incubation the samples were centrifuged at max. speed in a table-top microcentrifuge (13000rpm) at 4°C for 20min. The supernatant was carefully removed using a pipette, making sure not to disturb the pellet. The pellet was washed with 640µl 70% EtOH and centrifuged at max. speed at 4°C for 20min. The supernatant was removed and the pellet dried in a vacuum centrifuge for 5min. The pellet was subsequently resuspended with 22µl DEPC-$H_2O$ and put on a shaker at 1150rpm for 15min at RT. The concentration and the quality of the probe were measured with the Bioanalyzer QC using the RNA 6000 Nano-LabChip Kit. The probes were then stored at -80°C until use.

### 2.3.2 Whole mount *in situ* hybridization

The protocol used is based on protocols from A. McMahon's and R. Harland's laboratories.
Embryos were dissected in 1x PBS (DEPC-treated) and fixed in 4% PFA at 4°C overnight. Next day, the embryos were washed in 1x PBT (PBS with 0.1% Tween-20). With a very fine needle small holes were made in the embryo to facilitate the passage of the probe inside the embryo. Then they were dehydrated through a MeOH/PBT series (25%, 50% and 75% MeOH) and stored in 100% MeOH at -20°C until use.

Pretreatment and hybridization - Day 1

The embryos of the right genotype or other tissue were rehydrated through a MeOH/PBT series into PBT: 75%, 50%, 25% MeOH/PBT for 5-10min each, then 2x 10min in PBT. Then bleached with 6% $H_2O_2$ in PBT for 1h at RT and subsequently washed with PBT 3x 5min each at RT. Subsequently they were treated with 10µg/ml Proteinase K (Roche) in PBT at RT. Care was taken not to treat them for too long since that would have dissolved the embryo. Suggested times:

9.5 d embryo: 4min
11.5 d embryo: 8min

18.5 d kidney: 6min

14.5 d kidney: 3min

cultures, 8.5 d embryos: 1min

The embryos were then washed in 2mg/ml glycine in PBT, 2x for 10min at RT and rinsed 3x for 5min at RT. This was followed by postfixation in 4% PFA/0.2% Glutaraldehyde in PBT for 20min at RT. After this they were washed 3x with PBT for 5min each. 1ml Prehyb (50% Formamide, 5x SSC, pH 4.5, 1% SDS, 50µg/ml yeast tRNA, 50µg/ml Heparin) was added to the embryos and swirled around for 5min. The Prehyb was removed and 2ml fresh Prehyb added and incubated at 70°C for 1-2h. Prehyb was removed and 1-2ml (so that embryos were covered) Hyb was added with the probe (1µg probe per ml (10µl/ml) in Prehyp). The embryos were incubated in Hyb o/n at 70°C.

Post hybridization washes and antibody incubation - Day 2

The Hyb was removed and embryos were washed 3x 30min with prewarmed Solution I (50% Formamide, 5x SSC (20x SSC: 175g NaCl, 88.2g NaCitrate per L, pH with citric acid to 4.5), 1% SDS, in non-DEPC water) at 70°C. This was followed by 3x 5min washes in TNT (10mM Tris-HCl, pH 7.5, 0.5M NaCl, 0.1% Tween-20) at RT. Embryos were treated 2x 30min with 100µg/ml RNAse in TNT at 37°C and washed with TNT:Solution II (1:1) 5min at RT. Then they were washed 3x 30min with Solution II (50% Formamide, 2x SSC, pH 4.5, 0.2% SDS, in non-DEPC water) at 65°C. This was followed by 3x 5min washes in MAB (100mM Maleic acid, 150mM NaCl, 2mM Levamisole, 0.1% Tween-20, pH 7.5) at RT. Embryos were preblocked in 10% Sheep serum (Sigma) in MAB/2% blocking reagent (Roche) for 2-3h at RT. The blocking reagent had to be heated up in MAB and stirred until it dissolved before adding to the embryos. After incubation the blocking reagent was removed and 1-2ml of antibody mix (anti-Dig Alkaline Phosphatase (1:5000) from Roche in MAB/2%Block/1% sheep serum) were added. The embryos were rocked gently at 4°C o/n.

Post antibody washes - Day 3

The embryos were washed 3x with MAB for 10min at RT. Then hourly washes were done with MAB. All washes were done with agitation. The bigger the embryo the larger the

washing volume and the time should be. The washing was continued with MAB overnight at 4°C.

Color development and storage - Day 4 and 5

Next day the embryos were washed 3x 10min with NTMT (100mM TRIS HCl, pH 9.5, 50mM $MgCl_2$, 100mM NaCl, 0.1% Tween-20, 2mM Levamisole) at RT. Then they were incubated in 1-2ml BM Purple (Roche) with Levamisole at RT. Color development started after 1-2 hours of incubation. The embryos were checked regularly to stop reaction if necessary. Depending on the probe incubation time varied from 6 hours at RT to overnight at 4°C. Then they were washed 3x in PBT, pH 4.5, and fixed in 4% PFA/0.1% Glutaraldehyde from 1h at RT to o/n at 4°C. The embryos were stored in PBT, pH 4.5 or transferred into 80% Glycerol/PBT for photography or 70% ethanol/saline for sectioning.

### 2.3.3 *In situ* hybridization on sections

The *in situ* hybridization on sections was carried out with an automated High-Throughput-Procedure developed in this department (Herzig et al., 2001).
The embryos of the appropriate stage were washed with ice-cold phosphate buffered saline (PBS) (0.137M NaCl, 0.0027M KCl, 0.01M $Na_2HPO_4$, 0.002M $KH_2PO_4$, pH 7.4). The whole embryo or just the head in case of older embryos was put into ice-cold OCT (Tissue Tec$^{TM}$) and kept there for 5min. The head or embryos were then transferred to an OCT embedding chamber, in which they were frozen. The frozen blocks with embryos were kept at -80°C until use. Before sectioning they were put at -20°C for a few hours. The embryos were sectioned (20-25µm thick) with a Leica CM3050S cryostat (Germany) at -18°C. Sections were placed in appropriate positions on a Super Frost Plus microscopy slide and kept at -20°C until fixation. The sections on the slides were fixed for 20min with 4% PFA (EMS, USA) in 1x PBS solution. Then the slides were washed twice in 0,9% NaCl to remove the fixative and acetylated 2x 5min with acetylation solution (0,25% acetic anhydride, 0,1M Triethanolamine, pH8.0). The slides were washed in PBS and 0.9% NaCl for 5 minutes and dehydrated through a graded series of ethanol (30%, 50%, 70%, 80%, 95% and 2x 100%) for 2min each step at RT. The slides were air dried and stored at -20°C in air-tight desiccated chambers.

Prehybridization and hybridization – Day 1

The slides were mounted into slide chambers and treated 6x for 5min with MeOH solution (ROTH) containing 0.6% hydrogen peroxide (Fluka, Germany). The slides were then washed 6x with PBT (PBS with 0.05% Tween-20). Then they were treated 2x for 4min with 300µl of 0.2N HCl with 0.05% Tween and washed 8x with 300µl of PBT. Next Proteinase K buffer (50mM Tris, 5mM EDTA, pH 8.0 with 0.05% Tween-20) was added and incubated for 7min. The Proteinase K concentration depended on the stage of the tissue to be treated with 2µg/ml for embryonic tissue and 20µg/ml for adult brain. The slides were then washed 8x with 300µl of PBT. Next, treatment of the slides 2x 10min with 4% PFA followed and 8 washing steps with 300µl of PBT. The sections were then incubated in prehybridization buffer (Ambion, USA) 2x for 30min at 60°C. Finally they were incubated in hybridization buffer, which contained the probe (150ng/ml), overnight at 62°C.

Post hybridization washes and antibody incubation - Day 2

After hybridization the unspecifically bound probe was removed by washing 5x for 6min with 5x SSC (20x SSC: 3M NaCl, 0,3M NaCitrate, pH 7.0) in 50% Formamide at 65°C. This was followed by additional washing steps with 1x SSC, 0.5x SSC and finally in 0.1x SSC in 50% Formamide. The sections were then washed 3x with 250µl of NTE (0.05% Tween 20, 5mM EDTA, 10mM Tris, 500mM NaCl, pH 7.6) and incubated 2x in 20mM Iodoacetamide in NTE for 5min each at RT. The sections were washed again 3x with 250µl NTE. Next they were incubated 2x in 4% Sheep-serum in TNT buffer (0.05% Tween-20 in 100mM Tris/ 150mM NaCl, pH 7.6) for 30min at RT. Subsequently the slides were washed 8x with 250µl TNT solution. Two incubation steps were followed with 250µl of TNB blocking buffer (0.05% Tween 20 in 100mM Tris/ 150mM NaCl, 0.5% blocking reagent (PerkinElmer Lifesciences) pH 7.6; filter before use, 0.45µm) for 30min at RT. Then anti-Dig-POD (Roche) diluted in TNB blocking buffer (1:500) was added 2x to the slides, which were incubated for 45min each time at RT. 8 washing steps with TNT-buffer for 5min each followed. Then the sections were incubated with Tyramide-Biotin (T/B) in Tyramide buffer (PerkinElmer Lifesciences) 2x for 30min at RT. After 8 washes with 250µl Maleate washing buffer (MWB) (100mM Maleate,

150mM NaCl, 0.05% Tween 20, pH 7.5) the slides were incubated 2x for 30min with Neutravidin-AP-Conjugate (Pierce) (1:600 dilution in 1% blocking reagent (Roche) in MWB).

Post antibody washes and color development

The slides were washed 8x with MWB to remove unbound antibody. Then they were incubated 2x in TNT solution. Next the staining solution was added containing BCIP (1:250), NBT (1:200) (both from Roche) and 0.6µg/ml Levamisole in TMN. The incubation time was 3x 20min but could also be extended depending on signal strength. The slides were then washed 4x with 250µl $H_2O$ with 0.05% Tween and 2x with TNT. The stained sections were fixed for 20min with 4% PFA, 0.5% Glutaraldehyde (Sigma) in PBT. After 4 washes with 250µl PBT (with 0.05% Tween) and subsequently 4 washes with water, the slides were air dried overnight and coverslipped the next day with aqueous coverslipping medium (Hydromatrix).

## 2.4 LacZ/ X-Gal staining

By crossing the Foxb1-Cre mouse line with the ROSA26R mouse line cells expressing the Cre recombinase will also express the enzyme β-galactosidase, which catalyzes the conversion of the colorless substrate X-Gal into a blue product. This way Cre expressing cells can be detected as well as cells that have expressed Cre before, thereby also functioning as a lineage marker.

The embryos were taken out and washed in ice-cold 1x PBS. Next they were fixed depending on their age in FixA (1% Formaldehyd, 0.2% Glutaraldehyd, 0.02% NP-40 in 1x PBS). E9.5 and E10.5 embryos were fixed for 30min, E11.5 embryos for 40min, E12.5 for 50min and E13.5 embryos were fixed for 30min, then cut in the middle and fixed for an additional 30min in FixA. Postnatal brains of mice were fixed for 20min in FixB (1% Formaldehyd, 0.2% Glutaraldehyd, 0.2% NP-40, 0.1% DOC in 1x PBS), then cut in 100µm sections and fixed for an additional 1 hour and 45min. The fixation should always occurred on ice with shaking.

Subsequently the embryos were washed 2x for 20min in 1x PBS at RT. Then the embryos were transferred into the X-Gal staining solution (1mg/ml X-Gal (from Gibco), 4mM

$K_3Fe(CN)_6$, 4mM $K_4Fe(CN)_6$, 2mM $MgCl_2$) and incubated from 3 hours to overnight at RT. The staining was stopped by washing 2x with PBS, which was followed by fixation with 4% PFA in 1x PBS.

## 2.5 AP-staining

By crossing a Cre mouse line with the Z/AP reporter mouse line Cre recombinase expressing cells also produce human placental alkaline phosphatase, which catalyzes the conversion of the substrates NBT and BCIP into a blue product. The advantage of this reporter mouse line is that also the axons can be made visible by this staining.
The embryos were dissected in ice-cold 1x PBS and fixed on ice with 4% PFA for 30min. Then they were rinsed in 1x PBS 3x for 20min at RT. In case of older embryos (E18.5) 100µm sections were cut of the brains and fixed for an additional 30min on ice. The endogenous alkaline phosphatases were inactivated by heat treatment in PBS for 30min at 72°C in a water bath. The embryos were rinsed in PBS and washed in AP buffer (100mM Tris-HCl, pH 9.5, 100mM NaCl, 10mM $MgCl_2$) for 10min. The embryos were then incubated with the substrate (0.5mg/ml NBT, 0.18mg/ml BCIP in AP buffer) for 0.5-36 hours at 4-23°C. Lower staining temperature reduces backround but extends incubation time. After staining, the embryos or sections were washed with PBS.

## 2.6 Nissl staining

The staining was carried out on frozen sections. First they were fixed in 4% PFA for 20min and washed with PBS. Then the slides were placed directly into 1:1 EtOH/chloroform overnight and rehydrated through 100% and 95% EtOH to distilled $H_2O$ the next day. The staining was done in 0.1% cresyl fast violet in acetic buffer (2.721% Na-acetat, 1.2% Hac, pH 3.8-4.0) for 20min. For about 15sec the slides were put into 70% EtOH/ 0.1% $CH_3COOH$ to differentiate since too long incubation times in this solution could destained the sections again. Then the slides were transferred directly to 90% EtOH, followed by 100% EtOH and 2x Xylol for 5min each step. Next the slides could be mounted with a resinous medium.

## 2.7 Explant experiments

Wild type mouse embryos were dissected in ice-cold PBS at E9.5. The forebrains were cut into half along the rostral-ventral midline, placed neuroepithelium-down onto Millipore Millicell membranes and cultured for 40-48 hours in Neurobasal Medium (Gibco) supplemented with glutamine (2mM, Gibco) and B-27 (Gibco) at 37°C.
Explants were cultured with either D4476 (CK1 inhibitor, Rena et al 2005) (50µM in DMSO, Calbiochem), Cyclopamine (15µM in DMSO, Sigma), or LiCl (10mM) (Klein and Melton,1996). Control experiments were performed in parallel, cultured with the same amounts of DMSO or NaCl instead of LiCl. None of the added substances increased cell death in explants as assessed with the LIVE/DEAD Viability/Cytotoxicity kit (Molecular Probes). After culture the explants were fixed in 4% PFA overnight, dehydrated through increasing concentrations of methanol and stored at -20°C until used for *in situ* hybridization.

## 2.8 Electroporation of embryos

E9.5 or E10.5 wt embryos were dissected in ice-cold PBS. With a fine glass capillary the DNA (2µg/µl) was injected into the ventricles of the mouse embryo. The plasmid DNA solution contained the dye Fast Green (0.03%) to be able to see the injection of DNA into the embryo. Immediately afterwards the head of the embryo was placed between tweezer type electrodes (CUY650P5) and with a CUY21 electroporator (Nepagene) 3 pulses of a voltage of 22V were applied, with a puls length of 25msec. By applying a voltage the membranes of the cells become porous so that the negatively charged DNA can enter inside the cells in the direction of the positive electrode. In this way, only the side of the embryo, which is next to the positive electrode will be transfected with the plasmid-DNA, while the other side stays untransfected and therefore can be used as negative control. Usually only the still dividing cells of the neuroepithelium right next to the ventricles are

transfected this way. Subsequently the embryos were cut along the midline, placed with the neuroepithelial side down onto Millipore membranes and cultured as explants for 48 hours. After incubation the explants were analyzed for GFP expression under a fluorescent microscope. All cells that had been electroporated and expressed the gene of interest should have also expressed GFP that was on the same plasmid but separated by an IRES site from the gene of interest so that two separate proteins were produced. Next the explants, which were successfully electroporated were fixed in 4% PFA overnight at 4°C and processed for whole mount *in situ* hybridization.

## 2.9 Molecular biological methods for cloning experiments

### 2.9.1 Small-scale isolation of plasmid DNA

The Qiagen Qiaquick Plasmid kit was used according to the instructions of the manufacturer. 5ml of LB medium with the appropriate amount of antibiotic was inoculated with a single *E. coli* colony and incubated overnight at 37°C with shaking. Using 1-5ml of this culture the cells were pelleted in a microcentrifuge for 30sec at full speed. 250µl A1 was added and the cell pellet resuspended by vigorous vortexing. To lyse the cells 250µl A2 was added, mixed gently by inverting the tube 6-8 times and incubated at RT for a maximum of 5min. The sample was then mixed with 300µl A3 and the tube gently inverted 6-8 times for neutralization. The sample was centrifuged for 10min at full speed at RT. A plasmid column provided in the kit was placed in a 2ml collecting tube and the supernatant loaded on the column. This was centrifuged for 1min at full speed. The flowthrough was discarded and the plasmid column placed back into the 2ml collecting tube. Subsequently the column was washed with 600µl A4 and centrifuged 2x for 1min at full speed. Then the plasmid column was placed in a 1.5ml microcentrifuge tube and 50µl Elution buffer added to the center of the column. This was incubated for at least 5min at RT and centrifuged for 2min at full speed.

### 2.9.2 EndoFree plasmid Maxi protocol

The "QIAGEN Endofree Plasmid Maxi Kit" (QIAGEN, USA) was used according to the instructions of the manufacturer, since it yields endotoxin-free DNA, which improves transfection into sensitive eukaryotic cells.
A single colony was picked from a freshly streaked selective plate and a starter culture of 2-5ml LB medium containing the appropriate selective antibiotic inoculated. This was incubated in a shaker (250rpm) at 37°C over the day. The starter culture was diluted 1/500 to 1/1000 by pipetting 100-200µl of the culture into 100ml selective LB medium. This was incubated at 37°C overnight for 12-16h with rigorous shaking. Next day the bacterial cells were harvested by centrifugation at 6000x g for 15min at 4°C and the bacterial pellet resuspended in 10ml buffer P1. 10ml buffer P2 was added, mixed gently but thoroughly by inverting the tube 4-6 times and incubated at RT for 5min. During this incubation the QIAfilter Cartridge was prepared. Then 10ml chilled buffer P3 was added to the lysate and mixed immediately but gently by inverting 4-6 times. The lysate was poured into the barrel of the QIAfilter Cartridge and incubated for 10min at RT. The cap was removed from the QIAfilter outlet nozzle, the plunger gently inserted into the QIAfilter Cartridge and the cell lysate filtered into a 50ml tube. 2,5ml buffer ER was added to the filtered lysate mixed by inverting the tube approximately 10 times and incubated on ice for 30min. During this time a QIAGEN-tip 50 was equilibrated by applying 10ml buffer QBT and allowing the column to empty by gravity flow. The filtered lysate was applied to the QIAGEN-tip and allowed to enter the resin by gravity flow. The QIAGEN-tip was then washed 2x with 30ml buffer QC. The DNA was eluted with 15ml buffer QN from the column and precipitated by adding 10.5ml (0.7 volumes) RT isopropanol to the eluted DNA. This was mixed and centrifuged immediately at 9500x g for 30min at 4°C. The supernatant was carefully decanted and the DNA pellet washed with 5ml of endotoxin-free 70% ethanol. The pellet was air dried for 5-10min and redissolved in a suitable volume of endotoxin-free TE buffer.

### 2.9.3 Gel electrophoresis

By gel electrophoresis DNA fragments can be separated according to their size, since the negatively charged DNA migrates towards the anode in an electrical field. For a 1%

agarose gel 1g agarose was dissolved in 100ml of 1x TAE buffer (50x: 242g Tris base, 57.1ml glacial acetic acid, 100ml 0.5M EDTA) by boiling in the microwave. Then it was let cool down to about 50°C before adding 3µl ethidium bromide (10mg/ml). The gel was poured into a horizontal gel chamber in which it polymerized at RT. Before loading the samples into the wells of the gel, 10x loading buffer (0.4% bromophenol blue, 0.4% xylene cyanol FF, 50% glycerol, store at 4°C) was added to them and mixed. As a marker usually a 1kb or 100bp ladder from NEB was used. The gel was run at 100V in 1x TAE buffer.

### 2.9.4 Isolation of DNA fragments from agarose gels

For the isolation of DNA fragments from agarose gels the QiaQuick Gel Extraction Kit (Qiagen) was used. With a clean scalpel the DNA fragment was excised from the gel. The weight of the gel slice was determined and for each 100mg agarose gel 300µl QG added to the gel piece. The sample was incubated at 50°C until the gel piece had dissolved completely (5-10min). The column was placed into a 2ml collection tube and the sample was loaded. This was centrifuged for 1min at full speed (13000rpm). The flowthrough was discarded and the column placed back into the collection tube. 500µl QG buffer was added and centrifuged for 1min at full speed (13000rpm). The flowthrough was discarded and 750µl buffer PE added and centrifuged for 1min at full speed (13000rpm). To remove PE completely the sample was centrifuged again after discarding the flowthrough. The column was placed into a clean 1,5ml tube, 50µl Elution buffer EB were added, incubated for 5min at RT and centrifuged for 1min at full speed (13000rpm).

### 2.9.5 Determination of nucleic acid concentrations

The concentration of DNA was determined photometrically by measuring the absorption of the diluted sample at 260nm, 280nm and 320nm. The concentration was calculated according to the following formula:

c (µg/ml) = $OD_{260}$ x f x c

OD = optical density at 260nm

f = dilution factor

c = for double stranded DNA = 50µg/ml; for RNA = 40µg/ml

### 2.9.6 Restriction digestion of DNA

Restriction digestion was performed by incubating double-stranded DNA with a specific restriction enzyme (3-5U/µg DNA, from NEB) in its respective buffer for 3 hours to overnight at the optimal reaction temperature. The general reaction used:

1,0µg DNA

1,0µl 10x reaction buffer

0,5µl enzyme (10U/ul)

Add distilled $H_2O$ to a total volume of 10µl.

### 2.9.7 Ligation of DNA fragments

Normally a vector to insert ratio of 1 to 3 was used for cohesive ends. The amount of insert, which has to be added to 50ng of vector can be calculated with the following formula:

$Xng_{insert} = 50ng_{vector}$ x (size insert (bp)/size vector(bp)) x 3

To a 10µl reaction 1x ligase buffer with 1mM ATP (NEB) and 1U T4 DNA-Ligase was added. The ligation was incubated overnight at RT.

### 2.9.8 Transformation of bacteria

The electro-competent cells were thawed on ice. 2µl of the ligation mixture were added to 45µl of competent cells and mixed gently by pipetting up and down. The cells were then transferred to a cold electroporation cuvette, which was inserted into the Gene-Pulser (Bio-Rad) set to 1.8kV. Immediately after the voltage was applied, 1ml of LB medium was added to the bacteria and mixed by pipetting up and down. The cells were incubated for 1 hour at 37°C in the shaker (250rpm). After incubation 50µl and 200µl of the suspension were spread on agar plates with the appropiate antibiotic. The plates were incubated overnight at 37°C.

### 2.9.9 The In-Fusion™ PCR Cloning Method

The In-Fusion™ PCR Cloning Kit (Clontech) is designed for cloning of PCR products into vectors. The PCR template is amplified with gene-specific primers that have at least 15 bases of homology with sequences flanking the desired site of insertion in the cloning vector. The PCR product is then combined with the vector, which is linearized at the insertion site and the mixture is added to the In-Fusion cloning reaction. This reaction contains the poxvirus DNA polymerase with 3'–5' exonuclease activity, which excises nucleotides from the 3' ends of the molecules, thereby exposing the overlapping sequences. These overlapping ends are now free to anneal so that the resulting product is an assembled vector and insert (Fig.2.1).

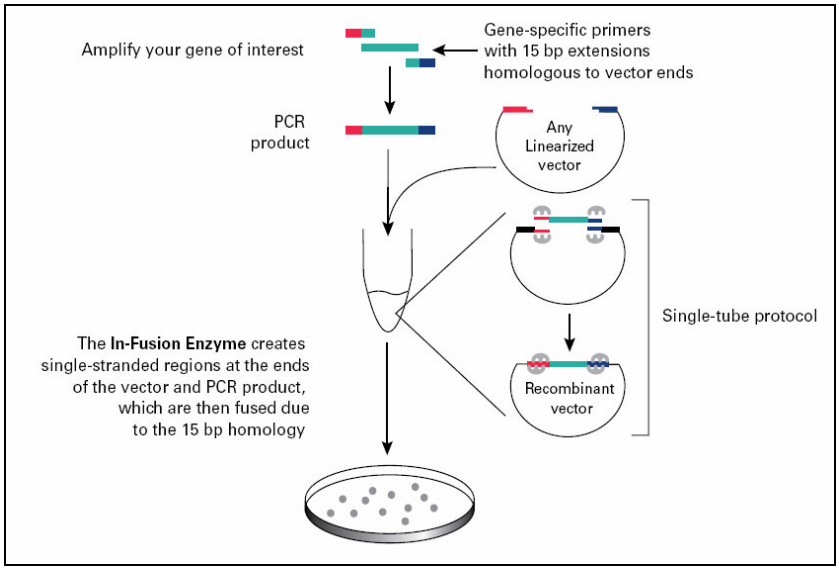


**Figure 2.1 The In-Fusion Method.** The gene of interest is amplified with primers with 15bp homology to the vector insertion site. The In-Fusion enzyme creates single-stranded regions at the ends of the vector and PCR product, which are then fused due to the homology. The assembled vector and insert is then used to transform bacteria (www.clontech.com).

#### 2.9.9.1 Cloning of the exon 2-deleted *Shh* construct

The In-Fusion method was used to clone the exon 2-deleted *Shh* cDNA into the pXL172 vector which contains eGFP that is separated from the multiple cloning site (MCS) by an IRES site and is driven by a pCAGGS promoter. *Shh* consists of 3 exons. To remove exon 2 of *Shh* a 3-step PCR was performed. First, exon 1 and exon 3 were amplified in two separate PCRs with the following primers:

| Exon amplified | Primer name | Primer sequence |
|---|---|---|
| Exon 1 | E1-for | TAC CGG ACT CAG ATC ACA GCT CAC AAG TCC TCA GGT TCC |
| | E1E3_rev | CGG AGT TCT CTG AGT CAT CAG CCG GTC TGC TCC |
| Exon 3 | E1E3_for | ATG ACT CAG AGA ACT CCG TGG CGG CCA AAT CCG |
| | E3_rev | CCG CGG TAC CGT CGA TCC CGT CGG GCT TCA GCT GGA CTT |

The forward primer of exon 1 and the reverse primer of exon 3 contained 15 bases of homology (bases in red) to the insertion site of the vector, which was cut with BglII and SalI (underlined bases are the restriction enzyme recognition sites in the vector after digestion). The reverse primer of exon 1 contained 9 bases of homology (in blue) to the beginning of exon 3 and the forward primer of exon 3 contained 9 bases homology (in blue) to the end of exon 1. The Pwo SuperYield DNA Polymerase Kit (Roche) for GC-rich sequences was used for the PCR reactions. As a template *Shh* cDNA in the pCMV-SPORT6.1 vector purchased from imaGenes (Berlin) was added.

xµl vector DNA (100ng)
5,0µl 10x buffer
1,0µl dNTP mix
1,5µl forward primer (10µM)
1,5µl reverse primer (10µM)
10µl 5x GC-rich solution
0,5µl polymerase (5U/µl)

Add up to 50µl with $dH_2O$.

PCR program:
94ºC, 4min+ 30 x (94ºC, 20sec + 62ºC, 30sec + 72ºC, 1min) + 72ºC, 5min

The resulting PCR products (exon 1, 300bp and exon 3, 750bp) were added as templates for the third PCR with the forward primer of exon 1 (E1_for) and the reverse primer of exon 3 (E3_rev). Since the two templates had overlapping sequences at the ends, they hybridized generating a PCR product of a fused exon 1/ exon 3 fragment (1050bp). This PCR product together with the linearized vector was added to the In-Fusion reaction using a 2:1 molar ratio of insert:vector. The reaction was incubated for 15min at 37°C followed by 15min at 50°C and transferred to ice. The sample was diluted 1:5 with TE buffer and subsequently 2,5µl of the diluted reaction mixture were added to the electro-competent cells for transformation.

# 3 Results

The results of this work have been organized into two manuscripts for their publication. The text and figures of the two articles can be found in the following pages.

## 3.1 First Manuscript

### Neuroepithelial *Sonic hedgehog* is essential to specify the hypothalamic subregions and to stabilize diencephalic against telencephalic fate

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### 3.1.1 Introduction

The hypothalamus is a ventral forebrain region regulating homeostasis and survival. Alterations of hypothalamic development can result in endocrine and metabolic disease (Caqueret et al., 2005; Michaud, 2001). Although hypothalamic development is under intense research it is not yet well understood. The developing hypothalamus is located in the rostral diencephalon, a region with particularly complex patterning, but whose subdivisions are shared by all vertebrates (Puelles and Rubenstein, 2003; Wullimann and Mueller, 2004). Hypothalamic regionalization is starting to be unveiled in the zebrafish (Mathieu et al., 2002; Rohr et al., 2001; Staudt and Houart, 2007), where the hypothalamic neuroepithelium can be divided into anterior-dorsal (AD) and posterior-ventral (PV) regions on the basis of expression patterns (Mathieu et al., 2002) which are conserved in the mouse (see Results). The adult hypothalamus consists of medial and lateral zones. The medial hypothalamus is a series of neuronal aggregates arranged rostro-caudally into four regions expressing specific markers. The AD generates the three most rostral regions, and the PV generates the most caudal or mammillary region. The adult derivatives of AD and PV form two independent units from the points of view of connectivity and function, marker gene expression (Puelles and Rubenstein, 2003) and patterning (Mathieu et al., 2002; Staudt and Houart, 2007).

The lateral hypothalamus is a continuum of neurons not forming identifiable aggregates and with enormous functional complexity, controlling ingestive, aggressive and reproductive behaviors (Simerly, 2004). Very little is known about lateral hypothalamus specification and it is not entirely clear from which neuroepithelial areas it derives. Specific lateral hypothalamus subpopulations expressing melanin-concentrating hormone (*Pmch*) and Hypocretin (*Hcrt*) are essential for the coordination of sleep-wake cycles and feeding behaviour (Burdakov et al., 2005).

Sonic hedgehog (Shh) is a signaling protein carrying out several essential functions in the development of the central nervous system (CNS) (Fuccillo et al., 2006). Ventral patterning of the vertebrate CNS is controlled by Shh secreted by non-neural tissues (non-neural Shh) ((Ericson et al., 1997; Gunhaga et al., 2000; Ingham and McMahon, 2001)). *Shh* is also expressed by the ventral midline of the CNS (neural Shh), forming a

continuous ventral CNS domain particularly complex and dynamic in the diencephalon (Shimamura and Rubenstein, 1997). A current question regards the functions of Shh secreted by different neural and non-neural sources (Dessaud et al., 2008). In the caudal diencephalon, neural Shh has well known patterning roles (Guinazu et al., 2007; Kiecker and Lumsden, 2004; Scholpp et al., 2006; Vieira et al., 2005). In the rostral diencephalon (hypothalamus), Shh (of any source) is essential for partition of the eye field and hypothalamic specification (Chiang et al., 1996; Mathieu et al., 2002). In the rostral diencephalon, neural *Shh* controls expansion of unspecified progenitors (in chicken; (Manning et al., 2006)). Its complex and dynamic expression domains suggest additional roles in patterning, which remain elusive.

Here we show that neural *Shh* is essential for the progressivity of fate acquisition through activation of differential regulatory pathways that specify sub-regions of the hypothalamus (AD, PV and LH). Neural Shh is also necessary to stabilize regional fate in the posterior-ventral hypothalamus and prethalamus. Additionally, neural Shh maintains expression of the hypothalamic survival factor *Foxb1* through an interplay with *Wnt8b*.

### 3.1.2 Methods

**Mutant mouse lines**

Animals were treated in ways that minimize suffering and under authorization Az 32.22/Vo from the "Ordnungsamt der Stadt Göttingen", according to the German Law of Animal Protection.

*Foxb1-Cre line.* This line expresses Cre recombinase in the mouse diencephalon (Zhao et al., 2007) and it is a knock-in-knock-out generating *Foxb1* heterozygous animals. These heterozygotes do not show haploinsufficiency and can be considered identical to wild type (Alvarez-Bolado et al., 2000b; Dou et al., 1997; Labosky et al., 1997).

*Foxb1-Cre / ROSA26R lineage reporter line*. In heterozygous *Foxb1-Cre* embryos carrying ROSA26 (Soriano, 1999) or Z/AP (Lobe et al., 1999) reporter alleles, all cells that express or have expressed *Foxb1*, and any cells derived from them, permanently produce β-galactosidase (ROSA26R) or human placental alkaline phosphatase (hPLAP; Z/AP), labeling the *Foxb1* lineage (Zhao et al., 2007).

*Conditional allele of Sonic hedgehog*. In this conditional allele, exon 2 of *Shh* is flanked by loxP sites (Dassule et al., 2000). Exon 2 encodes approximately half of the active N-terminal Shh signal, essential for Shh function (reviewed in (Mann and Beachy, 2004)).

*Shh-c mutant (Foxb1-Cre / Shh-fl conditional mutant).* Upon crossing *Foxb1-Cre* and *Shh-fl* mice, *Shh* conditional mutant mice are produced. We analyze here only mice heterozygous for *Foxb1-Cre* and homozygous for *Shh-floxed* (*Foxb1-Cre +/- Shh-fl -/-*), which we term *Shh-c* mutants. No double homozygotes were used for the analysis.

In *Shh-c* mutants, exon 2 of the *Shh* locus has been deleted in the entire *Foxb1* lineage, including the caudal diencephalon, the posterior-ventral hypothalamus and the diencephalic ventral midline (Zhao et al., 2007). In *Shh-c* mutants, whenever transcription from the *Shh* locus occurs (in cells of the *Foxb1* lineage), the recombined *Shh* locus produces a truncated, non-functional mRNA lacking exon 2. An exon 2-probe exclusively detects the functional *Shh* mRNA (full-length).

*Shh full mutants.* To generate full Shh mutants (see below, Fig.3. 1 D, E), we crossed the *Shh*-floxed (Dassule et al., 2000) with a transgenic mouse line carrying Cre under a ubiquitous promoter expressed in embryonic stem cells (Schwenk et al., 1995), in this way producing full mutant homozygous embryos (*CMV-Cre / Shh-floxed*).

*Gli3 mutants*. We used extra-toes J mouse mutants ($Xt^J/Xt^J$), which carry a deletion affecting only the *Gli3* gene (Maynard et al., 2002). $Xt^J$ mutant mice were kept as heterozygous animals in a mixed C57Bl6/C3H background and were interbred.

**Transfection constructs and electroporation**

In order to test the lack of function of Shh without exon 2, we prepared constructs expressing exon 2-deleted Shh-IRES-EGFP under the control of CAGGS promoter. We obtained a plasmid containing the full length *Shh* cDNA from imaGenes (Berlin, Germany), clone IRAVp968F10144D, and we cloned this cDNA into pXL172 (CAGGS promoter--multicloning site--IRES-EGFP). Next we used PCR to synthesize an exon 2-deleted *Shh* cDNA. In a first PCR step we used *Shh* cDNA as template to amplify exon 1 and exon 3 in separate reactions. In each reaction we used "bridge" primers so that the 3' primer for exon 1 contained 24 nucleotides of the 5' end of exon 3, and viceversa. We used the product of this amplification as template in a second PCR step, which produced a final continuous exon 1-exon 3 fragment (i.e. exon 2-deleted), which was also cloned into pXL172. The constructs were confirmed by sequencing.

E10.5 mouse brains were harvested, injected with 2µg/µL DNA, then electroporated with a CUY-21 Nepagene electroporator and the "tweezer electrodes" (22 V, 50 ms Pon, 950 ms Poff, 3 pulses). The brains were then cut open and cultured for 48 hours (see below "Explants"), then GFP expression was analyzed and photographed, and the explants were fixed and treated for whole mount in situ hybridization.

**In situ hybridization (ISH) and reporter detection**

Templates were PCR-amplified (primer sequence available upon request) from cDNA (from total RNA from newborn mouse brain plus E10.5-E11.5 embryos). Probes were synthesized using the Roche RNA transcription kit (DIG RNA Labelling Mix). ISH on whole-mount or cryostat sections has often been described. Stainings for alkaline phosphatase and for β-galactosidase activity were carried out as described (Koenen et al., 1982; Lobe et al., 1999).

**Explants**

Embryonic day (E)9.5 wild type mouse brains were cut open along the ventral midline, flattened (neuroepithelial side down) on Millicell-CM membranes (Millipore, Eschborn, Germany) and cultured under standard conditions in Neurobasal-glutamine (2mM)-B-27 (Gibco Invitrogen, Karlsruhe, Germany). Experimental reagents added: Wnt pathway-inhibitor D4476 (Bryja et al., 2007; Rena et al., 2004) (50µM in DMSO, Calbiochem, Darmstadt, Germany); Shh pathway-inhibitor cyclopamine (15µM in DMSO, Sigma-Aldrich); Wnt pathway-activator LiCl (Klein and Melton, 1996) (10mM, Sigma-Aldrich). Control experiments were performed in parallel with equal DMSO (NaCl to control for LiCl) concentrations. None of the reagents increased cell death ("Live/Dead" Viability/Cytotoxicity kit; Molecular Probes Invitrogen, Göttingen, Germany) (not shown). After 48 hours, explants were fixed (4% paraformaldehyde) and processed for ISH.

## 3.1.3 Results

### 3.1.3.1 Foxb1-driven Cre abolishes Shh full length expression in the anterior ventral neural plate and forebrain

We crossed *Foxb1-Cre* heterozygous mice with *Shh-floxed* (Dassule et al., 2000) mice in order to obtain *Foxb1-Cre* +/- */Shh-floxed* -/- animals, which we termed *Shh*-conditional (*Shh-c*) mutants. We did not use double homozygous animals for the analysis because a *Foxb1* -/- phenotype could alter the *Shh* -/- phenotype. *Foxb1* +/- are normal (see Methods). *Shh-c* mutants die around the time of birth. The patterning processes analyzed here take place much earlier.

The floxed *Shh* mouse line that we use has been crossed with other Cre lines to analyze the role of *Shh* in the development of other organs (Dassule et al., 2000; Komada et al., 2008; Machold and Fishell, 2002). The floxed *Shh* loses exon 2 upon Cre-mediated recombination (Dassule et al., 2000). Exon 2 encodes the amino-terminal which is essential for Shh function (Fan et al., 1995; Hynes et al., 1995; Lai et al., 1995; Lopez-Martinez et al., 1995; Miyashita-Lin et al., 1999; Roelink et al., 1995). However, we wanted to ascertain that, in our system (forebrain neuroepithelium), this was also the case. E10.5 explants transfected with a construct expressing full length Shh expressed ectopic *Ptch1* (diagnostic of *Shh* pathway activation, reviewed in (Dassule et al., 2000)), while transfection of Shh constructs in which exon 2 had been deleted did not have any effect (Fig.3.1 A-E).

In order to analyze our mutants, we needed to know if and when the mutant neural plate was able to express *Shh*. At E8.0, *Foxb1* was expressed in the ventral midline of the wild type anterior neural plate (Fig.3.1 F) (Zhao et al., 2007), while *Shh* was expressed in the prechordal mesendoderm but not yet in the neural plate of wild type or mutant (Fig.3.1 G, H). *Foxb1* was expressed in the neural plate of a *Shh* -/- embryo (full *Shh* knockout mutant), indicating that Shh is not required for initiation of *Foxb1* expression (Fig.3.1 I, J). At E8.5, both *Foxb1* and *Shh* were expressed in the wild type ventral neural plate (Fig.3.1 K, L). In the mutant, the *Shh* locus was active as revealed by ISH with a "long probe" detecting any *Shh* transcripts (i.e. wild type *Shh* mRNA as well as truncated *Shh* mRNA, the product of a Cre-recombined *Shh* locus) (Fig.3.1 M), but it produced

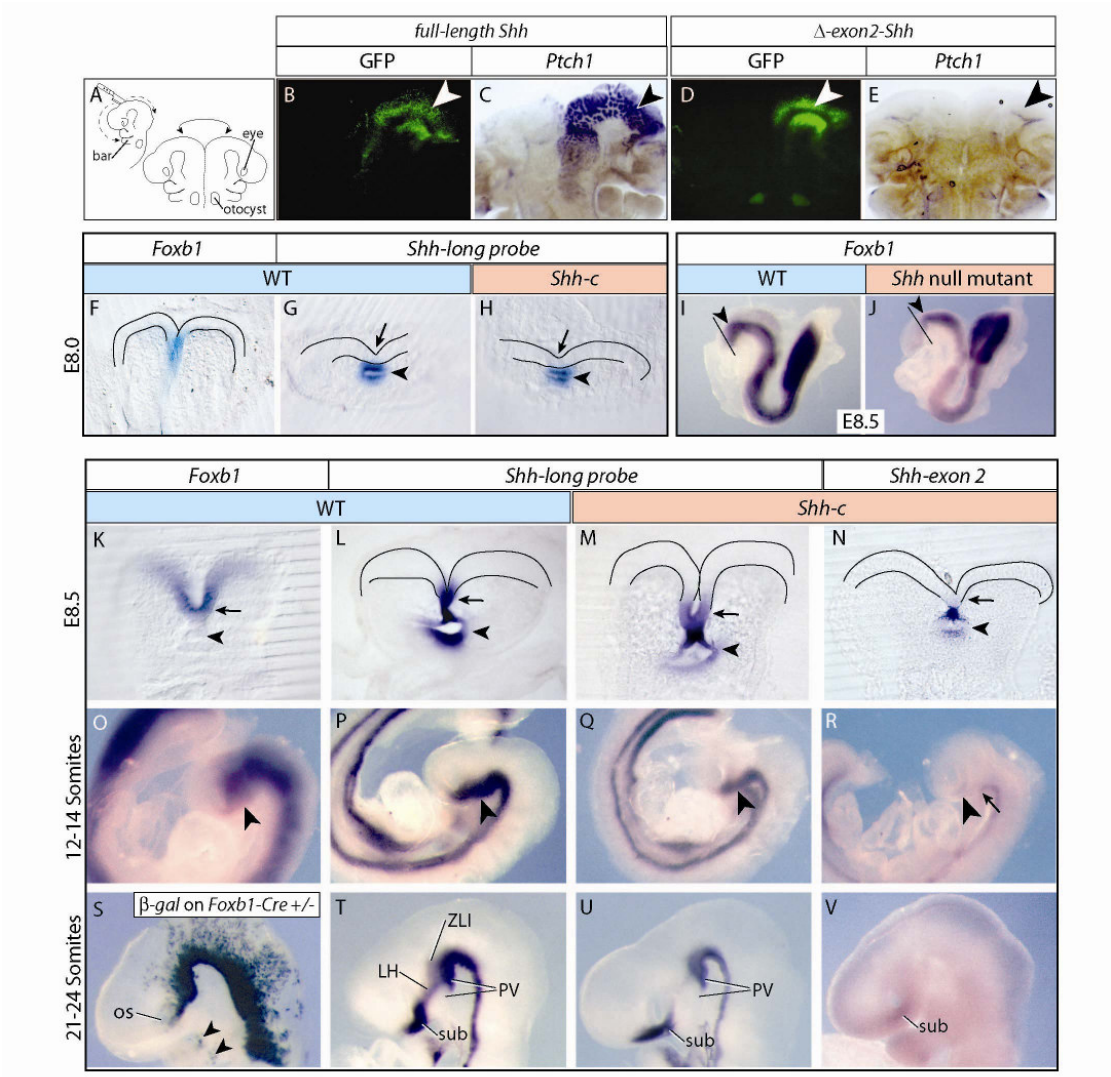


**Figure 3.1 *Foxb1*-driven Cre abolishes *Shh* expression in the anterior ventral neural plate and forebrain.** A-E) Explant cultures according to diagram (A) of E10.5 wild type mouse brains (B-E) electroporated with full-length *Shh* construct (B, C) or with exon 2-deleted *Shh* construct (D, E). GFP expression (B, D) shows the transfected areas, also indicated by arrowheads. Full-length *Shh* induced ectopic expression of *Ptch1* (C) but exon 2-deleted *Shh* did not (E). F-H) ISH for *Foxb1* (F) and *Shh*-long probe (G, H) on E8.0 mouse anterior neural plate in wild type (F, G) and *Shh-c* (H). The ventral neural plate shows *Foxb1* (F), but not yet *Shh* in wild type (G) or mutant (H). I, J) *Foxb1* is expressed in the early neural plate in wild type (I) and *Shh* -/- (full mutant) (J). Arrowhead indicates diencephalon, a line marks the di-telencephalic boundary. K-N) At E8.5, *Foxb1* and *Shh* are coexpressed in the wild type ventral anterior neural plate (arrow in K, L). *Shh* is expressed in the foregut also (arrowhead in K, L). In the mutant, *Shh* expression is activated in the ventral rostral neural plate (arrow in M) and foregut (arrowhead in M) but the transcript lacks exon 2 specifically in the neural plate (arrow in N) not foregut (arrowhead in N). O-R) At 12-14 somites, *Foxb1* (O) and *Shh* (P) expression overlap in the wild type ventral forebrain (arrowhead in O, P). In the *Shh-c* mutant, *Shh* expression was activated in a comparable domain (arrowhead in Q), but the transcript lacked exon 2 (arrowhead in R). Full-lenght *Shh* transcripts were found, very reduced, at tegmental levels (arrow in R). S-V) β-galactosidase detection of *Foxb1* lineage in *Foxb1-Cre / ROSA26R* mice at 21-24 somites (S) overlapped with *Shh* expression (T) (arrowheads in S show *Foxb1*-lineage cells in the branchial arches). *Shh* expression (T) showed several domains: suboptical (sub), lateral hypothalamus (LH), incipient ZLI and PV. In the mutant, *Shh* transcriptional activation was missing in the LH and ZLI domain (U), and functional *Shh* transcripts were produced only in the sub, in very reduced amounts (V). Abbreviations: LH: Lateral hypothalamus; os: optic stalk; PV: Posterior-ventral hypothalamus; sub: suboptical; ZLI: Zona limitans interthalamica.

non-functional *Shh* mRNA in the neural plate, as shown by an exon 2 probe, which detects only the product of the wild type *Shh* locus (non-Cre-recombined) (see Methods) (Fig.3.1 N). At the 12-14 somite stage, *Foxb1* and *Shh* were expressed in wild type ventral diencephalon with a rostral limit at the optic sulcus (Fig.3.1 O, P) (Shimamura and Rubenstein, 1997); (Zhao et al., 2007). In the mutant, the *Shh* locus was active in appropriate domains (Fig.3.1 Q) but full length, functional *Shh* transcripts were limited to the ventral caudal diencephalon (arrow in Fig.3.1 R), not rostral diencephalon (hypothalamus; arrowhead in Fig.3.1 R).

These results indicated complete elimination of functional *Shh* mRNA in the *Shh-c* hypothalamus from E8.5 on.

#### 3.1.3.2 Activation of Shh expression in the lateral hypothalamus depends on neural Shh

Since diencephalic *Foxb1* expression is mostly transient (Zhao et al., 2007) we used a lineage reporter mouse line to identify the regions where a phenotype can be expected. In these embryos, cells that have expressed *Foxb1*, as well as cells derived from them, express *β-galactosidase* permanently, allowing for the identification of the regions where *Foxb1*-driven Cre recombination has taken place (see Methods).

At 21-24 somites, *Foxb1*-lineage cells were found in the ventral part of rostral and caudal diencephalon, in the diencephalic ventral midline up to eye levels (Fig.3.1 S) and in the branchial arches (arrowheads in Fig.3.1 S). *Shh* at this age had disappeared from the ventral side (compare Fig.3.1 P and T) and was expressed in four distinct domains: suboptical, a longitudinal band termed basal forebrain cell column thought to give rise to the lateral hypothalamus (LH) (Altman and Bayer, 1986; Manning et al., 2006; Marchand et al., 1986), a small domain in the PV, and the incipient zona limitans interthalamica (ZLI) in the caudal diencephalon (Fig.3.1 T) (Shimamura and Rubenstein, 1997). In *Shh-c* mutants, *Shh* transcriptional activation was abolished in the lateral hypothalamus and ZLI domains, but maintained in the PV and suboptical (Fig.3.1 U). Functional *Shh* was expressed only in the suboptical domain, with reduced intensity (Fig.3.1 V). In the ZLI domain, maintenance of activity in the *Shh* locus requires Shh (feedback loop), as previously proposed (Kiecker and Lumsden, 2004; Zeltser, 2005), which could explain the lack of a ZLI in this mutant, and possibly also the lack of an LH domain. In contrast,

the suboptical domain and the PV domain can activate *Shh* expression independently of Shh of neural origin.

### 3.1.3.3 Early abolition of the Shh pathway in the Shh-c forebrain

Non-neural sources of Shh could still activate the *Shh* pathway in the mutant brain. Therefore we characterized the state of the pathway in *Shh-c* embryos by detecting expression of Shh receptor *Ptch1* and transcription factor gene *Gli1*, diagnostic markers of *Shh* pathway activity (reviewed in (Dassule et al., 2000)). At E8.5, the pathway was active in the mutant ventral midline (Fig.3.2 A-D), probably due to a response to non-neural Shh from the prechordal plate.

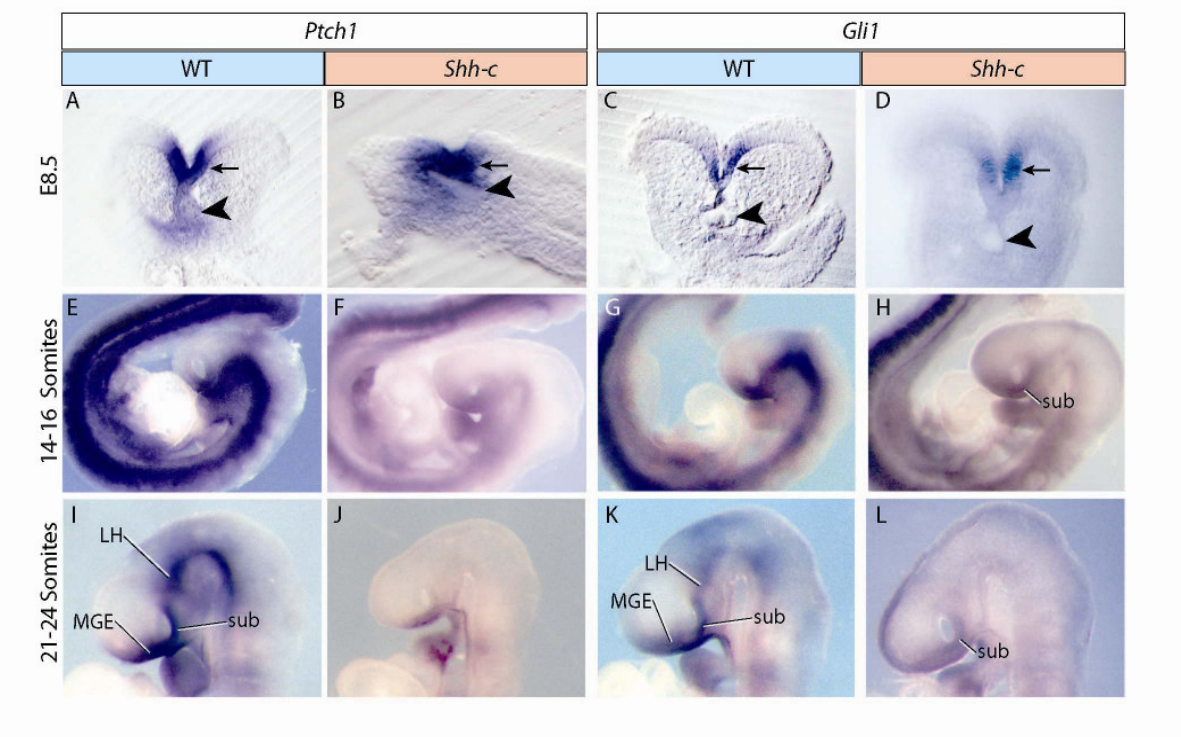


**Figure 3.2 The Shh pathway is gradually abolished in the early *Shh-c* forebrain.** *In situ* hybridization for Ptch1 and Gli1 on wild type and *Shh-c* mutant embryos. A-D) At E8.5, Ptch1 and Gli1 were expressed in the ventral rostral neural plate (arrow) in wild type (A, C) and mutant (B, D). Arrowheads indicate the foregut. E-H) At 14-16 somites, both Ptch1 and Gli1 were expressed in the entire ventral neural tube (E, G) but have disappeared from the mutant rostral neural tube (F, H) except in the sub. I-L) At 21-24 somites, the domain of expression of Ptch1 and Gli1 in wild type (I, K) showed domains similar to Shh: LH and sub, and an incipient telencephalic domain, MGE (compare with Fig.3.1 T), while in the mutant both markers were still absent from the forebrain (J, L) except for low intensity-expression of Gli1 in the sub domain (L). Abbreviations: LH: Lateral hypothalamus; sub: suboptical; MGE: Medial ganglionic eminence.

At 14 somites *Gli1* and *Ptch1* expression in the mutant disappeared from the ventral diencephalon (Fig.3.2 E-H) but was retained in the ventral telencephalon further demonstrating the specific ablation of *Shh* in the diencephalon (Fig.3.2 H).
The loss was maintained at 24 somites (Fig.3.2 I-L). These results show a shift in the dependence of the wild type neural plate from non-neural to neural sources of Shh, and indicate that in the *Shh-c* neural plate the Shh pathway is rendered inactive very early at diencephalic levels.

#### 3.1.3.4 Before and after ZLI and LH domain formation: early alteration of the hypothalamus and prethalamus in the Shh-c embryo

In order to explore the regionalization of the hypothalamus in the absence of neural Shh, we used marker genes *Nkx2-1* and *Dlx2* (Puelles et al., 2004), encoding transcription factor genes expressed in the presumptive hypothalamus and with important developmental roles (Kimura et al., 1996; Petryniak et al., 2007). The most prominent domains of expression of *Shh* in the diencephalon are the ZLI (caudal diencephalon) and the LH or lateral hypothalamus band (rostral diencephalon), which appear around E9.5-E10.0 and are absent in our mutant (Fig.3.1 T, U). Therefore, we labeled marker expression in wild type and mutant embryos at E9.5 and E11.5, i.e. at the onset of formation and after the formation of the ZLI and LH (Fig.3.3). *Nkx2-1* (*Titf1*) was expressed but its domain severely reduced in the mutant at E9.5 (Fig.3.3 A, B). The domain reduction was more intense at E11.5, and the PV was at this age abnormally devoid of *Nkx2-1* expression (Fig.3.3 C, D).
The expression domain of *Dlx2* includes a large domain in the hypothalamus as well as the prethalamus (Puelles et al., 2004). *Dlx2* is not expressed in the diencephalon at E9.5 (Fig.3.3 E, F). Its hypothalamic component was not yet present at E11.5, although an incipient prethalamic domain was present (Fig.3.3 G). This domain is known to depend on Shh from the ZLI (Kiecker and Lumsden, 2004) and was therefore absent in the mutant (Fig.3.3 H).
Intrigued by the defects in PV and prethalamus, we detected a specific marker for these regions, *Lhx5* (Fujii et al., 1994; Staudt and Houart, 2007). At E9.5 in wild type, *Lhx5* was expressed in an area encompassing PV and the presumptive prethalamus and had a sharp boundary at the caudal regions (arrow in Fig.3.3 I). In the mutant, the *Lhx5* domain was

similar but its caudal boundary lacked definition, constituting an early sign of alteration (arrow in Fig.3.3 J).

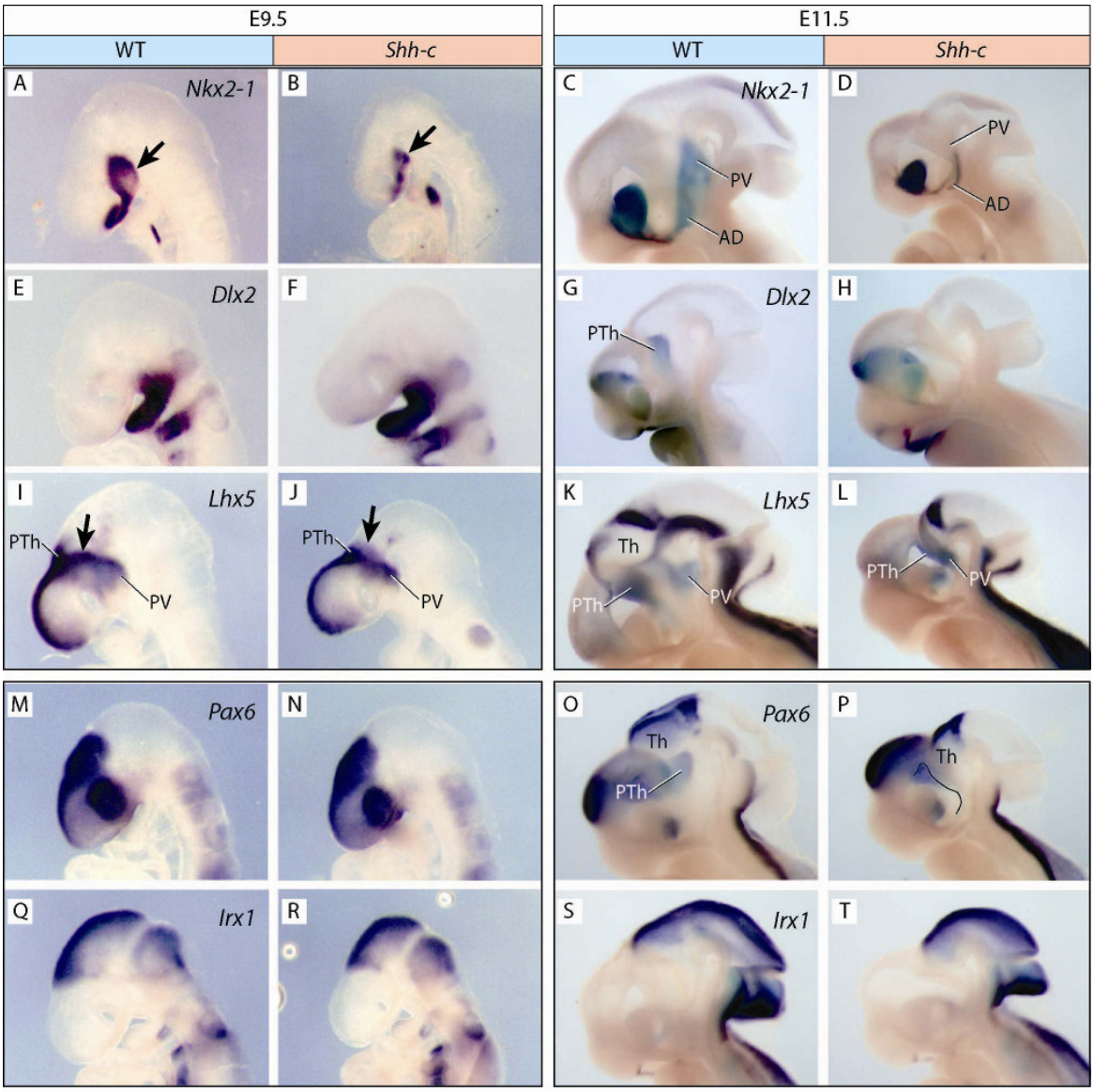


**Figure 3.3 Early and progressive hypothalamic and prethalamic alterations in the *Shh-c* embryo.** Whole mount ISH for the indicated markers on E9.5 (left columns) and E11.5 (right columns) wild type and *Shh-c* embryos. A-D) At E9.5 (A, B) the *Shh-c* shows reduced *Nkx2-1* expression in the hypothalamic domain (arrows). At E11.5 (C, D), expression is extremely reduced and abnormally absent in the PV domain (D). E-H) At E9.5 (E, F), no *Dlx2* expression in diencephalon. At E11.5 (G, H) the prethalamic domain present in the wild type (G) is absent in the mutant (H). I-L) At E9.5 *Lhx5* expression encompasses PV and Pth (I, J), but the caudal boundary is not sharply delimited in the mutant (arrows). At E11.5 both domains have separated in wild type (K) but not mutant brains (L). M-P) At E9.5 (M, N) *Pax6* expression shows no difference between wild type and mutant. At E11.5 the wild type (O) shows a PTh domain absent in the mutant (P) (interventricular border outlined in P for clarity). Q-T) The *Irx1* expression domain abuts the ZLI at E9.5 (Q, R) and starts to recede caudally at E11.5 (S, T) without major difference between wild type and mutant. Abbreviations: AD: Anterior-dorsal hypothalamus; Pth: Prethalamus; PV: Posterior-ventral hypothalamus; Th: Thalamus.

At E11.5, *Lhx5* labeled one prethalamic and one PV domain in the wild type (Fig.3.3 K) but, as a matter of fact, both domains appeared fused and severely reduced in size in the mutant (Fig.3.3 L).

To confirm this prethalamic defect we detected expression of a specific prethalamic marker, *Pax6* (Stoykova et al., 1996). At E9.5, expression of *Pax6* in a wide dorsal diencephalic area was the same for both, mutant and wild type mice (Fig.3.3 M, N). At E11.5, however, the well-defined prethalamic *Pax6* domain (Fig.3.3 O) was absent in the mutant (Fig.3.3 P). Finally, *Irx1* is expressed in a diencephalic domain caudally abutting the ZLI (Bosse et al., 1997), and appeared essentially unchanged at E9.5 and E11.5 in the mutant (Fig.3.3 Q-T).

In summary, while at E9.5 only slight alterations of marker expression were observed in our mutant, after the stage of ZLI and LH band formation, PV and prethalamus patterning alteration were evident.

#### 3.1.3.5 Division of the hypothalamic neuroepithelium into AD and PV

At E12.5, the major brain regions can be recognized by expression of specific markers (Shimamura and Rubenstein, 1997). This is also an optimal stage to compare brains across vertebrate species (Mueller et al., 2006), which is interesting, since much progress in hypothalamic regionalization has been made in species other than mouse.

At this age, thalamus, PV and scattered cells in prethalamus and hypothalamic floor plate were of *Foxb1*-lineage (Fig.3.4 A), indicating that those regions would not be able to express functional *Shh* in the mutant. In the wild type embryo at this age, a new *Shh* domain was present (medial ganglionic eminence, MGE) and the domains detected earlier were fully developed (Fig.3.4 B). Similar to what happens in zebrafish (Mathieu et al., 2002), the hypothalamic neuroepithelium is subdivided by *Shh* and *Emx2* expression into anterior-dorsal (AD) and posterior-ventral (PV) domains. The AD expresses *Shh* (Fig.3.4 B). The PV showed *Emx2*-expressing (Fig.3.4 B inset) and *Shh*-expressing subdomains. In the *Shh-c* brain, the lateral hypothalamus domain and the ZLI domain were absent (Fig.3.4 C), and most of the remnant *Shh* transcriptional activity produced inactive *Shh* transcripts, except for the MGE (Fig.3.4 D).

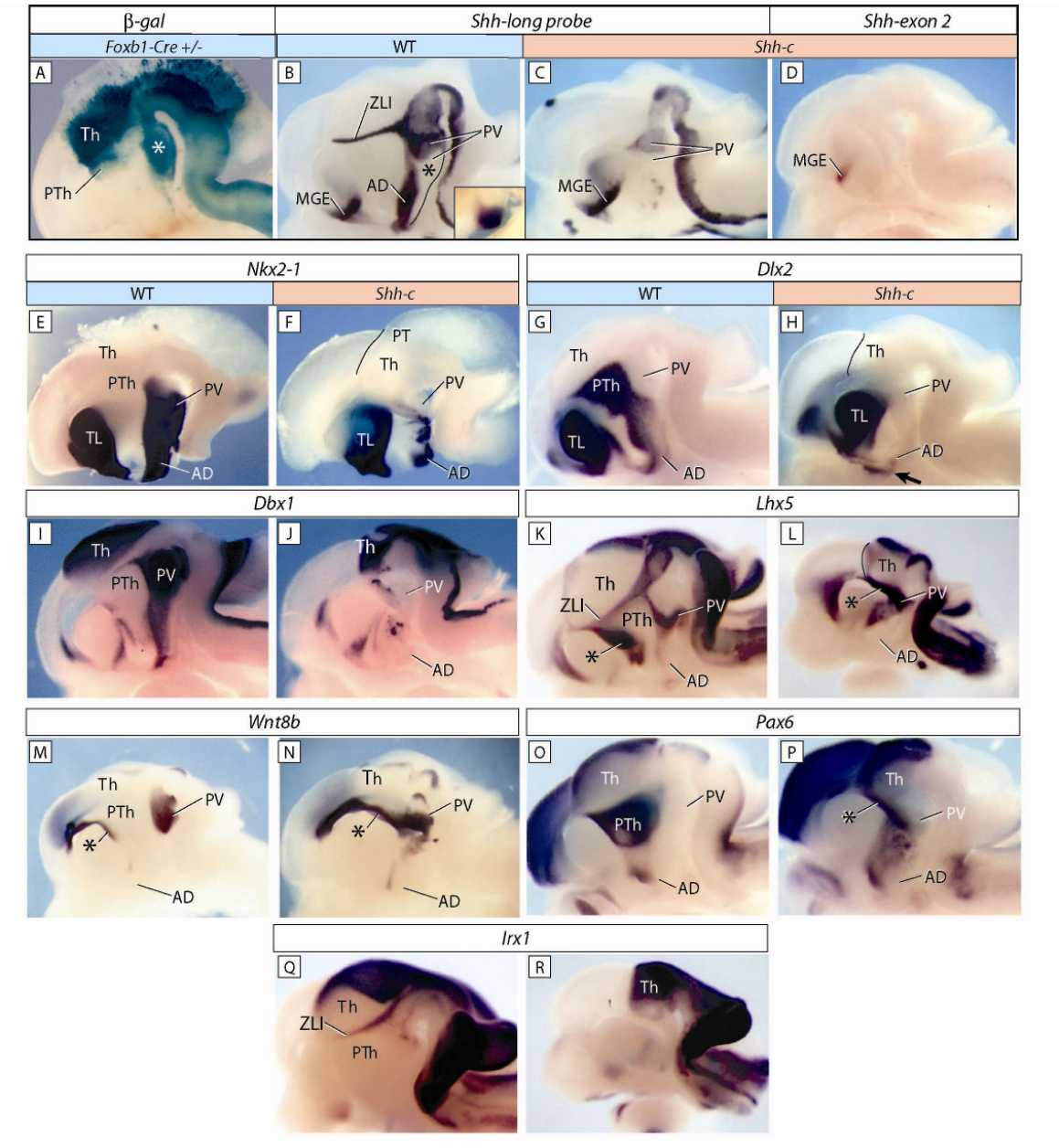


**Figure 3.4 Loss of prethalamus and reduction of hypothalamus in the *Shh-c* embryo at E12.5.**
A-D) At E12.5 the *Foxb1* lineage covers the thalamus and most of the prethalamus (Th and PTh in A), as well as the PV (asterisk in A, B). *Shh* expression (B) showed a new telencephalic domain (MGE). The PV showed two subdomains, one with *Shh* expression and one with no *Shh* expression (asterisk in B) but *Emx2* expression (inset in B). In the mutant (C) only the PV and MGE *Shh* domains remained, but only the MGE produced functional *Shh* transcripts (D). E-R) Whole-mount ISH detection of markers on hemisected brains of E12.5 wild type (E, G, I, K, M, O, Q) and *Shh-c* (F, H, J, L, N, P, R) mice. E, F) The hypothalamic domain of *Nkx2-1* (E) was very reduced in the mutant and absent from the PV (F). G, H) The PTh and hypothalamic domains of *Dlx2* (G) were absent in the mutant (H) (the arrow indicates a remnant). Upper border of Th outlined for clarity. I, J) *Dbx1* expression shows hypothalamic and thalamic domains (I). The hypothalamic domain was absent in the mutant (J). K, L) The PTh and PV domains of *Lhx5* (K) are reduced and did not separate in the mutant (L). Asterisks indicate the rostral domain of *Lhx5* in the PTh.
M, N) *Wnt8b* expression in the PV (M) was preserved in the mutant, but the PTh domain (asterisks) and the PV domain did not separate (N). O, P) The prethalamic domain of *Pax6* (O) was lost in the mutant (P) except for a narrow domain (asterisk). Q, R) The *Irx1* ZLI domain (Q) is absent in the mutant (R). Abbreviations: AD: Anterior-dorsal hypothalamus; MGE: Medial ganglionic eminence; Pth: Prethalamus; PV: Posterior-ventral hypothalamus; Th: Thalamus; TL: Telencephalon; ZLI: Zona limitans interthalamica.

#### 3.1.3.6 Hypothalamic specification is altered in the Shh-c embryo

In the mutant, the hypothalamic domain of *Nkx2-1* was extremely reduced and showed abnormal patches or islands of expression (Fig.3.4 E, F). *Dlx2* expression was abolished in the mutant hypothalamic domain (Fig.3.4 G, H) (it failed to appear also in the prethalamus as expected (Kiecker and Lumsden, 2004)). Transcription factor gene *Dbx1* has key roles in neuronal differentiation (Pierani et al., 2001) and labels thalamic and hypothalamic domains (Lu et al., 1992; Shoji et al., 1996) (Fig.3.4 I). The hypothalamic domain was lost in the mutant, but the thalamic domain was present (Fig.3.4 J). These results indicated a profound and specific alteration of hypothalamic specification in the *Shh-c* mutant.

#### 3.1.3.7 PV and prethalamus form one abnormal domain in the Shh-c embryo

*Lhx5* expression showed (Fig.3.4 K, L) disappearance of the ZLI domain and reduction and fusion of the PV and prethalamic domains in the mutant, confirming the prethalamic alteration observed at earlier ages (Fig.3.3 K, L). *Wnt8b* expression would be ideal to confirm this observation, since it labels PV in all vertebrates (Cui et al., 1995; Garda et al., 2002; Kelly et al., 1995; Lako et al., 1998; Staudt and Houart, 2007) and shows also a small prethalamic domain (asterisk in M). In the mutant, the prethalamic and PV domains were combined and very reduced in size (Fig.3.4 N) similar to what we observed with *Lhx5*. *Pax6* (Fig.3.4 O, P) confirmed the reduction of the prethalamus to a thin rostral domain (asterisk in P). *Irx1* showed loss of the ZLI domain (Fig.3.4 Q, R). Finally, expression of the tegmental marker *Foxa2* was not altered in the mutant (not shown).

#### 3.1.3.8 Cortical ectopia in the Shh-c embryo

*Emx1* is a very specific cortical marker not expressed in any other structure (Gulisano et al., 1996), and labels the entire cortex of the E12.5 mouse (Fig.3.5 A, B). In the *Shh-c* mutant, intriguingly, the caudal cortex seemed to grow towards the midline (red arrows in Fig.3.5 C, D). In coronal sections (E-H) of wild type and mutant we could ascertain ectopic expression of *Emx1* in the ventral side of the diencephalon (red arrows in Fig.3.5

G, H). Detection of *Emx1* at E18.5 (Fig.3.5 J, K) confirmed this finding (red arrows in Fig.3.5 K).

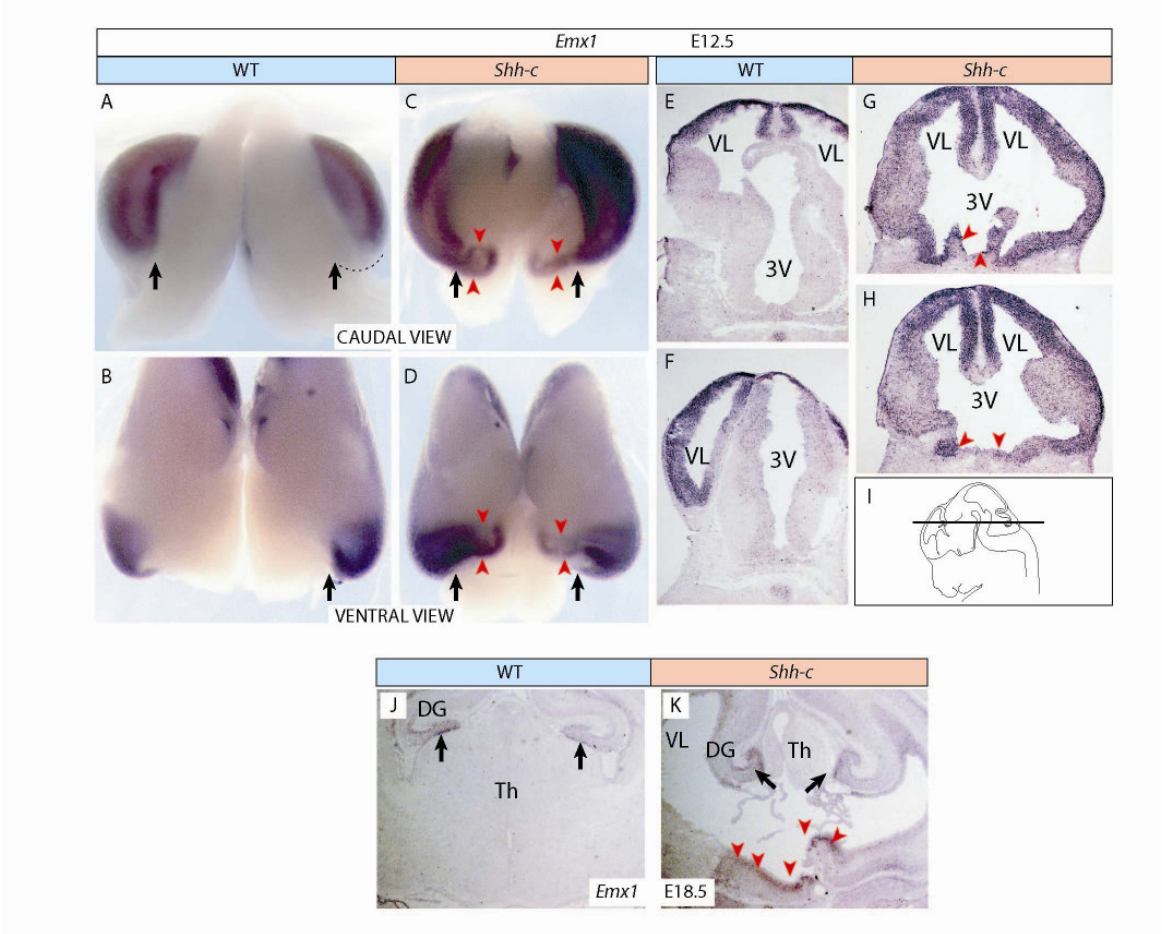


**Figure 3.5 Cortical expansion towards the ventral diencephalon in the *Shh-c* brain.** A-D) E12.5 wild type (A, B) and *Shh-c* (C, D) brains hybridized for *Emx1* in caudal (A, C) and ventral (B, D) view (the diencephalic ventral side was cut open longitudinally). Black arrows, medial limit of the cortex in wild type (A, B) and comparable point in the mutant (C, D). Red arrowheads, ectopic expression of *Emx1* in the mutant (C, D). E-I) Sections of E12.5 wild type (E, F) and *Shh-c* (G, H) brains hybridized for *Emx1* show ventral ectopic expression in the mutant (red arrowheads in G, H). Plane of section shown in (I). J, K) *Emx1* expression on sections of E18.5 wild type (J) and *Shh-c* (K) brains. Black arrows, normal expression in hippocampus. Red arrowheads (K), ectopic *Emx1* expression in the mutant. Abbreviations: DG: Dentate Gyrus; Th: Thalamus; 3V: Third Ventricle; VL: Lateral Ventricle.

### 3.1.3.9 Neural Shh vs Gli3 in diencephalic dorso-ventral patterning

The Gli3 transcription factor counteracts the ventralizing activity of Shh at different CNS levels including the ventral telencephalon and the spinal cord (see Discussion). If the same were true of the hypothalamus, in *Gli3*-deficient brains the region separating AD

from PV would become ventralized, generating a larger prethalamus and ZLI. The *extra-toes* ($Xt^J/Xt^J$) mouse mutant carries a *Gli3* null mutation ((Maynard et al., 2002)), and shows dorsal forebrain patterning alterations (Theil et al., 1999). Inspection of the *Gli3*-deficient diencephalon at E18.5 showed dorso-ventrally enlarged prethalamus and thalamus (Fig.3.6 A, B). *Emx2* is a specific PV marker (Fig.3.4 B, 3.6 C). Ventral expression of *Emx2* was strongly increased in the *Gli3* mutant, showing a larger ZLI and thalamus/prethalamus region (Fig.3.6 C, D). Detection of *Shh* in wild type and *Gli3* mutant confirmed the abnormal elongation of the ZLI (Fig.3.6 E, F). In keeping with it, the prethalamus, labeled by expression of specific marker *Pax6*, was also dorsally enlarged in the *Gli3* mutant (Fig.3.6 G, H).

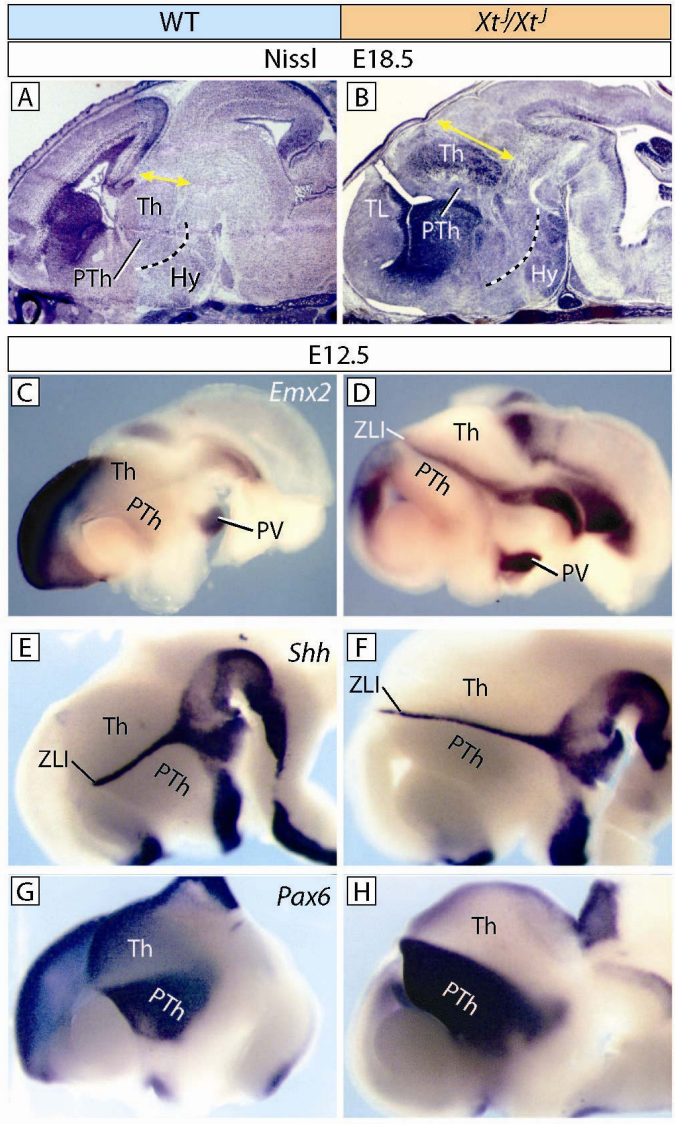


**Figure 3.6 Neural *Shh* vs *Gli3* in diencephalic dorso-ventral patterning.** A, B) Sagittal sections of E18.5 wild type (A) and $Xt^J/Xt^J$ (B) brains. The $Xt^J/Xt^J$ hypothalamus (Hy, separated by dotted line) looks normal, but the thalamus/ prethalamus region is dorsally enlarged (double arrows). C-H) Whole mount ISH of hemisected brains at E12.5. C, D) *Emx2* detection shows a mutant PTh and Th dorsally enlarged. The mutant ZLI is abnormally long and shows expression suggesting ventralization. E, F) *Shh* detection shows abnormally elongated ZLI. G, H) The *Pax6* domain in the mutant PTh is abnormally increased in keeping with the longer mutant ZLI. Abbreviations: Hy: Hypothalamus; Pth: Prethalamus; PV: Posterior-ventral hypothalamus; Th: Thalamus; TL: Telencephalon; ZLI: Zona limitans interthalamica.

#### 3.1.3.10 The Shh-c hypothalamus is transversally bisected by a dorsalized structure

Next we wanted to assess diencephalic differentiation at a later stage, when neurogenesis is over and the mantle layer has been produced and is differentiated. Since *Shh-c* mutants do not survive beyond the end of gestation, we analyzed E18.5 brains. The mutant showed an enlarged 3rd ventricle, suggesting tissue loss (Fig.3.7 A, B). The AD and PV were separated by an abnormal layered structure (asterisk in Fig.3.7 B), which in transverse sections was continuous with the cortex and expressed cortex-specific marker *Emx1* (Fig.3.5 K). The thalamus and PV were reduced in size (Fig.3.7 B). In order to confirm our identification of PV and thalamus in the mutant, we used marker genes *Pitx2* and *Gbx2*. *Pitx2* is a PV-specific homeobox transcription factor gene (Skidmore et al., 2007) (Fig.3.7 C) and its expression was maintained in the mutant (Fig.3.7 C, D). *Gbx2,* a transcription factor specifically expressed in the thalamus (Miyashita-Lin et al., 1999), confirmed our identification of the mutant thalamus (Fig.3.7 E, F). Prethalamic marker *Pax6* confirmed the absence of a prethalamus in the mutant, consistent with the enlarged 3rd ventricle (Fig.3.7 G, H).

To assess the rostro-caudal extent of the hypothalamus we wanted to identify the caudal boundary of the PV, which is the region of the midbrain tegmentum. *Nurr1*, an orphan nuclear receptor gene expressed by dopaminergic neurons identifies the rostral tegmentum, which is preserved but reduced in the mutant (Fig.3.7 I, J). *Nurr1* and *Pax6*, which are also cortical markers (Arimatsu et al., 2003; Stoykova et al., 1996), were expressed in the structure labeled with an asterisk (Fig.3.7 J, H), in agreement with the notion that this is an ectopic cortical structure.

These results (summarized in Fig.3.7 K, L) were surprising, since AD and PV are continuous in wild type adult brains, and in mutants earlier than E12.5 (Fig.3.3). This suggests that in the absence of neural Shh, a ventro-dorsal (transverse) slice of neuroepithelium initially properly regionalized is not maintained and becomes very small to virtually inexistent. This region would encompass PV and prethalamus, in keeping with our observations in Figs.3.3 and 3.4.

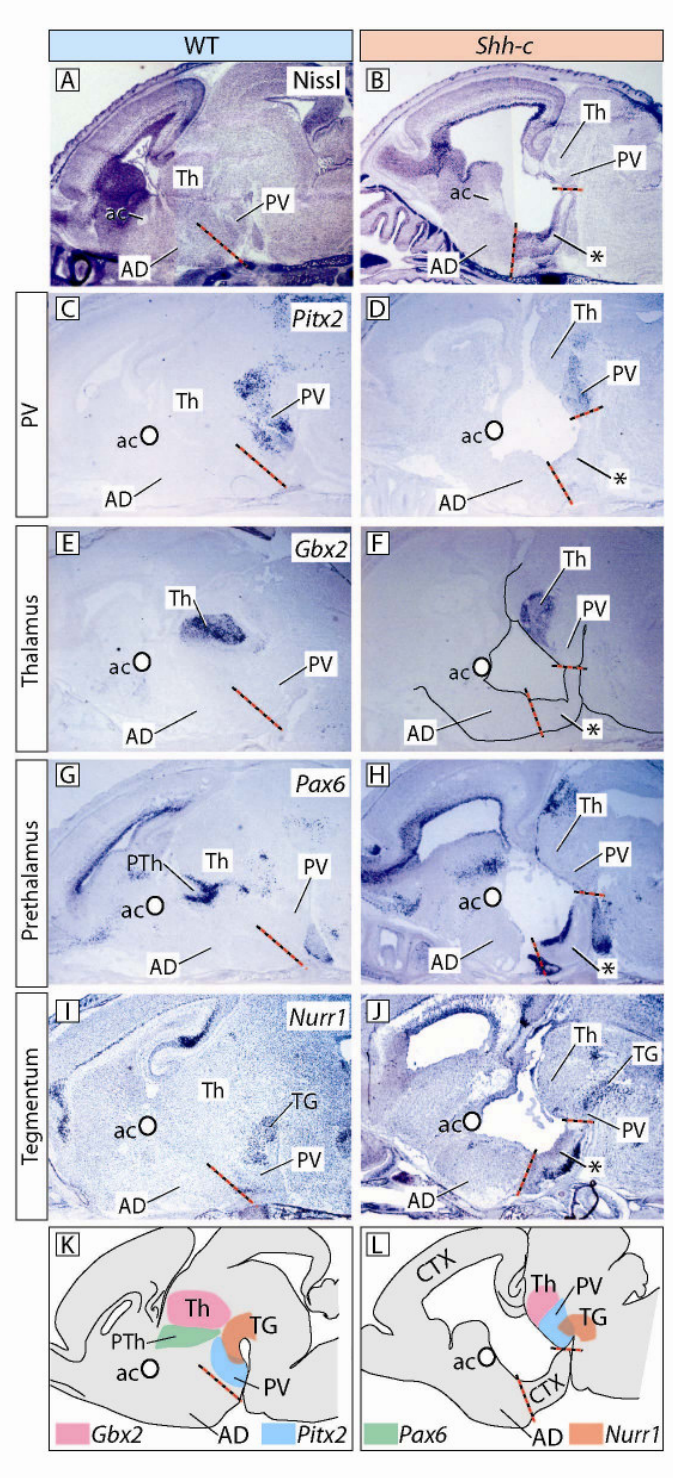


**Figure 3.7 The *Shh-c* hypothalamus is bisected by a dorsalized structure.** A, B) Nissl-stained sagittal sections of E18.5 wild type (A) and mutant (B) brains. Red-black line indicates AD-PV boundary in wild type (A) and AD and PV boundaries with abnormal layered structure (asterisk) in (B). C, D) *Pitx2* labels the PV and was preserved in the mutant. E, F) *Gbx2* labels the thalamus and was preserved in the mutant. G, H) *Pax6* labels the prethalamus and disappeared in the mutant. I, J) *Nurr1* labels the tegmentum and was preserved in the mutant. Results summarized in K, L. Abbreviations: ac: anterior commissure; AD: Anterior-dorsal hypothalamus; CTX: Cortex; Hy: Hypothalamus; Pth: Prethalamus; PV: Posterior-ventral hypothalamus; Th: Thalamus; TG: Tegmentum; TL: Telencephalon; ZLI: Zona limitans interthalamica.

#### 3.1.3.11 Neural Shh is required for the development of the lateral hypothalamus

Despite its functional importance, the development of the lateral hypothalamus is particularly elusive. Many of the neurons fated for the lateral hypothalamus are born in a longitudinal band of rostral diencephalic neuroepithelium showing *Shh* expression (Altman and Bayer, 1986; Manning et al., 2006; Marchand et al., 1986). Intriguingly, that band is missing in our mutant (Fig.3.1 U, Fig.3.4 C). In order to know if neural *Shh* is essential for the development of this region, we used transverse sections to explore the medio-lateral extension of the hypothalamus. Arginine-vasopressine *(Avp)* encodes a hormone specifically expressed in one medial nucleus, the paraventricular (PVN; Fig.3.8 A). In the mutant, *Avp* cells failed to form a tight PVN and were spread medio-laterally (Fig.3.8 B) indicating expansion of the medial hypothalamus and reduction of the lateral hypothalamus. Specific expression of marker genes *pro-melanin-concentrating hormone* (*Pmch*) (Fig.3.8 C) and *hypocretin-orexin* (*Hcrt*) (Fig.3.8 E) characterizes two independent neuronal subpopulations in the lateral hypothalamus. The number of *Pmch* neurons was extremely reduced in the mutant (Fig.3.8 D), while *Hcrt* neurons were completely absent (Fig.3.8 F). By crossing our *Shh-c* conditional mutants with the mouse reporter line Z/AP (Lobe et al., 1999), we labeled the neurons of *Foxb1*-lineage (see Methods), which are very abundant in the lateral hypothalamus (Fig.3.8 G) but were dramatically reduced in the mutant (Fig.3.8 H). These results (summarized in Fig.3.8 I, J) indicate that neural *Shh* is required for the growth and terminal differentiation of the lateral hypothalamus and in particular for the specification of particular and functionally important neuronal subpopulations.

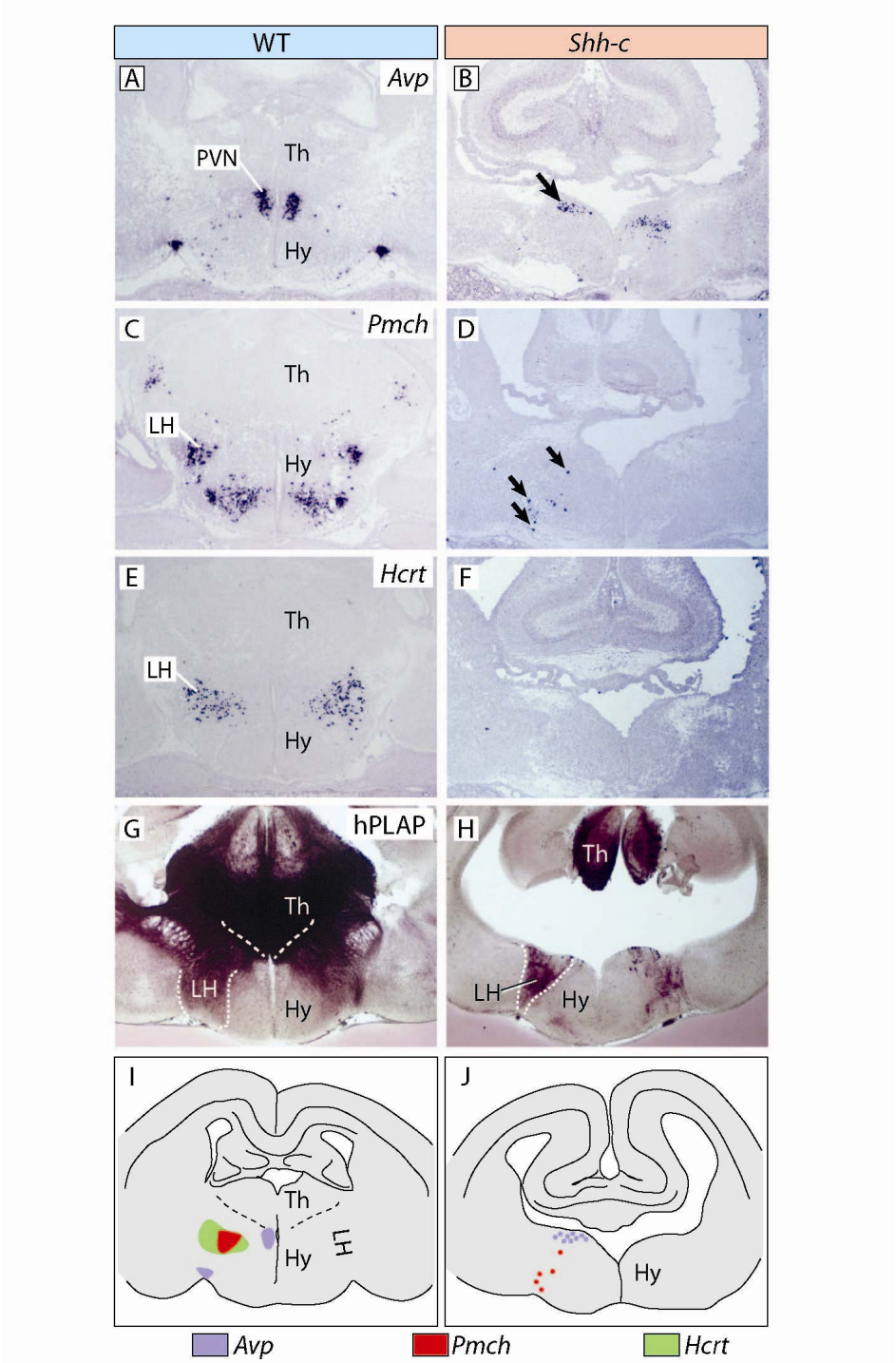


**Figure 3.8 Neural *Shh* is required for lateral hypothalamus development.** A-F) ISH on sections of wild type (A, C, E) and *Shh-c* (B, D, F) E18.5 brains. A, B) The *Avp* expression domain is broader in the mutant. C, D) *Pmch* neurons are very scarce in the mutant. E, F) *Hcrt* neurons cannot be detected at all in the mutant. G, H) hPLAP detection of the *Foxb1* lineage in *Foxb1-Cre / ZAP* reporter mice (see Methods) shows the normal extent of the lateral hypothalamus (G), very reduced in the *Shh-c* mutant (H). I, J) Summary diagrams of results. Expression is shown on the left side of each diagram, abbreviations on the right side. Abbreviations: hPLAP: human Placental Alkaline Phosphatase; Hy: Hypothalamus; LH: Lateral Hypothalamus; Pth: Prethalamus; PV: Posterior-ventral hypothalamus; Th: Thalamus.

#### 3.1.3.12 Differential alterations of AD and PV in the Shh-c hypothalamus

We then used specific markers to analyze the medial hypothalamus, which is divided from rostral to caudal into four regions containing discrete neuronal aggregates (Fig.3.9 A-H). The three most anterior regions of the medial hypothalamus correspond to AD and are the preoptic (PRO), anterior (AHA) and tuberal (TUB). The PRO expressed *Nkx2-1* in the wild type (Fig.3.9 A) and in the mutant (Fig.3.9 B). Immediately posterior to the PRO is the AHA, one of whose nuclei, the suprachiasmatic, can be identified by position (over the optic chiasm) and expression of transcription factor *Lhx1*, preserved in the mutant (Fig.3.9 C, D). The next rostrocaudal subdivision, the tuberal region (TUB), contains the ventromedial nucleus, specifically expressing *Calb1* in wild type and mutant (Fig.3.9 E, F). These results showed that, although much reduced in size, the mutant AD was able to specify neurons for its three major rostrocaudal subdivisions.

The most posterior subdivision of the medial hypothalamus corresponds to the PV and is the mammillary region (MAM), whose largest nucleus, the mammillary body (MBO), specifically expressed transcription factor gene *Foxb1* in wild type (Fig.3.9 G) but not mutant brains (Fig.3.9 H). The mutant MBO was missing also *Calb1* expression, but expressed *Lhx1* and *Nkx2-1* normally (Fig.3.9 B, D, F). Contrary to what happens in the AD, the mutant PV suffers a drastic size reduction as well as incomplete specification. The diagrams in Fig.3.9 I, J summarize the medial hypothalamic phenotypes.

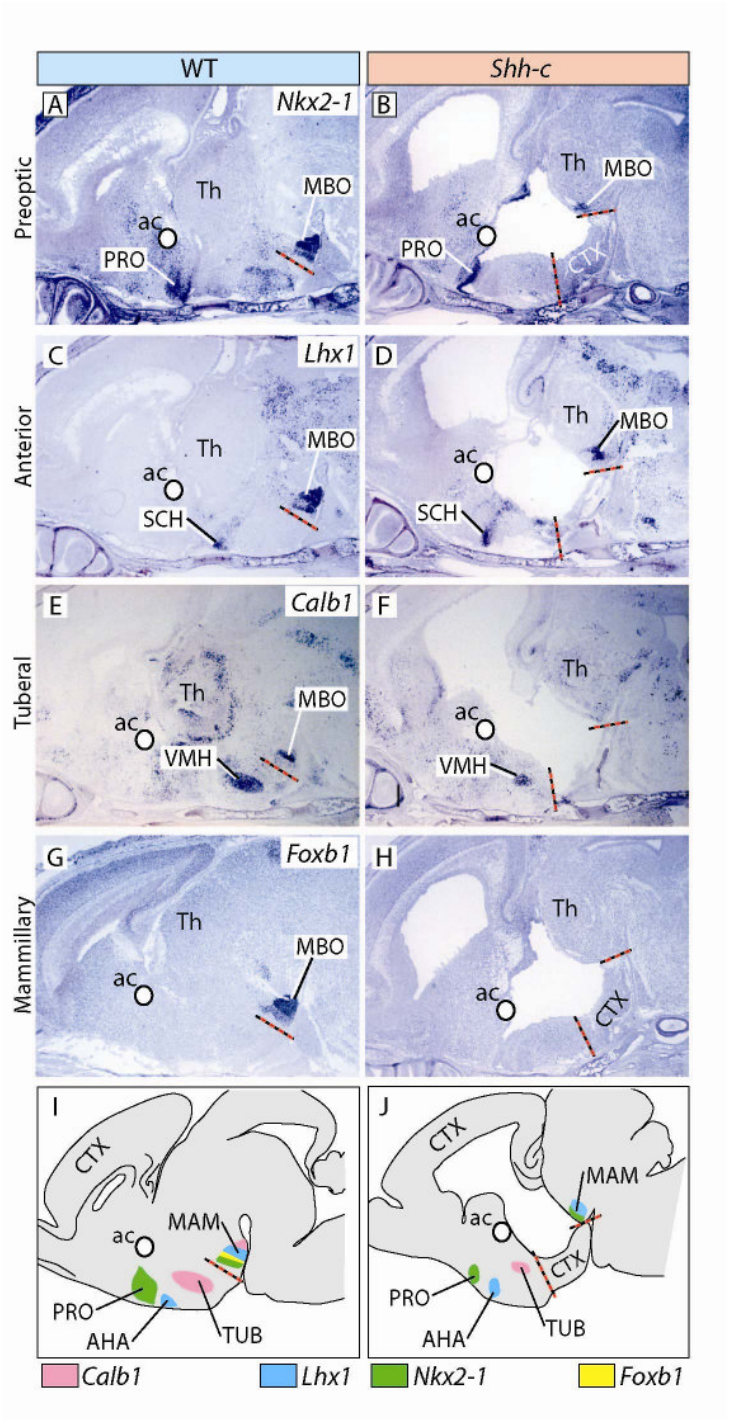


**Figure 3.9 Neural *Shh* is required for differentiation in the medial hypothalamus.** A-H) ISH on sections of wild type (A, C, E, G) and *Shh-c* (B, D, F, H) E18.5 brains. A white circle marks the anterior commissure (ac) for reference. A, B) Expression of *Nkx2-1* is preserved in the mutant PRO and MBO. C, D) *Lhx1* is preserved in the mutant SCH and MBO. E, F) *Calb1* is preserved in the mutant VMH but lost in the MBO. G, H) *Foxb1* disappears in the mutant MBO. I, J) Summary of results. Abbreviations: ac: anterior commissure; AHA: Anterior hypothalamic area; CTX: Cortex; MAM: Mammillary region; MBO: Mammillary body; PRO: Preoptic area; SCH: Suprachiasmatic nucleus; Th: Thalamus; TUB: Tuberal region; VMH: Ventromedial nucleus.

#### 3.1.3.13 Neural Shh is required to maintain expression of the PV survival factor Foxb1

Dependence of *Foxb1* expression on neural *Shh* was surprising, since *Foxb1* is expressed in the *Shh*-deficient neural plate (Fig.3.1 I, J), suggesting a feedback loop between both factors.

We analyzed this question on cultured explants of whole embryonic forebrain. For this purpose we dissected the forebrains of wild type E9.5 mouse embryos, cut them open following the ventral midline and flattened them on Millipore membranes with the neuroepithelial side down (Fig.3.10 A-C). The explants were cultured for 48 hours in control media or after adding one of several reagents to modify the activity of different signalling pathways. Shh pathway inhibitor cyclopamine abolished *Foxb1* expression in E9.5 explants (in 8 of 10 explants, 8/10) (Fig.3.10 D, E), but not in E12.5 explants (9/11) (Fig.3.10 F, G). Therefore, between E9.5 and E12.5, neural *Shh* maintains *Foxb1* expression, perhaps against inhibiting influences by other factors. A powerful signalling agent, *Wnt8b*, strongly expressed in the hypothalamus (Fig.3.10 H-J) is able to reduce the *Foxb1* PV expression domain in zebrafish (Suzuki et al., 1997). We therefore tested whether canonical Wnt signalling controls *Foxb1* expression. Wnt pathway activator LiCl (Stambolic et al., 1996) decreased the *Foxb1* PV expression domain (9/10) (Fig.3.10 K, L). D4476 is a casein kinase-1 inhibitor able to block the Wnt pathway (Bryja et al., 2007; Rena et al., 2004). In explants cultured with D4476 the *Foxb1* PV expression domain increased in size (9/11) (Fig.3.10 M, N). These results suggested that the Wnt pathway, probably activated by Wnt8b, restricts the *Foxb1* PV expression domain in the neuroepithelium. In agreement, as *Wnt8b* expression increases in the PV neuroepithelium during development, *Foxb1* is downregulated in the neuroepithelium and upregulated in the newly born PV neurons; by E12.5 the separation of the expression domains of both markers in the PV was complete (Fig.3.10 O, P). These results suggest that neural *Shh* maintains *Foxb1* PV expression while *Wnt8b* inhibits it in the neuroepithelium thereby restricting *Foxb1* to neurons. Our results, together with some data from the literature (see Discussion), are summarized in Fig.3.10 Q.

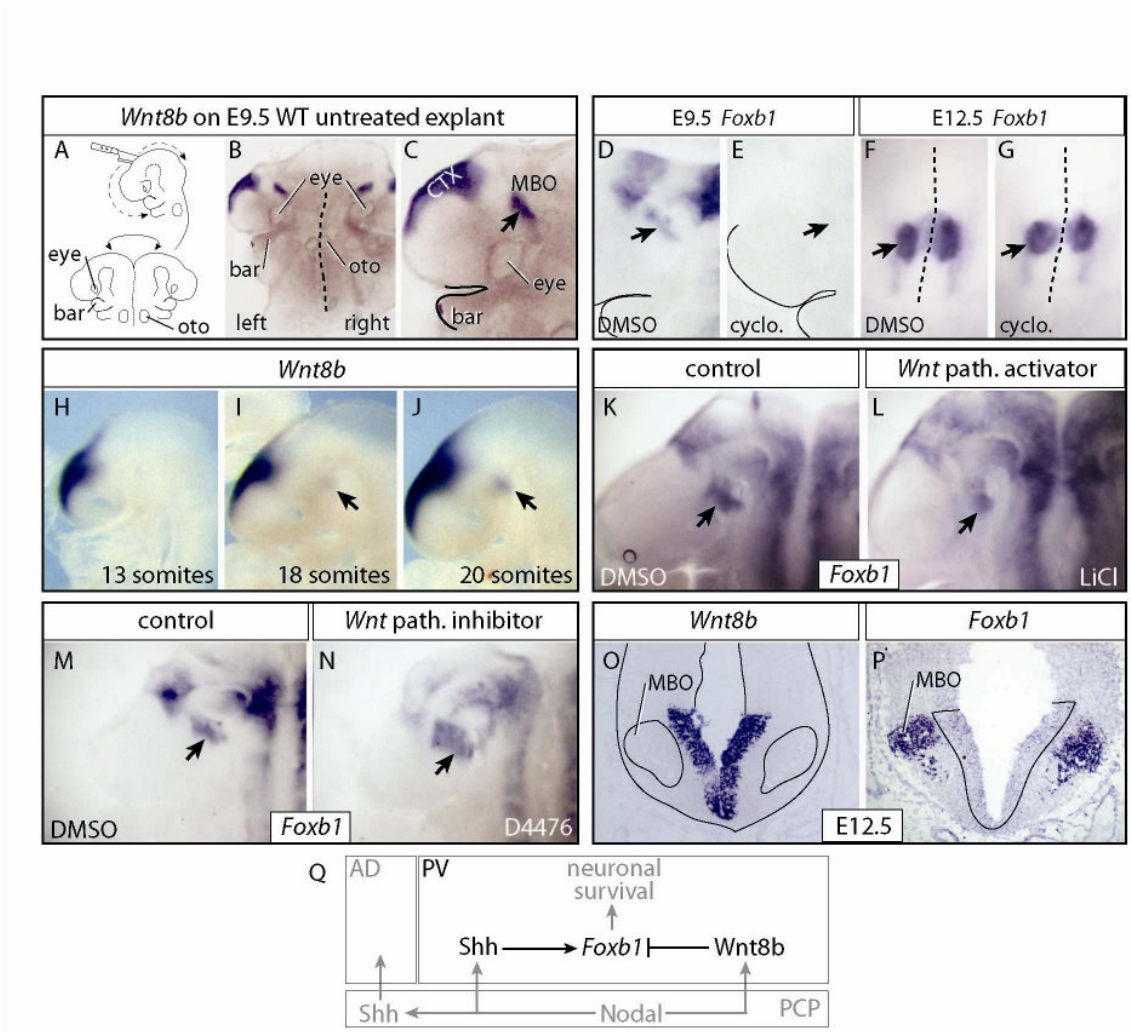


**Figure 3.10 Neural *Shh* and *Wnt8b* maintain and restrict expression of PV survival factor *Foxb1*.**
A) Diagrams showing forebrain explant preparation. B, C) Appearance of the E9.5 mouse brain explants at lower (B) and higher (C) magnification. This explant is labeled for *Wnt8b*. D-G) Treatment with *Shh* pathway blocker cyclopamine at E9.5 (D, E) eliminates *Foxb1* expression in the forebrain including PV (arrow). At E12.5 there is no effect on the MBO (arrow in F, G). H-J) Development of *Wnt8b* expression in the PV. Expression is absent at 13 somites (H), weakly detectable at 18 somites (arrow in I) and fully developed at 20 somites (arrow in J). K, L) Treatment with Wnt pathway activator LiCl reduces the *Foxb1* expression domain in the PV neuroepithelium (arrow). M, N) Treatment with *Wnt* pathway inhibitor D4476 increases the domain of *Foxb1* expression in the PV neuroepithelium (arrow). O, P) As *Wnt8b* expression (O) increases in the PV neuroepithelium, *Foxb1* (P) decreases and becomes restricted to the mantle layer. The section boundaries have been outlined in O, and the *Wnt8b* domain in P. Q) Diagram showing the control of *Foxb1* expression in the PV. In black, our data; in gray, data from the literature. Abbreviations: AD: Anterior-dorsal hypothalamus; CTX: cortex; MBO: Mammillary body; PCP: Prechordal Plate; PV: Posterior-ventral hypothalamus.

### 3.1.4 Discussion

**Hypothalamus development: dissecting the role of prechordal vs neural Shh**

*Shh* is required for hypothalamic development (Chiang et al., 1996), and a current question in Shh research relates to the differential contributions of different sources of Shh (Dessaud et al., 2008). In the ventral spinal cord model, notochordal *Shh* acts as a classical morphogen but neural *Shh* (from the floor plate) does not ((Chamberlain et al., 2008; Ding et al., 1998; Matise et al., 1998), reviewed in (Dessaud et al., 2008)). In thalamus and prethalamus (caudal diencephalon), neural *Shh* has patterning roles (Guinazu et al., 2007; Hashimoto-Torii et al., 2003; Hirata et al., 2006; Kiecker and Lumsden, 2004; Scholpp et al., 2006; Vieira et al., 2005). In hypothalamus (rostral diencephalon), neural *Shh* shows a dynamic expression pattern (Shimamura and Rubenstein, 1997) and promotes expansion of unspecified precursors (Manning et al., 2006). We have analyzed the roles of neural *Shh* in mouse hypothalamic development. Our results show that, after initial general specification of the hypothalamus by other factors, neural Shh signalling takes effect to activate differential regulatory pathways that specify sub-regions of the hypothalamus (AD, PV and LH). As a result, neural Shh is required for specification of the LH (including specification of hypocretin/orexin neurons) and PV, and to coordinate growth and patterning in the AD. Additionally, our data suggest that neural Shh is required to stabilize regional fates in a ventro-dorsal area including the PV and prethalamus. We have also uncovered an interplay between neural *Shh* and *Wnt8b* to regulate the expression of *Foxb1*, which is an essential regulator of hypothalamic development.

**Neural Shh vs non-neural Shh in the hypothalamus**

The roles of Shh from different sources in the diencephalon can be expected to be intricate, given the combinatorial nature of the genetic control of neuronal phenotype (Manning et al., 2006).

*Hypothalamus*. Full *Shh* mutant mice lack all ventral diencephalic structures including hypothalamus (Chiang et al., 1996), and in the absence of neural Shh *Nkx2-1* is expressed and the hypothalamus receives general regional specification (present work). Later, as we show here, there appear different neural *Shh*-dependent regulatory needs for the three

portions of the hypothalamus (LH or lateral hypothalamus, and AD / PV or medial hypothalamus).

In the PV, neural *Shh* maintains expression of *Nkx2-1* beyond E9.5, and activates *Dbx1*. In the LH, neural *Shh* activates *Dbx1* and *Dlx2*. In our mutant, both the PV and the LH are incompletely specified. Finally, early expression of *Nkx2-1* is enough to guarantee the development of the AD, which in the absence of neural Shh shows essentially lack of growth.

Fate acquisition is an inherently hierarchical process controlled by genetic regulatory networks (Davidson et al., 2002), and the networks involving *Shh* are being actively researched (Vokes et al., 2007). In the hypothalamus (as in prethalamus and thalamus) there is an initial specification conferring general regional fate through non-neural Shh (and/or other factors). At this point, neural Shh signalling gets underway to take the regulatory cascade to the next step, the specification of differential subregional identities.

**Neural Shh vs non-neural Shh in prethalamus and thalamus**

*Prethalamus*. The role of neural Shh, particularly from the ZLI, on prethalamus and thalamus has been analyzed in other models (Guinazu et al., 2007; Kiecker and Lumsden, 2004; Sampath et al., 1998; Scholpp et al., 2006; Vieira and Martinez, 2006; Zeltser, 2005). Early prethalamic specification (determined by *Lhx5* expression) does not depend on Shh from any source (zebrafish (Staudt and Houart, 2007)). However, neural Shh is necessary for correct prethalamic expression of *Pax6*, *Dlx2*, and *Six3* ((Kiecker and Lumsden, 2004; Scholpp et al., 2006; Staudt and Houart, 2007); present work).

*Thalamus*. Early thalamic fate, as opposed to what happens in the prethalamus, could depend on non-neural Shh since thalamic expression of *Dbx1* is missing in full *Shh* mutants (Ishibashi and McMahon, 2002) but present in neural Shh-deficient mutants (present work). However, experimental alteration of neural Shh activity causes thalamic defects (Kiecker and Lumsden, 2004; Kiecker and Lumsden, 2005; Vieira et al., 2005; Vieira and Martinez, 2006).

*Zona limitans.* The lack of a ZLI in our mutant (Fig.3.1 U, Fig.3.4 C) is somewhat puzzling, since ZLI formation (as determined by the characteristic Shh expression pattern) is independent from neural Shh expressed more ventrally. This has been shown in the chicken and zebrafish (Guinazu et al., 2007; Sampath et al., 1998; Scholpp et al., 2006; Zeltser, 2005). However, notice that in our mutant, *Foxb1*-driven Cre recombination

overlaps with the prethalamus and thalamus (revised Fig.3.4 A), preventing full length (active) *Shh* from being expressed there, and in this way interrupting the self-sustaining feedback loop that maintains the ZLI (Kiecker and Lumsden, 2004; Zeltser, 2005).

**Neural Shh is required for specification stability in an area formed by PV and prethalamus**

An unexpected consequence of neural Shh deficiency is the presence of an abnormal cortical structure separating the medial hypothalamus into AD and PV. The functions of AD and PV are quite dissimilar (homeostasis maintenance vs memory) and their development is differentially regulated ((Mathieu et al., 2002); present work). Accordingly, they originate from separate regions of the neural plate (zebrafish (Staudt and Houart, 2007)). Later, AD and PV grow and merge into one single medial hypothalamic structure, and their separation in our mutant could be partially due to decreased growth. Additionally, in the fate map (Staudt and Houart, 2007), the prethalamus occupies a dorsal position in register with that of the ZLI (separating AD from PV).

Therefore, failure of the prethalamus to grow in our mutant (after being only incompletely specified) would increase the AD/PV gap. This is borne out by the mutant expression pattern of *Lhx5* and *Wnt8b* (Fig.3.4), showing severely reduced and merged PV and prethalamus at E12.5. Reciprocally, an abnormally large prethalamus forms (Fig.3.6) in embryos deficient in *Gli3* (a Shh antagonist (Litingtung and Chiang, 2000; Persson et al., 2002; Rallu et al., 2002a; Rash and Grove, 2007)). The usefulness of zebrafish fate-mapping data to explain our mouse phenotype emphasizes the similarity of fate maps in vertebrate brains (Puelles and Rubenstein, 2003; Wullimann and Mueller, 2004).

As diencephalic tissues fail to expand in embryos lacking neural Shh, the dorsal telencephalic *Emx1* domain grows ectopically towards the ventral midline (Fig.3.5). This could mean that, if fate specification and growth do not progress beyond a certain point, eventually the region will be taken by an alternative fate. Genetic regulatory networks have feedback circuits that "lock in" the acquired specification state and secure the progressivity of development (Davidson et al., 2002). Another specific role of neural Shh would then be to stabilize the fate specification network in this area.

**An interplay between Shh and Wnt8b regulates expression of Foxb1, a central regulator of hypothalamus development**

Intriguingly, although the mutant MBO, the largest PV nucleus, expresses several specific markers (*Nkx2-1*, *Lhx1*), it fails to express the transcription factor gene *Foxb1*, which in mouse is essential for the survival of the MBO neurons beyond the day of birth (Alvarez-Bolado et al., 2000b). This result reveals an unexpected requirement for neural *Shh* in differentiation and survival of the PV, and is in keeping with data showing a role of *Shh* in neuronal survival in other systems (Miao et al., 1997; Oppenheim et al., 1999). A negative regulator of *Foxb1* expression in the PV of zebrafish is *Wnt8b* (Suzuki et al., 1997), which is expressed in the mouse PV and comparable regions of all vertebrates (Cui et al., 1995; Garda et al., 2002; Hollyday et al., 1995; Kelly et al., 1995; Lako et al., 1998; Richardson et al., 1999; Staudt and Houart, 2007). *Wnt8b* could be a local organizer of the PV (Erter et al., 2001; Staudt and Houart, 2007), whose neurogenesis it controls (Lee et al., 2006). We show that *Wnt8b* suppresses *Foxb1* expression in progenitors (neuroepithelium) and therefore it counterbalances or modulates the positive effect of *Shh* in *Foxb1* expression maintenance. The interplay *Shh* / *Wnt8b* in *Foxb1* control is presumably downstream the Nodal pathway (Erter et al., 2001; Mathieu et al., 2002; Rohr et al., 2001; Staudt and Houart, 2007; Strähle et al., 1996), as we illustrate in the diagram in Fig.3.10 Q.

**Neural Shh coordinates growth and pattern in the medial hypothalamus**

The medial hypothalamus develops in waves of neurogenesis precisely regulated through expression of transcription factors (Caqueret et al., 2005). *Shh* promotes dorsal and ventral diencephalic proliferation (Ishibashi and McMahon, 2002), and coordination of proliferation and patterning is a major role of *Shh* in the CNS (Agarwala and Ragsdale, 2002; Cayuso et al., 2006) requiring specific timing and duration of Shh action (Dessaud et al., 2007; Fuccillo et al., 2004; Matise et al., 1998) for precise activation of downstream targets. In the developing chicken hypothalamus, this is a stepwise process (Manning et al., 2006): 1) first, neural *Shh* promotes expansion of unspecified precursors; 2) then, neural *Shh* is downregulated, unspecified precursors become quiescent and a hypothalamic cell fate is specified; 3) finally, the newly specified hypothalamic precursors resume expansion. Accordingly, the small size of the medial hypothalamus in our mouse mutant can be explained so: 1) in the *Shh*-deficient hypothalamic neuroepithelium the still unspecified precursors do not expand due to the lack of *Shh*, but

their hypothalamic cell fate is specified; 2) these prematurely specified hypothalamic precursors are too few and generate only a small AD and PV. Therefore, in mouse (this study) and chicken (Manning et al., 2006), *Shh* expands rostral diencephalic precursors, but prevents them from receiving hypothalamic specification. In contrast, in the zebrafish model, *Shh* blocks expansion of PV precursors (Mathieu et al., 2002). This suggests that, in amniotes, hypothalamic growth/patterning regulation is a protracted and more complex version of that in zebrafish.

*Conclusions*: In the developing hypothalamus of the mouse, Shh of neuroectodermal origin has the following roles: activating differential regulatory pathways that specify sub-regions of the hypothalamus (AD, PV and LH), stabilizing regional fates in a ventro-dorsal area including the PV and prethalamus and regulating the expression of *Foxb1*, which is an essential regulator of hypothalamic development.

### 3.1.5 Authors' contributions

I generated and maintained the *Shh-c* mutant mouse line.

The following experiments were performed by me:

- Collection of all embryos, generation of all the probes and subsequently the whole mount *in situ* hybridizations
- Collection of all embryos, cutting of the sections of the embryos and generation of the probes for ISH on sections
- The explant culture experiments
- Cloning of the full-length *Shh* and the exon 2-deleted *Shh* construct and the electroporation of these constructs into embryos for explant culture

## 3.2 Second Manuscript

### The role of *Shh* of neural origin in thalamic differentiation in the mouse

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### 3.2.1 Introduction

Brain nuclei differentiate stepwise, receiving general regional specification during gastrulation and progressing down a genetic regulatory hierarchy to the effector genes conferring mature functional attributes to neurons. Transient embryonic structures called organizers and their secreted products are key in this process. The best known model is the specification of ventral spinal cord neurons through a morphogenic concentration gradient of Sonic hedgehog (Shh) protein secreted by the notochord (Dessaud et al., 2008). *Shh* is expressed in the forebrain neuroepithelium (neural *Shh*) and the underlying notochord and prechordal plate. If and how the mechanisms found in the ventral spinal cord have become modified to specify the convoluted three-dimensional neuronal assemblies found in the forebrain is not well understood. The thalamus (dorsal thalamus) is a forebrain region constituted mostly by neurons projecting to cortex and striatum, and in rodents it can be subdivided into several nuclear groups harboring altogether some 38 nuclei and subnuclei (Swanson and Cowan, 1977). These differ from each other specifically in position, morphology, marker expression, membrane properties and cortical targets (Jones, 2007). Thalamic subdivisions originate as five undifferentiated cell masses (pronuclei; (Labosky et al., 1997)), gradually resolving into specific nuclear groups: central pronucleus (anterior, intralaminar and ventral groups), dorsal pronucleus (lateral and posterior groups), medial pronucleus (medial group), lateral geniculate pronucleus and medial geniculate pronucleus.

The thalamic neuroepithelium is bounded by a *Shh* expression domain, consisting of ventral and rostral parts, called respectively diencephalic tegmentum (TG) and zona limitans interthalamica (ZLI). Shh from the ZLI is essential for prethalamus (ventral thalamus) specification (Kiecker and Lumsden, 2004; Scholpp et al., 2006; Vieira et al., 2005; Vieira and Martinez, 2006; Zeltser, 2005). The role of neural Shh in thalamic development, however, is still unclear, although Shh (of any origin) promotes thalamic growth (Ishibashi and McMahon, 2002). *Shh* controls thalamic expression of *Gbx2* (Hashimoto-Torii et al., 2003), a transcription factor gene expressed in the entire thalamic neuroepithelium starting around E11.5 and required for thalamic development (Hevner et al., 2002; Miyashita-Lin et al., 1999). The relative contributions of Shh of different sources as well as *Gbx2* to thalamic development is an unresolved question.

Here we have used a Cre-lox system to abolish all Shh function in the mouse diencephalic neuroepithelium and we have analyzed the differentiation of the mutant thalamus, as well as the *Gbx2* mutant thalamus. We have found that neural *Shh* is required for the emergence of nuclear-specific phenotypical traits in fate-specified thalamic cells but does not act as a classical morphogen. Additionally, *Gbx2* is expressed by default in the absence of neural *Shh* and is specifically responsible for the development of the medial and intralaminar nuclear groups. Finally, the individual thalamic pronuclei show differential molecular identity and are differentially specified.

### 3.2.2 Methods

We have used a *Foxb1*-Cre mouse line (Zhao et al., 2007) and a conditional *Shh* allele (Dassule et al., 2000) in order to generate mouse embryos with alterations in thalamic development. Together with the Z/AP reporter line, we have been able to trace the lineage of thalamic cells.

**Mouse lines**

Experiments with animals were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and under authorization Az 32.22/Vo (Ordnungsamt der Stadt Göttingen).

*Foxb1-Cre mouse line*. Homologous recombination was used to replace the *Foxb1* coding sequence by the Cre recombinase cDNA (Zhao et al., 2007), and a mouse line was created (kept in the C57BL/6 background). This line expresses Cre under the control of the regulatory sequences of *Foxb1*.

*Reporter lines*. To map the embryonic brain region where Cre-mediated recombination takes place, we crossed our mice with the *ROSA26R* reporter mouse line (Soriano, 1999). In *ROSA26R* animals, the reporter gene β-galactosidase is inserted in the constitutively active ROSA locus downstream a floxed stop codon. Upon Cre-mediated recombination, the stop codon is deleted and β-galactosidase is constitutively produced. This reporter is a lineage marker because in mice carrying both the $Foxb1^{Cre}$ and the *ROSA26R* alleles, cells expressing *Foxb1* and any cell derived from them will permanently express β-galactosidase. To label the axons of neurons of the *Foxb1* lineage, we crossed our $Foxb1^{Cre}$ mice with the Z/AP (Lobe et al., 1999) reporter mouse line (C57BL/6) (see below). In Z/AP animals, Cre-mediated recombination activates constitutive expression of human placental alkaline phosphatase (hPLAP). This reporter is also a lineage marker: in mice carrying both the $Foxb1^{Cre}$ and the Z/AP alleles, cells expressing *Foxb1* and any cell derived from them will permanently express hPLAP.

All mutant mice used for our study were heterozygous for $Foxb1^{Cre}$ and therefore they were heterozygous for *Foxb1* and showed normal phenotype as expected (Alvarez-Bolado et al., 1999; Alvarez-Bolado et al., 2000b; Kloetzli et al., 2001; Labosky et al., 1997). No homozygotes were used in this study.

*Floxed Shh mouse line.* This line was generated in the laboratory of Andy McMahon and has been previously described (Dassule et al., 2000). In these mice, exon 2 of the Shh locus, which encodes approximately half of the active N-terminal Shh signal which is essential for Shh function; (Fan et al., 1995; Hynes et al., 1995; Lai et al., 1995; Lopez-Martinez et al., 1995; Miyashita-Lin et al., 1999; Roelink et al., 1995) is flanked with loxP sites. Upon crossing this line with a Cre line, a conditional allele of *Shh* (*Shh-c*) is generated. The same floxed line has been used for the study of limb development (Dassule et al., 2000) and cortical development (Komada et al., 2008).

To obtain embryos and fetuses for the analysis, timed-pregnant females of the appropriate crossings were killed by cervical dislocation.

*Gbx2 mutant mouse line.* Was provided by Dr. Alex Joyner (Miyashita-Lin et al., 1999). A portion of the 3' UTR of *Gbx2* remains unaltered in the targeted *Gbx2* locus (Li et al., 2002), and *in situ* hybridization probes against this sequence can label the expression of *Gbx2* mRNA (although truncated and non-functional) in the *Gbx2* mutant (see Results).

**Foxb1-Cre abolishes the expression of functional Shh mRNA, but truncated Shh mRNA can still be detected**

When *Shh* expression is activated, the recombined *Shh* locus produces a truncated mRNA lacking exon 2. In situ hybridization probes can be designed that detect either all *Shh* mRNA forms --full-length (functional) and truncated (not functional)-- or only the full-length form. Therefore, we can test in our mutant if full-length *Shh* is being expressed (in territories not co-expressing *Foxb1-Cre*) or if expression of the *Shh* is activated but producing a non-functional mRNA (in territories where Shh expression colocalizes with *Foxb1-Cre*).

**Axonal labeling by alkaline phosphatase activity**

In *Foxb1*$^{Cre}$ Z/AP animals, *Foxb1* lineage neurons will express hPLAP. Since hPLAP attaches to axonal membranes, it is a very good marker of axons of lineage-labeled neurons (Fields-Berry et al., 1992; Gustincich et al., 1997; Leighton et al., 2001).

*Alkaline phosphatase activity detection.* This protocol has been described (Lobe et al., 1999). Briefly, brains of the appropriate genotypes were collected, fixed (4% paraformaldehyde) on ice 60 min., agarose-embedded, cut into 150 micrometer-thick sections, fixed again (4% paraformaldehyde/0.2% glutaraldehyde) on ice 60 min., incubated 30 min at 72°C to inhibit endogenous phosphatase, rinsed in alkaline

phosphatase-buffer (100mM Tris-Cl, pH 9.5, 100mM NaCl, 10mM $MgCl_2$), incubated with staining solution (250µl NBT +187.5µl BCIP per 50ml in alkaline phosphatase-buffer) overnight (4°C), and fixed (4% paraformaldehyde) 60 min (4°C).

***In situ* hybridization (ISH) in whole mount**

Was performed as described (Wilkinson, 1992). The probes were cloned by PCR (primers available upon request). Briefly, the embryos were fixed in formaldehyde 4% in phosphate buffer saline (PBS) overnight (4°C), washed in PBS + 0.1%Tween-20 (PBT) and stored at -20°C in methanol. For ISH the embryos were rehydrated, bleached (6% $H_2O_2$), digested (10 µg/ml Proteinase K in PBT at RT), washed (2 mg/ml glycine/PBT), postfixed (4% PFA/0.2% glutaraldehyde/PBT), prehybridized for 1-2h (70°C) and hybridized overnight (70°C). They were then washed (50% formamide, 5x SSC, pH4.5, 1% SDS at 70°C), rinsed (100 mM Maleic acid, 150 mM NaCl, 2 mM Levamisole, 0.1% Tween-20 (MAB)) and incubated in 10% sheep serum in MAB/2% Blocking Reagent (Roche Diagnostics GmbH, Mannheim, Germany) for 2-3h (RT), then in anti-DIG AP antibody (Roche) overnight (4°C). The embryos were rinsed, then left in MAB overnight (4°C). The embryos were then incubated in BM-Purple (Roche) with Levamisole (RT) and, after color developed, washed in PBT (pH 4.5), fixed in 4% formaldehyde/0.1% glutaraldehyde overnight (4°C), and transferred into 80% Glycerol/PBT.

**ISH on sections**

Nonradioactive ISH on cryosections (25 micrometer thick) which were fixed in 4% paraformaldehyde and acetylated after sectioning. Prehybridization, hybridization and washing steps were performed with the help of an automatic liquid-handling unit Genesis RSP 200 (Tecan, Männedorf, Switzerland), and the digoxigenin-labeled probe was detected by a dual-amplification procedure (Reymond et al., 2002; Visel et al., 2004; Yaylaoglu et al., 2005).

## Abbreviations used for thalamic structures

| Pronucleus | Abbreviation | Name |
|---|---|---|
| Central | ATN | anterior thalamic nuclei |
| | VEN | ventral group |
| | VM | ventromedial |
| | VMb | ventromedial basal |
| | VP | ventral posterior |
| Medial | PV | paraventricular |
| | PT | paratenial |
| | MV | medioventral |
| | MD | mediodorsal |
| | CM | centromedial |
| | CL | centrolateral |
| Dorsal | LD | laterodorsal |
| | LP | lateral posterior |
| | Po | posterior |
| Lateral Geniculate | LGd | lateral geniculate dorsal |
| Medial Geniculate | MG | medial geniculate |
| Other structures | Ctx | cortex |
| | em | external medullary lamina |
| | H | habenula |
| | ic | internal capsule |
| | MB | midbrain |
| | PTh | prethalamus |
| | RT | reticular nucleus |
| | Th | thalamus |

**Table 3.1 Abbreviations used for thalamic and other structures**

## 3.2.3 Results

### 3.2.3.1 Abolition of functional Shh expression and Shh signaling in the caudal diencephalon of Shh-c mutants

At E10.0 Foxb1 is expressed in the prethalamus and thalamus (Fig.3.11 A) overlapping the domain of the incipient zona limitans interthalamica (ZLI; arrow in Fig.3.11 B). In *Shh-c* mutants at this age, *Shh* expression has been abolished in the ZLI as well as in domains of the rostral diencephalon (Fig.3.11 C). As expected (Ishibashi and McMahon, 2002), the caudal diencephalon is smaller in the mutant (compare dotted line in Fig.3.11 B and C). An in situ probe that specifically identifies exon 2 of the *Shh* locus failed to label the diencephalon (Fig.3.11 D), demonstrating that the *Shh-c* neuroepithelium does not produce any functional *Shh* (see Methods). The telencephalic domains of Shh expression are intact (Fig.3.11 D), since they do not overlap with the *Foxb1* expression domain (Fig.3.11 A). At E12.5, all major regions of the brain can be recognized by specific marker expression (Shimamura and Rubenstein, 1997). At this age, *Foxb1* drives Cre recombination activity in the caudal diencephalon, dorsal and ventral (as reflected by lineage labeling in ROSA26R crossings; Fig.3.11 E). In wild type embryos, *Shh* showed a very pronounced and characteristic expression pattern in the caudal diencephalon (ZLI and diencephalic tegmentum) as well as the rostral diencephalon (hypothalamus) (Fig.3.11 F). In the *Shh-c* mutant, expression of *Shh* was absent from the diencephalon (Fig.3.11 G), and this was confirmed by an exon 2 probe (Fig.3.11 H). Expression of Shh receptor *Ptch1* is diagnostic of a functioning Shh signaling pathway (see (Dassule et al., 2000) for citations). *Ptch1* is expressed in both prethalamus and thalamus in the wild type at this age (Fig.3.12 A), but was completely absent in the *Shh-c* mutant caudal diencephalon (Fig.3.12 B), indicating abolition of the Shh pathway.

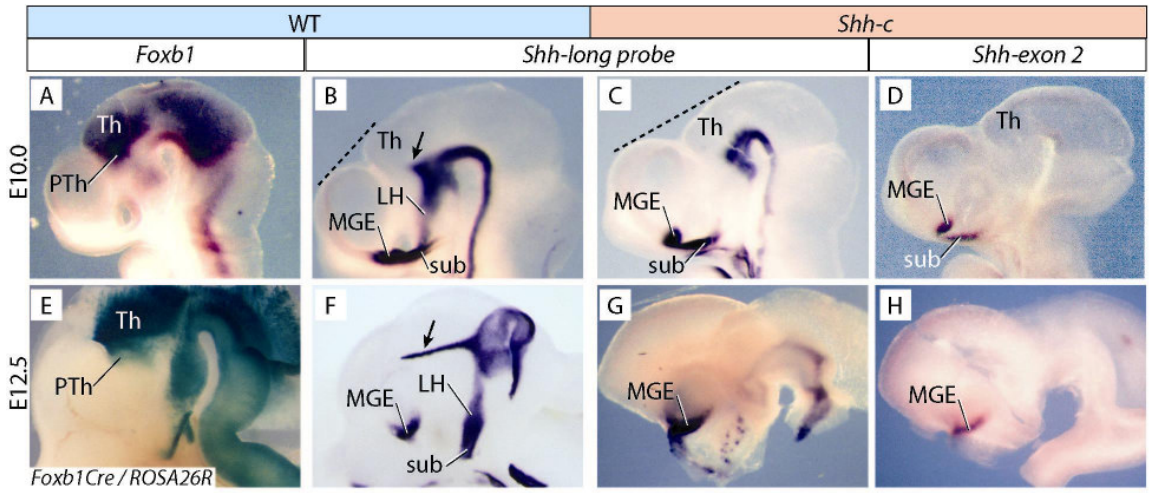


**Figure 3.11 Abolition of functional *Shh* expression in the caudal diencephalon of *Shh-c* mutants.**
A-D and F-H) Whole mount in situ hybridization for the genes and genotypes indicated. (E) *Foxb1* lineage-mapping by β-galactosidase detection in *Foxb1*$^{Cre}$*/ROSA26R* heterozygotes. A, B) At E10.0, *Foxb1* expression overlaps with the incipient ZLI as labeled by *Shh* (arrow in B). C, D) In the *Shh-c* mutant, *Shh* transcription is not active in the ZLI but in the TG (C). All expression of *Shh* at this age is truncated (inactive) in the diencephalon (D). E, F) At E12.5, *Foxb1* lineage labeling is present in most of the thalamic region (E), overlapping thalamus and ZLI as labeled by *Shh* (F). The *Shh-c* mutant shows almost no *Shh* transcriptional activation in the caudal diencephalon (G) and no functional *Shh* at all (H).
Abbreviations: LH: lateral hypothalamus; MGE: medial ganglionic eminence; PTh: prethalamus; sub: suboptical domain; Th: thalamus.

### 3.2.3.2 Abolition of the ZLI in the Shh-c mutant mouse

Shh from the ZLI is essential for prethalamic development as has been demonstrated in chicken (Kiecker and Lumsden, 2004; Vieira and Martinez, 2006) and zebrafish (Scholpp et al., 2006). As expected, expression of ZLI marker gene *Nkx2-2* (Kiecker and Lumsden, 2004; Kitamura et al., 1997) was absent from the *Shh-c* mutant (Fig.3.12 C, D). Transcription factor gene *Lhx1* is a marker of the zona limitans, prethalamus and thalamic eminence (Bachy et al., 2001), and was also absent from our mutant (Fig.3.12 E, F). In order to evaluate the thalamus of the *Shh-c* mutant, we used expression of specific markers *Dbx1* and *Gbx2* (Hevner et al., 2002; Miyashita-Lin et al., 1999). *Dbx1* was still expressed in the mutant (not shown), but *Gbx2* was very reduced although it showed one restricted domain of expression in the thalamus (Fig.3.12 G, H).

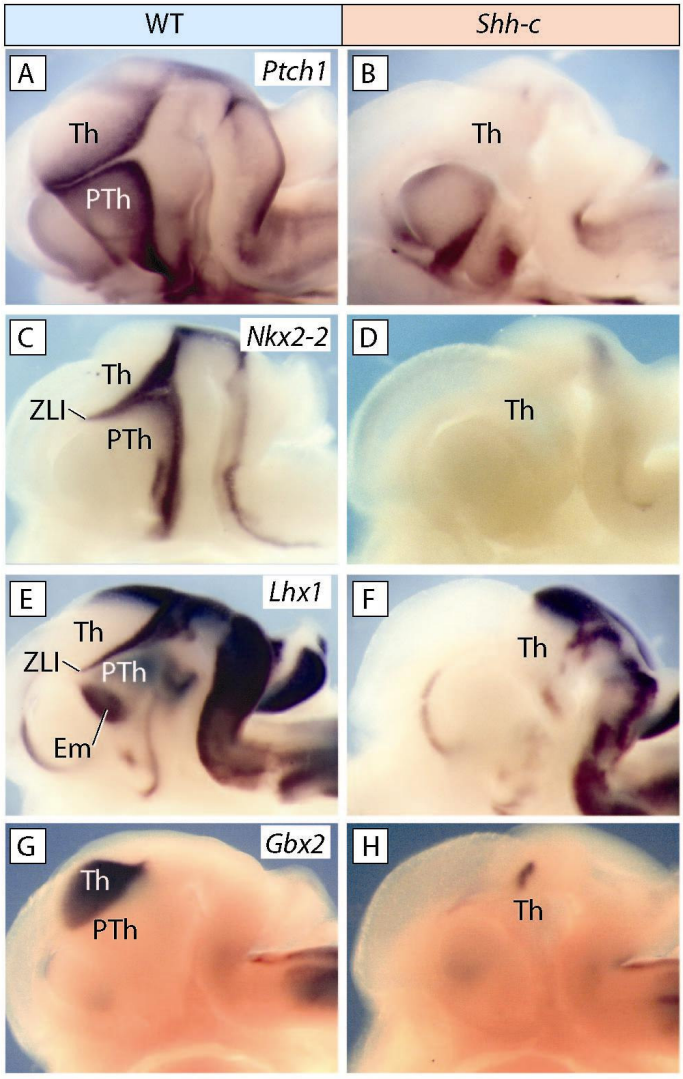


**Figure 3.12 Abolition of the ZLI and prethalamus in *Shh-c* mutants.**
Whole mount in situ hybridization of hemisected E12.5 mouse brains, probes and genotypes as indicated. A, B) Lack of *Ptch1* expression indicates that the *Shh* signaling pathway is abolished in the *Shh-c* mutant diencephalon. C-F) The zona limitans and prethalamus have disappeared in the *Shh-c* mutant, as determined by expression of *Nkx2-2* and *Lhx1*. G, H) *Gbx2* is expressed in a small restricted thalamic domain at E12.5 in the *Shh-c* mutant. Abbreviations: Em: thalamic eminence; PTh: prethalamus; sub: suboptical domain; Th: thalamus; ZLI: zona limitans interthalamica.

#### 3.2.3.3 Specific markers of pronuclei in the wild type thalamus

To explore thalamic differentiation, we chose five marker genes whose expression in the thalamus has been well established: *Calb2-Calretinin* (Arai et al., 1994; Arai et al., 1991; Frassoni et al., 1998; Winsky et al., 1992), *Gbx2* (Jones and Rubenstein, 2004), *Lhx2*

(Nakagawa and O'Leary, 2001), *Neurog2* (Nakagawa and O'Leary, 2001) and *Cdh6* (Jones and Rubenstein, 2004). Since the *Shh-c* mutants survive up to E18.5, we carried out our analysis at this age. We first determined marker expression on transverse sections of E18.5 wild type thalamus, at rostral, intermediate and caudal thalamic levels (Fig.3.13; Tables 3.1, 3.2). Our findings on the wild type match on the whole those of the cited literature (see above) except in minor discrepancies due either to differences in in situ probe or sensitivity of the method, or to species (mouse vs rat) or to age of analysis (E18.5 vs adult, P2 or P0).

Through this work we show our data on thalamic differentiation in wild type, *Shh-c* and *Gbx2* mutant in Figs. 3.13, 3.14 and 3.16 and Tables 3.2-3.4. While the Figures show individual examples of specific marker expression in some nuclei at four different rostrocaudal levels, the Tables summarize information gathered from collections of sections through the brains of several individuals.

*Central pronucleus (anterior and ventral nuclear groups).* The anterior group was labeled by *Lhx2* (weakly), *Neurog2* and *Cdh6* (Fig.3.13 C-E, H-J). The ventral group was labeled by *Lhx2*, *Neurog2* and *Cdh6* (Fig.3.13 M-O). *Calb2*, *Gbx2*, *Lhx2* and *Cdh6* labeled the "tail" of the central pronucleus, represented at this level by the basal portion (parvicellular) of the ventromedial nucleus (Fig.3.13 P-R, T).

*Medial pronucleus (medial and intralaminar nuclear groups).* The intralaminar group (centromedial and centrolateral nuclei) was labeled by *Calb2*, *Gbx2*, *Lhx2* and *Cdh6* (Fig.3.13 K-M, O). The medial group was labeled by *Calb2*, *Gbx2* and *Lhx2* (Fig.3.13 A-C, F-H). Notice that we include the intralaminar nuclei in the medial pronucleus (not in the central, (Labosky et al., 1997) because of marker and phenotype similarity (see below and Discussion).

*Dorsal pronucleus (lateral and posterior nuclear groups).* The lateral posterior nucleus was labeled by *Calb2*, *Gbx2* and *Cdh6* (Fig.3.13 K, L, O). The posterior nucleus was labeled by *Cdh6* (Fig.3.13 O).

*Lateral geniculate pronucleus.* Was labeled by *Neurog2* and *Cdh6* (Fig.3.13 N, O).

*Medial geniculate pronucleus.* Was labeled by *Calb2*, *Gbx2*, *Lhx2* and *Cdh6* (Fig.3.13 P-R, T).

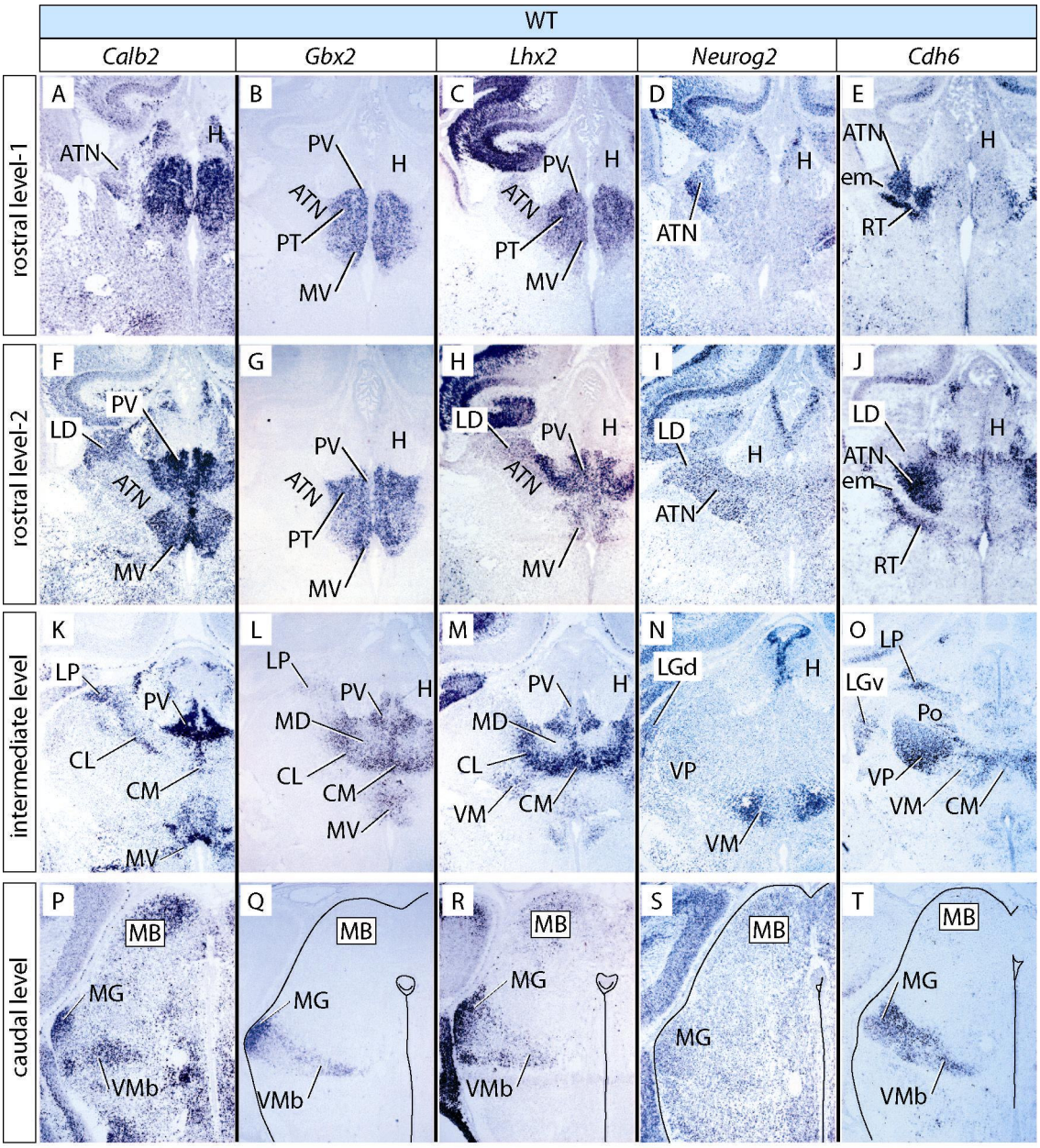


**Figure 3.13 Marker expression in the E18.5 wild type thalamus.**
In situ hybridization on E18.5 mouse brain sections for five marker genes as indicated on top of each column. Rows show sections at given rostro-caudal levels, as indicated on the left side, labeled for each of the five markers. Columns correspond to four rostro-caudal levels labeled with the same probe.

| Pronuclei | Nuclei | *Calb2* | *Gbx2* | *Lhx2* | *Neurog2* | *Cdh6* |
|---|---|---|---|---|---|---|
| Central | ATN | | | | + | + |
| | VP | | | | + | + |
| | VM | | | + | + | + |
| | VMb | + | + | + | | + |
| Medial | PV, PT, MV | + | + | + | | |
| | MD | | + | | | |
| | CM | + | + | + | | + |
| | CL | + | + | + | | |
| Dorsal | LD | + | | + | + | |
| | LP | + | + | + | | + |
| | Po | | | | | + |
| Lat. Genic. | LGd | | | | + | + |
| Med. Genic. | MG | + | + | + | | + |

**Table 3.2 Pronuclear markers in the wild type thalamus.**
+ indicates expression in a recognizable structure (listed in the left column). Intensity of expression has not been recorded. *Cdh6*, *Calb2* and *Gbx2* label respectively the entire central, dorsal and medial pronuclei. The same genes plus *Lhx2* and *Neurog2* are partial markers of other pronuclei

#### 3.2.3.4 General appearance of the Shh-c thalamus

In the *Shh-c* brain, the prethalamus is absent so that the thalamus seems to hang over the third ventricle, particularly on rostral sections (Fig.3.14 A-J). At caudal levels, the *Shh-c* thalamus seemed "embedded" into the midbrain (Fig.3.14 K-T). By counting the number of histological sections we estimated that the *Shh-c* thalamus is smallar than the wild type, on the rostrocaudal axis, by about 50%, as could be expected (Ishibashi and McMahon, 2002).

Expression of *Lhx2, Gbx2*, *Calb2* and *Cdh6* was maintained in the *Shh-c* thalamus, while expression of *Neurog2* was almost completely abolished. The general position of the nuclear groups in the rostro-caudal and dorso-ventral axes was correct. The results are summarized in Table 3.3.

#### 3.2.3.5 Medial and intralaminar nuclei are preserved in the Shh-c thalamus

*Central pronucleus (anterior and ventral nuclear groups).* The mutant anterior thalamic nuclei could be recognized by their preserved *Cdh6* expression and their relation to the external medullary lamina (compare Figs. 4.3 E and 4.4 E). They lost however *Lhx2* and *Neurog2* expression (Fig.3.14 C, D). Notice also that, since this mutant lacks a prethalamus, the reticular nucleus (a prethalamic derivative), which expresses *Cdh6*, was absent (compare Figs. 4.3 E and 4.4 E).

The ventral group lost the expression of *Neurog2* (Fig.3.14 I) and preserved only the most reduced expression of *Cdh6* (Fig.3.14 J, O). The entire central pronucleus (anterior plus ventral groups) seemed reduced to a rostrocaudal row of *Cdh6*-expressing cells (arrows in Fig.3.14 J, O). The "tail" of the central pronucleus (the ventromedial basal) was an exception, since it lost *Calb2* and *Cdh6* but still expressed *Gbx2* and *Lhx2* in the mutant (Fig.3.14 Q, R).

*Medial pronucleus (medial and intralaminar nuclear groups).* This pronucleus was well preserved in morphology and marker expression, including *Calb2* (Fig.3.14 A, F, K), *Lhx2* (Fig.3.14 C, H, M) and *Gbx2* (Fig.3.14 B, G, L). Expression of *Gbx2* labeled the entire pronucleus and is an argument towards including the intralaminar nuclei together with the medial group in the medial pronucleus (not the anterior, (Labosky et al., 1997)).

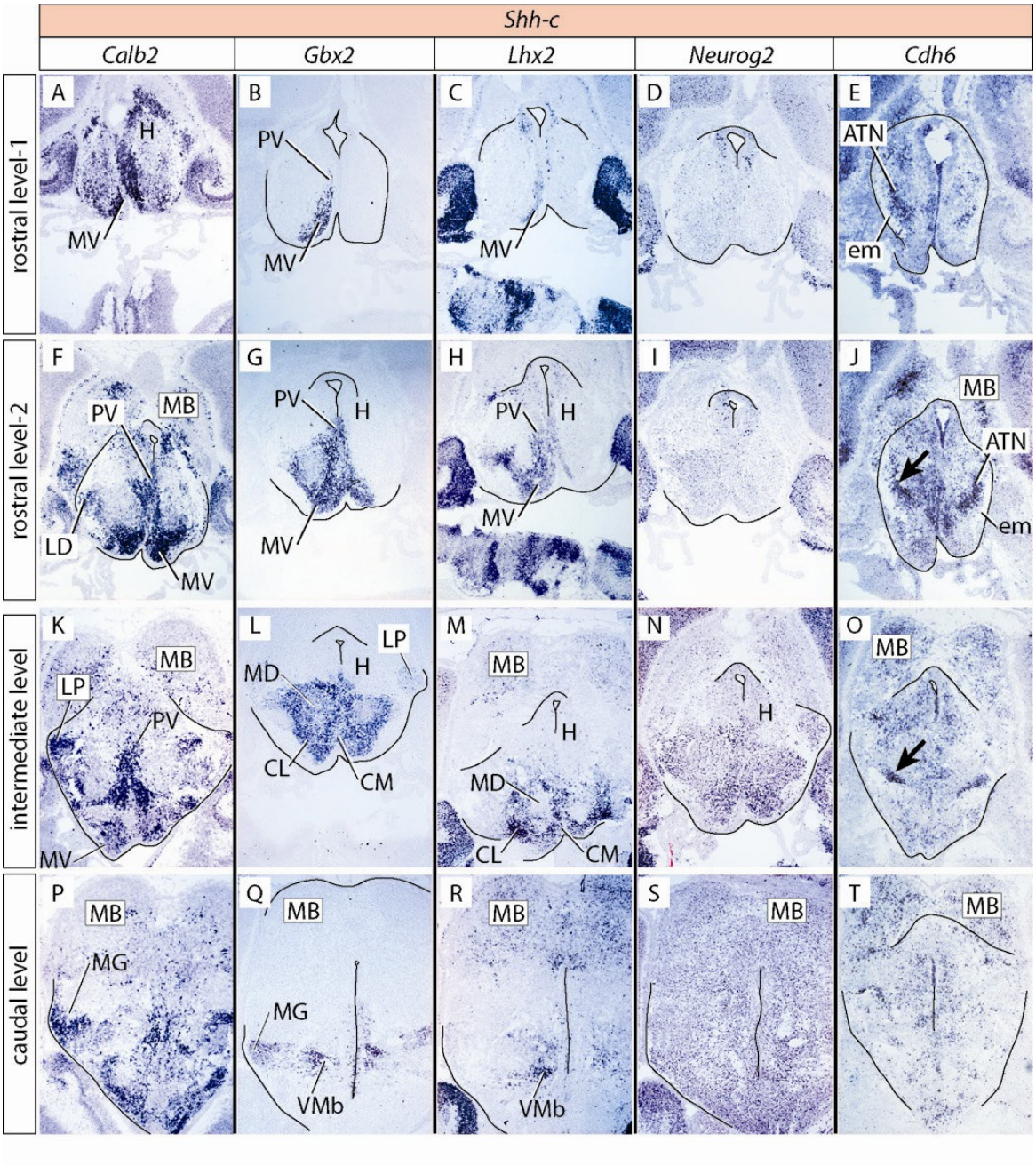


**Figure 3.14 Marker expression in the E18.5 *Shh-c* thalamus**
In situ hybridization on E18.5 mouse brain sections for five marker genes as indicated on top of each column. Rows show sections at a given rostro-caudal level, as indicated on the left side, labeled for each of the five markers. Columns correspond to four rostro-caudal levels labeled with the same probe. The arrows in J, O mark continuous rostro-caudal expression of *Cdh6* in the anterior group.

*Dorsal pronucleus (lateral and posterior nuclear groups).* The laterodorsal nucleus, expressing *Calb2*, was preserved in the mutant (Fig.3.14 F). The lateral posterior nucleus, labeled by *Calb2* and (weakly) by *Gbx2* was also preserved (Fig.3.14 K, L). The posterior nucleus appeared lost, to judge by *Cdh6* expression (Fig.3.14 J).

*Lateral geniculate pronucleus.* We could not identify a lateral geniculate nucleus in the *Shh-c* thalamus.

*Medial geniculate pronucleus.* Preserved expression of *Calb2* and *Gbx2* (Fig.3.14 P, Q) but lost *Lhx2* and *Cdh6* in the mutant (Fig.3.14 R, T).

In summary, the medial pronucleus (including the intralaminar group, in our view) was comparatively little affected by lack of neural Shh, while the other four pronuclei showed major alteration.

| Pronuclei | Nuclei | Calb2 | Gbx2 | Lhx2 | Neurog2 | Cdh6 |
|---|---|---|---|---|---|---|
| Central | ATN | | | | (-) | ? |
| | VP | | | | ? | ? |
| | VM | | | ? | ? | ? |
| | VMb | ? | + | ? | | ? |
| Medial | PV, PT, MV | + | + | + | | |
| | MD | | + | | | |
| | CM | | + | + | | ? |
| | CL | | + | + | | |
| Dorsal | LD | ? | | (-) | (-) | |
| | LP | + | + | (-) | | ? |
| | Po | | | | | ? |
| Lat. Genic. | LGd | | | | ? | ? |
| Med. Genic. | MG | + | + | (-) | | ? |

**Table 3.3 Markers and nuclei in the *Shh-c* thalamus.**
? means "normal" marker expression in a morphologically altered part of the thalamus identified by its position along the AP and DV axes and comparison with wild type.
(-) means abnormal lack of expression
+ means normal expression in a recognizable structure.
Expression is usually present, but the morphology is very much altered in all pronuclei except the medial.

### 3.2.3.6 No thalamocortical axons in the Shh-c mutant

Extension of axons projecting to the cortex is an important differentiation trait of thalamic neurons (reviewed by (Lopez-Bendito and Molnar, 2003)). In order to explore this capability in the *Shh-c* mutant thalamus, we crossed our *Shh-c* line with the reporter mouse line Z/AP (Lobe et al., 1999). In these mice, *Foxb1*-expressing cells express also the gene for the enzyme human placental alkaline phosphatase (hPLAP). This protein attaches to the membrane and constitutes an excellent marker of axons (Fields-Berry et al., 1992; Gustincich et al., 1997; Leighton et al., 2001). *Foxb1-Cre* Z/AP mice showed staining of thalamocortical axons as expected (Fig.3.15A), while the *Shh-c* mutant had a completely blank cortex (Fig.3.15B). The axons had a place to pass to the telencephalon, albeit abnormal (arrow in Fig.3.15B). Sagittal sections of *Shh-c* thalamus labeled with Nissl or antineurofilament antibody 2H3 (not shown) showed tangles of axons in the thalamus, indicating that some cells could make axons but these could not find a way out. The external medullary lamina observed before (Fig.3.13 J, T) argues in the same direction.

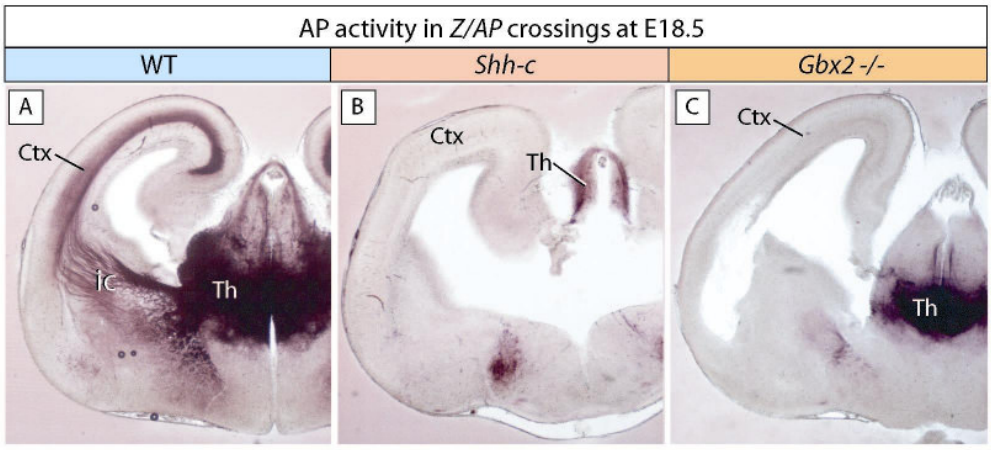


**Figure 3.15 No thalamic axons reach the cortex in the *Shh-c* brain.**
Detection of alkaline phosphatase activity in transverse sections of wild type (A), *Shh-c* (B) and *Gbx2 -/-* (C) brains at E18.5. The wild type cortex (A) shows abundant labeled thalamic axons in the cortex, while the *Shh-c* (B) and the *Gbx2 -/-* (C) cortices are completely unlabeled.
Abbreviations: Ctx: Cortex; IC: Internal Capsule; Th: Thalamus.

### 3.2.3.7 No regionalization defect in the Gbx2 mutant

*Gbx2* is a transcription factor specifically expressed in the mouse thalamic primordium starting at E11.5 (Bulfone et al., 1993a; Bulfone et al., 1993b; Hashimoto-Torii et al., 2003) and essential for thalamic development (Hevner et al., 2002; Miyashita-Lin et al., 1999). Since thalamic expression of *Gbx2* is under the control of *Shh* (Hashimoto-Torii et al., 2003), we were surprised to find a small domain of *Gbx2* expression in the mutant at E12.5 (Fig.3.12 G, H), as well as essentially normal thalamic expression at E18.5 (Fig.3.14 B, G, L, Q). We asked if this late, default expression of *Gbx2* in the absence of neural Shh was able to rescue some traits of the wild type phenotype in the *Shh-c* mutant, making its phenotype milder. Therefore, we wanted to compare the *Gbx2 -/-* thalamus with the *Shh-c* thalamus. We used a *Gbx2* mutant in which exon 2, containing the homeobox-coding sequence, has been deleted (Li et al., 2002).
First we detected regional marker genes at E12.5 to assess regionalization in this mutant. Probes against *Ptch1, Gli1, Nkx2-2, Lhx1, Emx2, Pax6, Dlx2, Dbx1 and Irx3* detected no changes in the regionalization of the caudal diencephalon, except for the smaller size of the thalamus (not shown). We then proceeded to analyze thalamic differentiation in the *Gbx2* mutant at E18.5.

### 3.2.3.8 Abolition of the medial pronucleus in the Gbx2 mutant

As was the case for the *Shh-c* mutant (see above), the *Gbx2*-deficient thalamus was smaller than normal, but has correct dorso-ventral and antero-posterior axes, as judged from the presence of a morphologically recognizable habenula (epithalamus) dorsally and the medial geniculate nucleus caudally (see below) (Fig.3.16, Table 3.4). Prethalamic derivatives are present in this mutant, making the thalamic region look less abnormal than in the *Shh-c*. The shape of the *Gbx2 -/-* thalamus has changed into a sort of truncated pyramid (very different from the shape of the *Shh-c* thalamus).

*Central pronucleus (anterior and ventral nuclear groups).* The anterior group, although very altered morphologically, showed ectopic *Calb2* expression (Fig.3.16 A), lost *Neurog2* and *Lhx2* (Fig.3.16 D, H) and preserved *Cdh6* expression (Fig.3.16 E). Of the

ventral group, only the most caudal nuclei, the ventromedial nucleus (Fig.3.16 I, N, J, O) and its basal portion (Fig.3.16 P, Q, R, T) seemed essentially unchanged.

*Medial pronucleus (medial and intralaminar nuclear groups).* Truncated, non-functional *Gbx2* mRNA can still be detected in the *Gbx2* mutant (see Methods), allowing for recognition of the medial pronucleus. This marker as well as *Lhx2* show complete morphological abolition of the medial and intralaminar groups in the *Gbx2* mutant (arrows in Fig.3.16 B, C, G, H, L, M).

*Dorsal pronucleus (lateral and posterior nuclear groups).* The laterodorsal nucleus had disappeared, while the lateral posterior preserved *Gbx2* expression (i.e. transcriptional activation of the mutated *Gbx2* locus) (not shown; Table 3.4) and *Cdh6* expression (Fig.3.16 O), but lost *Calb2* and *Lhx2* expression. The posterior nucleus preserved *Cdh6* (not shown; Table 3.4).

*Lateral geniculate pronucleus.* Preserved its two markers, *Neurog2* and *Cdh6* (Fig.3.16 N, O).

*Medial geniculate pronucleus.* Preserved all its markers, *Calb2* (Fig.3.16 P), *Gbx2* (not shown), *Lhx2* (Fig.3.16 R) and *Cdh6* (Fig.3.16 T).

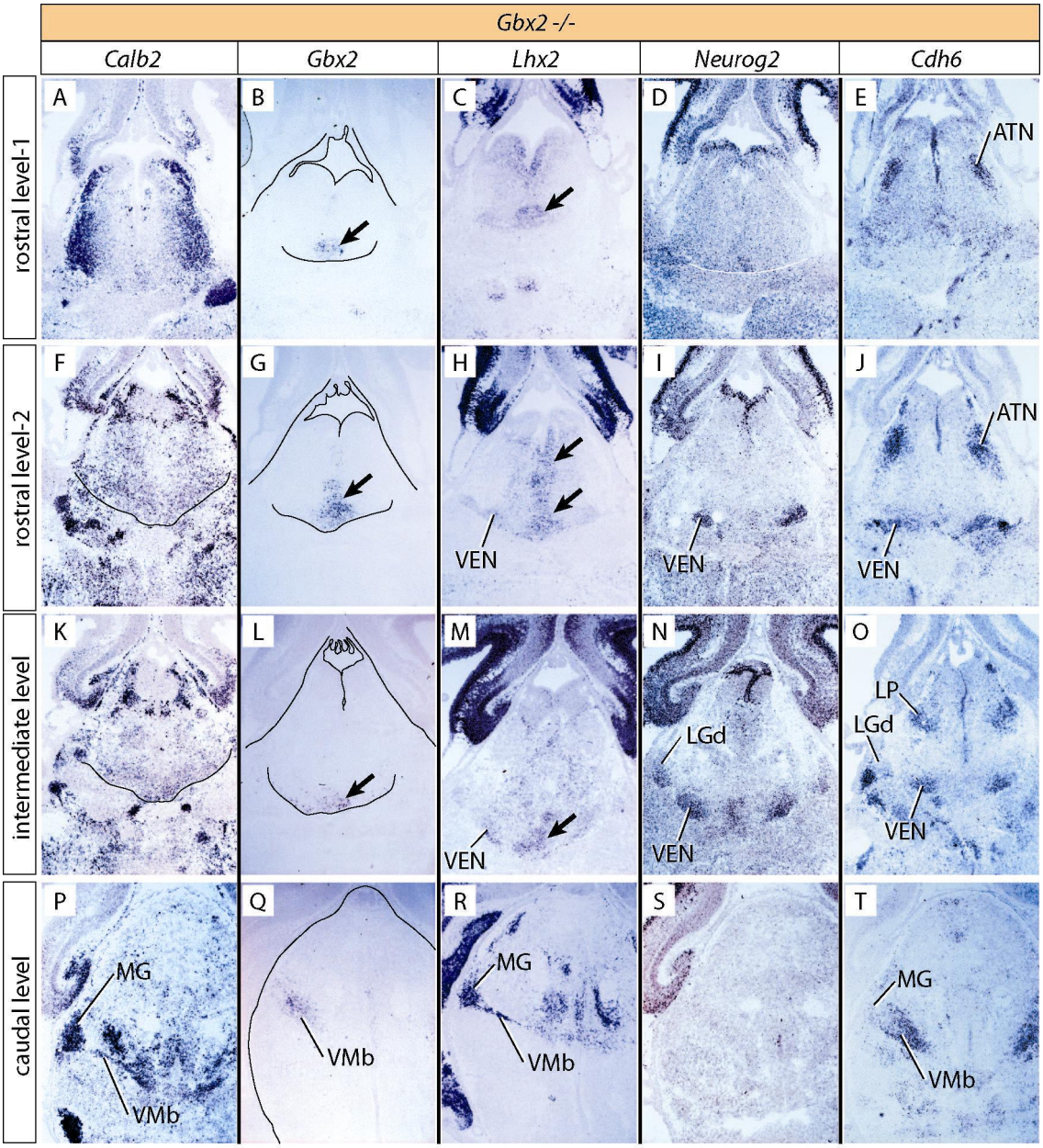


**Figure 3.16 Marker expression in the E18.5 *Gbx2* thalamus.**
In situ hybridization on E18.5 mouse brain sections for five marker genes as indicated on top of each column. Rows show sections at a given rostro-caudal level, as indicated on the left side, labeled for each of the five markers. Columns correspond to four rostro-caudal levels labeled with the same probe. The arrows in B, C, G, H, L, M indicate marker expression in morphologically abolished medial pronucleus.

| Pronuclei | Nuclei | *Calb2* | *Gbx2** | *Lhx2* | *Neurog2* | *Cdh6* |
|---|---|---|---|---|---|---|
| Central | ATN | ! | | | (-) | ? |
| | VEN | | | ? | ? | ? |
| | VMb | + | + | + | | + |
| Medial | PV, PT, MV | (-) | ? | ? | | |
| | MD | | ? | | | |
| | CM | | ? | ? | | ? |
| | CL | | ? | ? | | |
| Dorsal | LD | (-) | | (-) | (-) | |
| | LP | (-) | (-) | (-) | | + |
| | Po | | | | | + |
| Lat. Genic. | LGd | | | | + | + |
| Med. Genic. | MG | + | + | + | | + |

**Table 3.4 Markers and nuclei in the *Gbx2 -/-* thalamus.**
? means "normal" marker expression in a morphologically altered part of the thalamus identified by its position along the AP and DV axes and comparison with wild type.
(-) means abnormal lack of expression
+ means normal expression and the exclamation mark
! means ectopic expression.
Asterisk indicates ISH detection of the truncated, non-functional *Gbx2* mRNA in the *Gbx2* mutant.

### 3.2.4 Discussion

**Neural Shh and Gbx2 are required for the emergence of nuclear-specific phenotypical traits in fate-specified thalamic cells**

We have assessed the role of Shh generated in the ZLI and TG in thalamic development by eliminating all functional *Shh* expression in the diencephalic neuroepithelium and analyzing all thalamic nuclear groups with specific markers. The *Shh-c* thalamus shows important divergence between morphology and size on one hand and gene expression on the other --the neurons are in position and express mostly the marker genes, but do not form identifiable nuclei (this translates into many "question marks" but few "minus" signs in Tables 4.2 and 4.3). The most characteristic trait of fully differentiated neurons, their connectivity pattern, is also altered in the *Shh-c* thalamus. Formation of thalamocortical connectivity is controlled by regulatory cascades involving several essential transcription factors and effector genes (Lopez-Bendito and Molnar, 2003; Price et al., 2006). The *Shh-c* thalamus produces some axons which are not able to abandon the primordium (not shown) and do not reach the cortex (Fig.3.15 B). The *Gbx2* mutant thalamus is mostly unable to generate thalamocortical axons (Hevner et al., 2002; Miyashita-Lin et al., 1999) (Fig.3.15 C).
Therefore, the pronuclei depend on *Shh* and/or *Gbx2* for terminal differentiation, but not for earlier aspects of fate specification.

**Dissecting out the roles of neural and non-neural Shh in thalamic regional specification**

Combining our results with previous work clarifies the relative contributions of Shh of different sources to thalamic specification. The *Shh-c* mutant expresses thalamic marker *Dbx1* correctly (not shown), but full *Shh* mutant embryos lack specific thalamic expression of *Dbx1* (Ishibashi and McMahon, 2002), indicating that thalamic specification starts earlier than neural *Shh* expression and depends on non-neural Shh. This control has to be indirect, since the *Shh* pathway is not active in the presumptive dorsal diencephalon during gastrulation (as determined by *Ptch1* expression; not shown). Expression of transcription factor gene *Pax6*, essential for thalamic development (Pratt et al., 2002; Pratt

et al., 2000; Warren and Price, 1997), is activated independently from Shh of any origin (Ishibashi and McMahon, 2002).

After gastrulation, neural Shh signalling kicks in activating expression of *Gbx2*, *Nkx2-2* and *Sox14* and repressing expression of *Pax6* (Hashimoto-Torii et al., 2003; Ishibashi and McMahon, 2002; Kiecker and Lumsden, 2004) on a regionally specified thalamus.

Therefore, non-neural Shh (plus other factors) confers general thalamic specification to a region, and then neural Shh gets underway to move the thalamic specification cascade forward by activating expression of transcription factors responsible for final differentiation.

**The individual thalamic pronuclei show differential molecular identity**

We show that the morphologically defined thalamic pronuclei have distinct genetic identities which translate into specific combinations of markers. Expression of *Cdh6*, *Gbx2* and *Calb2* labels respectively the derivatives of the central, medial and dorsal pronuclei. The same markers, together with *Lhx2* and *Neurog2*, label certain derivatives of other pronuclei, as well as the lateral and medial geniculate pronuclei (Table 3.2). Since the marker genes encode effectors (*Cdh6*, *Calb2*) as well as transcription factors (*Lhx2*, *Neurog2*, *Gbx2*), pronuclear specificity can probably be found at every level in the differentiation network. The distinct genetic personalities of the pronuclei suggests early subdivision of the thalamic neuroepithelium into pronucleus-specific areas. This agrees with the separate neuroepithelial origins ("sublobules") for certain nuclear groups suggested by birthdating studies (Altman and Bayer, 1986). Since the intralaminar nuclear group was more similar to the medial group than to the anterior group in markers (Table 3.1) and phenotypes (see below; Tables 4.3, 4.4), we have included it in the medial pronucleus.

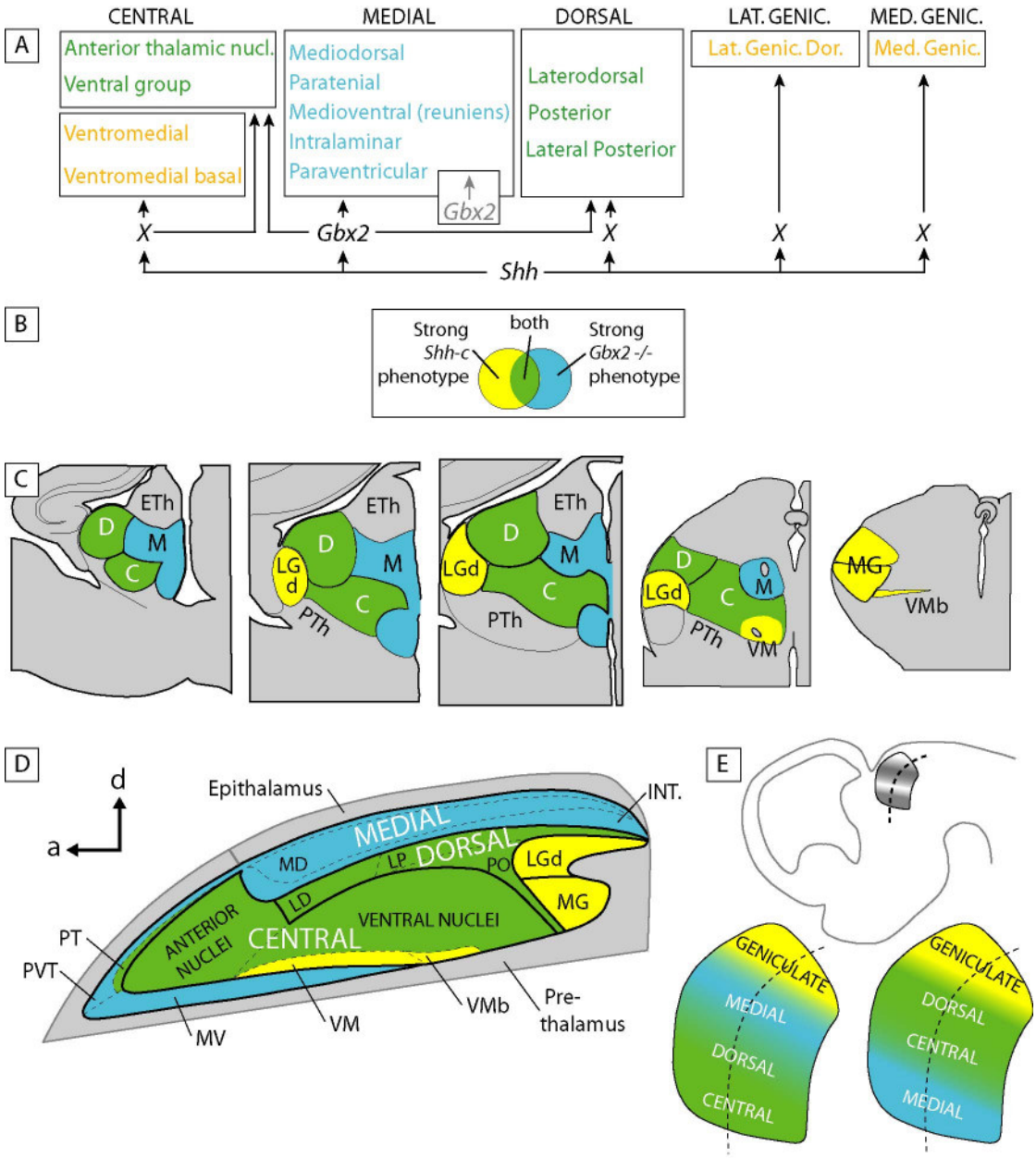


**Figure 3.17 Summary of results.** A) Involvement of *Shh*, *Gbx2* and unknown factors (*X*) in the differentiation of thalamic nuclear groups inferred from the mutant phenotypes. The *Gbx2* label in a closed box symbolizes default expression of *Gbx2*. We include the intralaminar nuclei in the medial pronucleus (see Results). Color code as in (B). B) Color code for this figure. C) Diagram showing the thalamic pronuclei in five rostro-caudal levels labeled after the severity of their phenotype in the *Shh-c* and *Gbx2* mutants. D) Dependence of thalamic nuclear groups on *Shh* and/or *Gbx2* mapped on a flat map (Swanson and Cowan, 1977) shows the groups are continuous. E) Localization of the thalamic neuroepithelium (upper panel) and the presumptive regions that will give rise to the different nuclear groups (lower panels). Dotted line, AP axis of thalamic primordium.

**The individual thalamic pronuclei show differential regulation**

We have also found differentially controlled pronuclear development, contributing to the distinctive "genetic personality" of each pronucleus. Three kinds of thalamic nuclei can be defined according to their dependence on *Shh* and *Gbx2* for their differentiation (Fig.3.17 A).

In both the *Shh-c* and the *Gbx2* mutant thalamus, the central and dorsal pronuclei showed severe morphological alteration. The exception is the ventromedial nucleus (VM + VMb), not very altered in the *Gbx2* mutant. The lateral and medial geniculate pronuclei are dependent on *Shh*, not *Gbx2*, for differentiation. Finally, in the *Shh-c* mutant, the medial pronucleus preserved general shape and gene expression, including expression of its specific marker *Gbx2*. Since *Gbx2* is initially expressed at E11.5 in the entire thalamic primordium downstream *Shh* (Hashimoto-Torii et al., 2003), this observation indicates delayed restricted default expression of *Gbx2* independent of Shh. Default *Gbx2* expression in the *Shh-c* mutant could be partially rescuing the wild type phenotype in the medial pronucleus. In agreement, the *Gbx2* mutant showed complete and specific morphological abolition of the medial pronuclear derivatives.

The resultant *Shh-Gbx2*-thalamic differentiation cascade (Fig.3.17 A) misses (at least) one component (*X* in Fig.3.17 A), that would be responsible for the specific effects of *Shh* independently of the default expression of *Gbx2* (which is restricted to the medial group). That component could simply be the early expression of *Gbx2* in the entire primordium.

**Prepattern in thalamus**

The pronuclei, labeled according to their differential *Shh* or *Gbx2* dependence (Fig.3.17 B), have a complex tridimensional relation to each other (Fig.3.17 C). However, when depicted on a "flat map" of the adult thalamus (Fig.3.17 D; after (Swanson and Cowan, 1977)), it is obvious that each of the three groups encompasses adjacent nuclei. This suggests a compartmentation of the thalamic primordium (Fig.3.17 E, upper panel) into three areas according to *Shh*-, *Gbx2*- or *Shh*-and-*Gbx2*-dependence (Fig.3.17 E, lower panels). Based on birthdate data (the anterior and dorsal group would originate more

rostrally than the medial group; (Altman and Bayer, 1986)), the left panel (Fig.3.17 E, lower left panel) would seem more likely.

In any case we have uncovered heterogeneity in the thalamic primordium leading to specific formation of nuclei. The existence of an early, Shh-independent pattern ("prepattern") in the spinal cord has been suggested to explain why the phenotype of *Gli* compound mutants is less severe than expected (Ruiz i Altaba et al., 2003). Thalamic prepatterning is carried out by Wnt activity, which activates *Irx3* (Braun et al., 2003) conferring to the thalamic neuroepithelium competence to respond to Shh (Kiecker and Lumsden, 2004). The default expression of *Gbx2* that we have uncovered here, responsible for the differentiation of two nuclear groups, suggests still other prepatterning influences at work in the thalamus, independent from neural Shh from ZLI or TG.

**Is neural Shh a morphogen for the thalamus?**

Shh from the notochord confers cell fate to the ventral spinal cord through positional information encoded in a concentration gradient (reviewed in (Dessaud et al., 2008; Jessell, 2000)), i. e., it acts as a classical morphogen (as defined for instance in (Lander, 2007; Wolpert, 1969)). Neural Shh (from the floor plate) does not act as a morphogen in the spinal cord or midbrain (Chamberlain et al., 2008; Ding et al., 1998; Matise et al., 1998; Roelink et al., 1995). Therefore it would be an exception if neural Shh was a morphogen in the thalamus. Shh expression in the ZLI appears too late (5-7 somites; (Shimamura and Rubenstein, 1997)) to be involved in the fate specification of the principal brain regions, which occurs during gastrulation (reviewed by (Fraser and Stern, 2004)). In agreement, the onset and maintenance of thalamic *Dbx1* expression is independent from neural Shh (see above).

Other evidence against neural Shh acting as morphogen in the thalamus: expression of *Gbx2* on the entire thalamic primordium obeys to one single Shh concentration threshold (Hashimoto-Torii et al., 2003); ectopic *Shh* expression in the thalamus causes ectopic *Gbx2* expression but does not rostralize the primordium (Vieira et al., 2005); and blocking Shh release from the ZLI causes some growth and differentiation alterations, but does not caudalize the primordium (Vieira and Martinez, 2006). Consistent with those reports, we have not detected "directionality" (e.g. rostral-to-caudal fate changes) in the thalamic defects of the *Shh-c* mutant.

Finally, since *Gbx2* default expression in the *Shh-c* mutant can rescue the medial and intralaminar groups, it follows that these nuclei undergo cell fate and positional specification in the absence of neural Shh. That a nuclear group situated in the middle of the thalamus be controlled only by non-Shh-dependent *Gbx2* expression contradicts any simple gradient hypothesis, so much if the *Gbx2*-dependent area is located in the center of the primordium as if it is located at the rostral end (see Fig.3.17 E, lower panels).

### 3.2.5 Authors' contributions

I generated/maintained the following mouse mutant lines: *Shh-c*, *Shh-c*-Z/AP, Gbx2 and Gbx2-FoxCre-Z/AP.

The following experiments were performed by me:

- Collection of all embryos, generation of all the probes and subsequently the whole mount *in situ* hybridizations
- Collection of all embryos, cutting of the sections of the embryos and generation of the probes for ISH on sections

# 4 General Discussion

## 4.1 Dissecting the role of prechordal vs neural Shh

During mouse embryonic development *Shh* is expressed in the notochord and prechordal plate from E7.5 on and activates its own expression in the ventral anterior neural plate at E8.5. The differential contributions of these two sources of *Shh* in forebrain development are still elusive. By crossing the Foxb1 Cre line with the floxed Shh line we were able to remove the expression of functional Shh specifically from the anterior neural plate and forebrain from E8.5 on, leaving the expression in the prechordal plate and notochord intact (Fig.3.1). In this way we were able to analyze the functions of neural Shh, which has a very dynamic expression pattern in the forebrain, focusing on hypothalamic development (rostral diencephalon) in the first manuscript and on thalamus development (caudal diencephalon) in the second manuscript.

## 4.2 Neural Shh in hypothalamic development

Shh from the prechordal mesendoderm is necessary for the partition of the eye field (Chiang et al., 1996). It is furthermore required for hypothalamic specification by inducing the ventral forebrain marker *Nkx2-1* (Pera and Kessel, 1997). *Nkx2-1* demarcates the ventral forebrain, which later becomes the hypothalamus and hypophysis and is essential for their formation (Kimura et al., 1996). The analysis of the *Shh-c* mutant showed that there was general regional specification of the hypothalamus as shown by *Nkx2-1* expression in the forebrain at E9.5 (Fig.3.3 B), which was induced by non-neural Shh. Also the Shh pathway was activated in the neural plate of the *Shh-c* mutant by Shh secreted from the prechordal plate (Fig.3.2). But as development proceeds the Shh pathway in the neural plate seems to become more dependent on neural sources of Shh since the pathway was gradually lost in the absence of neural Shh in the *Shh-c* mutant. This subsequently led to the progressive alterations in the forebrain of the *Shh-c* embryo (Fig.3.3). Further analysis of the *Shh-c* embryo showed that neural Shh is essential for the specification of the hypothalamic subregions and for growth.

The results demonstrate that the three subregions of the hypothalamus (the AD, PV and LH) show differential requirements for neural Shh.

In the LH neural Shh activates *Dbx1* and *Dlx2* and specifies Hypocretin/Orexin neurons. Hypocretins/Orexins are highly excitatory neuropeptide hormones produced by a small population of cells in the lateral hypothalamus that send projections throughout the brain. These hormones are involved in stimulation of food intake, wakefulness and energy expenditure.

In the PV neural Shh activates *Dbx1* and maintains *Nkx2-1* expression since it disappeared mostly in that region at E11.5 in the mutant (Fig.3.3 D).

At E18.5 *Nkx2-1* expression in the PV is restricted to the MBO. In the *Shh-c* mutant *Nkx2-1* expression could still be detected in that region albeit reduced, but *Foxb1* and *Calb1*, also markers of the MBO, had disappeared showing impaired specification of the PV in the *Shh-c* mutant.

However, Shh is not necessary for the activation of *Foxb1* since *Foxb1* was expressed in the *Shh* null mutant (Fig.3.1 J). Therefore, Shh seems to be required for maintenance of *Foxb1* expression between E9.5 and E12.5 which could be demonstrated with explant experiments (Fig.3.10). Therefore, neural Shh is not only required for the differentiation but also the survival of the PV since Foxb1 was shown to be essential for the survival of MBO neurons (Alvarez-Bolado et al., 2000b).

A negative regulator of Foxb1 is Wnt8b, which is expressed in the neuroepithelium of the PV and has been shown to regulate neurogenesis in the zebrafish hypothalamus (Lee et al., 2006). Wnt8b seems to counterbalance the positive effect of Shh by downregulating *Foxb1* expression in the PV neuroepithelium thereby restricting it to the MBO neurons.

Therefore, this work uncovers an interplay of neural Shh with Wnt8b downstream of Nodal to maintain hypothalamic survival pathways.

In contrast to the PV, the AD was correctly specified as shown by marker expression in its major nuclei (Fig.3.9). But the nuclei just like the whole hypothalamus were very much reduced in size. This is in agreement with data showing that Shh is required for CyclinD1 expression and the subsequent growth of both ventral and dorsal regions of the diencephalon (Ishibashi and McMahon, 2002). Also in the telencephalon Shh regulates proliferation and survival of neurons (Komada et al., 2008). Manning et al. reported that Shh promotes proliferation of unspecified precursors in the chicken hypothalamus (Manning et al., 2006). Subsequently *Shh* downregulation is required for the progression of the precursors to hypothalamic progenitors and their further proliferation. A possible

explanation of the *Shh-c* phenotype therefore would be that in the absence of Shh the precursors do not expand but become specified as hypothalamic progenitors too early. The subsequent proliferation of these hypothalamic progenitors results only in a small hypothalamus. It is possible that additionally increased cell death is also responsible for the reduced size of the hypothalamus.

## 4.3 Neural Shh is required for cell fate maintenance

In the *Shh-c* mouse the abnormal alterations progressively accumulate as the brain grows. The full extent of the loss of neural Shh was most apparent when the brain was fully differentiated at E18.5. In sagittal sections an enlargment of the third ventricle and the separation of the two hypothalamic parts, the AD and PV, by an abnormal cortical structure was observed.

The separation of AD and PV in the *Shh-c* mutant can be well explained by fate mapping done in zebrafish (Staudt and Houart, 2007). The neural plate contains distinct domains with well-defined cell populations that develop into specific forebrain regions (Fig.4.1). The precursors of a given forebrain region are located in a very defined area, implying very little or no cell mixing during early brain development and express a certain set of genes showing that those cells are strictly committed to distinct fates already in the nascent neuroepithelium. In zebrafish the ZLI is specified as a medial compartment between AD and PV and with the prethalamus laterally on each side (Fig.4.1 A). Later, the AD and PV grow and merge into one single hypothalamic structure but they still show different expression patterns and functions (Mathieu et al., 2002).

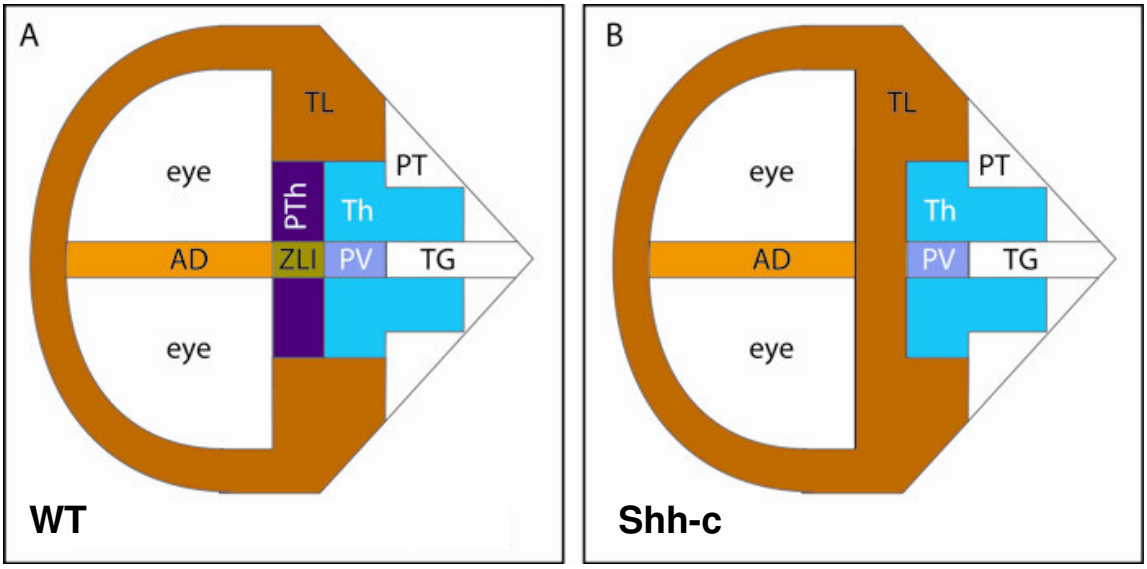


**Figure 4.1 Neural Shh is required to maintain and stabilize fate specification in the diencephalon.** A) Fate map in the neural plate of the zebrafish showing the distinct domains that develop into specific brain regions. The AD and PV of the hypothalamus are separated by the domain giving rise to the ZLI. B) In the *Shh-c* mutant the prethalamus/ ZLI compartment is incorrectly specified and acquires cortical fate during development because of lacking stabilizing influences. Therefore cortical tissue separates the AD from the PV in the *Shh-c* mutant (Picture adopted and simplified from (Staudt and Houart, 2007)). Abbreviations: AD: anterior-dorsal hypothalamus; PT: Pretectum; PV: posterior-ventral hypothalamus; TG: Tegmentum; Th: Thalamus; TL: Telencephalon;

In the *Shh-c* mutant the prethalamus was detected by *Lhx5* expression at E9.5 which is in agreement with Staudt and Houart who reported that prethalamic specification does not depend on Shh (Staudt and Houart, 2007). However, in the *Shh-c* mutant the prethalamus was only incompletely specified since it failed to express other prethalamic markers like *Dlx2* and *Pax6*. It furthermore failed to grow, so that by E18.5 it could not be identified anymore. The ZLI, which normally appears at E10.0 was abolished in the *Shh-c*. This is probably due to the loss of *Shh* expression in that region since the ZLI was shown to require *Shh* expression for self-maintenance by a positive feedback mechanism (Kiecker and Lumsden, 2004). Therefore, in the absence of neural Shh the prethalamus/ZLI compartment was not correctly specified, failed to be maintained and disappeared completely at a later stage (Fig.4.1 B). The results are also in agreement with reports showing that the ZLI and its signaling component Shh are necessary for the maintenance of prethalamic identity (Kiecker and Lumsden, 2005; Scholpp et al., 2006).

Not only the loss of the prethalamus/ZLI compartment but also the reduced growth of the hypothalamic subregions possibly account for the separation of the AD and PV and the enlargment of the third ventricle in the *Shh-c*.

Instead the extension of the cortex all the way through the hypothalamus was observed as shown by *Emx1* expression (Fig.4.1 B). *Emx1* is normally restricted to the dorsal telencephalon corresponding to the neocortex and is suppressed by Shh (Cecchi and Boncinelli, 2000; Chiang et al., 1996; Kuschel et al., 2003).

The results show that there is general regional fate very early in the neural plate of the *Shh-c* probably conferred by non-neural sources of Shh and other factors. But neural Shh seems to be required to stabilize and maintain that fate specification. Gene regulatory networks (GRNs), which are comprised of several TFs, are involved in fate acquisition. They secure the progression of development by stabilizing and locking cells into certain specification states. In the absence of neural Shh this is not achieved so that the prethalamic/ZLI region is taken by an alternative fate, in this case by cortical fate because of dorsalizing influences. These observations also emphasize the similarity of fate maps in vertebrates.

## 4.4 Gli3 vs Shh in diencephalic patterning

In the ventral spinal cord all the effects of Shh are mediated by the combined activity of the three Gli transcription factors (Lei et al., 2004). Gli3 acts primarily as a transcriptional repressor whereas Gli1 and Gli2 function as activators. Gli activity is also the mediator of patterning in the diencephalon since the diencephalon is partially absent in the *Gli1/Gli2* deficient mutant (Park et al., 2000). However, the *Gli1/Gli2* double homozygous mutants have a considerably milder phenotype than *Shh* mutants showing that these two transcription factors are not required in all tissues downstream of Shh. These double mutants have for example no major telencephalic defects. Instead, the repressor form of Gli3 is primarily important for telencephalic cell specification (Fig.4.2). It is expressed in the dorsal telencephalon, whereas *Shh* is expressed ventrally in the MGE and the balance between these two genes is crucial in the establishment of dorso-ventral patterning within the telencephalon (Rallu et al., 2002a). In the *Xt/Xt* mutants, which carry a null mutation of the *Gli3* gene, ventral telencephalic markers expand dorsally into the cortex whereas in the *Shh* deficient mice the telencephalon is strongly dorsalized. Interestingly, in the absence of both *Shh* and *Gli3* general aspects of dorso-ventral patterning are restored (Rallu et al., 2002b). On the one hand this shows that there are other pathways acting in

the establishment of dorso-ventral patterning in the telencephalon and on the other hand this demonstrates that Shh acts indirectly through the inhibition of Gli3 repressor activity.

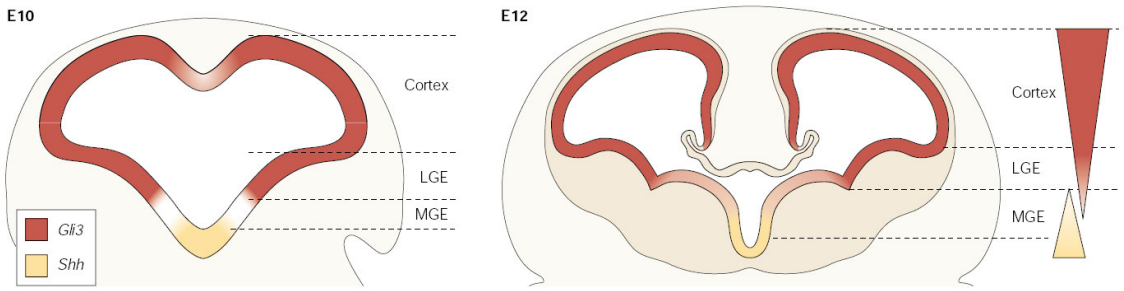


**Figure 4.2 Interaction between Shh and Gli3 in patterning of the mouse telencephalon.** The drawing is showing a coronal section through a mouse telencephalon at E10 on the left and E12 on the right. The expression domain of *Gli3* is marked in red, the *Shh* expression domain ventrally in yellow. Their expression pattern is complementary and they antagonize one another during development (Rallu et al., 2002a). Abbreviations: LGE: lateral ganglionic eminence; MGE: medial ganglionic eminence.

Therefore Shh signaling functions negatively to prevent dorsalization of ventral telencephalic domains. This explains why in the *Shh-c* mouse dorsalizing influences probably mediated by Gli3 are not repressed and cortical tissue is able to grow inside the diencephalon (Fig.4.1 B). This cortical tissue showed *Emx1* expression, which is a marker of cortical identity. The *Gli3* gene has been indicated as an upstream regulator of *Emx* genes (*Emx1* and *Emx2*) in controlling dorsal telencephalon development since both *Emx* genes were reported to be absent in the dorsal forebrain of the *Xt/Xt* mutant mouse (Theil et al., 1999). This is in favor of *Gli3* being the dorsalizing influence in the *Shh-c* mouse. Interestingly, analysis of the *Xt/Xt* mutant showed an enlargement of the prethalamus/ZLI compartment and the thalamus while the hypothalamus seemed normal (Fig.3.6). This is demonstrating that the antagonism between Shh and Gli3 is reciprocal in the diencephalon since gene expression domains expand dorsally in the absence of *Gli3* gene function. Surprisingly, even the ventral expression domain of *Emx2* was enlarged in the *Xt/Xt* mutant with new expression in the ZLI suggesting that there is differential regulation of *Emx2* expression in the dorsal and ventral forebrain.

## 4.5 Neural Shh in thalamic development

*Shh* is expressed ventrally in the diencephalic tegmentum and lateral hypothalamic band, encompassing the thalamus and prethalamus respectively. *Shh* in the ZLI extends dorsally abutting thalamus and prethalamus (Fig.1.3). The ZLI was reported to function as a signaling center by releasing Shh and thereby regulating the acquisition of identity for these regions (Kiecker and Lumsden, 2004). This is achieved by the distinct competence of prethalamus and thalamus for the same signaling molecule leading to different responses of the cells depending on their intrinsic properties. This competency is conferred by the adjacent but nonoverlapping expression patterns of *Six3* and *Irx3*, which encode Iroquois-type transcription factors and demarcate the location of the ZLI. *Six3* is expressed anteriorly above the prechordal plate and its expression results in the induction of anterior forebrain-specific genes like *Dlx2* whereas *Irx3* is expressed posteriorly to the ZLI and leads to expression of posterior forebrain-specific genes like *Gbx2* (Kobayashi et al., 2002) (Fig.4.3).

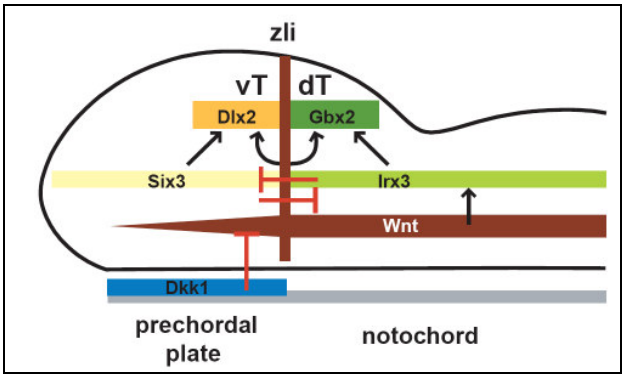


**Figure 4.3 Anteroposterior patterning in the diencephalon.** A Wnt signal induces the expression of *Irx3* posterior to the presumptive ZLI. This leads to the expression of *Gbx2* in the dorsal thalamus by signals released from the ZLI. The prechordal plate secretes Wnt antagonists that lead to expression of *Six3* and subsequently *Dlx2* in response to ZLI derived signals. *Six3* additionally represses the expression of *Irx3*, *Wnt* and *Gbx2*. Abbreviations: vT: ventral thalamus or prethalamus; dT: dorsal thalamus or thalamus; zli: zona limitans interthalamica.

It was shown in chicken that Wnt signalling is sufficient to induce *Irx3* expression and suppress *Six3* expression, thereby demonstrating an important role of Wnts in early anteroposterior patterning (Braun et al., 2003). Braun et al. also showed that a Wnt signal is necessary and sufficient for specifying thalamic identity by inducing *Irx3* and *Gbx2* expression. Inhibiting the Wnt response caused prospective thalamic tissue to acquire a prethalamic fate (Braun et al., 2003).

In the *Shh-c* mutant anteroposterior patterning was established and the thalamus specified as shown by *Dbx1*, *Irx3*, *Irx1* and the downregulation of *Pax6* expression. Even *Gbx2* was

expressed in the mutant thalamus only in a smaller domain at E12.5 but which expanded during development. This was surprising since Gbx2 has also been reported to be regulated by Shh signaling from the ZLI (Kiecker and Lumsden, 2004). Therefore, thalamic regional identity is acquired in the absence of neural Shh or any other ZLI-derived signals, possibly due to Wnt signalling and/or non-neural Shh. This also demonstrates that the forebrain region anterior to the ZLI is more dependent on neural Shh for regional identity than the region posterior to it.

### 4.5.1 Neural Shh in thalamic differentiation

Neurogenesis of the thalamus starts at E10.5 for cells that form the caudal thalamus, while the neurons of the more rostral and medial thalamic nuclei start to be born only 1-2 days later (Angevine, 1970). Several regulatory genes have been shown to be expressed in distinct but overlapping patterns in the thalamus suggesting that they cooperate to control the specification and differentiation of thalamic nuclei. The differential expression patterns of the genes which mark the nuclei at later ages are already seen at E12.5 or earlier (Nakagawa and O'Leary, 2001) and their expression patterns correlate with the histologically defined borders of nuclei. Therefore, they seem to negatively regulate each other to prevent mixing of cells and to remain as distinct cell groups (Hashimoto-Torii et al., 2003; Nakagawa and O'Leary, 2001). This combinatorial expression pattern of regulatory genes parcellates the thalamus and is reminiscent of the GRNs of the spinal cord that establish dorsoventral patterning.

To characterize the thalamus of the *Shh-c* mutant a set of thalamic markers with distinct but overlapping expression patterns was used. Expression of the markers was still present albeit reduced and the genes showed distinct expression patterns as in the wiltype thalamus suggesting parcellation of the mutant thalamus. However, many nuclei failed to differentiate correctly, demonstrating a major requirement for neural Shh in thalamic differentiation. The least affected nucleus in the mutant with preserved morphology and marker expression, including expression of *Calb2*, *Lhx2* and *Gbx2,* was the medial pronucleus. *Gbx2* was reported to be expressed in a subset of nuclei of the thalamus which require it for their differentiation, as well as for the development of the TCA projections (Hevner et al., 2002; Miyashita-Lin et al., 1999). Interestingly, all nuclei that showed *Gbx2* expression in the wt, also preserved *Gbx2* expression in the mutant. It is possible

that these *Gbx2* positive neurons were also able to send thalamocortical axons in the *Shh-c* mutant but due to the abnormal environment with a lack of regions and guidance cues and probably due to defects in the thalamic cells themselves they were not able to reach the cortex.

By analyzing the thalamus of *Gbx2* deficient mice it was demonstrated that the right differentiation of the medial pronucleus in the *Shh-c* was mediated or rescued by the expression of *Gbx2* since the complete morphological abolition of the medial pronucleus was observed in the *Gbx2* mutant. Besides that, the dorsal pronucleus and part of the central pronucleus were affected in both, the *Gbx2* and the *Shh-c* mutant, demonstrating requirement of both factors for correct differentiation of these nuclei. Therefore, three different kinds of pronuclei could be defined according to their dependence on either *Shh*, *Gbx2* or both (Fig.3.17 A).

### 4.5.2 Does Shh act as a morphogen in the thalamus?

A morphogen is defined as a secreted substance that emanates from a localized source and spreads through the tissue to establish a gradient of activity. Therefore it acts on cells at a distance from its source by inducing differential gene expression in a concentration-dependent manner (Dessaud et al., 2008). These criteria are met by Shh signaling from the notochord. It forms a dynamic gradient in the neural target field and induces ventral cell identities in the adjacent neural tube by a concentration-dependent mechanism (Chamberlain et al., 2008). It furthermore induces a second organizing center of Shh activity within the floor plate at the ventral midline of the neural tube. Interestingly, *Gli2* mutants lack a floor plate in the spinal cord and therefore this second center of Shh activity. However, they are still capable of specifying all primary Shh dependent neural progenitor populations demonstrating that notochord derived Shh is primarily responsible for setting up dorsoventral pattern in the spinal cord (Matise et al., 1998). Neural Shh therefore seems to have different functions than non-neural Shh in the spinal cord. It has been suggested that the induction of neural *Shh* expression in the floor plate of the spinal cord normally serves an autocrine function to inhibit floor plate cell differentiation. It has also been shown in the telencephalon that neural Shh does not act in a concentration-dependent manner as non-neural Shh from the notochord but rather induces fates depending on the timing of Shh exposure. Therefore, temporal changes in the competence of cells to respond to the same signal lead to basic ventral telencephalic patterning (Kohtz

et al., 1998). In the thalamus neural Shh does not seem to act as a morphogen either since a concentration-dependent effect could not be observed in the *Shh-c* thalamus with regions closer to the *Shh* expression domain being more affected than regions further away. The rostral thalamic nulei were affected to the same extend as the caudal nuclei in the *Shh-c* mutant. Neither a caudalization of the mutant thalamus was observed. Therefore, it seems likely that in the thalamus the timing of Shh exposure is rather crucial in specifying different nuclei. This is in agreement with the duration of neurogenesis of thalamic cells from E10.5 to E14.5 and caudal nuclei being born 1-2 days earlier than rostral and medial nuclei.

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# 6 Abbreviations

| | |
|---|---|
| A | Adenine |
| ac | anterior commissure |
| AD | anterior-dorsal hypothalamus |
| AP | alkaline phosphatase |
| ATP | Adenosine triphosphate |
| bar | branchial arch |
| BCIP | 5-bromo-4-chloro-3-indolyl-phosphate |
| bp | base pairs |
| C | Cytosine |
| °C | degree celsius |
| cDNA | complementary deoxyribonucleic acid |
| CMV | cytomegalovirus |
| CNS | central nervous system |
| Ctx | cortex |
| D | day |
| DEPC | Diethylpyrocarbonate |
| DIG | Digoxigenine |
| DMSO | Dimethylsulfoxid |
| DNA | deoxyribonucleic acid |
| dNTP | deoxynucleotide triphosphate |
| DTT | Dithiothreitol |
| EtOH | Ethanol |
| E | Embryonic day |
| e.g. | exempli gratia (Latin; for example) |
| EGFP | enhanced green fluorescent protein |
| ES | embryonic stem cells |
| FB | forebrain |
| for | forward primer |
| G | Guanine |
| GFP | green fluorescent protein |
| GRN | gene regulatory network |
| h | hour |
| HB | hindbrain |
| hPLAP | human placental alkaline phosphatase |
| Hy | hypothalamus |
| IC | internal capsule |
| IRES | internal ribosomal entry site |
| ISH | *in situ* hybridization |
| kb | kilobases |
| ko | knockout |
| L | liter |
| lacZ | ß-galactosidase |
| LB | Luria-Bertani Medium |
| LH | lateral hypothalamus |
| M | molar |
| MB | midbrain |
| MBO | mammillary body |
| MeOH | Methanol |
| mg | milligram |
| MGE | medial ganglionic eminence |
| min | minute |

| | |
|---|---|
| ml | milliliter |
| mM | millimole |
| mtc | mammillotectal tract |
| mtg | mammillotegmental tract |
| mtt | mammillothalamic tract |
| NBT | 4-nitro-blue-tetrazoliumchloride |
| NEB | New England Biolabs |
| neo | neomycin |
| ng | nanogram |
| NP40 | nonyl phenoxylpolyethoxylethanol |
| NTP | Nucleotide triphosphate |
| OD | optical density |
| o/n | overnight |
| os | optic sulcus |
| oto | otocyst |
| P | postnatal day |
| PBS | phosphate buffered saline |
| PBT | PBS with Tween-20 |
| PCP | prechordal plate |
| PCR | polymerase chain reaction |
| PFA | Paraformaldehyde |
| pH | potential Hydrogenii |
| pm | picomole |
| PT | pretectum |
| Ptch1 | patched1 |
| Pth | prethalamus |
| PV | posterior-ventral hypothalamus |
| PVN | paraventricular nucleus |
| rev | reverse primer |
| RNA | ribonucleic acid |
| rpm | rounds per minute |
| RT | room temperature |
| RT | Reverse transcriptase |
| RT-PCR | Reverse transcriptase polymerase chain reaction |
| SC | superior colliculus |
| sec | seconds |
| Shh | Sonic hedgehog |
| *Shh-c* | Shh conditional mutant |
| sub | suboptical domain |
| T | Thymine |
| TAE | Trisacetate-EDTA |
| TCA | thalamocortical axons |
| TD | touchdown PCR |
| TE | Tris-EDTA |
| TF | transcription factor |
| TG | tegmentum |
| TH | thalamus |
| TL | telencephalon |
| µg | microgram |
| µl | microliter |
| U | Unit |
| V | Volt |
| VL | lateral ventricle |
| wt | wild type |
| X-Gal | 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside |

| | |
|---|---|
| ZLI | zona limitans interthalamica |

## Curriculum Vitae

| | | |
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# Publications

Zhao T, Kraemer N, Oldekamp J, Cankaya M, **Szabó N**, Conrad S, Skutella T, Alvarez-Bolado G.
**Emx2 in the developing hippocampal fissure region**
Eur J Neurosci. 2006 Jun;23(11):2895-907.

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Zhao T*, **Szabó N***, Ma J, Luo L, Zhou X and Alvarez-Bolado G.
**Genetic mapping of Foxb1-cell lineage shows migration from caudal diencephalon to telecephalon and lateral hypothalamus**
Eur J Neurosci., **Accepted**
* Equal contribution

**Szabó N**, Zhao T, Çankaya M, Theil T, Zhou X and Alvarez-Bolado G.
**Neuroepithelial *Sonic hedgehog* is essential to specify the hypothalamic subregions and to stabilize diencephalic against telencephalic fate**
Submitted

**Szabó N**, Zhao T, Zhou X and Alvarez-Bolado G.
**The role of *Shh* of neural origin in thalamic differentiation in the mouse**
Submitted

## Conference contributions

**Szabó N**, Zhao T, Zhou X and Alvarez-Bolado G.
**Sonic hedgehog in mouse diencephalic regionalization**
Horizons in Molecular Biology- 4th International PhD student Symposium, Göttingen, Sept.2007
Abstract and Poster

Zhao T, Zhou X, **Szabó N**, Leitges M and Alvarez-Bolado G**.**
**Foxb1-driven Cre expression in somites and the neuroepithelium of diencephalon, brainstem, and spinal cord**
Horizon in molecular biolgy - 4Th International PhD student Symposium, Göttingen, Sept 2007,
Abstract and Poster

**Szabó N**, Zhao T, Zhou X and Alvarez-Bolado G.
**Roles of Sonic hedgehog of neuroepithelial origin in hypothalamic development**
Horizons in Molecular Biology- 5th International PhD student Symposium, Göttingen, Sept.2008
Abstract and Poster

## Erklärung

Hereby I declare that I did all of the work described in this thesis myself and that I indicated all means that were used and help that was provided by others. The results included in this work were never used for another thesis (e.g. Diploma) and, except the indicated partial publications, have not been published otherwise.

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Nora Szabó

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