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THE MORPHOGENETIC ROLE OF WNT1 IN THE DIENCEPHALIC REGIONALIZATION

Tesis Doctoral presentada por

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LIST OF ABBREVIATIONS

Arx: Aristaless related homeobox

Ascl1: Achaete-scute complex homolog 1

Bmp4: Bone morphogenetic protein family 4

Bmp7: Bone morphogenetic protein family 7

Dbx1: Developing brain homeobox 1

DCC: Deleted in Colorectal Cancer

EGFP: Enhanced green fluorescent protein

Emx1: Empty spiracles homeobox 1

Fgf8: Fibroblast growth factor family 8

Fgf15: Fibroblast growth factor family 15

Fgf19: Fibroblast growth factor family 19

Gbx2: Gastrulation brain homeobox 2

GFP: Green fluorescent protein

Gli1: Glioma-associated oncogene homolog 1

Gli2: Glioma-associated oncogene homolog 2

Gli3: Glioma-associated oncogene homolog 3

Hh: Hedgehog family

Irx3: Iroquois homeobox 3

L-fng: Lunatic fringe

Msx1: Msh homeobox 1

Ngn2: Neurogenin 2

Nkx2.2: NK2 homeobox 2

Otx2: Orthodenticle homolog 2

Pax6: Paired box 6

PH3: Phosphohistone-3

Shh: Sonic hedgehog

Six3: Sine oculis-related homeobox 3

Tcf4: Transcription factor 4

Wnt1: Wingless-Int protein family 1

Wnt2b: Wingless-Int protein family 2b

Wnt3a: Wingless-Int protein family 3a

Wnt5a: Wingless-Int protein family 5a

Wnt8b: Wingless-Int protein family 8b

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Abstract

The diencephalon is a complex brain area which derives from the caudal region of the prosencephalon. This structure is divided in horizontal zones along the dorso-ventral axis. The mechanisms that govern the formation of this axis during development remain unclear. Different signaling molecules have been described as involved in this process, known as morphogenes, which induce a specific neural fate to the neighbouring epithelium. In order to demonstrate that one of these signaling molecules, Wnt1, has a critical role in the correct establishment of the diencephalic territories, we carried out gain- and loss-of-function experiments. Our results indicate that Wnt1 signal has a key role in the structuration of the pretectal territories during embryonic development, as well as in the correct development of some diencephalic dorsal structures. Moreover, we demonstrate that Wnt1 signal is required for the diencephalic dorso-ventral regionalization. We further show that Wnt1 is directly induced by Bmp4, unraveling the molecular regulatory code modulating this regionalization in the diencephalon.

Resumen

El diencéfalo es una compleja región del cerebro que deriva del prosencéfalo caudal. Esta estructura esta subdividida en zonas horizontales en el eje dorso-ventral. Los mecanismos que gobiernan la formación de este eje durante el desarrollo no se conocen aún en profundidad. Diferentes moléculas de señalización, conocidas como morfógenos, han sido implicadas en este proceso, las cuales inducen destinos neurales específicos en el epitelio adyacente. Con el objetivo de demostrar que una de esas moléculas señalizadoras, Wnt1, tiene un papel fundamental en el correcto establecimiento de los territorios diencefálicos, hemos llevado a cabo experimentos de ganancia y pérdida de función. Nuestros resultados indican que la señal de Wnt1 tiene un papel fundamental en la estructuración de los territorios pretectales durante el desarrollo embrionario, así como en el correcto desarrollo de determinadas estructuras dorsales del diencéfalo. Además, demostramos que la señal de Wnt1 es necesaria para la regionalización dorso-ventral del diencéfalo, teniendo un papel fundamental en este proceso. También mostramos que Wnt1 es directamente inducido por Bmp4, revelando el código regulatorio molecular que modula la regionalización del diencéfalo.

Chapter I

INTRODUCTION

I.1. Development of the neural plate and tube

The process of generating the neural tube involves a complex mechanism dependent on inductive molecules derived from specific signaling centers. This process is called neural induction and neurulation and it occurs in four steps: formation of the neural plate, shaping of the neural plate, bending of the neural plate and closure of the primitive neural tube (Colas and Schoenwolf, 2001). All these stages are harmonized with particular movements of the primitive strike, whose caudal progression will lead to the correct formation of the primitive neural tube. Then, to better understand the molecular interactions and cellular contributions that take place during neurulation, it is necessary to date back to the early steps of gastrulation.

The precursor of the brain is a planar lamina of pseudostratified neuroepithelium induced during the gastrulation in the central region of the epiblast (the original epithelium that will generate the three blastodermal layers) (Stern et al., 2006). This lamina is known as neural plate, where vertical and planar signals from neighboring regions lead to the specification of its antero-posterior and dorso-ventral axes, which will be maintained in the embryo.

Following the process of neurulation, the neural plate will be converted in the neural tube. Spemann and Mangold (1924) described the cellular processes underlying neural induction in amphibian embryos. They performed grafting experiments between differently pigmented species in order to clarify the interactions established between the blastopore and the neighboring ectoderm. Spemann named the blastopore as “organizer” and demonstrated that this region induces the nervous system in the adjacent ectoderm. In the absence of the induction from this blastopore, they showed how the ectoderm is not able to develop as a nervous system and instead differentiates as epidermis. The equivalent region of the blastopore in amphibians was identified in most vertebrate species. In birds, it was called the Hense’s node and in mammals, the node. This area is also able to achieve neural induction, suggesting that this mechanism is conserved among vertebrates (Waddington and Schmidt, 1933). Due to these morphogenetic properties the node is known as the primary organizer, having a key role in the induction of neural anlage at early stages of the development.

The primary organizer segregates inducing signals that regulate temporo-spatial patterns of gene expression and subsequent changes in cellular morphology and cellular rearrangements inside the ectoderm, which finally will generate the structural differentiation between the neural and the non-neural primordial (that is, between neuroectoderm and ectoderm, respectively). The neural plate will extend along its antero-posterior axis by means of normal gastrulation

movements and the progressive regression of the primitive streak towards the caudal embryonic pole (Voiculescu et al., 2007).

Subsequently, the bending of the neural plate takes place, involving the formation of the neural folds at the lateral sides of the neural plate, which will be elevated and will converge at the dorsal midline level. This step will form the lumen of the primitive neural tube. This bending is driven by variations not only in neuroepithelial cells, but also in the adjacent cells of the epidermal ectoderm. In the absence of signals coming from these lateral ectodermal tissues, the neural plate starts its bending but folding fails. In addition to that, the neural folds also play a key role during the bending of the neural plate, since each fold is a bilaminar structure (a layer of neuroepithelium covered with a layer of epidermal ectoderm) and the correct development of both lamina is necessary to transduce the mechanical forces generated by expansion of the lateral ectoderm allowing the correct bending of the neural plate (Colas and Schoenwolf, 2001). Indeed, the expansion of the interface between both laminas will generate forces inherent to the neural folds, helping to their convergence in the dorsal midline. The fusion between the neural folds at this point determines the establishment of the roof plate of the neural tube (Figure 1).

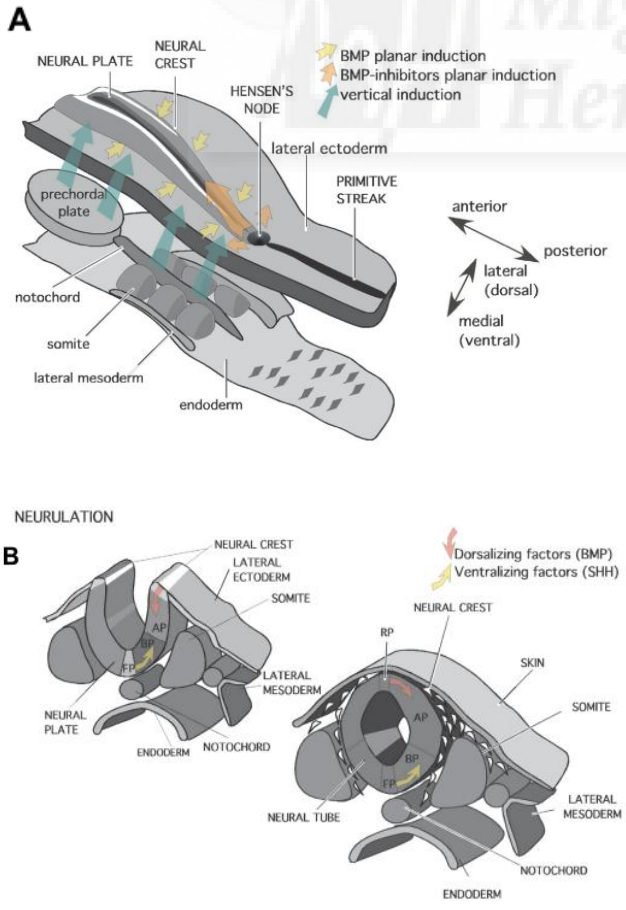

Figure 1. The neurulation process.

(A) Neural plate stage. Dorso-ventral polarity and the first steps of determination of the antero-posterior axis are regulated by inductive signals from the axial mesoderm (notochord and prechordal plate, blue arrows), from the Hense's node (orange arrows) and ectoderm (yellow arrows). (B) In the process of neurulation, the cells of the neural crests migrate from the neural fold which finally fuse together forming the lumen of the neural tube. The involvement of dorsalizing and ventralizing factors in the establishment of the dorso-ventral axis has a key role in this regionalization. As a consequence of these process, the lateral wall of the neural tube is subdivided into four domains: alar plate (AP), roof plate (RP), basal plate (BP) and floor plate (FP) (see bellow for more details). From *Vieira et al., 2010*.

I.2. Regionalization of the neural tube

The development of the neural tube is an orchestrated process where many molecules are involved, among which specific interactions take place in a perfectly accurate temporal and spatial manner. These interactions act as inductive signals that influence the formation of the two axis along the neural tube: an antero-posterior (longitudinal) and a dorso-ventral (tranverse) axis. This neuroepithelial organization could therefore be represented as a simple Cartesian coordinate model along which the neural progenitor cells will acquire their identity in relation to their localization in the map. Cell identity will be regulated by positional information that usually is coded by a gradiental distribution of morphogenetic signals (Meinhardt, 2008), which are traduced in the epithelial cell as differential distribution of receptors and intracellular processes. Derived cells from epithelial progenitors will follow properly different pathways accordingly with their position that finally specifies its final fate.

The recent combination of genetic and experimental embryological approaches using mouse and chick embryos has resulted in a tremendous progress in understanding the molecular and cellular mechanisms that orchestrate for the specification of these two axis, which will determine the final structure of the neural tube.

- Dorso-ventral regionalization

The regionalization of the dorso-ventral axis in the neural tube is established by the antagonism created between signals produced by two polarized cell groups: signals coming from the dorsal region (the roof plate territory), which assist the dorsal properties in the neighboring cells, against factors generated from the ventral areas (the floor plate territory), which induce ventral properties (Lee and Jessell, 1999; Tanabe and Jessell, 1996).

The major ventralizing signal is Sonic hedgehog (Shh), which is produced first in the cells of the notochord and then, induced in the ventral midline of the neuroectoderm. From these ventral midline structures Shh signal is distributed in a ventral to dorsal gradient, emanating its activity directly from the floor plate (Figure 2. A) (Chiang et al., 1996; Wijgerde et al., 2002). On the contrary, the dorsal identities are induced mainly by Bone morphogenetic protein family (Bmp) and Wingless-Int protein family (Wnt) members, which are secreted directly from the roof plate cells of the neural tube and distributed in a dorso-ventral gradient (Figure 2. A) (Nguyen et al., 2000; Timmer et al., 2002).

Once this axis is properly established, the neural tube will be subdivided in four longitudinal columnar domains known as (from ventral to dorsal): floor plate (FP), basal plate (BP), alar plate (AP) and roof plate (RP) (Figure 2. B). As a consequence of the differential positional information between the neuroepithelial progenitors in relation to ventralizing and dorsalizing signals, the molecular specification of each columnar domain show a different and specific gene expression pattern. Therefore, along the different structures of the entire neural tube, each columnar plate have common characteristics but they also could have important differences, imposed by antero-posterior regionalization, with respect to the cellular identity and morphogenesis, among other aspects, in each single domain of the Cartesian neuroepithelial plane (Placzek and Briscoe, 2005).

The FP is composed by the ventral midline cells and it has been described as an organizing center of the neural tube, due to the secretion of determined signaling molecules which are involved in the regionalization process. The RP is situated in the dorsal midline and the progenitors located within this domain have a key role in the closure of the neural tube during the neurulation. In the same way as the FP, the RP is also considered as a signaling center since from them emanate signaling molecules which will determine the dorso-ventral regionalization of the neural tube

In summary, the FP is composed from the ventral midline cells that represent an organizing center of the neural tube, due to the secretion from this region of Shh, the ventralizing signal. The RP is situated in the dorsal midline and the progenitors located within this domain have a key role in the closure of the neural tube during the neurulation and represent the dorsal organizer center because the expression of dorsalizing signals, such as Bmp and Wnt. Antagonistic and gradiental activities of these molecular signals operate in single neuroepithelial cells, which will regulate their expression pattern and fate in relation to the position into the columns according to a more or less well defined activity threshold of each signal. Therefore, the homogeneously graded distribution of ventro-dorsal signaling activity will be transformed at the cellular level into columnar subdomains of differentially specified cellular populations. Finally, the BP and the AP are located in the middle of the territory, between the RP and the FP, and most of the nuclei of the developed diencephalon are derived from these two plates (Figure 2. C-F).

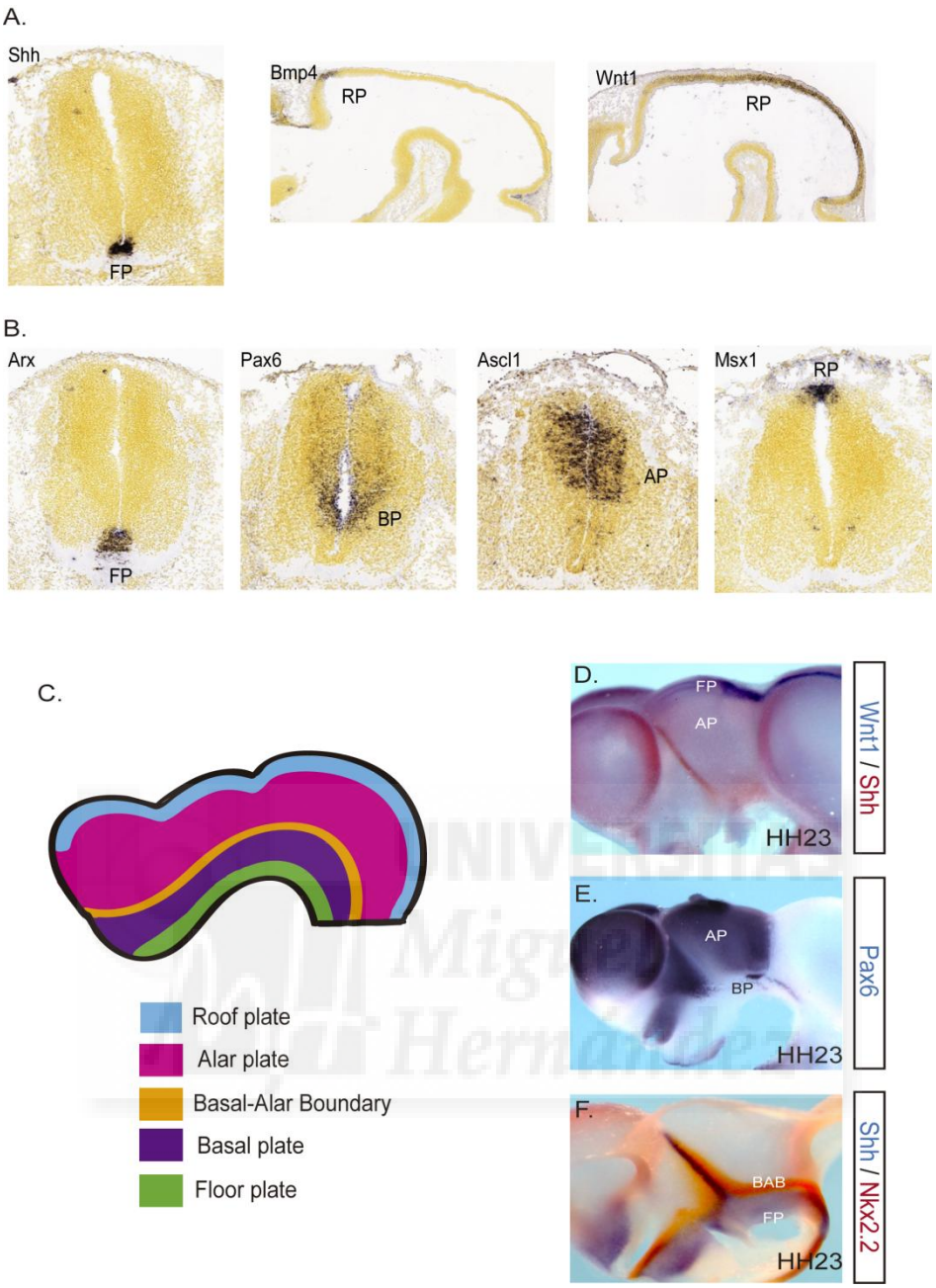

Figure 2. Longitudinal domains along the dorso-ventral axis of the neural tube.

(A) On the right, a frontal section of a caudal region of mice neural tube at E11.5 stage of development where the expression of Shh in the FP is showed. In the middle, a sagittal section of a rostral region of mice neural tube at E11.5 stage of development where the expression of Bmp4 in the RP is showed. On the left, a sagittal section of a rostral region of mice neural tube at E11.5 stage of development where the expression of Wnt1 in the RP is showed. From Allen *Atlas Brain*. (B) Frontal sections of a caudal region of mice neural tube at E11.5 stage of development where is showed the expression of a set of genes which are expressed in the different columnar domain: Arx (FP), Pax6 (BP), Ascl1 (AP) and Msx1 (RP). From *Allen Atlas Brain*. (C) Four domains have been established along the dorso-ventral axis: the roof plate, the basal plate, the alar plate and the floor plate. The basal-alar boundary is situated between the alar plate and the basal plate. (D-F) Lateral view of chick embryos at HH23 stage of development, showing *in situ* hybridization for different genes which are markers for the different longitudinal domains. The most dorsal domain, the roof plate (RP) is characterized by the expression of Wnt1 (D), whereas the most ventral domain, the floor plate (FP), together with the basal plate (BP) is characterized by the expression of Shh (D). The alar plate (AP) is occupied by the expression of Pax6 (E). The boundary between the AP and the BP (BAB, basal-alar boundary) is marked by Nkx2.2 (F).

- Antero-posterior regionalization

The process of the antero-posterior axis definition in the neural tube entails a high complexity due to the high structural heterogeneity present along this axis. This specification starts during gastrulation, when the primary organizers (blastopore, Hense's node) begin to generate key signals that determine differences along the antero-posterior axis of the neural tube (Beddington and Robertson, 1998; Stern, 2001). Later on, other signaling centers, known as secondary organizers, will be involved in the refinement of the antero-posterior axis specification (Echevarria et al., 2003), giving rise first to the differentiation of three primary vesicles in the anterior area of the neural tube. These three domains are called forebrain (or prosencephalon), midbrain (or mesenphalon) and hindbrain (or rhombencephalon) (Rubenstein et al., 1998). Once these three vesicles are established, inductive morphogenetic signals coming from the secondary organizers will determine additional gradients, which will be superimposed over the previous ones (involved in ventro-dorsal regionalization), and locally specify and generate the structural subdivisions inside these principal areas (Shimamura et al., 1995; Martinez, 2001; Martinez and Puelles, 2000). The secondary organizers, therefore, have a key role during the regionalization of the neural tube. There are three specific areas distributed longitudinally along the neural tube that act as secondary organizers.

Rostrally, the anterior neural ridge (ANR) is one of these secondary organizers. It has been described that the morphogenetic information coming from this region plays a critical role in the specific development of the most rostral vesicle, the forebrain, which will be subdivided in two different domains called the secondary prosencephalon (most rostrally situated, which will give rise later to the telencephalon and the hypothalamus) and the diencephalon (Rubenstein and Puelles, 2003).

Caudally, situated in the boundary between the midbrain and the hindbrain, it is found the isthmic organizer (IsO) (Martinez et al., 1991; Li and Joyner, 2001). This secondary organizer regulates cell specification in the midbrain and rostral hindbrain.

Situated between the ANR and the IsO, in the middle of the diencephalon, is located the third secondary organizer. It is called zona limitans intrathalamica (ZLI) and is a prominent structure which is projected from the basal plate at the boundary between the prospective ventral thalamus and the dorsal thalamus (Vieira et al., 2005; Vieira and Martinez, 2006; Kiecker and Lumsden, 2004). From this singular structure, it is regulated antero-posterior regionalization of the diencephalon.

I.3. The prosomeric model of the diencephalon

Experimental embryologists have analyzed in depth the structuration, boundaries and anatomical reliefs present in the neural tube. From the results obtained in these studies, two major different paradigms to identify and to understand the different cytoarchitectonic domains of the neural tube have been postulated: the *columnar model* and the *prosomeric model*.

The first paradigm, the columnar model, was postulated by Herrick (1910) and is based in the establishment of longitudinal functional columns which are extended from the prosencephalon until the rhombencephalon (revised by Alvarez-Bolado et al., 1995; Swanson, 2003). The second paradigm, the prosomeric model, was postulated by Bergquist (1932) and recently re-formulated and reviewed by Puelles and Rubenstein (2003). The modern neuroanatomy based on phylogenetic correlations and the causal analysis of morphogenesis is progressively adding new data in support of the prosomeric model. Therefore we take this model as a reference to connect anatomical structures with experimental data, in order to approach study of the primordial molecular mechanisms underlying the regionalization of the diencephalon.

The prosomeric model proposes that the forebrain is subdivided in consecutive transverse domains named prosomeres. These prosomeres are enumerated from p1 to p3 in the diencephalon and, rostral to it, other additional three prosomeres are situated in the secondary prosencephalon (formed by the hypothalamus and telencephalon). As a summary diagram, the prosomers could be grouped in two major subdivisions of the forebrain: the rostral forebrain is known as secondary procensephalon and includes the prosomere 4, 5 and 6, whereas the caudal forebrain is known as diencephalon and consists of the prosomeres 1, 2 and 3 (Rubenstein et al., 1994; Puelles, 2001; Puelles and Rubenstein, 2003).

Therefore, the prosomeric diencephalon is situated between the secondary prosencephalon and the midbrain and is subdivided in three transverse segments in the antero-posterior axis, and in four longitudinal columns, which correspond with the RP, AP, BP and FP. The morphologic segmentation of the diencephalon begins at E9.5, between E10-11 the diencephalic prosomeres are morphologically structured and at E12.5 the diencephalon presents a complete segmental structure (Martinez-Ferre and Martinez, 2012).

- Prosomere 1: Pretectum

The prosomere 1 (p1) is situated caudally in the diencephalon, limiting with the mesenphalon in its posterior limit and with the prosomere 2 in the anterior limit. The alar plate of p1 develops the pretectum, a region that is still not well known in mammals, which molecular regionalization and genoarchitecture is actively subject of study in the present. It is implicated in the regulation of the visual information and in the establishment of visual reflexes. Three subdomains are described as components of the pretectum, which from caudal to rostral are: the commisural pretectal domain (CoP), juxtacommisural pretectal domain (JcP) and precommisural pretectal domain (PcP) (Redies et al., 1997; Ferran et al., 2007; Ferran et al, 2009; Merchan et al., 2011; Puelles et al, 2012). The CoP, located caudally, is characterized by the development of the posterior commisure at the roof plate, and nuclei mainly related to it, and the analysis of visual information. The JcP, situated in the middle, is a narrow area where specific pretectal nuclei are distributed, which is functionally related to the mesencephalic tectum. Finally, the PcP is the most rostral structure, limiting with the thalamus (p2), and is the domain which produces the most voluminous pretectal population (Figure 3).

In the basal plate of p1 are located the pretectal tegmentum, which will develop part of the ventral tegmental and sustantia nigra, the magnocellular part of red nucleus and the interstitial nucleus of Cajal, among other less characterized neuronal regions (Puelles et al., 2008).

- Prosomere 2: Thalamus

The prosomere 2 (p2) is situated in the central region of the diencephalon, limiting caudally with p1 and rostrally with p3. The alar plate of this prosomere will grow much more than the anterior p3 and posterior p1 in most of vertebrates and develops the thalamic nuclear complex. This dominant expansion of p2 alar plate will compress and elongate p3 alar plate, which is distributed as a narrow territory between thalamic and telencephalic domains. Dorsal to the thalamus, the epithalamus continues dorsally the alar plate towards the choroid plexus at the p2 roof plate.

The thalamus covers almost the complete alar plate of p2, and acts as a relay station of the sensory information, before being projected to the cerebral cortex. Moreover, it is formed by several nuclei, which have been classified in agreement with their location inside this thalamic nuclear complex (Figure 3, Puelles et al., 2008; Puelles et al., 2012; Martinez-Ferre and Martinez, 2012). The most rostral nuclei are the *anterior nuclei of the thalamus*, which are implicated in the

emphasis of alertness and in learning and memory processes. Situated in the medial region of this complex are the *medial dorsal thalamic nuclei,* implicated in emotional behaviors and the sensations of pleasure. Moreover, the *centromedian thalamic nuclei* have a key role in adjusting the circadian rhythms, whereas the *medial geniculate thalamic nuclei* and the *lateral geniculate thalamic nuclei* are involved in the projection to the cerebral cortex of auditory and visual information, respectively. Finally, situated ventrally, there are the *ventral thalamic nuclei*, whose nuclei from the anterior region have motor roles whereas the nuclei from the posterior region are involved in sensory systems.

The epitathalamus occupies the dorsal region of the alar plate of p2, and include the habenula, the stria medullaris and the pineal gland. The habenula is involved in emotional responses to sensorial and motor stimuli by means of the reception of afferent information from several regions of the septum (located in the telencephalon) and the efferent sending information through the habenulo-interpeduncular tract (retroflexus tract). This tract is located in the boundary between p1 and p2, descends dorsoventrally from the habenular nuclei, follows then a longitudinal direction in the basal plate and arrives to the interpeduncular region located in the rostral rhombencephalon. The pineal gland is involved in the control of the circadian internal rhythms.

In the ventral region of p2 is situated the prerubral tegmentum, that develops as poles of ventral tegmental area and the sustantia nigra, as well as the rostral interstitial nucleus and other reticular structures.

The rostral boundary of this prosomere is the region that will generate the ZLI organizer at early postneurulation stages (Figure 3).

- Prosomere 3: Prethalamus

The prosomere 3 (p3) is situated in the most rostral part of the diencephalon. In this prosomere is situated the prethalamus, a region that have been very recently defined clearly as an independent region between the anterior thalamus, the caudal peduncular telencephalon and the hypothalamus, by molecular expression profiles and characteristic genoarchitectural development (Puelles and Martinez, 2013; Puelles, 2013). This structure recieves collaterals of the reciprocal connections between the thalamic nuclei and the cortex, as well as motor information in connection to the striatum and substantia nigra.

The dorsal region of the alar plate of this prosomere contains the prethalamic eminence and the choroid plexus, which correspond to the anterior extension of the epithalamus. The basal region contains the retromammillary region (Garcia-Lopez et al., 2004; Martinez-Ferre and Martinez, 2012), the p3 tegmentum and the most rostral part of sustantia nigra and ventral tegmental area (Figure 3).

The knowledge of the prosomeric model allows us to deeply analyze each morphogical structure and the potential molecular mechanisms that pattern the development of the forebrain.

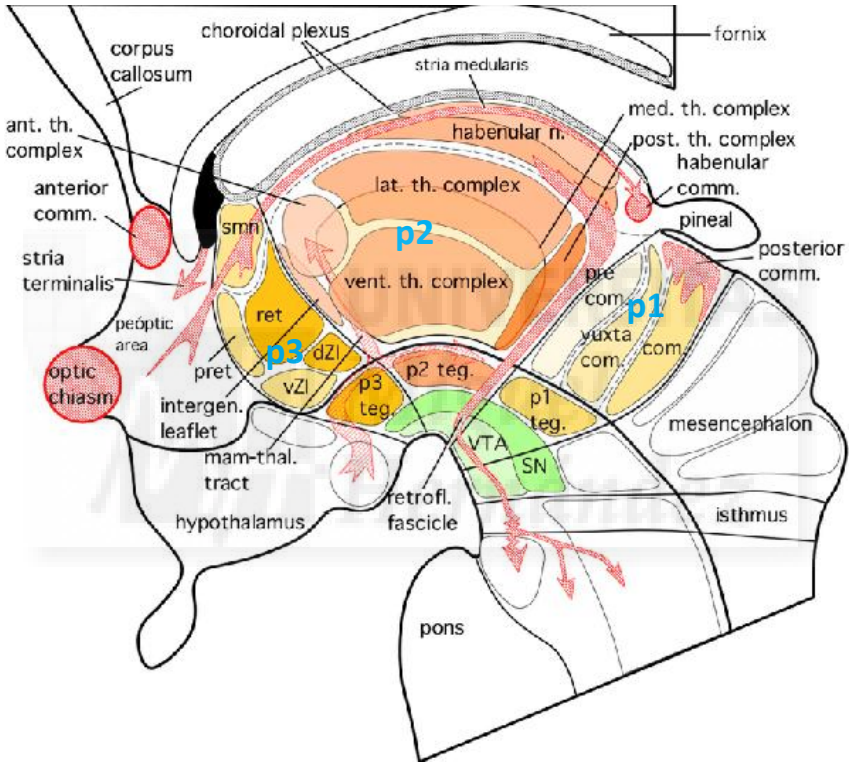

Figure 3. Representation of the prosomeric model of the mammalian diencephalon, including the major nuclei of each prosomere.

The three prosomeres (p1, p2 and p3) are illustrated in the figure, with their major structures of the roof, alar and basal plate, with the main axonal tracts and derivatives of each structure (modified from *Martinez-Ferre and Martinez, 2012*). Abbreviations: p1, prosomer 1; p2, prosomer 2; p3, prosomer 3; posterior comm., posterior commisure; com, commissural pretectum; yuxta com., yuxtacommisural pretectum; pre com., precommisural pretectum; p1 teg., p1 tegmentum; SN, sustantia nigra; VTA, ventral tegmental area; retrofl.fascicle, retroflexus fascicle; habenular n., habenular nucleus; Ant. th. complex, anterior thalamic complex; Anterior comm., anterior commissure; dZI, dorsal zona incerta; vZI, ventral zona incerta; Habenular comm., habenular commissure; Lat. th. complex, lateral thalamic complex; Mam-thal. tract, mammillo-thalamic tract; Med. th. complex, medial thalamic complex; p2 teg, prosomer 2 tegmentum; p3 teg, prosomer 3 tegmentum; Post.th. complex, posterior thalamic complex; Vent. Th. Complex, ventral thalamic complex; Pret, prethalamus; Ret, reticular nucleus; Smn, stria medullaris.

I.4. Signaling molecules

As it has been mentioned before, morphogenetic information that will specify the progressive development of the neural tube is originated from the secondary organizers, which exert their activity by producing and segregating signaling molecules that act as morphogenes. These morphogenes induce specific characters in the neighboring epithelium, refining the anatomical and histological organization of the developing brain (Jessell and Sanes, 2000; Colas and Schoenwolf, 2001).

The most important signalling molecules belong to four different families: *Fibroblast growth factor family* (Fgf), *Hedgehog family* (Hh), *Bone morphogenetic protein family* (Bmp) and *Wingless-Int protein family* (Wnt). The interactions which take place between some members of each family and between them and other signalling molecules, as well as their specific temporal and spatial distribution during the process of the neural tube development are essential for the correct regionalization of the neural tube and, therefore, for the precise brain morphogenesis.

- *Fibroblast growth factor family* (Fgf)

This is a family of polypeptides that are able to activate fibroblast proliferation and epithelial cell growth (Armelin, 1973), and have an essential role during the development of the neural tube.

After neurulation, the prosencephalon expresses one of the most important members of this family, Fgf8. Its expression starts in the ANR cells at neural plate stage in the anterior pole of neuroepithelial sheet, and it has been described as essential for the proper development of the forebrain (Crossley and Martin, 1995; Martinez et al., 1999; Crossley et al., 2001; Chi et al., 2003; Storm et al., 2006). Then, this expression extends caudally through the alar plate of the diencephalon, as well as becoming activated in the isthmus, where plays a key role in the regionalization and histogenetic development of these areas (Crossley and Martin, 1995; Martinez et al., 1999; Crossley et al., 2001; Chi et al., 2003; Martinez-Ferre and Martinez; 2012). It has been shown that alterations in the physiological levels of Fgf8 produce structural changes in the embryonic and postnatal diencephalon in mouse (Kataoka and Shimogori, 2008). Later on, it has been demonstrated that the development of specific diencephalic regions as the habenula and the pineal gland could be altered in a dose-dependent manner by reducing Fgf8 signaling activity (Martinez-Ferre and Martinez, 2009).

Another member of the FGF family is also important in the regionalization of the neural tube. Fgf15, a Fgf molecule ortolog to human and chick Fgf19 (Nishimura et al., 1999), has been

described as a signal which suppresses proliferation and promotes differentiation in the developing telencephalon (Borello et al., 2008). Moreover, Fgf15 is proposed as a gene which diencephalic-mesencephalic expression is regulated by Shh expressed in the ZLI and in the midbrain basal plate (Miyake et al., 2005, Ishibashi and McMahon, 2002, Gimeno and Martinez, 2007).

- *Hedgehog family* (Hh)

The most important morphogene of the hedgehog family is Sonic Hedgehog (Shh). Shh has been described as necessary and sufficient for the determination of ventral identities in the neural tube (Echelard et al., 1993; Wijgerde et al., 2002).

The transduction of Shh signalling is mediated by two transmembrane proteins: Patched (Ptc), which acts as a Shh receptor, and Smoothened (Smo) which initiates the intracellular signaling (Alcedo et al., 1996; Ulloa and Marti et al., 2010). Smo activation leads to the regulation of the members of Gli family transcription factors (Dahmane et al., 2001, Rallu et al., 2002; Meyer and Roelink, 2003; Wang et al., 2007). Three Gli proteins have been described in vertebrates and their response to the Shh signal differs between them. Gli1 only has a transcriptional activator role, whereas Gli2 and Gli3 could develop both an activator or a repressor role (Ruiz i Altaba, 2003; Jacob and Briscoe, 2003). Specifically, Gli3 is proteolitically processed to give rise to a transcriptional repressor in the absence of Shh, however in the presence of Shh, this processing of Gli3 is inhibited and the full-length protein acts as a weak transcriptional activator, inducing the expression of the Shh pathway target genes (Wang et al., 2000; Aza-Blanc et al., 2000). However, the expression of Gli3 gene is inhibited by Shh, therefore, Gli3 is expressed in a dorsal to ventral gradient, showing a pattern completely opposite to Shh expression, which is expressed in a ventral to dorsal gradient (Jacob and Briscoe, 2003). Therefore, it has been described that this antagonism between Gli3 and Shh plays a major role in the development of the dorso-ventral axis in the neural tube (Meyer et al., 2003; Ruiz i Altaba et al., 2007).

In addition to Shh-Gli interactions, Otx genes are also regulators of Shh. These homeobox transcription factors play important roles in the regionalization and specification of the vertebrate brain. Lack of Otx2 expression during embryonic development leads to an absence of rostral neuroectoderm (Acampora et al, 1995; Matsuo et al., 1995; Tian et al., 2002). Moreover, it has been proposed that Otx activity regulates in a dose-dependent manner the correct pattern of Shh

and Fgf8 expression along the longitudinal and transversal axis of the neural tube (Puelles et al., 2003).

- *Bone morphogenetic protein family* (Bmp)

This is a secreted family of proteins which belongs to the transforming growth factor β (TGF-β) superfamily of peptides (Gazzerro and Canalis, 2006). They are implicated in the dorso-ventral regionalization of the neural tube and are secreted from the dorsal regions of the neural tube, including the roof plate (Liem et al., 1995, Liem et al., 1997; Lim et al., 2005; Lim and Golden, 2007; Ulloa and Marti, 2010; Le Dreau and Marti, 2012).

The expression of the different Bmp members during the neural tube development is highly dynamic. At early stages of development, after neurulation, several Bmps (Bmp4, Bmp5 and Bmp6) are expressed in the RP of the neural tube.

Despite the fact that Bmp signals play a role in dorso-ventral patterning of the neural tube and that several Bmps are expressed in the dorsal diencephalon, the role that these morphogenes play in diencephalic patterning remains unclear.

It has been reported in numerous studies in the spinal cord that Bmp activity is required for the development of the dorsal cellular identities, antagonizing the role of Shh (Liem et al., 1995; Barth et al., 1999; Lim et al., 2000; Mekki-Dauriac et al., 2002; Liu and Niswander, 2005; Wilson and Maden, 2005). In this context, it has been described that Bmp7 activity is not required for the dorsal patterning, however Bmp4 activity is required for the proper formation of the RP (Le Dreau et al., 2012). Therefore, these two members of the Bmp family have non-redundant functions during neural tube development. In contrast with the spinal cord, several Bmp ligands and receptors are expressed not only in the dorsal region, but also in the ventral area (Lim and Golden, 2007) of the diencephalon. This suggests the existence of a more complex molecular mechanism involving Bmps in the diencephalon than in spinal cord.

In parallel to these data obtained from studies in spinal cord, several studies have suggested the involvement of Bmp signaling in the forebrain dorso-ventral patterning (Furuta et al., 1997; Golden et al., 1999; Ohkubo et al., 2002; Shimamura and Rubenstein, 1997). It has been described that ectopic activation of Bmp signal in the diencephalon disturbs the development of the alar diencephalic nuclei (Lim et al., 2005).

The difficulty in the elucidation of the role of these family proteins is increased given that Bmp mutant mice are poorly informative due to the early embryonic lethality and/or a possible redundant function between different Bmps (Luo et al., 1995; Lyons et al., 1995; Zhang and Bradley, 1996; Dudley and Robertson, 1997; Solloway et al., 1998; Hebert et al., 2003;).

- *Wingless-Int protein family (Wnt).*

This family of proteins has been always a matter of study due to their multiple roles in the metazoan embryonic development, from being involved in the establishment of the polarity of a single cell within a tissue until specifying the entire body axis of an organism (Willert and Nusse, 2012).

Wnt genes encode a large family of cysteine-rich glycoproteins which are secreted as palmitolayted proteins. Nineteen different Wnt proteins have been identified in the murine and human genome. Two different pathways can mediate the signaling activity of these proteins: a canonical and a non-canonical pathway (Logan and Nusse, 2004; Strutt 2003, Veeman et al. 2003). The main difference between them is the implication or not of β-catenin and Wnt receptors in the transduction of Wnt signals.

The canonical pathway implies the cellular receptors of Wnt signalling *Frizzled* (Fz) and *Low density lipoprotein receptor-related protein* (LRP). These proteins are in the cell surface and, after binding of secreted Wnt protein, transduce the signal by means of the activation of several intracellular proteins. These are *Dishevelled* (Dsh), *Glycogen synthase kinase-3 β* (GSK-3), *Axin*, *Adenomatous Polyposis Coli* (APC), and the transcriptional regulator, β-catenin. In the absence of Wnt, GSK-3, APC and Axin form a complex which keeps the levels of β-catenin in the cytoplasm low by means of continuous proteosomal degradation. In the presence of Wnt signal, the protein Dsh binds to Axin, and the complex GSK-3/APC/Axin is inhibited causing and increase of the citoplasmatic levels of β-catenin its translocation into the nucleus, where it will interact with transcription factors such as *lymphoid enhancer-binding factor 1/T cell-specific transcription factor (*LEF/TCF) to activate the transcription of the target genes (Figure 4).

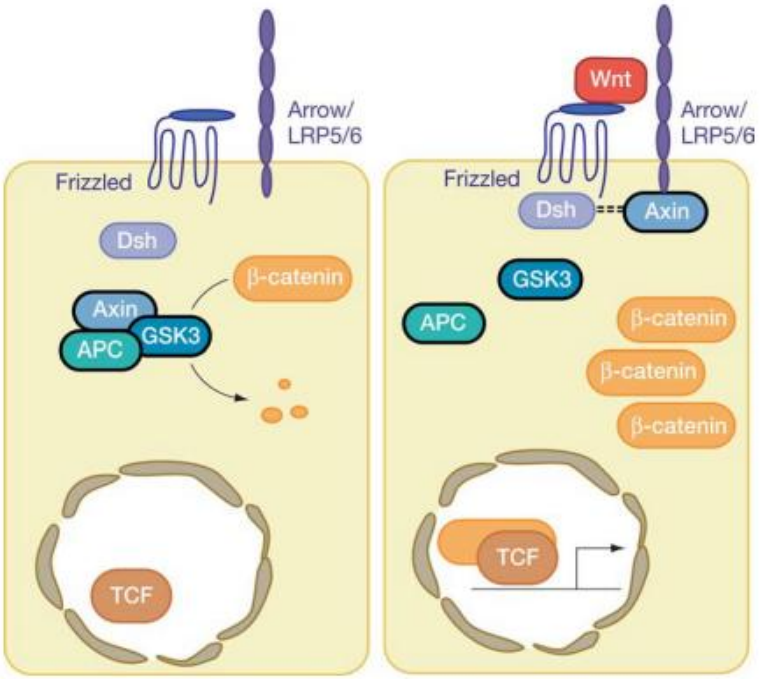

Figure 4. The canonical Wnt signaling pathway.

In this figure is illustrated how Wnt signal is transduce, by means of the involvement of Dsh, GSK-3, APC, Dsh and β-catenin. The signal is transducer directly to Dsh and Axin, with the consequence of the inhibition of the degradation of β-catenin, which will be accumulated in the cytoplasm and in the nucleus, where will interact with TCF to regulate the transcription. From *Logan and Nusse, 2004*.

As antagonists of Wnt signal, it has been described a family of secreted proteins, which negatively regulates by competitive binding of Wnt receptors, and are known as *secreted Frizzle-related proteins* (SFRP, Rattner et al. 1997). In addition to this family, *Wnt inhibitory factor* (WIF) proteins have also been described as secreted molecules that act as extracellular Wnt inhibitors (Uren et al. 2000). Other potent inhibitors of Wnt signaling are *Wise* (Itasaki et al., 2003) and *Dickkopf* (Dkk, Glinka et al. 1998; Bafico et al., 2001), both of them are able to bind LRP receptors.

Although several Wnt molecules are expressed in similar temporo-spatial patterns, functional specificity of each one is suggested by the presence of severe phenotypes due to the loss of expression of a single member (Amerongen and Berns, 2006). The expression patterns which are exhibited by these genes are extremely dynamic and frequently overlaping and are often localized in morphological boundaries between different longitudinal and transversal compartments of the embryonic neural tube (Parr el at., 1993; Hollyday et al., 1995; Quinlan et al., 2009; Martinez-Ferre, Navarro-Garberi et al., 2013).

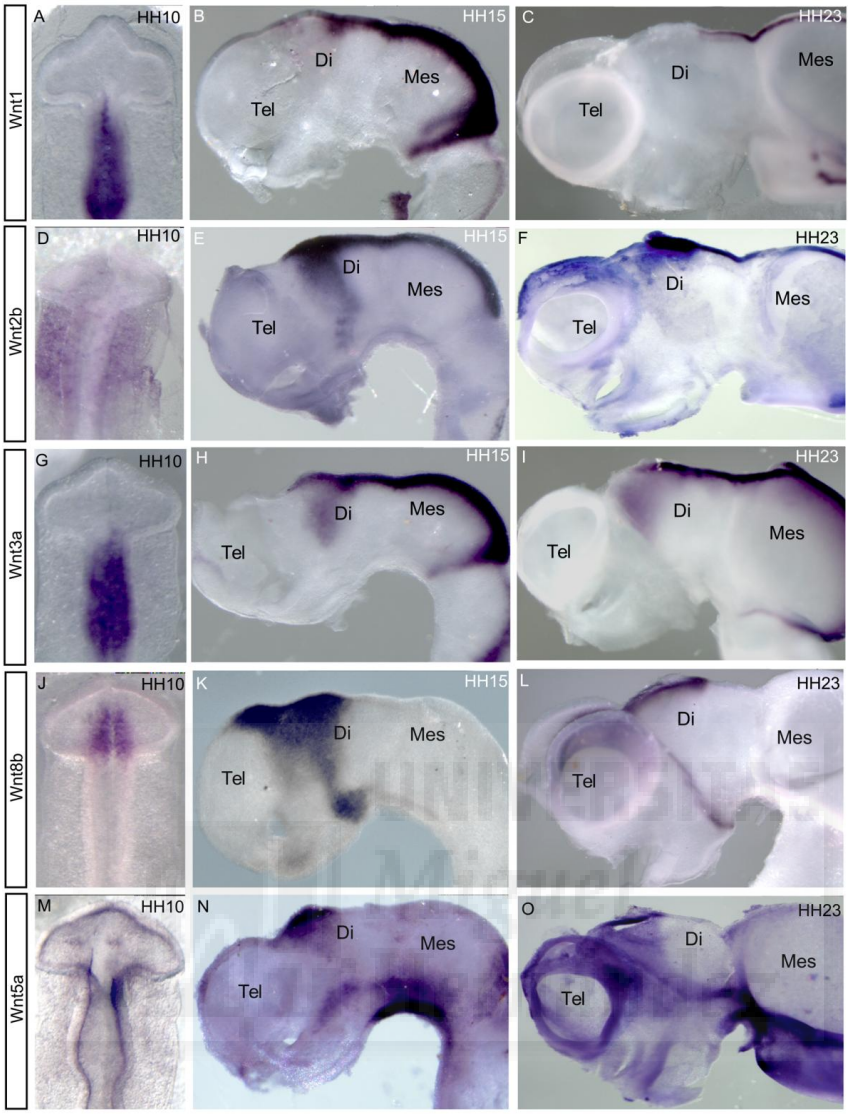

Figure 5. Expression pattern of Wnt genes in the chick prosencephalon.

Dorsal view in HH10 stage (A,D,G,J,M) and lateral view in HH15 (B,E,H,K,N) and in HH23 (C,F,I,L,O) of Wnt1 (A-C), Wnt2b (D-F), Wnt3a (G-I), Wnt8b (J-L) and Wnt5a (M-O) in whole mount chick embryos. Di, Diencephalon; Tel, telencephalon; Mes, mesencephalon. Modified from *Martinez-Ferre, Navarro-Garberi et al 2013*.

The first Wnt protein that was isolated in 1982 was *int-1*, which was the founder member to be called later as Wnt1 (Nusse and Varmus, 1982). This protein is first expressed in the presumptive midbrain of neural plate epithelium at early stages of development, and later, its expression becomes restricted to the dorsal midline of the mesencephalon and extended until the prosomere 2/3 boundary of the diencephalon (Hollyday et al., 1995; Martinez-Ferre, Navarro-

Garberi et al., 2013). There are some differences in the expression of Wnt1 between chick and mouse species. In chick, the expression of Wnt1 is present also in the telencephalon and in the rostral diencephalon at HH19 (Quinlan et al., 2009), expression which is absent in mice, whereas a weak Wnt1 expression domain in the ventral midline of the diencephalon and the mesencephalon (Parr et al., 1993; Echevarria et al., 2003) is present in mice but not in chick embryos.

Wnt1 signaling induces cell proliferation (Dickinson et al., 1994; Megason and McMahon, 2002) and it is essential for the development of both the midbrain and adjacent rhombomere 1 in the hindbrain (McMahon and Bradley, 1990; Thomas and Capecchi, 1990), as well as for the formation of the mid-hindbrain boundary (Bally-Cuif et al., 1995).

In general, the implication of Wnt signal during the development of the neural tube has been deeply analyzed (Mulligan and Cheyette, 2012) and several experimentation lines demonstrate that Wnts are involved in the dorso-ventral patterning of the neural tube. It has been described that the role of Wnt during this regionalization antagonizes the Shh pathway, inducing dorsal identities in neuroepithelial cells (Braun et al., 2003; Yu et al., 2008; Lavado et al., 2008).

Moreover, several studies indicate a role for Wnt signaling in the early antero-posterior patterning of the neural tube (Nordstrom et al., 2002). Wnt signal has also been described, during early steps of neural tube development, as a factor which induces, in a dosage-graded manner, the neural identities of the posterior regions of the neural tube (caudal forebrain, midbrain and hindbrain), and suppresses anterior identities during the regionalization of the neural tube (Kiecker et al., 2001; van de Water et al., 2001), whereas the absence of Wnt signaling allows the differentiation of the anterior forebrain identities (Mukhopadhyay et al., 2001; Houart, 2002). More specifically, Wnt signaling seems to be crucial for the initial antero-posterior organization of the forebrain at the limit between secondary prosencephalon/prethalamus and thalamus (Martinez-Ferre and Martinez, 2012; Puelles and Martinez, 2013) by acting upstream of locally expressed transcription factors: coding inhibition of the anteriorly expressed factor Six3 in the prethalamus and the induction of the posteriorly expressed factor Irx3 in the thalamus (Braun et al., 2003).

The activity of Wnt signal have been studied also in relation to Bmps (Guo and Wang, 2009; Chesnutt et al., 2004; Ille et al.,2007). In the spinal cord, it has been described that the Wnt pathway induces the expression of Bmp7 (Alvarez-Medina et al., 2008). However, the relationship between Wnt and Bmp signalling during the dorso-ventral patterning remains unclear since some

studies suggest that Wnts could induce some Bmp expression while others show how the activity of Wnt signaling do not activate Bmp signaling activity (Yu et al., 2008).

Recently, we have published that Wnt signal is involved in the ZLI development, in a molecular mechanism where Wnt signal regulates the expression domains of Gli3 and Lunatic fringe (L-fng) in the alar plate of the diencephalon, generating a permissive territory where Shh will be progressively activated ventro-dorsally within the ZLI (Martinez-Ferre, Navarro-Garberi et al., 2013).

Although multiple studies have been performed in order to unravel the role of Wnt signalling in the developing diencephalon, here we demonstrate by means of experimental embryology techniques that Wnt signal, and more specifically Wnt1 signal, is directly involved in the establishment of the dorso-ventral patterning of the diencephalic epithelium. This function depends on a novel mechanism not based in Shh antagonizing mechanisms, but in the direct regulation of dorsal genes expression coding dorsalizing specification, which will determine the proper formation of the four different longitudinal plates of the diencephalic neural tube.

Chapter II

AIMS

The general aim of this thesis experimental program is to unravel the cellular and molecular mechanisms underlying the development of the diencephalon, and more specifically, it is focused in analyzing the role of Wnt signaling in this process. Wnts have been described as an essential family of proteins, which acting as secreted signaling molecules code morphogenetic information in many different processes during embryonic development. Nevertheless, its specific function in the development of the diencephalon as well as the interactions between Wnt family members with other signaling molecules expressed in this prosencephalic region remains to be properly established.

Specific aims:

1.- To describe the prosencephalic phenotype of Wnt1 mutant mice in order to analyze the role of Wnt1 during diencephalon development.

The role of Wnt1 in the development of the neural tube has been analyzed by several authors, but the specific function of Wnt1 in the diencephalon regionalization is still unclear. We want to analyze the structural anomalies of diencephalic territory in Wnt1 mutant embryos and wild type embryos, by means of molecular, inmunohistochemical techniques and classical structural staining.

2.- To unravel the molecular mechanisms underlying the regionalization of the diencephalon.

We also analyze the expression pattern of the development active genes involved in the morphogenesis and cytogenesis of the diencephalon in wild type and Wnt1 mutant embryos. The gene expression patterning has been analyzed by means of *in situ* hybridization techniques, trying to further investigate the molecular mechanism underlying the diencephalic regionalization.

3.- To elucidate the role of Wnt1 in the diencephalic regionalization by means of gain of function studies.

The segmentation of the mouse diencephalon starts at E9.5, and then at E12.5 stage of development, the diencephalon displays the complete segmental structure. For that reason, in order to study the molecular mechanisms underlying the role of Wnt1 in this process, *in ovo* electroporations of chick embryos were performed.

4.- To understand the molecular interactions which take place between Wnt1 and the different morphogenetic molecules implicated in the diencephalic regionalization in the experimental chick embryos.

The expression patterns of the major important genes in the diencephalic regionalization were analyzed in the experimental chick diencephalon and compared with control embryos.

5.- To study the relationship between Wnt and Bmp proteins during the diencephalic regionalization.

Bmps proteins are secreted by the roof plate of the diencephalon and have been described as key molecules in neural tube regionalization. However, the interactions between Bmps and Wnts families of morphogenes have not been sufficiently analyzed and needs to be clarified.

Chapter III

MATERIALS AND METHODS

III.1. Experimental embriology

All animal experiments were performed in agreement with the Spanish and European Union laws on animal care in experimentation (Council Directive 86/609/EEC). They have been analyzed and approved by the Animal Experimentation Committee of the Miguel Hernández University. Wnt1 transgenic mice line (*Mus musculus*), used for this thesis project, were bred in the animal facilities of the Instituto de Neurociencias.

III.1.1. Wnt1 mice line

A transgenic mouse Wnt1 (C57BL/6 background) was used for this experimental study (McMahon et al. 1992). Heterozygous animals were treated as wild type embryos and homozygous animals were used for the experimental cases.

Embryos were collected at the desire stage of gestation. The morning when vaginal plug is visualized was considered as embryonic day 0.5 (E0.5). Female pregnant mice were sacrificed by cervical dislocation. Embryos were collected and dissected in cold 0.1 M phosphate buffered saline (PBS) and were fixed overnight in paraformaldehide (PFA) 4%. Then, they were washed in PBS, dehydrated in metanol and stored at -20 ºC.

For identification of the mutant allele in adults, we performed DNA isolation from tail. The tail was digested with 430 µl of lysis buffer (composed by 0,2% sodium dodecyl sulfate (SDS) (Sigma-Aldrich), 100 Mm Tris buffer pH=8 (Sigma-Aldrich), 5 Mm EDTA (Sigma-Aldrich), 200 mM NaCl (Sigma-Aldrich)) plus 20 µl of proteinase K (25 µg/ml , Roche). The samples were incubated overnight at 65ºC at 500 r.p.m. Afterwards, the samples were centrifugated 5 minutes at 14000 r.p.m. The supernatant was separated in a new eppendorf and 1 µl was used for the PCR reaction.

For the identification of the mutant allele in embryos, we performed DNA isolation as follows: each tail was digested in 72 µl of sterile water (Sigma-Aldrich) and 8 µl of proteinase K (25 µg/ml) at 65ºC overnight. After this digestion, the samples were vortexed twice and, afterwards, were centrifugated 5 minutes at 14000 r.p.m. The supernatant was separated in a new eppendorf and 1 µl was used for the PCR reaction.

All the samples were stored at -20ºC until used for PCR reaction. For PCR amplification, two different pairs of primers were used to screen the mutant alleles. The oligonucleotides for allele specific genotyping for the Wnt1 mutant allele (496 bp) were: 5' ATA GCC TCC TCC ACG AAC CT 3' (sense) and 5'TCT TGA CAA AAA GAA CCG GG 3' (antisense). The oligonucleotides for allele specific genotyping for the wild type allele (394 bp) were: 5' CAG GCA AGG ATA GGG TGG TA 3' (sense) and 5' AGA TGA CCC TGC TTT TCC CT 3' (antisense). The mix for the PCR reaction includes 12 µl of a master mix solution for PCR (Ready Mix Thermo Scientific), mixed with 1 µl of each pair of primer (forward and reverse from mutant and wild type pairs at 10 µM) and 1 µl of DNA (100ng/µl). Amplifications were performed as follows: 5 min at 95ºC; 35 cycles of 1 min at 95ºC, 1 minute at 57ºC and 1 min at 72ºC; 10 min at 72ºC. PCR amplification products were separated through electrophoresis on a 1% agarose gel and identified by their size.

III.1.2. In ovo electroporation

Chick (*Gallus gallus*) fertilized eggs used for these experiments were obtained from commercial sources, and were incubated at 37ºC in a forced-air incubator until desired developmental stage according to Hamburger and Hamilton (Hamburger and Hamilton, 1951) was reached. The electroporation was performed on stage HH10. Plasmidic DNA solution was injected into the lumen of the neural tube. Platinum electrodes were used with 5 x 10 ms pulses at 25 V generated by a TSS20 Ovodyne electroporator.

Embryos were incubated for further 3 days after the electroporation. Afterwards, the embryos were collected and dissected in cold 0.1 M phosphate buffered saline (PBS) and were fixed overnight at 4% PFA. Then, they were washed in PBS, dehydrated in metanol and stored at -20 ºC.

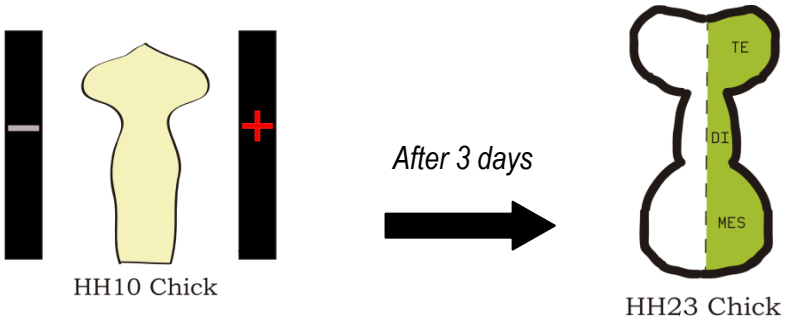

Figure 6. Schematic illustration of the *in ovo* electroporation

This scheme illustrates a dorsal view of the experimental design of the electroporation *in ovo*. The electroporation was carried out in HH10 and they embryos were allowed to develop until HH23. After these days, the right side of the embryos will express the protein of interest plus the reporter protein GFP. TE=Telenphalon; DI= diencephalon; MES=mesenphalon.

III.2. Expression constructs

Plasmids electroporated in our experiments were obtained from different sources. The plasmid CAG-Wnt1-IRES-EGFP (this study) was constructed by inserting a mouse Wnt1 sequence into the commercial plasmid CAG-IRES-EGFP (Addgene). This plasmid was electroporated at 5 µg/µl. The plasmid pCAGGS-Bmp4 (kindly provided by K.N. Kishimoto), was electroporated at 5 µg/µl together with the commercial plasmid CAG-IRES-EGFP at 1 µg/µl. All of them were injected in 0,02% fast green in PBS.

III.3. Histological analysis

The embryos that were destined to obtain paraffin sections, once they were dehydrated until metanol 100%, exposed to Butanol 30 minutes - 2 hours (depending on the stage of development), and included in paraffin with the desired orientation. The sections (7 µm) were obtained using a microtome (Leica) and processed.

The embryos destined to *in situ* hybridization and posterior immunohistochemistry in whole mount, were rehydrated until PBS, were peeled from the external tissue of the neuroepithelium and processed with the *in situ* hybridization in whole mount protocol (III.3.2) or dehydrated until methanol 100% and stored at -20ºC.

III.3.1. Cresyl violet staining

The sections of paraffine destined to Cresyl violet (Acros organics) staining, were deparaffined for 1 hour at 65ºC and twice for 30 minutes in xilol. Then, they were rehydrated through a descending series of etanol solutions: 10 minutes 2 times in etanol 100%, 10 minutes 2 times in etanol 96% (diluted in water), 10 minutes 2 times in etanol 70% (diluted in water) and finally, 10 minutes in water. Afterwards, paraffin sections were submerged in cresyl violet staining for 10 minutes. Then, the sections were dehydrated in an ascending series of etanol until etanol 100%. Finally, sections were submerged in xilol for 30 minutes and mounted with Eukitt (O. Kindler GmbH). The sections were left at 37ºC overnight.

III.3.2. In situ hybridization

- Antisense mRNA probes preparation

Circular DNA plasmids kindly provided by several laboratories (Table 1) were used to obtain RNA probes. Competent *E.Coli DH5α* cells were used to amplify plasmids. These cells were transformed by heat-shock at 42ºC for 1 minute in contact with the plasmid (100-300ng) and then were exposed to ice for 2 minutes. Afterwards, the cells were mixed with 1 ml of LB in order to increase the number of cells for 1 hour at 37ºC at 200 r.p.m. shaking. After growth incubation, the cells were plated into an LB-Agar petri plate with Amplicillin (50mg/ml Sigma-Aldrich) for clone selection and incubated overnight at 37ºC. The next day, a single colony was selected and grown into 100 ml of LB medium with Amplicillin at 37ºC for colony amplification. Plasmid extraction and purification were performed using Genopure plasmid midi Kit (Roche) ant then stored at -20ºC. The circular plasmids were linearized using specific restriction enzymes (Table 1). 10µg of plasmidic DNA was mixed with 5µl of linearization buffer (Fermentas), 2µl of enzyme (Fermentas) and sterile water (Sigma-Aldrich) up to 50µl and was incubated at 37ºC for 2 hours.

Then, 1 µg of this linear DNA plasmid was incubated for in vitro transcription at 37ºC for 2 hours. Together with it, we added 2µl of transcription buffer (Roche), 2µl of RNase inhibitor (Takara), 2µl of a mixture of labeled dNTPs (composed of 10mM ATP, 10mM CTP, 10mM GTP, 6.5mM UTP and 3.5mM of digoxigenin- or fluorescein-labeled UTP from Roche) and 2µl the appropriate RNA-Polymerase enzyme (Roche) and sterile water (Sigma-Aldrich) up to 20 µl.

After these 2 hours, 1µl of DNase enzyme (Roche) was added and after 15 minutes, 1 µl of RNase inhibitor was finally added. Afterwards, probes were cleaned using RNAeasy Mini Protocol for RNA Cleanup (Quiagen) and used for *in situ* hybridization.

PROBE	***ANTISENSE***	***LABORATORY***
mSix3	XbaI+T7	A.Mailhos,G.Oliver
mPax6	EcoRI+ sp6	C. Bonsch
mDbx1	SmaI+T7	J.Guimera
mWnt1	EcoRI + T7	J.Guimera
mNgn2	BamHI+T7	J.Rubenstein
mIrx3	XbaI + T3	P.Gruss
mFgf8	BamHI + T7	G.R.Martin
mShh	HindIII + T3	A.McMahon
mTcf4	XhoI + T7	J.Galceran
mGli3	EcoRI+sp6	A.Simeone
mBmp4	EcoRI + sp6	J.Rubenstein
cOtx2	BamHI+T7	A.Simeone
cShh	HindIII + T3	S.Martinez
cGbx2	EcoRI + sp6	J.Ferran
cPax6	ClaI + T7	A.Garda
cGli3	EcoRV + T3	C.Tabin
cWnt8b	EcoRI + sp6	A.McMahon
cL-fng	ClaI +T3	A.Rodriguez
cFgf8	EcoRI + T7	G.R.Martin
cNkx2.2	BamHI+T7	J.Rubenstein
cFgf19	BamHI+T7	L.Gimeno
cSix3	HindIII + T3	P.Bovolenta
cBmp4	BamHI + T3	P.Bridzell
cWnt1	ClaI + T7	S.Martinez

Table 1. *In situ* hybridization probes.

- *In situ* hybridization in whole mount

If the embryos were dehydrated in methanol, they were rehydrated until PBS with a descending scale of methanol solutions (100%-75%-50%-25% methanol). Then, they were bleached with 30% hydrogen peroxide diluted in PBS for 1 hour at room temperature. After washing three times with PBS Tween 0,1%, the embryos were treated with proteinase K (10µg/ml) for 5 minutes at RT. After washing tree times more in PBS Tween 0,1%, they were postfixed in PFA 4% for 10 minutes at RT. Then, after washing two times with PBS Tween 0,1%, the embryos were incubated with the prehybridation buffer (Deionized Formamide 50%, 5X SSC buffer pH 7, Heparin 50µg/ml, Tween 0.1%, tRNA 50µg/ml, salmon sperm DNA 50µg/ml, completed with distilled DEPC treated milliQ water) at 65ºC for 3 hours. Afterwards, the embryos were incubated with the different probes (Table 1), at a final concentration 100-300ng/ml. The probes were diluted in hybridation buffer (Deionized Formamide 50%, 5X SSC buffer pH 7, Heparin 50µg/ml, Tween 0.1%, salmon sperm DNA 50µg/ml, completed with distilled DEPC treated milliQ water) and first denaturalized at 80ºC. Then, they were poured in the correspondent well. The incubation was performed overnight at 65ºC. The next day, they embryos were rinsed three times with solution I (Formamide 50%, 4X SSC pH 5, SDS 20%, Tween 1%, in autoclaved water) and three times with solution II (Formamide 50%, 2X SSC pH 4.5, Tween 1% and autoclaved water) at 65ºC. Subsequently, the embryos were washed in MABT 1x 0.1% solution at pH 7.5 (MAB 5x: 21.75gr NaCl, 29gr $C_4H_4O_4$, 19gr NaOH; Tween 0.1%) at RT. Then, the embryos were incubated with blocking buffer (10% of Sheep serum diluted in MABT 1x 0.1%) for 2 hours at RT. After this step, the embryos were incubated at 4ºC with 1:3500 dilution of alkaline-phosphatase coupled anti-digoxigenin Fab fragment antibody (Roche Applied Science). The next day, the embryos were rinsed 8 times (for 45 min each) in MABT 0,1%. After these washes, the embryos were washed 3 times in NTMT (NaCl 0.1M, TrisHCl pH=4.5 0.2M, $MgCl_2$, Tween20 0.1% and autoclaved water). Then, NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate, Fermentas/Roche) solution was used as a chromogenic substrate (0.45µl NBT + 3.4µl BCIP per ml of NTMT solution) to develop the signal from digoxigenin-labeled probes. Once the signal was completely developed, washes with PBS were used to stop the chromogenic reaction. After that, the embryos were dehydrated and rehydrated in order to inactivate the alkaline-phosphatase from the anti-digoxigenin antibody, and rinsed 3 times in MABT 0,1%. Then, the embryos were incubated again with blocking buffer (10% of Sheep serum diluted in MABT 1x 0.1%) for 2 hours at RT. After this step, the embryos were incubated at 4ºC with 1:2000 dilution of alkaline-phosphatase coupled

anti-fluorescein Fab fragment antibody (Roche Applied Science). The next day, the embryos were rinsed 8 times (for 45 min each wash) in MABT 0,1%. After these washes, the embryos were washed 3 times in NTMT. Then, INT/BCIP (iodonitrotetrazolium/5-bromo-4-chloro-3-indolyl phosphatase, Roche Diagnostics) solution was used as a chromogenic substrate (7 µl INT/BCIP per ml of NTMT solution) to develop the signal from fluorescein-labeled probes. Once the signal was completely developed, the embryos were rinsed in PBS and postfixed in PFA 4% for 15 minutes. Then, the mice embryos were photographed in a Leica loupe and the chicken electroporated embryos were processed with the immunohistochemistry in whole mount embryos protocol (III.3.3).

III.3.3. Immunohistochemistry

- In paraffin sections

Sections were exposed to 65ºC during 30 minutes to deparaffin the tissue. Then, they were exposed to xilol during 1 hour and rehydrated until PBS. Afterwards, they were exposed to boiling during 15 minutes in sodium citrate Buffer 0.1M pH 6, and they were rinsed in PBS with TritonX-100 0,1% and blocked in bovin serum albumin (BSA) 1% and Lysin 0.1M pH 6. Incubation were done overnight at 4ºC with DCC primary antibody (1:100, Santa Cruz) or PH3 primary antibody (1:500, Upstate, Lake Placid NY, USA). After washes in PBT 0.1% the sections were incubated with the correspondent biotinilated secondary antibody: rabbit anti-goat (1:200, vector) to detect DCC and goat anti-rabbit (1:200, vector) to detect PH3. Then, the slides were incubated with the ABC complex (1:500, ABC Elite Kit). After rinsed in PBT 0.1%, the sections were incubated with Diaminebenzidine and hydrogen peroxide. The sections were finally mounted with Eukitt (O. Kindler GmbH) and dried overnight at 37ºC.

- In whole mount embryos

After whole mount *in situ* hybridization, the embryos were prepared to perform immunohistochemistry to detect the expression of GFP. The embryos were washed 3 times in PBS Tween 0,1% at RT. Then, the embryos were incubated with the blocking buffer (10% Sheep serum diluted in PBS Tween 0,1%) for 3 hours at RT and afterwards, they were incubated

overnight at 4ºC with the antibody anti GFP chicken IgY (1:1000, Aves). The next day, the embryos were rinsed 6 times with PBS Tween 0,1% (30 minutes each wash) and then, were incubated with the secondary antibody Donkey-anti-chicken Alexa 488 (1:1000, Molecular probes) for 2 hours at RT. Subsequently, the embryos were rinsed again with PBS Tween 0,1%, postfixed in PFA 4% for 10 minutes and photographed in a Leica loupe.

III.4. Quantifications and statistical analysis

Adobe photoshop was used to measure the Shh domains in experiments of loss and gain of function. The measures were taken following the scheme E of the Figure 16 (see Results). Microsoft Excel was used to statistically analyze experimental data.

Chapter IV

RESULTS

IV.1. Histological and anatomical analysis of Wnt1 mutant embryos

IV.1.1. Several diencephalic structures are altered in Wnt1 mutant embryos

To investigate the role of Wnt1 in the diencephalic development, we first analyzed the embryonic phenotype of Wnt1 deficient mice at 17.5 days of development (E17.5), a moment when the diencephalic cytological structure is almost entirely formed. In order to achieve this goal, we have used the mice line generated by McMahon and Bradley (1990), which have null alleles for Wnt1 gene. Consecutive and serial sagittal sections of Wnt1 mutant and WT embryonic brains were processed using specific methods for structural analysis.

Observation of these sections, using the cresyl violet staining, show that the transversal regionalization, represented by the three diencephalic prosomeric subdivisions (p1, p2 and p3, from caudal to rostral), are not significantly changed in Wnt1 mutant embryos in comparison with WT embryos. The thalamus maintains similar structure in mutant and WT, as well as the pretectum, although it should be pointed out that these two structures seemed to be displaced towards the caudal region (Figure 7. A, B), however this difference was very subtle and could be consequence of a change in relative proportions secondary to the strong reduction of midbrain alar plate. The mid-hindbrain phenotype of Wnt1 mutant mouse has been previously described in several publications (McMahon and Bradley, 1990; McMahon et al., 1992; Thomas and Capecchi, 1990). This region is severely compromised in the absence of Wnt1 during neural tube development: the whole or posterior half of the mesencephalic alar plate and the anterior-most rhombencephalon are absent (Figure 7. B).

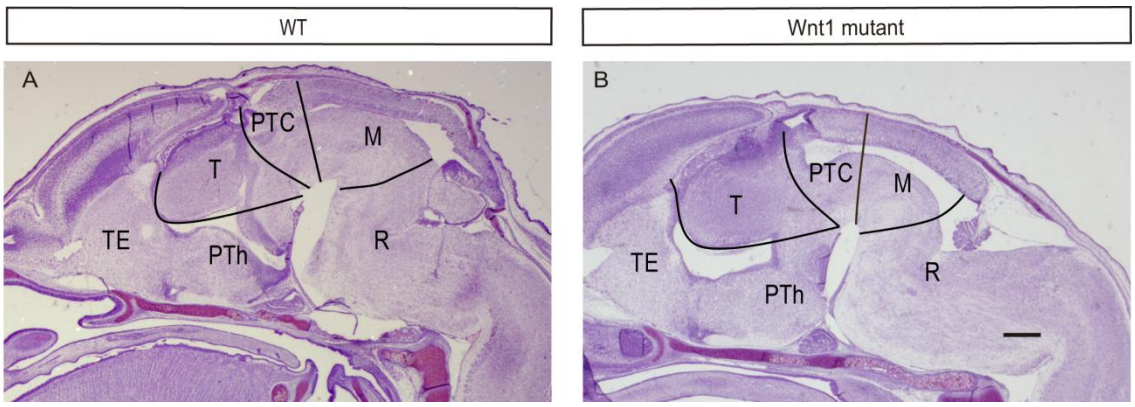

Figure 7. Anatomical phenotype of the diencephalon of Wnt1 mutant embryos.

Lateral view of sagittal sections of the neural tube with cresyl violet straining in wild type (A) and Wnt1 mutant embryo (B) at E17.5. Abbreviations: TE, telencephalon; PTh, prethalamus; T, thalamus; M, mesencephalon; R, rhombencefalon. Scale bar A,B: ~ 1mm.

After this structural study, we wanted to deeply analyze this mature phenotype by specifically revealing the fine structure of each diencephalic region at different stages of development.

We continued analyzing the anatomy of the diencephalon in brain sagittal sections. We observed that the retroflexus tract, an axonal tract situated between p2 and p1, is disrupted in Wnt1 mutant embryos. Even using cresyl violet staining it is clear that the retroflexus tract is enlarged and subdivided in two or three sub-fascicles in Wnt1 mutant embryos (Figure 8. B, D), showing a wider size than in WT embryos (Figure 8. A, C).

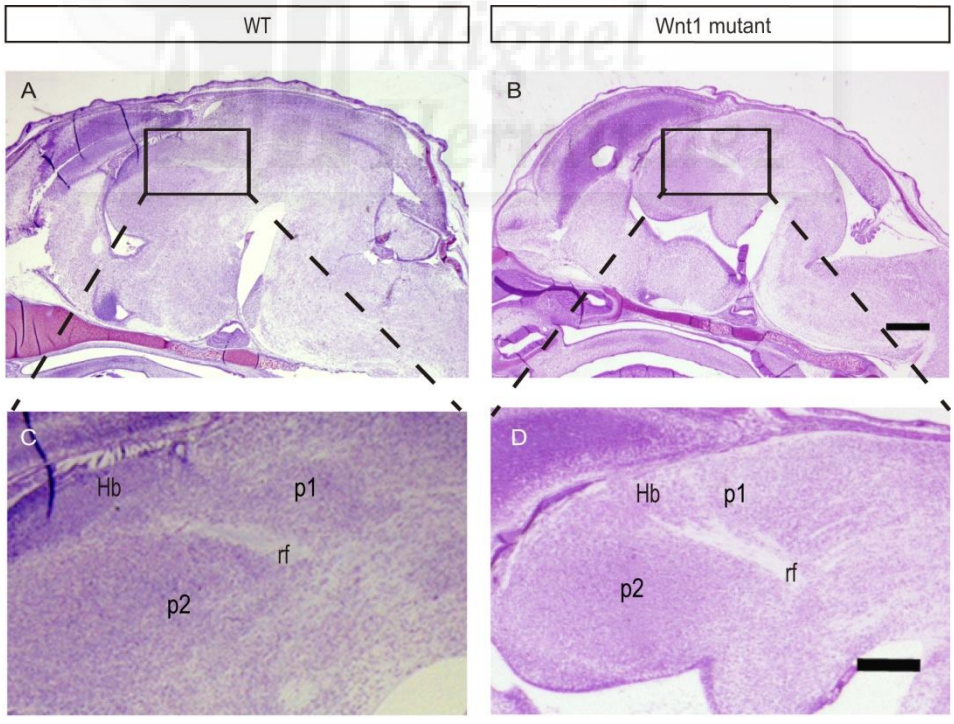

Figure 8. Analysis of retroflexus tract in Wnt1 mutant embryos.

Lateral view of sagittal sections of the neural tube with cresyl violet straining in wild type (A, C) and Wnt1 mutant embryo (B, D) at E17.5. C and D are higher magnifications from the retroflexus tract from A and B, respectively. Abbreviations: Hb, habenula; rf, retroflexus tract; p1, prosomere 1; p2, prosomere 2. Scale bar A,B: ~ 1mm. Scale bar C,D: ~ 500µm.

Then, we hypothesized that this alteration of axons projecting from the habenular to the interpeduncular nuclei could be due to two different reasons. The first reason could be an increase in the number of fibers of this tract, which means an increased number of projecting neurons in the epithalamus region (where these fibers are generated). The second reason, on the other hand, could be an alteration in the degree of fasciculation of the fibers of this tract as a consequence of anomalies in the substrate of axonal growth or an autonomous axonal problem of axon-axon interaction.

In order to test the first hypothesis, we analyzed cell proliferation in Wnt1 mutant and WT embryos in coronal sections at E13.5 by detecting the expression of the M-phase cell cycle marker PH3. We did not observed changes in the proliferation rate in the epithalamus between Wnt1 mutant and WT embryos (WT: 37 ± 3,78 cells; Wnt1 mutant: 35,33 ± 4,17 cells , n=3, p=0,39; Figure 9. A, B). Once this option was discarded, we analyzed the fasciculation of the retroflexus tract by detecting the expression of the marker DCC, which is a receptor that mediates the effects of Netrin, in coronal and sagittal sections at E13.5. This immunostaining demonstrates that, indeed, the retroflexus tract is defasciculated in Wnt1 mutant embryos, showing that the dorsal anchoring point of this tract, the habenular region, seems to occupy a more extensive area in comparison with that of the wild type (Figure 9. C-F).

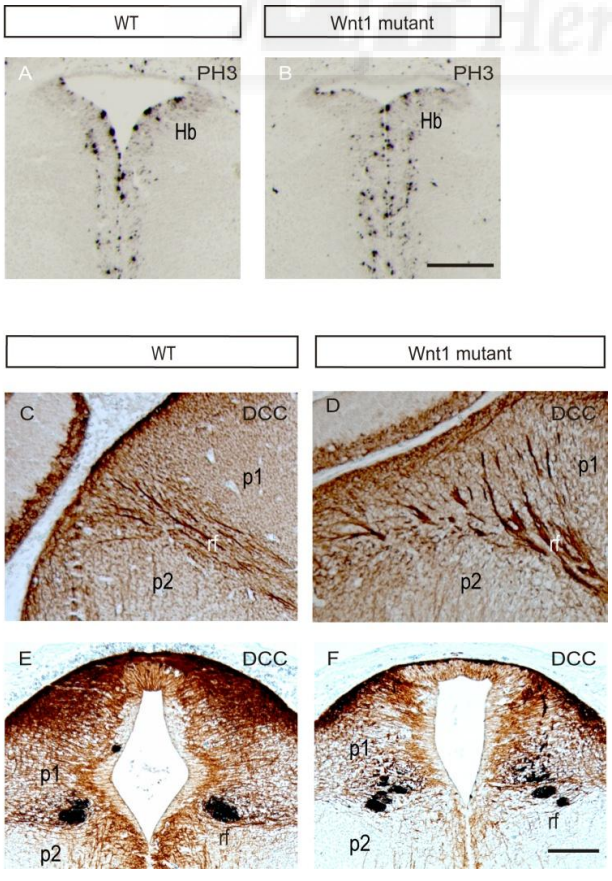

Figure 9. Detection of PHA and DCC in Wnt1 mutant and WT embryos to analyze the retroflexus tract.

(A,B) Inmunohistochemistry against PH3 in coronal sections at E13.5 shows no differences in cell proliferation rate in the epithalamus region between WT (A) and Wnt1 mutant embryos (B).
(C-D) Inmunohistochemistry against DCC in sagittal sections (C,D) and coronal sections (E,F) at E13.5 shows that in Wnt1 mutant embryos the region where the retroflexus tract is initiated in the habenula is bigger than in WT embryos (C,D), and the fasciculation of this tract is lower in the mutant embryos than in WT embyos (E,F). Abbreviations: Hb, habenula; rf, retroflexus tract; p1, prosomere 1; p2, prosomere 2. Scale bar A,B: ~ 250 µm. Scale bar C-F: ~ 500 µm.

As it has been explained in the introduction of this manuscript, Wnt1 is mostly expressed in the dorsal midline of the neural tube. This lead us to think that the structures situated in the dorsal diencephalon should be the most affected areas when Wnt1 signaling is altered. Therefore, we now focused our study in the diencephalic roof plate. Cresyl violet staining in brain sagittal sections at E14.5 stage of development shows that, indeed, two of the most important structures of the diencephalic roof plate are altered.

The most caudal of these structures is the posterior commissure, situated along the caudal half of the pretectal region and ending at the diencephalon-mesencephalon boundary. This structure shows a disorganized positioning of the axon fascicles in homozygous Wnt1 mutants (Figure 10. D, black arrowheads) in comparison with control embryos (Figure 10. C, black arrowheads). Actually, the commissural area is enlarged towards the thalamus without a clear separation between commissural and precommissural roof plates. Rostrally to the posterior commissure and located in the caudal half of p2 roof plate, is situated the pineal gland which is also defective in Wnt1 mutant embryos, being almost absent (Figure 10. D, red arrowheads) in comparison with the normal pineal gland present in control embryos (Figure 10. C, red arrowheads).

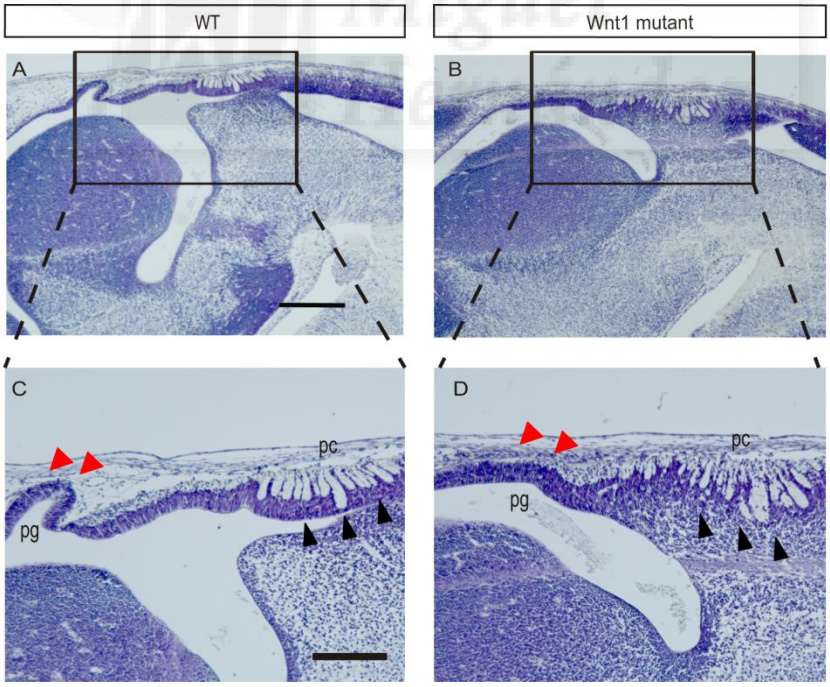

Figure 10. Analysis of pineal gland and posterior commisure in Wnt1 mutant embryos.

Cresyl violet staining in sagittal sections at E14.5 shows the differences between the WT (A, C) and Wnt1 mutant embryos (B, D) in the pineal gland (red arrowheads) and posterior commisure (black arrowheads). Abbreviations: pg, pineal gland; pc, posterior commisure. Scale bar A,B: ~ 750 µm. Scale bar C,D: ~ 250 µm

Therefore, Wnt1 signal seems to be required for the normal patterning of the caudal half of the diencephalic roof plate, from the pineal gland to the posterior commissure, where actually the gene is expressed. Moreover, by paracrine activity, the neighboring paramedian dorsal territories of the p2 alar plate, that is the epithalamic habenular nuclei, where the gene is not expressed, are also affected. These phenotypes in p1 and p2 dorsal territories suggest anomalies in the regional patterning of the diencephalon.

IV.2. Diencephalic patterning in Wnt1 mutant embryos

IV.2.1. Alteration of the antero-posterior pretectal axis

To investigate the importance of the role of Wnt1 signal in the organization of the diencephalon, we studied how the different territories of this area are patterned in the absence of Wnt1 protein. We analyzed embryos at early stages of development (from E11.5 to E12.5), a time when the diencephalon shows an entirely segmental structure (Suzuki-Hirano et al., 2010; Puelles et al., 2012; Martinez-Ferre and Martinez, 2012).

We first wanted to determine the global prosomeric structure (size and anatomical relations) of the diencephalic prosomeres. We started performing a double *in situ* hybridization for Six3 and Pax6. Six3 is a transcription factor whose expression domain is extended through the diencephalon, as a columnar parabasal band that runs dorsally to the expression of Shh in the basal plate, along the midbrain and p1 at E11.5 (Figure 11. A), extending into the p2 at E12.5 (Figure 11. C, E). Rostrally, the longitudinal expression abruptly ends at the ZLI (p3/p2 boundary) where it changes the direction at E12.5 towards the dorsal thalamic epithelium, as a Six3-expressing band beside the caudal edge of the ZLI. Another transversal domain of Six3 appears also at E12.5 running dorsally, from basal to alar pretectum and covering the juxtacommisural pretectal territory (JcP; Figure 11. C, E), allowing us to visualize the three pretectal subdivisions (Braun et al., 2003; Ferran et al., 2008). Moreover, Six3 expression analysis has been selected not only because is a good marker of diencephalic subdivisions, but also because Six3 and Wnt1 have a mutual crossinhibition relationship (Lavado et al., 2008). Regarding Pax6, this is an early regulatory gene of the paired-box family, whose expression appears in the entire pretectal and thalamic alar plate ventricular zone (with highest expression at the pretectal region, having a gradiental distribution which from the commissural domain is decreasing rostrally into the

precommisural domain). Moreover, Pax6 expression is a perfect marker of the p1-mesencephalon boundary in the alar plate (Figure 11. A, C, E), and is negatively regulated by Shh in spinal cord and in the thalamus, but not in the prethalamus (Ericson et al., 1997; Kiecker and Lumsdem; 2004).

At E11.5 stage of development, the expression of Six3 is clearly increased in the parabasal band of mesencephalic and diencephalic alar plate of Wnt1 mutant embryos (Figure 11. A, B). At E12.5, the expression level of this gene remains upregulated in the absence of Wnt1 signal: the expression domain which lines the ZLI has a higher expression of Six3 than in WT embryos, as well as the domain expression located in the JcP (Figure 11. C-F). This increase in the expression of Six3 was expected due to the mutually repressive behavior between Wnt1 and Six3 (Braun et al., 2003).

The expression of Pax6 remains unaltered and shows that the structure of p2 seems to be normal. The pretectal subregions PcP and CoP are also correctly located and organized (Figure 11. C-F). In addition to that, it is clear that the diencephalic parabasal band, close to the basal plate has an abnormal structure in Wnt1 mutant embryos, since the longitudinal domain of Six3 expression in the parabasal p2 band is almost absent and the ZLI-related expressing band coincides with JcP band at their ventral origin in an acute angle (Figure 11. D, red arrowheads). Moreover, the alteration of the retroflexus tract is clearly evident in this figure, as a Pax6 negative transversal band, showing that this structure occupies a wider territory in Wnt1 mutant embryos (Figure 11. E, F, black arrowheads), probably due to its defasciculation, as we previously described (Figure 9).

However, the increase of Six3 expression domain in the JcP leads us to think that pretectal regionalization could be also altered. Therefore, the next step was to unravel whether Six3 expressing domain was invading the precommisural domain, given a more extended juxtacommisural pretectal region.

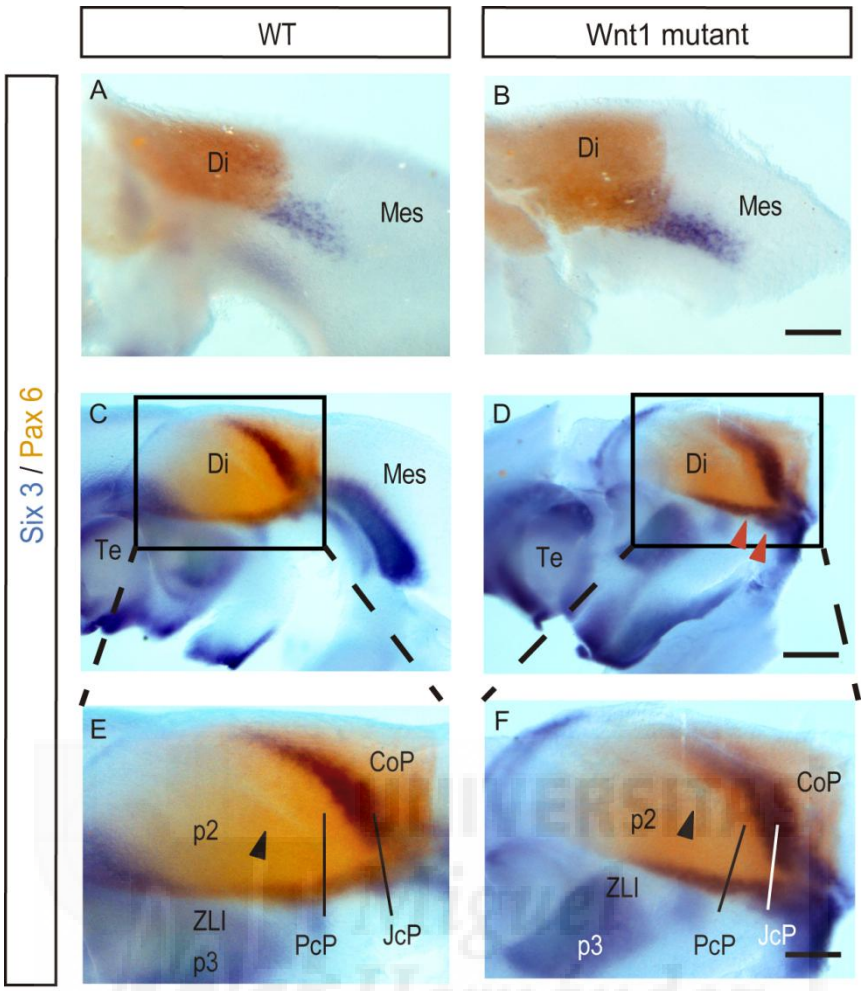

Figure 11. Expression domains of Six3 and Pax6 in WT and Wnt1 mutant embryos.

Lateral view of *in situ* hybridization for Six3 and Pax6 in whole mount embryos at E11.5 (A, B) and E12.5 (C-F) of WT (A,C,E) and Wnt1 mutant embryos (B,D,F). E and F are high magnifications of the diencephalic territory from C and D, respectively. Red arrowheads in D indicate the alteration of Six3 in the basal plate. Black arrowheads in E and F indicate the location of the retroflexus tract, which is clearly wider in Wnt1 mutant embryos. Abbreviations: Di, diencephalon; Mes, mesencephalon; Te, telencephalon; p2, prosomere 2; p3, prosomere 3; PcP, precommisural domain; JcP, juxtacommisural domain; CoP, commissural domain. Scale bar A,B: ~ 300 µm. Scale bar C,D: ~ 750 µm. Scale bar E,F: ~ 250 µm.

In order to unravel if the boundary between the precommissural and the juxtacommissural pretectal domain is correctly established or, alternatively, the expression of Six3, whose expression is restricted normally to the juxtacommissural domain, is also localized in the precommissural areas in Wnt1 mutant embryos, we analyzed the expression of Dbx1. Dbx1 is a transcription factor selectively expressed in the precommissural domain (Shoji et al., 1996; Vue et

al., 2007), and its study let us clearly differentiate both domains of the pretectum. The results show that the expression of these two genes is no co-segregated, and the identity and boundary between these domains is clearly established (Figure 12. A, B, white discontinued line). Thus, we conclude that the juxtacommisural domain seems to be enlarged in Wnt1 mutant embryos, while the precommissural domain is not altered.

We also observed that the parabasal column of the alar plate and the basal plate are abnormally organized. The parabasal column of CoP is strongly reduced in Wnt1 mutant embryos, as it is showed by the lacking or strong reduction in length of the caudal tail of JcP-Six3 expression domain (Figure 12. A, B). In addition, the p1 basal plate under the ventral expression of Six3 occupies a smaller area, which is especially observable by its reduction in size (Figure 12. A, B, black arrows) and the narrowing of the cephalic flexure. Therefore, these results indicate that the expression of Wnt1 could be also necessary for the correct establishment of the diencephalic dorso-ventral axis.

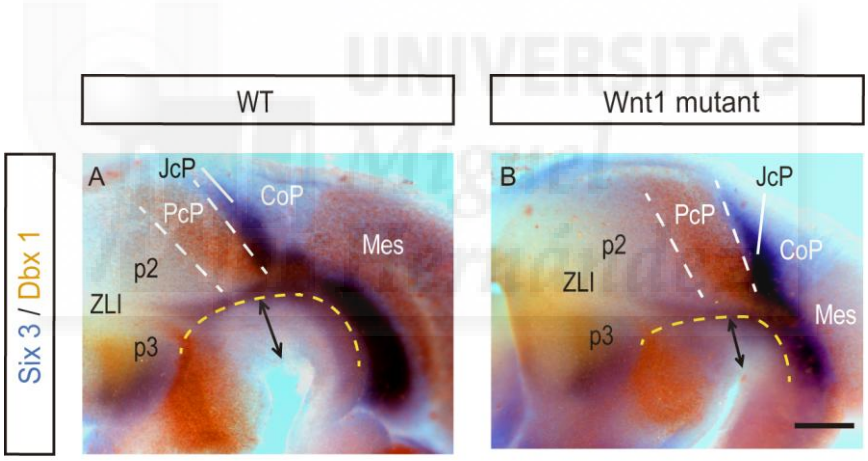

Figure 12. Expression domains of Dbx1 and Six3 in WT and Wnt1 mutant embryos.

(A,B) Lateral view of *in situ* hybridization for Dbx1 and Six3 in whole mount embryos at E12.5 in WT (A) and Wnt1 mutant embryos (B). White discontinued lines indicate the boundaries between the different prectal domains, showing that Six3 and Dbx1 are no co-segregating. Yellow discontinued lines indicate the boundary between the basal and the alar plate. Black arrows indicate the differences of size in the basal plate, which is decreased in Wnt1 mutant embryos. Abbreviations: Mes, mesencephalon; p1, prosomere 1; p2, prosomere 2; p3, prosomere 3; PcP, precommisural domain; JcP, juxtacommisural domain; CoP, commissural domain. Scale bar A,B: ~ 500 µm.

IV.2.2. Antero-posterior thalamic axis is unaltered in Wnt1 mutant embryos

To further investigate how these observed alterations are affecting anterior diencephalic prosomeres, we performed *in situ* hybridization of different markers for each altered region in order to analyze potential changes in the regionalization pattern of the whole diencephalon. We have observed that conversely to the p1 described alterations, p2 seems to be properly formed. In order to assess whether the p2 territory is altered or not, we analyzed the expression of Ngn2, which is broadly expressed in p2 neuroepithelium (Nakagawa and O'Leary, 2001; Seibt et al., 2003; Vue et al., 2007). Ngn2 is expressed in the thalamus (alar p2), the precommissural pretectum (alar PcP) and weakly in the commissural pretectum (alar CoP). Indeed, in a lateral view of the E12.5 whole embryonic diencephalon, we observed that the Ngn2 expression domain in the thalamus remains mainly unaffected in Wnt1 mutants (Figure 13. A, B).

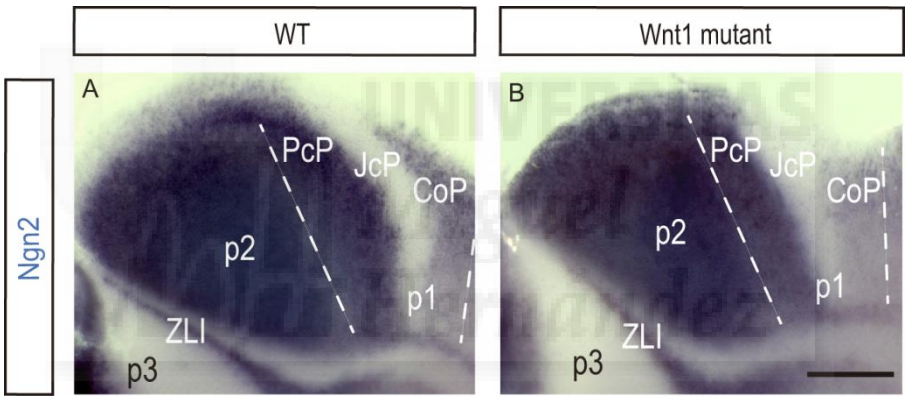

Figure 13. Expression domains of Ngn2 in WT and Wnt1 mutant embryos.

Lateral view of *in situ* hybridization for Ngn2 in whole mount embryos at E12.5 in WT (A) and Wnt1 mutant embryos (B) shows that the thalamus is properly structured in Wnt1 mutant embryos. Abbreviations: p1, prosomere 1; p2, prosomere 2; PcP, precommisural domain; JcP, juxtacommisural domain; CoP, commissural domain. Scale bar A,B: ~ 500 µm.

IV.2.3. Irx3 and Fgf8 expression domains are altered in Wnt1 mutant embryos

It has been published that Six3, together with Irx3, have key roles during diencephalic development and in the specification of the ZLI, the diencephalic organizer (Braun et al., 2003; Kobayashi et al., 2002). The expression of Six3 (rostrally) and Irx3 (caudally) at early stages of development are regulated by Wnt signaling, which directs the initial expression of Irx3 and repression of Six3 in the early forebrain, delineating the posterior and anterior forebrain domains

(Braun et al., 2003). Given this established relationship, we decided to analyze the expression of Irx3 in Wnt1 mutant embryos. As it was expected, Irx3, which is expressed in the dorsal alar region of p2 (epithalamus) and in the PcP, is strongly reduced in the absence of Wnt1 protein (Figure 14. A, B, black arroheads). Moreover, Irx3 expression domain in the basal plate of the prethalamus and retrommamillar tegmentum is decreased as well, showing that the anterior diencephalic basal plate seems to be also reduced in Wnt1 mutant embryos (Figure 14. B white arrowheads).

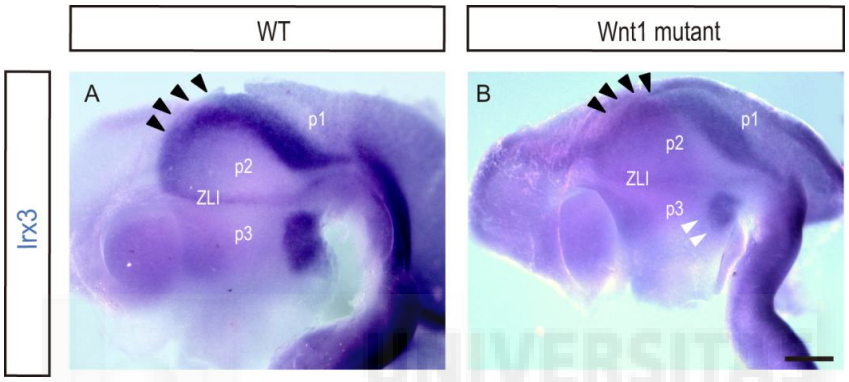

Figure 14. Expression domains of Irx3 in WT and Wnt1 mutant embryos.

Lateral view of in situ hybridization for Irx3 in whole mount embryos at E12.5 in WT (A) and Wnt1 mutant embryos (B) show that Irx3 expression is clearly downregulated in the absent of Wnt1. Black arrowheads indicate the reduced Irx3 expression in the dorsal thalamus. White arrowheads show the alteration of Irx3 expression in the basal plate of the hypothalamus. Abbreviations: p1, prosomere 1; p2, prosomere 2; p3, prosomere 3. Scale bar: ~ 750 µm.

We analyzed the expression of Fgf8, another gene that has been described as a fundamental morphogenetic signal during diencephalic development. This gene is expressed in the dorsal midline of the diencephalon, and its loss has been associated with alterations in the habenular region and pineal gland, which are reduced or absent in Fgf8 mutant mice (Martinez-Ferre et al., 2009). As we showed before, the pineal gland formation is altered in Wnt1 mutant embryos, so we decided to analyze the expression of Fgf8 in this mutant. As expected, the expression of Fgf8 in the anterior diencephalic roof plate is clearly expanded from the prethalamus to more caudal levels into the p2 in the dorsal midline, reaching the rostral region of p1 (Figure 15. B, black arrowheads) and suggesting that Fgf8 expression in the epithalamic roof plate could be controlled by Wnt1 signal.

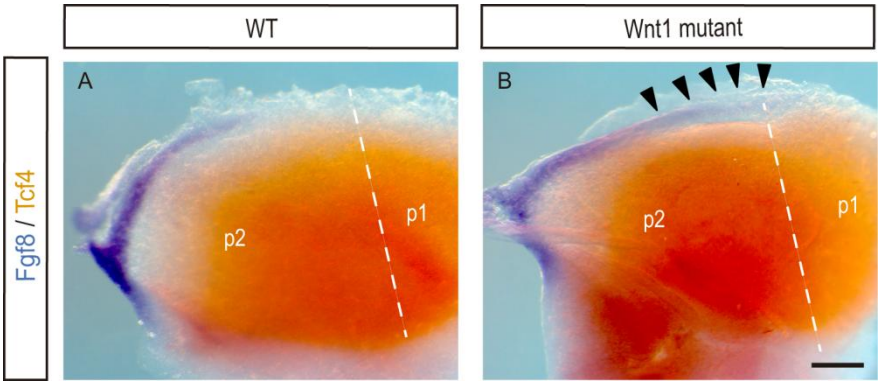

Figure 15. Expression domains of Fgf8 and Tcf4 in WT and Wnt1 mutant embryos.

Lateral view of *in situ* hybridization for Fgf8 and Tcf4 in whole mount embryos at E12.5 in WT (A) and Wnt1 mutant embryos (B). The expression of Fgf8 is expanded caudally in the diencephalon in the absence of Wnt1 (black arrowheads). Abbreviations: p1, prosomere 1; p2, prosomere 2. Scale bar: ~ 250 µm.

IV.2.4. Shh expression domains are altered, suggesting misspatterning of the dorso-ventral axis in Wnt1 mutant embryos

The next step was to analyze the expression of Shh, a member of the hedgehog family of secreted signaling molecules. At early stages of development, Shh is expressed in the ventral midline along the entire neuraxis (Echelard et al., 1993). From the isthmic region to the rostral pole of neural plate, Shh expression is activated in the basal plate at early neural stages and then its expression expands dorsally into the ZLI (Echevarria et al., 2003; Kiecker and Lumsdem, 2004; Vieira et al., 2006, Vieira et al., 2010; Scholpp and Lumsden, 2010). In combination with the analysis of Shh expression, we use the expression of Tcf4 as a marker of the alar plate of p2 and p1, in order to clearly visualize the boundary between the pretectum and the mesencephalon.

We observe that the expression of Shh is altered in Wnt1 mutant embryos. The ventral expression domain of Shh in the diencephalon, which is extended along the FP and BP from the rostral to caudal neural tube and delineates the basal-alar boundary (Figure 16. A, B), is reduced in the absence of Wnt1, suggesting that the basal plate region is narrower in Wnt1 mutants than in WT (Figure 16. C, D, red line of points). Moreover, the length of the expression domain of Shh in the ZLI is reduced, that is, the dorsal most point of Shh expression within the ZLI is situated more ventral in the absence of Wnt1 signal (Figure 16. C, D, black arrowheads).

We provide quantitative data which supports these differences visualized in the expression of Shh in the diencephalon. We measured the size of the Shh expression domain in the BP, from the FP to the basal-alar boundary, (Figure 16. E, green discontinued line Shh-BP) and also the distance from the dorsal limit of Shh expression within the ZLI to the RP of the diencephalon (Figure 16. E, orange discontinued line ZLI-RP). There is a significant reduction (12, 81 ± 2,40 %, n=5) in the Shh expression domain of the BP in mutant embryos compared with control embryos (Figure 16. F). The distance between the RP and the dorsal limit of Shh expression in the ZLI is increased (20,19 ± 5,09 %, n=5) in Wnt1 mutant embryos, suggesting that the Shh expression domain within the ZLI does not reach the same dorsal level as in WT conditions (Figure 16. G).

These results suggest that Wnt1 signal is essential for the correct formation of the dorso-ventral axis of the diencephalon, and its loss leads to an abnormal patterning of the horizontal domains of the diencephalon.

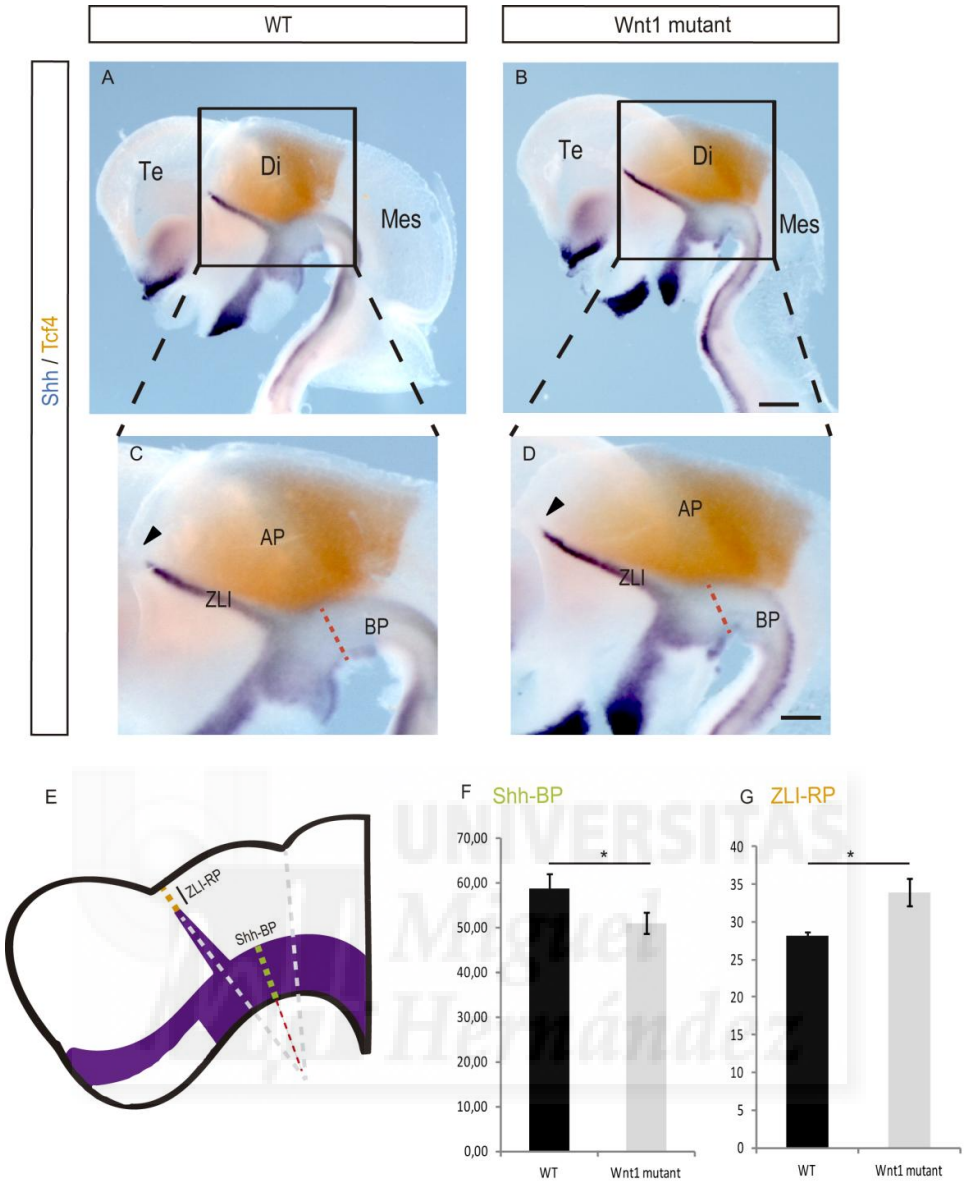

Figure 16. Expression domains of Shh and Tcf4 in WT and Wnt1 mutant embryos.

(A,B) Lateral view of control side (A) and Wnt1 mutant (B) embryos at E12.5 showing *in situ* hybridization for Shh and Tcf4. (C) and (D) are higher magnifications of A and B, respectively. Black arrowheads indicate the end of the Shh domain expression within the ZLI, which is situated further from the roof plate in Wnt1 mutant embryos. Red discontinued lines indicate the extension of the basal plate. (E) Schematic representation of the measures that have been taken in order to obtain quantitative data. Orange discontinued line indicates the distance measured between the end of Shh expression within the ZLI to the roof plate. Red discontinued line indicates the bisector of the angle which results from the extension of the line which delineates the ZLI and the line which delineates the boundary between the diencephalon and the mesencephalon. Green discontinued line indicates the size of the basal plate. (F) Histogram representing mean ± SEM percentage of the size of Shh expression domain in the basal plate, measuring the distance between the floor plate and the basal-alar boundary. Asterisk indicates statistical significance by T-test analysis (n=5). The Shh expression domain in the basal plate is reduced (12,81 ± 2,40 %) in Wnt1 mutant embryos (p=0,04). (G) Histograms representing as the mean ± SEM percentage of the distance between the roof plate and the end of Shh expression within the ZLI in the alar plate. Asterisk indicates statistical significance by T-test analysis (n=5). The distance between the RP and the dorsal limit of Shh expression in the ZLI is increased (20,19 ± 5,09 %) in Wnt1 mutant embryos (p=0,005). Abbreviations: Te, telencephalon; Di, diencephalon; Mes, mesencephalon; AP, alar plate; BP, basal plate. Scale bar A,B: ~ 750 µm. Scale bar C,D: ~ 250 µm.

IV.2.5. Gli3 expression is increased in Wnt1 mutant embryos

It has been published that the Shh/Gli pathway plays a major role in the dorso-ventral patterning of the neural tube (Jessell, 2000; Ruiz i Altaba et al., 2007). It has been demonstrated that Wnt1 signal, by means of the inhibition of Gli3 and the regulation of L-fng at the p3-p2 boundary, has a fundamental role in the complete formation of the ZLI (Martinez-Ferre, Navarro-Garberi et al 2013). We analyzed the expression of Gli3 in the Wnt1 mutant embryos in order to clarify these interactions in the diencephalon. Interestingly, Gli3 expression is clearly increased in the alar plate in Wnt1 mutant embryos (Figure 17. A, B), suggesting that, indeed, Wnt signal negatively regulates the expression of Gli3 in the diencephalic AP.

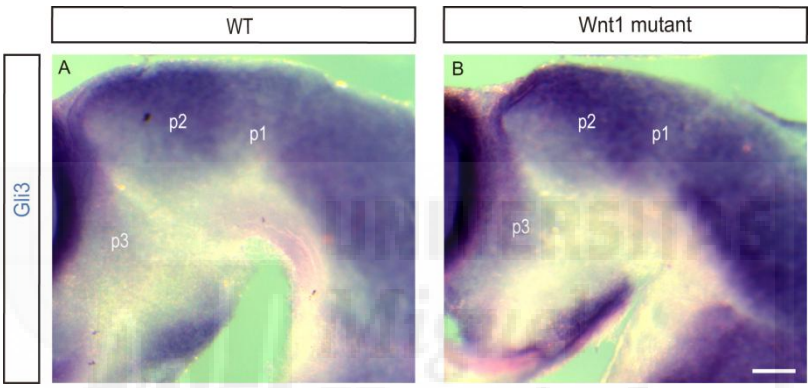

Figure 17. Expression domain of Gli3 in WT and Wnt1 mutant embryos.

Lateral view of *in situ* hybridization for Gli3 in whole mount embryos at E12.5. (A) Control embryos show the normal expression of Gli3 in the AP of the diencephalon. (B) Expression of Gli3 in Wnt1 mutant embryos shows that this expression is upregulated in the absence of Wnt signal. Abbreviations: p1, prosomere 1; p2, prosomere 2; p3, prosomere 3. Scale bar: ~ 250 µm.

IV.2.6. Bmp4 expression is absent in Wnt1 mutant

The participation of the Bmp-mediated signaling in the determination of the dorso-ventral axis has been extensively described along the neural tube (Liem et al., 1997; Nguyen et al., 2000; Timmer et al., 2002; Lim et al., 2005). Since the interactions between Wnt and Bmp signals during the dorso-ventral diencephalic patterning process are still unclear, we analyzed the expression of Bmp4 in Wnt1 mutant embryos. Interestingly, we observed that the expression of Bmp4, which is expressed in the caudal region of the diencephalic roof plate at the level of the

pineal gland (Figure 18. A, black arrowheads), is missing in Wnt1 mutant embryos (Figure 18. B, black arrowheads).

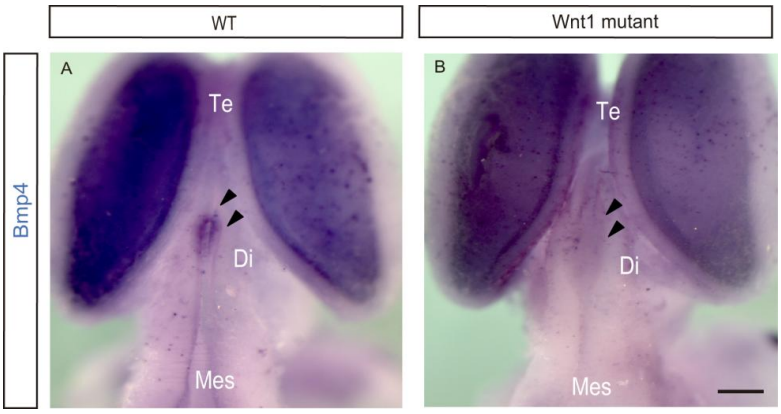

Figure 18. Expression domain of Bmp4 in WT and Wnt1 mutant embryos.

Dorsal view of *in situ* hybridization for Bmp4 in whole mount embryos at E12.5 show that the expression of Bmp4 situated in the diencephalic RP in control embryos (A, black arroheads) is absent in Wnt1 mutant embryos (B, black arrowheads). Abbreviations: Te, telencephalon; Di, diencephalon; Mes, mesencephalon. Scale bar: ~ 750 µm.

IV.3. Diencephalic patterning after Wnt1 gain of function

IV.3.1. Wnt1 overexpression displaces dorsally the boundary between the diencephalic basal and alar plates

To elucidate the molecular mechanisms underlying the role of Wnt1 expression in the formation of the dorso-ventral axis, we performed *in vivo* gain-of-function experiments by means of *in ovo* electroporation which allows manipulation at early stages of development, when the diencephalon is not yet properly patterned. A plasmid expressing Wnt1 and EGFP proteins was unilaterally introduced into the neuroepithelium of chick embryos at three vesicles stage (HH10). After 3 days, embryos were sacrificed and the expression of markers for the different diencephalic areas was analyzed.

Analyzing the expression of Shh, we observed that the Shh ventral expression domain is clearly increased, since this domain has a bigger size in the electroporated side than in the control side (black arrows in Figure 19. A and Figure 21. A, B). The dorsal expansion of Shh

within the ZLI is also increased, reaching the spike more dorsal levels in the electroporated size (white arrowhead in Figure 19. A).

We also provide quantitative data which supports these differences observed in the expression of Shh. We measured the size of the Shh expression domain in the BP, from the FP to the basal-alar boundary (Figure 16. E, green discontinued line Shh-BP) and also the distance from the dorsal limit of Shh expression within the ZLI to the RP of the diencephalon (Figure 16. E, orange discontinued line ZLI-RP). In the case of this gain of function experiments, there is a significant increase (40,84 ± 10,99 %, n=4) in the Shh expression domain of the BP in the electroporated compared with the control side (Figure 19. E). Moreover, the distance between the RP and the dorsal limit of Shh expression in the ZLI is clearly reduced (49,33 ± 0,96 %, n=4) in the electroporated side, which suggests that the Shh expression domain within the ZLI reaches more dorsally levels when Wnt1 is overexpressed (Figure 19. F).

We also analyzed the expression of Otx2, a transcription factor expressed in the entire forebrain and midbrain neuroepithelium in vertebrate embryos (Rhinn and Brand, 2001) This expression does not show any visible alteration along the dorso-ventral axis.

In addition, the expression domains of Gbx2 and Pax6 transcription factors are also altered (Figure 19. C). Gbx2 is a homeobox gene described as a thalamic marker, expressed in the mantle layer cells where it is essential for the development of many of its dorsal thalamic nuclei and possibly regulated by Shh (Miyashita-Lin et al., 1999, Hashimoto-Torii et al., 2003; Kiecker and Lumsden, 2004; Vieira et al., 2005; Vieira and Martinez, 2006). In the electroporated side, the ventral edge of Gbx2 thalamic expression domain is repelled from the basal plate (Figure 19. C, brown arrowhead). Dorsally, the expression of Gbx2 is clearly expanded in the dorsal thalamus (Figure 19. C, white arrowhead) in the electroporated side. When Wnt1 is overexpressed, Pax6 suffers a strong patterning modification since, in addition to the expected reduction of its dorso-ventral alar expression (Figure 19. C, black arrows), the Pax6 positive cells situated as a longitudinal stripe in the boundary between the basal and alar plate of the caudal diencephalon disappears (Figure 19. C, pinks arrowheads).

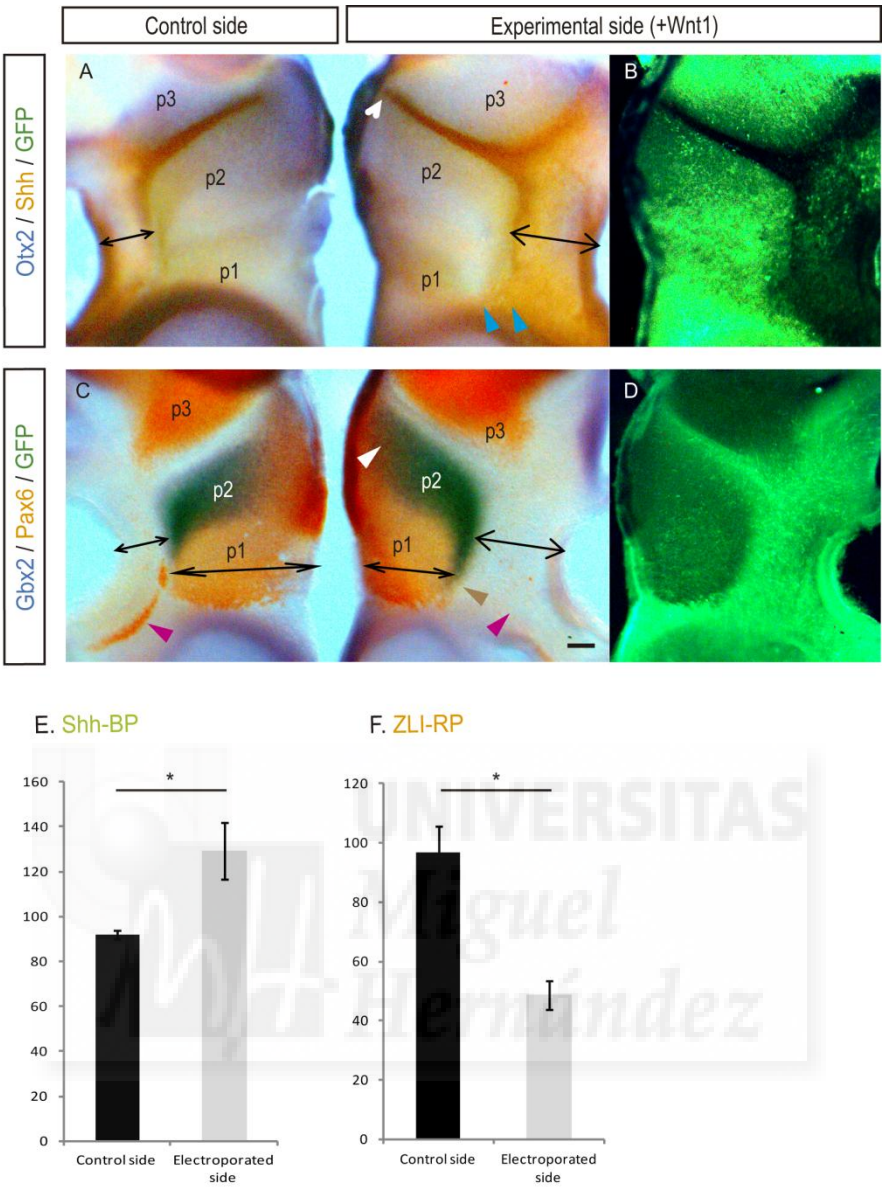
Control side
Experimental side (+Wnt1)
Otx2 / Shh / GFP
Gbx2 / Pax6 / GFP
A
B
C
D
p1
p2
p3
E. Shh-BP
F. ZLI-RP
Control side
Electroporated side
*

Figure 19. Wnt1 signal *in ovo* electroporation avoid the proper formation of the dorso-ventral axis.

(A) Dorsal view of the opened embryos (HH23) through the ventral midline showing *in situ* hybridization for Shh and Otx2. Right side is the control side and left side is the experimental side. The black arrows indicates the different size of the Shh ventral expression domain, showing that the boundary between the alar and the basal plate has been dorsally displaced. White arrowheads indicate the end of the Shh domain within the ZLI, arising more dorsally levels in the experimental side. Blue arrowheads indicate the initial formation of an ectopic spyke of Shh (this issue will be explained later). (B) Dorsal view of the experimental side of the opened embryos of picture A, showing the expression of GFP along the experimental side. (C) Dorsal view of the opened embryos through the ventral midline showing *in situ* hybridization for Gbx2 and Pax6. Right side is the control side and left side is the experimental side. The black arrows indicate the different size between the BP and the AP, which are increased and decreased in the experimental side, respectively. White arrowheads indicate the expansion of Gbx2 through the dorsal thalamus. Pink arrowheads indicate the expression domain of Pax6 in the ventral boundary between diencephalon and mesencephalon, which is missed in the experimental size. Brown arrowhead indicates the Gbx2 repulsion from the basal plate. (D) Dorsal view of the experimental side of the opened embryos of picture C, showing the expression of GFP along the experimental side. (E) Histogram representing mean ± SEM percentage of the size of the Shh expression domain in the basal plate, measuring the distance between the floor plate and the basal-alar boundary. Asterisk indicates statistical significance by T-test analysis (n=4). The Shh expression domain in the basal plate is increased (40,84 ± 10,99 %) in the electroporated side (p=0,013). (F) Histograms representing as the mean ± SEM percentage of the distance between the roof plate and the end of Shh expression within the ZLI in the alar plate. Asterisk indicates statistical significance by T-test analysis (n=4). The distance between the RP and the dorsal limit of Shh expression in the ZLI is reduced (49,33 ± 0,96 %) in the electroporated side (p=0,004). Abbreviations: p1, prosomere 1; p2, prosomere 2; p3, prosomere 3. Scale bar: ~ 500 μm.

These results demonstrate that, when Wnt1 is overexpressed, the structure of the diencephalic dorso-ventral axis is abnormally formed, since the boundary between the basal and the alar plate is dorsally displaced, due to an increase in the size of the basal plate, whereas the alar plate is significantly reduced with the disappeareance of its most ventral longitudinal domain.

IV.3.2. Wnt1-dependent regulation of Gli3 through L-fng

It has been reported that the Shh/Gli pathway plays a major role in the dorso-ventral patterning of the neural tube (Jessell., 2000; Ruiz i Altaba et al., 2007). Our own results have demostrated how Wnt signal, by means of the inhibition of Gli3 expression and the regulation of L-fng at the p3-p2 boundary, has a fundamental role in the activation of Shh expression and the complete formation of the ZLI (Martinez-Ferre, Navarro-Garberi. et al 2013). As we have shown previously, Gli3 is clearly increased in the diencephalic alar plate of Wnt1 mutant embryos (Figure 17. A, B). In order to confirm this molecular interaction between Wnt1 and Gli3, we analyzed the expression of Gli3 in Wnt1 electroporated chick embryos. Interestingly, under Wnt1 missexpression in the diencephalic alar plate, Gli3 expression is restricted to the dorsal half of the

diencephalic alar plate (Figure 20. A, B, discontinued black line). These results strongly suggest that Gli3 expression in the diencephalon is under the repressive control of Wnt1 signal, which restricts its expression in the alar plate. That is, it seems that the dorso-ventral extension of Gli3 in the alar plate is regulated by Wnt1 signal.

Several studies demonstrate a positive mutual regulation between Wnt signal and Gli3 expression (Mullor et al., 2001; Alvarez-Medina et al., 2008; Fotaki et al., 2011). In contrast, in the diencephalic region, it has been described that Wnt signal has an inhibitory behavior over Gli3 (Martinez-Ferre, Navarro-Garberi et al., 2013). This led us to postulate that this effect could be mediated by another factor, which would be induced by Wnt signaling and could repress the expression of Gli3. A strong candidate for that role is L-fng. Previous work has suggested that Shh is repressed by L-fng (Zelster et al., 2001) and that L-fng is under the control of Wnt signals (Martinez-Ferre, Navarro-Garberi et al., 2013). L-fng is expressed throughout the forebrain, flanking the compartment where the ZLI will be situated and has a complementary expression with Wnt8b (Garcia-Lopez et al., 2004). Interestingly, L-fng expression after Wnt1 overexpression is slightly increased in the diencephalon and expanded caudally beyond the boundary between JcP and CoP, extending ventrally into the CoP (black arrowheads in Figure 20. E). These results suggest that the increased levels of L-fng, induced by Wnt1 overexpression, could be the cause of Gli3 repression in the ventral half of experimental alar plate and, as a consequence, allow the increase of Shh expression where Gli3 is repressed.

In order to investigate whether the expression of other Wnts members could be altered due to the diencephalic altered patterning consequence of Wnt1 ectopic expression, we also analyzed the expression of Wnt3a and Wnt8b in electroporated embryos. These results show that both expression patterns are altered in agreement with the ventralization of the dorso-ventral axis which results from the missexpression of Wnt1. Wnt8b is expressed within the ZLI and at the basal plate in the anterior limit of diencephalic tegmentum, with a longitudinal band following the alar-basal boundary in p3. This pattern is unaltered in the electroporated side, but it occurs at more dorsal levels (Figure 20. D, E, brown arrowheads), due to the alar-basal plate boundary dorsal displacement. In a similar way, Wnt3a is normally expressed in the dorsal diencephalic alar plate, showing a descending gradient of expression from dorsal to ventral and from rostral, next to the ZLI, to caudal, reaching ventrally till the basal-alar plate boundary. In the electroporated side, while the antero-posterior gradient is maintained, the expression of Wnt3a is

restricted to more dorsal levels due to the dorsal displacement of the alar-basal boundary (Figure 20. G, H, black arrows).

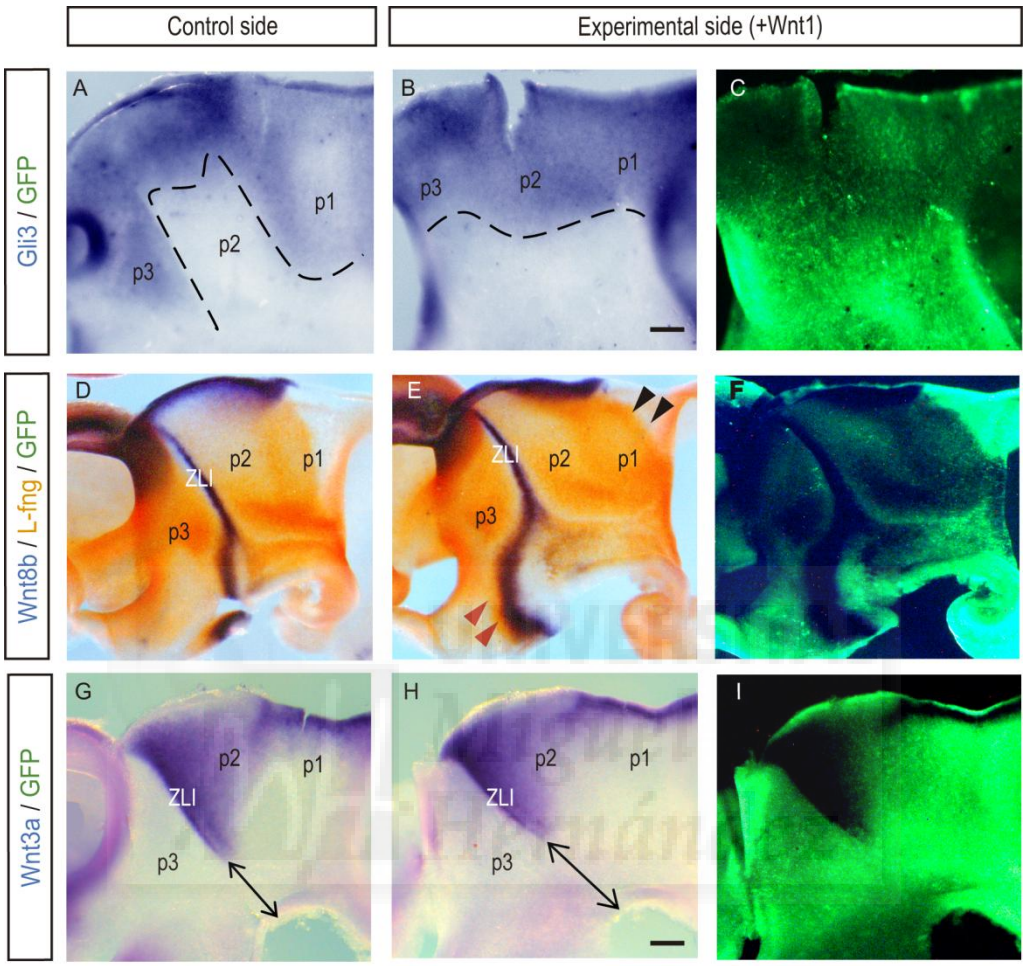

Figure 20. Expression of Gli3 and L-fng are altered in Wnt1 electroporated side.

(A,B) Lateral view of control side (A) and electroporated side (B) embryo (HH23) showing in situ hybridization for Gli3. The black discontinued line indicates the different expression domain present in the control and the experimental side, showing that Gli3 expression in the electroporated side is restricted to the dorsal diencephalic levels. (C) Lateral view of the experimental side of the embryo (B), showing the expression of GFP along the electroporated side. (D,E) Lateral view of control side (D) and electroporated side(E) embryo (HH23) showing in situ hybridization for L-fng and Wnt8b. Black arrowheads indicate the alteration in the L-fng expression, which is increased and expanded caudally. Brown arrowheads indicate the alteration in the expression of Wnt8b, due to the displacement of the basal-alar plate boundary. (F) Lateral view of the experimental side of the embryo (E), showing the expression of GFP along the electroporated side. (G,H) Lateral view of control side (G) and electroporated side(H) embryo (HH23) showing in situ hybridization for Wnt3a. Black arrow line indicates the different size between the end of Wnt3a expression and the FP, showing that this size is bigger in the electroporated side. (I) Lateral view of the experimental side of the embryo (H), showing the expression of GFP along the electroporated side. Abbreviations: p1, prosomere 1; p2, prosomere 2; p3, prosomere 3. Scale bar A-C: ~ 250 µm. Scale bar D-I: ~ 500 µm.

IV.3.3. Wnt1 overexpression induces an ectopic dorsal expansion of Shh at the caudal diencephalon

To further elucidate whether other regions of the diencephalon are altered when Wnt1 signal levels are changed, we analyzed the expression of some major genes involved in diencephalic development. Previously, we have described that Shh expression domains are altered both in Wnt1 loss of function (Wnt1 mutant mouse) and Wnt1 gain of function (*in ovo* electroporation) experimental paradigms. In addition to that, we observed that the Shh expression domain was not only extended into more dorsal domains than in the control side, due to the expansion of the diencephalic basal plate, but also it was induced the formation of an ectopic Shh expressing small spike, similar to the ZLI, close to the basal plate at the caudal diencephalon (blue arrowheads in Figure 19. A and Figure 21. B, C). This effect was confirmed when we analyzed the expression of Nkx2.2. This is a transcription factor that defines the ventral (parabasal) domains of the alar plate and flanks the expression of Shh in the ZLI and the basal plate (Shimamura et al., 1995). In our gain-of-function experiments, as expected, Nkx2.2 shows at the same point a disruption in its expression, following the outline of the Shh ectopic caudal spike (red arrowhead in Figure 21. E).

In agreement to Matsunaga et al. (2002) we observed that Wnt1 is involved in the induction of the expression of Fgf8 in the isthmus (Figure 21. E), which is known to be a fundamental protein in the development of the forebrain (Shimamura and Rubenstein, 1997; Storm et al., 2006). Moreover, we found that the expression pattern of another member of FGF family, Fgf19, was altered. Experimental data suggests that Shh and Fgf8 positively regulate the expression of Fgf19 (Kurose et al., 2005; Gimeno et al., 2007). Indeed, as a consequence of the increase and ectopic localization of Shh expression domain in our experimental paradigm, the expression of Fgf19 was also clearly increased in a parallel pattern (Figure 21. G, H).

Moreover, the expression of Six3 confirms ours observations. The ventral expression domain of this gene in the parabasal column is almost abolished when Wnt1 is overexpressed, as well as in the ventral pretectum expressing patch situated in the boundary between the forebrain and the midbrain (pink arrowheads Figure 21. J, K). This disruption, similar to the disappeareance of Pax6 longitudinal expression in P1 and mesencephalon, is probably caused by a ventralizing induction due to the invasion of Shh expression in this region of the alar plate, forming the ectopic spike, which simulates a caudal ZLI. Moreover, the JcP is aberrantly structured as well. The dorsal induction of Shh expression together with Nkx2.2 and Fgf19 ectopic expression suggest a

repaterning process at this ventral zone of the p1 alar plate, confirming that Wnt1 signal has a key role in pretectal patterning.

These results suggest that Wnt1 signal has a potential role in the induction of the initial formation of the prosencephalic basal plate and the ZLI, regulating the control of the dorso-ventral axis diencephalic development.

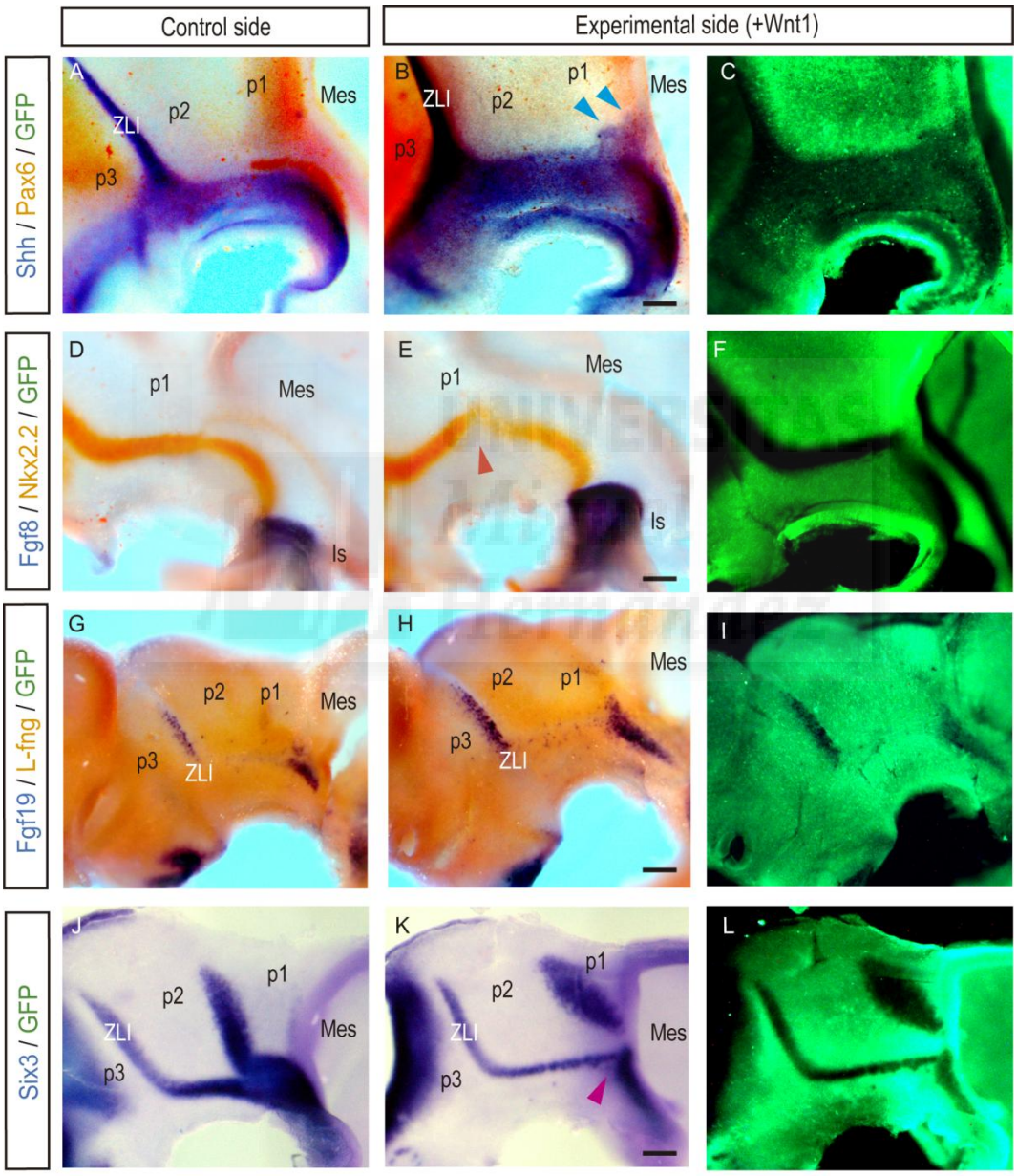

Figure 21. Ectopic expression of Wnt1 induces an ectopic spyke of Shh at the caudal diencephalon.

(A,B) Lateral view of control side (A) and electroporated side(B) embryo (HH23) showing in situ hybridization for Shh and Pax6. Blue arrowheads indicate the formation of an ectopic expansion of Shh from the BP, invading the AP, forming a short spyke in the electroporated size. (C) Lateral view of the experimental side of the embryo (B), showing the expression of GFP along the electroporated side. (D,E) Lateral view of control side (D) and electroporated side (E) embryo (HH23) showing in situ hybridization for Fgf8 and Nkx2.2. Red arrowhead indicates the alteration in the Nkx2.2 expression at the point where Shh generates the ectopic spyke in the electroporated side. (F) Lateral view of the experimental side of the embryo (E), showing the expression of GFP along the electroporated side. (G,H) Lateral view of control side (G) and electroporated side (H) embryo (HH23) showing in situ hybridization for Fgf19 and L-fng. (I) Lateral view of the experimental side of the embryo (H), showing the expression of GFP along the electroporated side. (J,K) Lateral view of control side (J) and electroporated side (K) embryo (HH23) showing in situ hybridization for Six3. Pink arrowheads indicate the disrtuption of the ventral pretectal expression domain of Six3. (L) Lateral view of the experimental side of the embryo (K), showing the expression of GFP along the electroporated side. Abbreviations: p1, prosomere 1; p2, prosomere 2; p3, prosomere 3; Mes, mesencephalon; Is, isthmo. Scale bar A-C: ~ 250 µm. Scale bar D-F: ~ 500 µm. Scale barg G-I: ~ 600 µm. Scale bar J-L: ~ 500 µm.

IV. 4. Diencephalic patterning after Bmp4 gain of function

IV.4.1. Wnt1 and Bmp4 inductive mutual interaction during diencephalic dorso-ventral axis development

The involvement of the Bmp family of molecular signals in the determination of the dorso-ventral axis has been described (Liem et al., 1997; Nguyen et al., 2000; Timmer et al., 2002). The interactions between Wnts and Bmps during the dorso-ventral patterning process are still unclear. We show previously that Bmp4 expression is lost in Wnt1 mutant mice (Figure 18). Thus, we decided to analyze the expression of Bmp4 in Wnt1 gain of function experiments. We show that Bmp4 was unaltered when Wnt1 gene was ectopically expressed by electroporation at neural tube stages (Figure 22 A, B). All together, our results suggest that Wnt1 could be necessary for the maintance of Bmp4 but not for its initial expression.

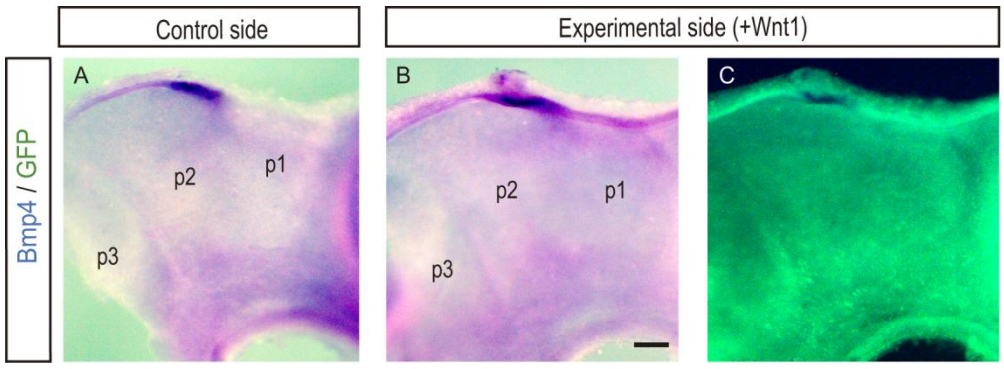

Figure 22. Bmp4 expression is not altered when Wnt1 is overexpressed

(A,B) Lateral view of control side (A) and electroporated side (B) embryo (HH23) showing *in situ* hybridization for Bmp4. It can be observed that this expression does not change in the electroporated side. Abbreviations: p1, prosomere 1; p2, prosomere 2; p3, prosomere 3. Scale bar: ~ 250 µm.

Thus, in order to explore the role of Bmp4 and its interaction with Wnt1, we performed *in vivo* gain-of-function experiments by means of *in ovo* electroporation. A plasmid enconding Bmp4 full length gene was coelectroporated with a GFP expressing plasmid into the neuroepithelium of chick embryos at HH10. After 3 days, embryos were sacrificed and were analyzed with diencephalic markers.

Our results show that the expression of Wnt1, usually restricted to the dorsal midline of the diencephalon, is significantly increased when Bmp4 is overexpressed, being extended through the dorsal alar plate of the diencephalon in the electroporated side (Figure 23 and Figure 24. A, B). Thus, our results suggest that Wnt1 is induced by the expression of Bmp4, and in turn, Wnt1 expression is essential for the maintenance of Bmp4 expression. This interaction would control the correct establishment of the diencephalic dorso-ventral axis.

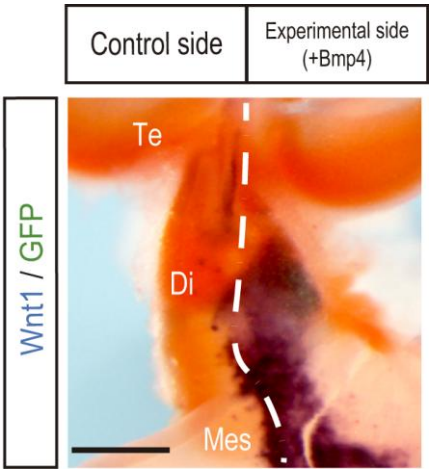

Figure 23. Bmp4 ectopic expression causes an increase of Wnt1 expression.

Dorsal view of a chick embryo electroporated with Bmp4 (HH23). On the right is the electroporated side and on the left is the control side. The expression of Wnt1 is induced when Bmp4 expression is increased by means of the electroporation. The black discontinued line indicates the midline of the neural tube. Abbreviations: Te, telencephalon; Di, diencephalon; Mes, mesencephalon. Scale bar: ~ 500 µm.

IV.4.2. Bmp4 overexpression causes the same phenotype as Wnt1 overexpression

Since the overexpression of Bmp4 induced the ectopic expression of Wnt1 in the alar plate, we thought that this should have an effect in the establishment of the horizontal domains, expecting the same phenotype that we see in the overexpression experiments of Wnt1. Thus, we analyzed the diencephalic markers in electroporated embryos with Bmp4.

Indeed, we see how the alar plate of the diencephalon is abnormally formed when Bmp4 is overexpressed. The expression pattern of Pax6 is disrupted. We show how the parabasal stripe of Pax6 expressing cells situated in the boundary between the basal and the alar plate in the caudal diencephalon is aberrant but not totally abolished. Moreover, the expression of Pax6 in the alar plate is also disrupted, invading the gap situated in the boundary between p3 and p2, where is located the ZLI (Figure 24. A, B).

In relation to Gbx2 expression, it has a normal expression in the diencephalic alar plate but the ventral region of this domain is altered: it can be observed that the ventral tail lacked its most caudal part (Figure 24. E, purple arrowhead). This restriction should be a consequence of the alteration of Shh ventral domain, which this time invades the alar plate and forms the ectopic spyke of Shh at more rostral levels (Figure 24. E, H, white arrowheads). As it was expected, there is an increase of the Shh domain in the basal plate and in ZLI lenght, although this increase is present at lower levels (Figure 24. G, H, white arrows), due to the lower degree of Wnt1 expression modification than achieved after direct Wnt1 overexpression.

The expression of Gli3 is also changed, showing a restriction to more dorsal levels in the alar plate (Figure 24. G, H, black discontinued line), possibly restricted by the increase in the expression of L-fng, which indeed shows an invasion of mesencephalic ventral areas (Figure 24. J, K, black arrowheads). Finally, Wnt8b is also altered at the level of the basal-alar plate boundary, due to the displacement of this boundary to more dorsal levels (Figure 24. J-L brown arrowheads).

Thus, as expected, the overexpression of Wnt1 and Bmp4 causes a similar aberrant dorso-ventral axis phenotype, although less severe in the case of Bmp4 missexpression. This suggests that Bmp4 acts as inductor of Wnt1 expression and Wnt1 plays the main role in the establishment of the diencephalic dorso-ventral axis. Moreover, in turn, Wnt1 seems to be involved in the maintenance of Bmp4 expression in the diencephalic roof plate.

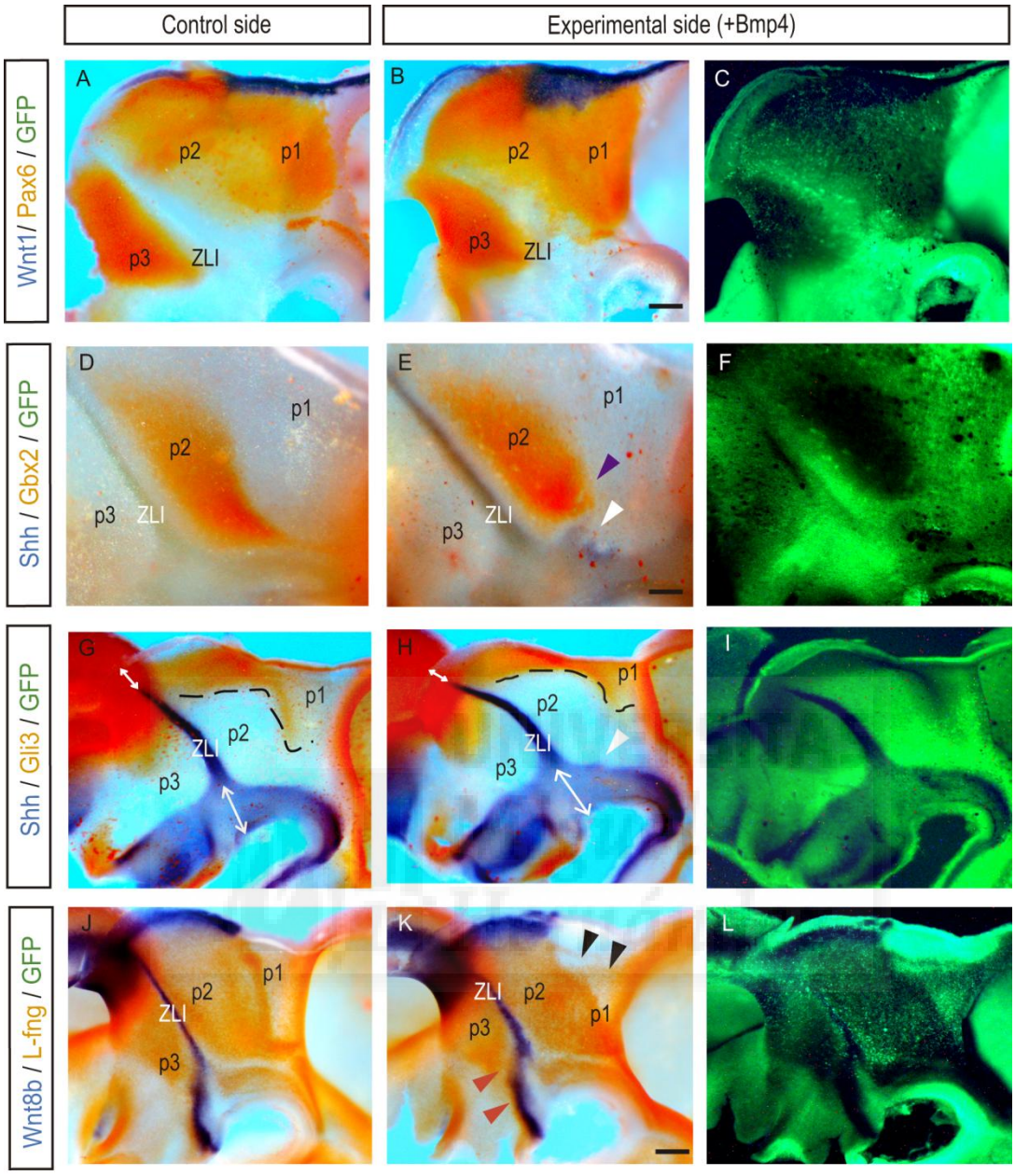
Control side
Experimental side (+Bmp4)
Wnt1/ Pax6 / GFP
Shh / Gbx2 / GFP
Shh / Gli3 / GFP
Wnt8b / L-fng / GFP
A
B
C
D
E
F
G
H
I
J
K
L
p1
p2
p3
ZLI

Figure 24. Ectopic expression of Bmp4 induces the same phenotype, but less severe, than the ectopic expression of Wnt1

(A,B) Lateral view of control side (A) and electroporated side(B) embryo (HH23) showing in situ hybridization for Wnt1 and Pax6. (C) Lateral view of the experimental side of the embryo (B), showing the expression of GFP along the electroporated side. (D,E) Lateral view of control side (D) and electroporated side (E) embryo (HH23) showing in situ hybridization for Gbx2 and Shh. White arrowhead indicated the ectopic Shh spyke formed as a consequence of the electroporation. Purple arrowhead indicate the alteration in the ventral region of Gbx2 expression domain. (F) Lateral view of the experimental side of the embryo (E), showing the expression of GFP along the electroporated side. (G,H) Lateral view of control side (G) and electroporated side (H) embryo (HH23) showing in situ hybridization for Shh and Gli3. White arrows indicate the different size of the basal plate and the level arised by the Shh spyke within the ZLI between control and electroporated side. White arrowhead indicate the ectopic Shh spyke formed as a consequence of the electroporation. (I) Lateral view of the experimental side of the embryo (H), showing the expression of GFP along the electroporated side. (J,K) Lateral view of control side (J) and electroporated side (K) embryo (HH23) showing in situ hybridization for L-fng and Wnt8b. Brown arrowheads indicate the alteration in Wnt8b nirmal expression due to the displacement of the basal-alar plate boundary. Black arrowheads indicate the aberrant expression of L-fng, which is completely unstructured and invading the mesencephalon at ventral levels. (L) Lateral view of the experimental side of the embryo (K), showing the expression of GFP along the electroporated side. Abbreviations: p1, prosomere 1; p2, prosomere 2; p3, prosomere 3. Scale bar A-C: ~ 500 µm. Scale bar D-F: ~ 250 µm. Scale bar G-L: ~ 600 µm.

Altogether, these data demostrate that Wnt1 signal plays important roles during diencephalic development. First of all, it has a key role in the organization of pretectal subdivisions, probably mediated by Six3 expression. Secondly, it is also esential for the development of some diencephalic dorsal structures, as the pineal gland and the posterior commisure, and also for the axonal tract situated in the middle of the diencephalon, the retroflexus tract. And finally, Wnt1 signal may be a key molecular system to code positional information essential for the proper establishment of the diencephalic dorso-ventral axis and seems to be also important in the positioning of the ZLI along the antero-posterior axis.

Chapter V

DISCUSSION

V.1. Wnt1 signal is necessary for the formation of diencephalic dorsal structures

During the embryogenesis of the neural tube, several morphogenetic signals emanating from morphogenetic organizers underlie regionalization and neural histogenesis (Martinez, 2001; Echevarria et al. 2003; Meinhardt, 2008). These signals code for positional information and regulate basic cellular processes, such as proliferation, adhesion and cell death. We decided to study the role of Wnt1 signaling in the diencephalic development since this morphogene is a determinant signal involved in this process but which specific functions remain unclear (McMahon and Bradley, 1990; Fischer et al., 2007; Martinez-Ferre and Martinez, 2009; Martinez-Ferre, Navarro-Garberi et al., 2013).

The mice line of Wnt1 mutant used for this project was generated by McMahon and Bradley (1990). They generated null alleles of Wnt1 by homologous recombination (Thomas and Capecchi, 1987) in embryonic stem cells (Evans and Kaufman, 1981) in order to generate this mice line. They described that Wnt1 is essential for the correct formation of the midbrain and the rostral rhombencephalon, but in their study, they considered normal the development of the diencephalic structure (McMahon et al., 1992; Parr et al., 1993). In this thesis memory, we show evidence demonstrating that Wnt1 signal is also necessary for the correct establishment of the different diencephalic territories.

It has been described that at later stages of gestation, Wnt1 homozygotes embryos show a strong structural alteration in their phenotype: the midbrain and the cerebellum are not formed, while the rest of the brain was described as normal. The mutation is lethal within 24 hours after-birth (McMahon and Bradley, 1990).

In a previous work of our group analyzing the role of Fgf8 in the regionalization and morphogenesis of the diencephalon (Martinez-Ferre and Martinez, 2009), Wnt1 expression pattern in the diencephalic roof plate appeared in complementary equilibrium with Fgf8. The possible interaction between these two signals suggested to us the present analysis of the potential role of Wnt1 signal in dorsal diencephalic development. We have analyzed the diencephalic development in two experimental paradigms: in mutant mouse embryos as a model of loss of function, and in Wnt1 electroporated chick embryos, as a model of gain of function.

The retroflexus tract, formed mainly by descending axons from the habenular nuclei to the interpeduncular nucleus, is clearly altered in the absence of Wnt1 (Figure 9. D, F). In Wnt1

mutant embryos, the tract is less compact than in WT embryos and divided in several fascicles, while in WT is always formed by a single fascicle running from dorsalventrally following the limit between p2 and p1. A possible relationship between Wnt signal and guidance factors in the axonal growth cones and/or migration substrate is unknown. Therefore, further investigations are necessary to determine the molecular mechanism underlying this axonal phenotype. It has been suggested that Wnt/β-catenin signaling is important for the correct formation of the habenula, specifically for its characteristic left/right asymmetry in zebrafish (Hüsken and Carl, 2013). Recently, it has been described that Semaphorin 5A and Sempahorin 3F, members of a family of secreted and membrane-anchored guidance cues, have a key role in the guidance of the axons of this tract during development (Kantor et al., 2004). We propose that future research should study a possible regulation of this molecules by Wnt1 signal in the diencephalon.

Other dorsal structures are altered in the absence of Wnt1. The pineal gland, at the caudal p2 roof plate, is absent and the posterior commisure, in the p1 roof plate seems to be enlarged at E14.5 (Figure 10). This is in agreement with what has been previously described in a different strain of Wnt1 mutant mice, $Wnt1^{sw/sw}$ (*swaying*) mutants, where the formation of the posterior commisure is compromised (Louvi and Wassef, 2000). These observed alterations in Wnt1 mutant embryos could be related to the altered equilibrium between Wnt1 and Fgf8 in this area. In normal conditions, Fgf8 is expressed only in the most rostral diencephalon, including p3 and the rostral half of p2 (from the prethalamic eminence until the epithalamus) and Fgf8 signal is required in a dose-dependent manner for the proper formation of the pineal gland (Martinez-Ferre and Martinez, 2009). Under loss of Wnt1 signal, Fgf8 domain is expanded caudally at E12.5, covering all of the dorsal p2 and the most rostral regions of p1 (Figure 15). Therefore, our results suggest that not only Fgf8 signal is required for the development of these structures, but also Wnt1 seems to be necessary for their development.

In physiological conditions, Wnt1 is expressed in a complementary domain to that of Fgf8 in the diencephalic roof plate. It has been postulated that Fgf8 signal may control the establishment of the rostral end of Wnt1 expression in the diencephalic roof plate, since the expression of Wnt1 is rostrally expanded in Fgf8 mutant mice (Martinez-Ferre and Martinez, 2009). This result could be complementary to our results where the expression of Fgf8 is extended caudally in the absence of Wnt1. Therefore, in an auto-regulative loop, Fgf8 could be restricting the rostral limit of Wnt1 but, in turn, Wnt1 seems to be controlling the caudal limit of Fgf8 expression in the diencephalon. A mechanism of mutual dose-regulation of their expression could be, moreover,

regulating different regional domains (choroidal plexus, pineal gland and habenular-posterior commissural plate, from rostral to caudal), and required for the proper formation of the dorsal diencephalon. However, this mutually repressive interaction seems to be specific of diencephalic epithelium, since the electroporation of Wnt1 in chick embryos leads to the overexpression of Fgf8 in the isthmic region (Matsunaga et al., 2002; our results: Figure 21. E).

The requirement of Wnt1 signal in the diencephalic roof plate development is in agreement with the loss of Bmp4 expression in the diencephalon of Wnt1 mutant embryos. Bmp4 is expressed in the pineal neuroepithelial anlage and it has been described as a signalling molecule involved in the differentiation of the dorsal prosencephalon, playing an important role in the epithalamus development (Lim et al., 2005; Storm et al., 2006). The absence of this gene should be correlated with misspecification of this domain due to the expansion of Fgf8 expression as a consequence of the lack of Wnt1 signal, according with the reported antagonistic interaction described between Bmp4 and Fgf8 (Ohkubo et al., 2002; Shimogori et al., 2004; Storm et al., 2006). However, it has also been described that in Fgf8 mutant embryos, Bmp4 expression is reduced (Storm et al., 2006; Martinez-Ferre and Martinez, 2009), which although incompatible with a simple mechanism of mutual repression between Fgf8 and Bmp4 signals, it could suggest that abnormal regionalization underlies pineal anlage misspecification and Bmp4 reduced expression. Therefore, further studies are needed in order to clarify this regulatory mechanism to distinguish the primary molecular interaction between Fgf8 and Bmp4 from a secondary phenotype due to regional misspecification of the diencephalic roof plate.

In summary, our results demonstrate that Wnt1 signal in the roof plate is necessary for the proper patterning of several diencephalic structures such as the retroflexus tract, the pineal gland and the posterior commissure. Wnt1 and Fgf8 mutual repressive interaction articulate an equilibrium that may code positional information in the roof plate. Moreover Wnt1 signal seems to be necessary for the maintenance of Bmp4 expression. Regarding to the physiological importance of these altered structures found in Wnt1 mutant embryos, further investigations are needed to better understand the observed phenotype.

V.2. Wnt1 has a key role in the proper establishment of caudal diencephalic territories

It has been described that specifically Wnt1 signalling is repressed by Six3 transcription factor, which seems to be required to ensure a proper alar plate patterning of the diencephalon, since Six3-deficient mice display a strong reduction of the neural tissue rostral to the ZLI (Lagutin et al., 2003; Lavado et al., 2008). Moreover, the expression of Six3 (rostrally) and Irx3 (caudally) in the forebrain (Kobayashi et al., 2002) has been described as controlled by Wnt signal, delineating the posterior and anterior forebrain domains (Braun et al., 2003). Therefore, these reports suggest that the rostro-caudal polarity of the forebrain through the regulation of Six and Irx3 by Wnt signaling, and the mutually inhibitory interactions between them, confers differential competence to respond to the inductive signals to form the diencephalic territories. It is necessary to take account that these theories are fundamentally based on the results of misexpression studies or explants assays, and that there is not sufficient genetic *in vivo* demonstration using physiological signal concentrations, as well as genetic evidence to support it.

In agreement with this antagonistic mechanism, we demonstrate how in Wnt1 mutant embryos the expression of Six3 is slightly increased (Figure 11) and Irx3 is strongly downregulated (Figure 14). This observation confirms the relation between Wnt1 signal expressed in the caudal thalamic and pretectal roof plate and the expression of Irx3 in the alar plates of these neuromers. However, our observations in the gain and loss of function experiments do not show severe changes in the antero-posterior diencephalic structures: except for the JcP size, which is altered in Wnt1 mutant embryos (Figure 12), the other territories remain mainly unaltered.

V.3. Wnt signal regulates the dorso-ventral axis in the diencephalon

The establishment of the dorso-ventral axis during the regionalization of the neural tube is regulated by morphogenetic signals coming from the ventral and dorsal midline. Shh is the major morphogenetic signal, originated in the floor plate, which induces ventral properties in the neuroepithelium, whereas Bmps and Wnts morphogenetic signals have been described as dorsalizing factors (Braun et al., 2003; Lim et al., 2005; Lim and Golden, 2007; Lavado et al., 2008; Ulloa and Marti, 2010).

In order to properly establish conclusions and possible hypothesis to understand how Wnt signal regulates the diencephalic dorso-ventral regionalization, it is important to keep in mind that the RP, AP, BP and FP of the different regions in the neural tube (forebrain, midbrain, hindbrain and/or spinal cord) could have different properties. A particular molecular mechanism described in one of these regions could be different in another area. For example, it has been described that the forebrain FP is somehow different from the spinal cord FP, regarding their morphology, cell types, ontogeny and molecular constituents, among others (Placzek and Briscoe, 2005; Puelles, 2013).

Our results clearly demonstrate that Wnt1 signal has a key role in the dorso-ventral diencephalic regionalization. Recent studies have demonstrated that Wnt/β-catenin signalling is involved in the dorso-ventral telencephalic regionalization (Backman et al., 2005; Danesin et al., 2009). Backman et al. (2005) demonstrated that Wnt signalling is involved in the positive regulation of the expression of dorsal markers and in the suppression of ventral programs in the telencephalon. In diencephalon, it has been published that Wnt signalling, specifically Wnt3/Wnt3a, was sufficient and required to induce the expression of dorsal thalamic specific genes (Irx3 and Gbx2) and to suppress specific genes of the ventral thalamus (Six3), whereas the absence of Wnt signaling drives ventral thalamus specific differentiation (Braun et al., 2003). Our results are in agreement with this role of Wnt signalling, suggesting that also Wnt1 signal is able to induce Irx3 and to repress Six3 (which are downregulated and upregulated, respectively, in Wnt1 mutant embryos. Figure 14 and 11).

The role of Wnt signalling has been decribed as completely opposite to Shh signal, which emanates from the ventral areas of the neural tube and has been reported as necessary and sufficient for the determination of ventral identities in the neural tube (Echelard et al., 1993; Wijgerde et al., 2002). Moreover, Shh signalling from the ZLI is required to downregulate Wnt signaling (specifically, Wnt4) in the presumptive thalamus (Quinlan et al., 2009). Our results are paradoxical in the light of these reports, but they do not necessarily have to be contradictory to this antangonistic mechanism described between the two morphogenes. Indeed, we demonstrate that Wnt1 signal, originated in the diencephalic roof plate, regulates the establishment of the boundary between the alar plate (dorsal diencephalon) and the basal plate (ventral diencephalon), causing an increase of the Shh basal plate domain by means of the negative regulation of the expression of other molecules which are expressed in the diencephalic alar plate. A positive direct regulation of Wnt signal over Shh expression is discarded.

Several investigations have reported a regulation of Gli3 transcription factor over Wnt signalling (Mullor et al, 2001). It has been published that Gli3 inhibits canonical Wnt signaling (Ulloa et al., 2007). However, exhaustive examination of mutant mice with no functional Gli3 (extra-toes, $Gli3^{Xt/Xt}$ mutants) has revealed that Gli3 is a required transcription factor for maintaining the expression of Wnts and the Wnt/β-catenin activity at early stages of the development in the forebrain (Grove et al., 1998; Fotaki et al., 2011). Moreover, it has been published that the antagonism between Gli3 and Shh plays a major role in the development of the dontro-versal axis in the neural tube (Jessell, 2000; Meyer et al., 2003; Ruiz i Altaba et al., 2007).

In our previous report (Martinez-Ferre, Navarro-Garberi., et al 2013), we demonstrated that Wnt signal, by means of the inhibition of Gli3 and the upregulation of L-fng at the p3-p2 boundary, has a fundamental role in the complete formation of the ZLI. This Wnt-mediated inhibition of Gli3 is now, with the results obtained in this thesis project, shown to be important not only to determine the proper progression of the ZLI, but also determining the dorso-ventral axis of the whole diencephalic structure.

However, the opposite regulation of Wnts over Gli3 has been described in other regions of the neural tube. In the spinal cord, it has been reported that Wnt canonical activity had the ability to induce Gli3 expression in chick and mouse (Alvarez-Medina et al., 2008; Yu et al., 2008; Ulloa and Marti, 2010). In agreement with this idea, several Tcf-binding sequences have been recognized in the human Gli3 locus (Abbasi et al., 2007; Alvarez-Medina et al., 2008). Albeit further studies are necessary to clarify and ensure if this induction is direct or indirect.

In our results, we clearly show that when Wnt1 signal is absent, the expression of Gli3 is significantly increased in the alar plate of the diencephalon (Figure 17), and due to the antagonism between Shh and Gli3, Shh expression in the basal plate is reduced (Figure 16). In the same way, when Wnt1 signal is missexpressed, the expression of Gli3 is partially inhibited and remains restricted to the most dorsal levels of the alar plate (Figure 20. B), which allows the expansion of Shh expression (Figure 19. A), displacing the basal-alar boundary to more dorsal levels (Figure 25). Therefore, here we postulated a model where Wnt signalling may control the diencephalic dorso-ventral regionalization, through a mechanism that involves Gli3 restriction in the dorsal alar plate.

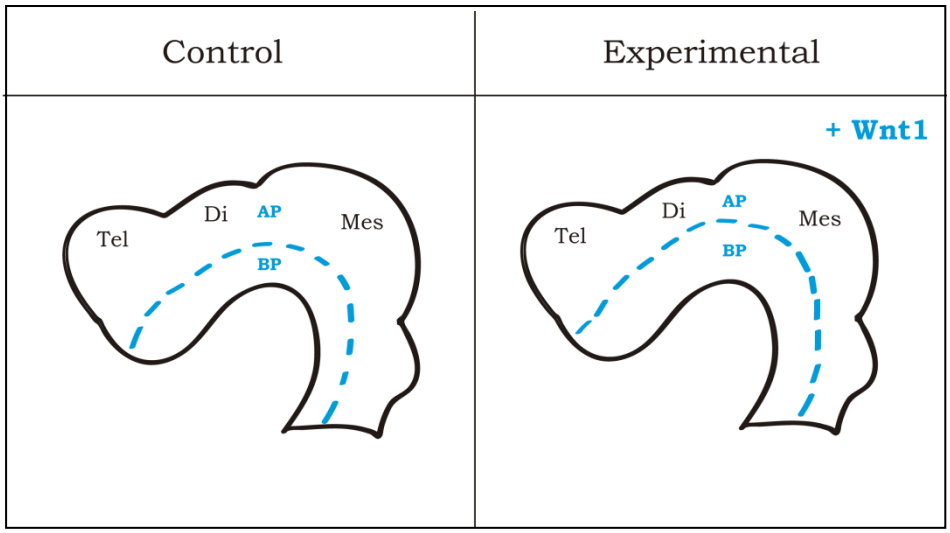

Figure 25. Schematic representation of Wnt1 overexpression phenotype observed.

Lateral view of control side (on the left) and electroporated side (on the right) embryos showing the effect caused by the overexpression of Wnt1 in chick embryos. The alar-basal boundary is dorsally displaced when Wnt1 is missexpressed, resulting in an aberrant formation of the dorso-ventral axis. Abbreviations: Tel, telencephalon; Di, diencephalon; Mes, mesencephalon, AP, alar plate; BP, basal plate.

Therefore, we propose two possible explanations for this phenotype. The first of them is that, due to the differences aforementioned between diencephalon and other regions of the neural tube, as the spinal cord, Gli3 is inhibited by Wnt signal by a diencephalic specific mechanism, which happens exclusively, so far, in this area. The second hypothesis is that the inhibition of Wnt over Gli3 is achieved in an indirect manner, mediated by L-fng, which has been demonstrated to be under the control of Wnt signals (Martinez-Ferre, Navarro-Garberi et al., 2013). The importance of this gene has been previously decribed in the ZLI development, suggesting that the encroachment of L-fng is modulated by Wnt8b signal in the presumptive ZLI territory; this progressive encroachment of L-fng may be a fundamental signal for the induction of Shh expression within the ZLI (Zelster et al., 2001; Martinez-Ferre, Navarro-Garberi et al., 2013).

In experiments of Wnt signal loss of function from Martinez-Ferre, Navarro-Garberi et al, (2013) we demostrated that the encroachment of L-fng in the diencephalic alar plate is blocked when Wnt signal is inhibited (Figure 26). Therefore, Wnt signal from the ZLI (Wnt8b expressing territory) and from the roof plate (Wnt1 expressing territory) is needed for L-fng expression. As was expected, in the experiments of gain of function of Wnt1 signal presented in this thesis project, L-fng expression is clearly increased and its encroachment in the diencephalic alar plate

is disorganized (Figure 20. D-F). All together, these observations suggest that L-fng is a strong candidate to mediate Gli3 repression: Wnt1 signal would induce L-fng which, in turn, would inhibit the expression of Gli3.

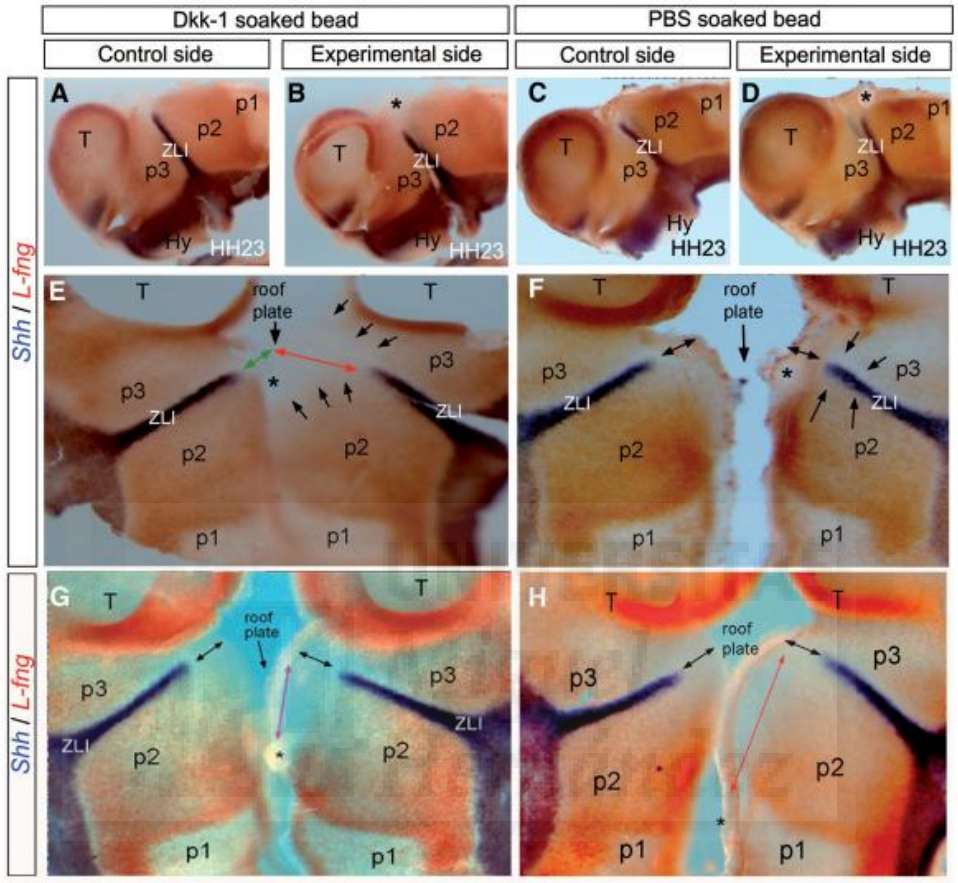

Figure 26. Wnt signal loss of function experiments demonstrate that Wnt signaling is required for L-fng expression in the alar diencephalon.

(A-D) Lateral view of the whole mount prosencephalic neural tube (HH23) stained for Shh and L-fng. (A) and (C) are control side and (B) and (D) are experimental side of the embryos. (E-H) Dorsal view of the opened embryos through the ventral midline showing in situ hybridization for Shh and L-fng. Asterisk in B, E and G indicates the region in which a Dkk-1-soaked bead was implanted at HH10. Asterisk in D, F, and H indicates the region in which the PBS-soaked bead was implanted. Green and red arrows in E indicate the distance between the dorsal midline and the dorsal level of Shh expression in the control (green) and experimental (red) side of an embryo in which a Dkk-soaked bead was implanted (asterisks).Black arrows in E indicate that L-fng encroachment was disrupted in the experimental side of the embryo. Black arrows in F and H indicate the similar distance between the dorsal midline (roof plate) and the dorsal level of Shh expression in the control and experimental side of an embryo in which the PBS-soaked bead was implanted. Black arrows in G indicate the same distance between the dorsal midline (roof plate) and the dorsal level of Shh expression in the control and experimental side of an embryo in which the Dkk-1-soaked bead was implanted in the most caudal position of the alar/roof plate of p2. From *Martinez-Ferre and Navarro-Garberi et al., 2013*.

One more important issue has to be discussed. A weak change has been observed in the expression of Shh in the ZLI in Wnt1 mutant embryos, where the Shh expression of this domain ends at more ventral levels in Wnt1 mutant embryos. However, a clear and evident dorsal expansion of this expression domain when Wnt1 is ectopically expressed is observed. Moreover, the displacement of the basal-alar plate boundary in Wnt1 mutant embryos is not as evident as in the experiments of gain of function. That means that the phenotype caused by the lack of Wnt1 expression is much less severe than the one observed in Wnt1 gain of function experiments in chick embryos. A possible explanation could be the differences in the expression of Wnt1 between chick and mouse species. In both of them, Wnt1 is expressed along most of the dorsal midline of the neural tube and in the mid/hindbrain junction, however in mouse, the expression of Wnt1 is excluded from the telencephalon and the anterior diencephalon, regions where expression of Wnt1 was detected at HH19 in chick embryos (Quinlan et al., 2009). This pattern of expression in the most rostral brain of the chick embryos is absent in mice, but interestingly, it appears in Emx2 mutant mice, which suggests that this homeodomain transcription factor repress this Wnt1 expression domain in WT mice embryos (Ligon et al., 2003). Another important difference in the expression of Wnt1 in mice compared to that of chick, it is that Wnt1 expression is not only restricted to the dorsal midline, but also there is a weak expression in the ventral midline of the mesencephalon and the diencephalon (Parr et al., 1993; Echevarria et al., 2003). This differences could explain why the phenotype observed in the experiments of gain of function, where the dorso-ventral axis is aberrantly positioned given an important increase of the BP and clear reduction of the AP, whereas in Wnt1 mutant embryos this axis is also altered with the opposite effect (reduction of the BP and increment of the AP) but not with the same seriousness. The missexpression of Wnt1 in the ventral regions of the chick embryos diencephalon is a very aggressive change since in control conditions this genes is completely absent in this area.

V.4. Wnt1 signal is involved in the positioning of the ZLI

The molecular mechanism underlying the positioning of the ZLI involve several molecules which, at early stages of the development, are located in the anterior (Six3 positive) and posterior (Irx3 positive) neuroepithelium which form the interface where the prospective ZLI will be developed (Kobayashi et al., 2002; Vieira et al., 2005; Puelles and Martinez, 2013). Moreover, it has also been published that the specification of this organizer is dependent on the interactions

between rostral Fez and caudal Otx expressing domains in zebrafish (Scholpp and Lumsden, 2010).

In our previous publication (Martinez-Ferre, Navarro-Garberi et al., 2013) we demonstrated that Wnt signal is also involved in the regulation of the ZLI, generating a permissive territory where the ZLI will be developed. The ZLI represents a singular and specific brain area where Shh expression is expanded ventro-dorsally in the alar plate in addition to the longitudinal expression in the basal and floor plates. Moreover, a planar induction from Shh expression in the BP has been described as necessary for the progression of Shh expression within the ZLI (Vieira and Martinez 2006; Martinez-Ferre and Navarro-Garberi et al, 2013). However, the dorsal signal of Wnt has been described as sufficient for the activation of Shh at the ZLI locus in one-eye-pinhead zebrafish mutant (Scholpp et al., 2006).

Our results presented in this thesis project suggest that Wnt signal is not only involved in the ZLI structuring, but also in the ZLI positioning, since the overexpression of Wnt1 leads to the formation of a new position where Shh is expanded ventro-dorsally, in a manner that mimics the first steps of the ZLI development. This ectopic ZLI–like structure develops in the ventral pretectum, at pretectal-mesencephalic boundary (Figure 21. B). This boundary coincides with a misspecification of the ventral p1, where Six3 and Gli3 disappear. Further investigations should be performed in order to unravel which molecular mechanism, involving Wnt1 signal, underlies the positioning and activation of Shh in this event.

V.5. Wnt1 and Bmp4 inductive mutual regulation

The roof plate of the diencephalon expresses several inductive signaling molecules such as Bmps and Wnts. In general, Bmp signaling has been described as an inductor of dorsal cell fates (Furuta et al., 1997; Lee et al., 1998; Liem et al., 1995, 1997; Lim et al., 2005; Lim and Golden, 2007; Ulloa and Marti, 2010; Le Dreau and Marti, 2012). Specifically in the forebrain, several studies have described that some Bmps are implicated in forebrain patterning in mouse and chick embryos (Furuta et al., 1997; Shimamura and Rubenstein, 1997; Golden et al., 1999; Ohkubo et al., 2002). Moreover, explants or grafts of chick neural plate tissue placed in contact with tissue expressing Bmps induce the expression of dorsal markers in the neural tissue (Liem et al., 1995, 1997). Specifically, it has been reported that two members of this family, Bmp4 and

Bmp7 have this function, whereas a Bmp antagonist, Noggin, have the opposite function and act in a inhibitory manner (Knecht and Harland, 1997; Liem et al., 1997). However, Gunhaga et al. (2003) have recently suggested that Bmp4 is not able to induce dorsal specific markers in chick telencephalon. On the other hand, the requirement of Bmps in the brain was suggested in studies where the roof plate was ablated (Millonig et al., 2000; Monuki et al.,2001).

The existence of a cross-talk between Wnt and Bmp have been described along the neural tube, however these interactions remain a subject of controversial hypothesis. Bach et al. (2003) proposed a mechanism in the dorsal midline of the diencephalon where Msx1 would be an intermediary between Bmp and Wnt signalling. In the spinal cord, it has been described that Bmp4 was weakly expanded ventrally from the RP, whereas Bmp7 was clearly induced by Wnt canonical pathway (Alvarez-Medina et al., 2008). Our results show that the expression of Bmp4 is not altered after overexpression of Wnt1 in diencephalon (Figure 22), which suggests that the interactions between these two families of molecules could differ between spinal cord and diencephalon. Moreover unlike in the spinal cord, in the diencephalon Bmp ligands and Bmp receptors are expressed not only in the dorsal midline, but also in the ventral midline, suggesting a more complex role of these molecules in the dorsoventral diencephalic patterning (Cho et al., 2008).

Our results are in agreement with Yu et al (2008), where mice embryos expressing stable β-catenin in specific regions of the spinal cord show that Bmp sinaling activity is not changed. However, expression of Bmp4 is not maintained in Wnt1 mutant embryos (Figure 18), which suggest that indeed, Wnt1 signal could be necessary for Bmp4 expression maintenance along development.

Therefore, despite the clear evidences that Bmp signaling is required for dorso-ventral patterning at other levels of the neural tube, its specific role in diencephalic patterning and its control over Wnt protein remain uncertain. For that reason, we decided to analyze the direct role of Bmp by means of a direct electroporation of Bmp4, since it has not been studied so far. It has been reported that ectopic activation of Bmp4 perturbs diencephalic nuclear development (Lim et al., 2005), but how Wnt signaling could be involved in this perturbation has not been clarified.

Here, we demonstrate that ectopic induction of Bmp4 signal clearly induced Wnt1 expression in the roof plate, suggesting that Wnt1 is directly or indirectly downstream Bmp4 signal. Moreover, this overexpression of Bmp4 causes the same global phenotype, albeit less

severe due to the lower levels of Wnt1 achieved, as when Wnt1 itself was extensively missexpressed. Therefore, these data suggest that Bmp4 has a key role in the development dorso-ventral axis since mediates the expression of Wnt1 from the roof plate, which regulates (directly or indirectly) the expression of Gli3, which in turn, antagonizes Shh activity and determines the establishment of the basal-alar boundary along the dorso-ventral axis (Figure 27).

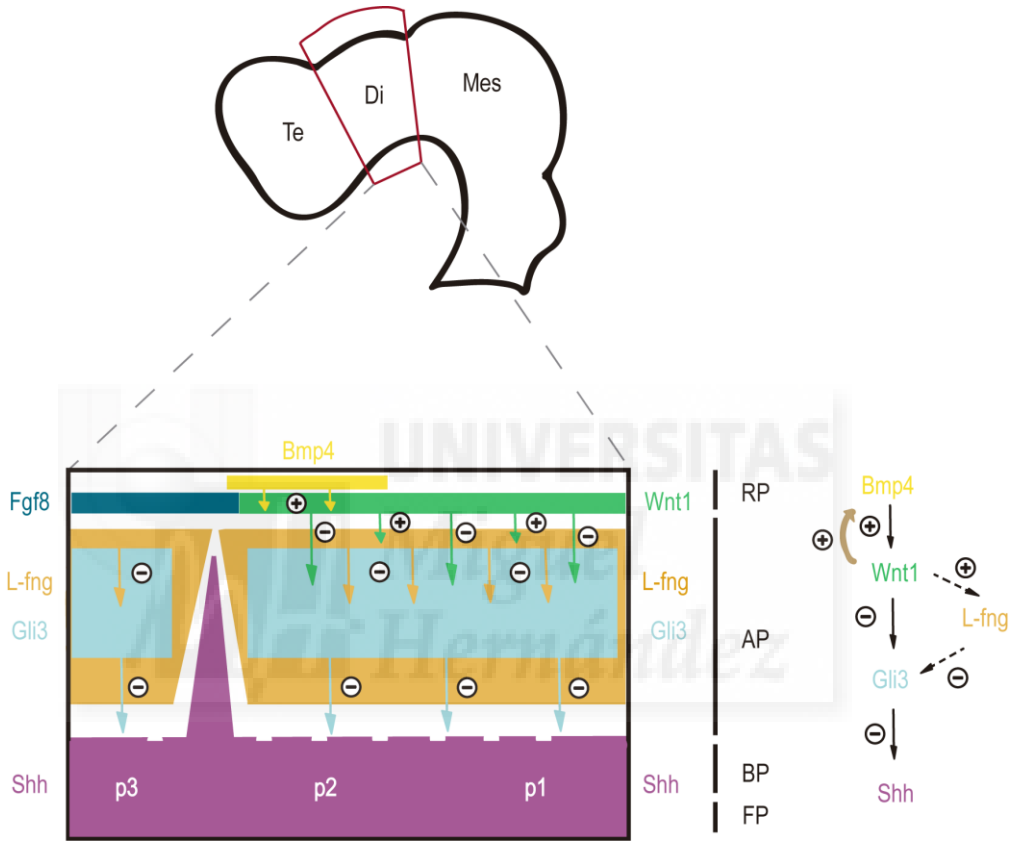

Figure 27. Schematic representation of the proposed mechanisms underlying the dorso-ventral axis regionalization.

Diencephalic representation during the neural tube development where the major molecules involved in the regionalization for this territory are represented. Bmp4 is expressed in the roof plate and induces the expression of Wnt1. Wnt1 signal seems to be necessary for the maintance of Bmp4 expression and, moreover, represses, directly or indirectly through L-fng, the expression of Gli3, which antagonizes the Shh functions. Abbreviations: Te, telencephalon; Di, diencephalon; Mes, mesencephalon; RP, roof plate; AP, alar plate; BP, basal plate; FP, floor plate; p1, prosomere 1; p2, prosomere 2; p3, prosomere 3.

Chapter VI

CONCLUSIONS

1. Wnt1 signal is essential for the proper development of diencephalic roof plate structures, such as the pineal gland and the posterior commissure, by means of molecular mechanisms which may involve other determinant molecules of the diencephalic development, such Fgf8 and Bmp4.

2. Wnt1 seems to have an important role in the proper fasciculation of the retroflexus tract. In the absence of Wnt1, this tract is clearly de-fasciculated. The molecules that determine the way of this tract should be influenced by Wnt1 expression.

3. Wnt1 signal in the diencephalon is important for the proper subdivision of the pretectum territory, especially for the correct formation of the juxtacommisural pretectal domain. This effect involves the expression of Six3, which Wnt1-mediated repression seems to be essential for the pretectal development.

4. Wnt1 signal in the diencephalon is involved in the correct development of the dorso-ventral axis. The expression of Wnt1 regulates the establishment of the basal-alar plate boundary by means of the negative regulation of Gli3, which antagonizes functions of Shh. The repression of Gli3 by Wnt1 could be direct or indirect in a molecular mechanism that is still not well known in the diencephalon. However, we propose a possible mediator for this regulation in the diencephalon, L-fng, which would be induced by Wnt signal in order to repress Gli3.

5. Wnt1 signal is involved in the positioning of the Shh expressing mechanisms in the ZLI at the p2-p3 boundary, mediantig the formation of the singular spike of Shh determinant of the ZLI.

6. Bmp4 induces the expression of Wnt1, regulating the proper formation of the diencephalic dorso-ventral axis. The overexpression of Bmp4 shows the same phenotype as the overexpression of Wnt1, which demonstrates that both molecules are involved in the regionalization of the dorso-ventral diencephalic territories.

7. Wnt1 does not induce the expression of Bmp4. However, it is important for maintenance of its expression in the diencephalic roof plate.

1. La señal de Wnt1 es esencial para el desarrollo apropiado de las estructuras diencefálicas de la placa del techo, como la glándula pineal y la comisura posterior, a través de mecanismos moleculares que podrían implicar otros moléculas determinantes en el desarrollo diencefálico, como Fgf8 y Bmp4.

2. Wnt1 parece tener un papel importante en la correcta fasciculación del tracto retroflejo. En ausencia de Wnt1, este tracto está claramente de-fasciculado. Las moléculas que determinan la trayectoria de este tracto parecen estar bajo la influencia de la expresión de Wnt1.

3. La señal de Wnt1 en el diencéfalo es importante para la correcta subdivisión del territorio pretectal, especialmente para la formación del dominio pretectal yuxtacomisural. Este efecto implica la expresión de Six3, cuya represión mediada por Wnt1 parece ser esencial para el desarrollo pretectal.

4. La señal de Wnt1 en el diencéfalo está implicada en el correcto desarrollo del eje dorso-ventral, regulando el establecimiento del límite entre la placa basal y la placa alar, mediante la represión de Gli3, el cual antagoniza la función de Shh. La represión de Gli3 por Wnt1 podría estar mediada directa o indirectamente mediante un mecanismo molecular que todavía no ha sido claramente descrito. Sin embargo, proponemos un posible mediador para esta regulación en el diencéfalo, L-fng, el cual podría ser inducido por la señal de Wnt con el objetivo de reprimir a Gli3.

5. La señal de Wnt1 está implicada en el posicionamiento de la expresión de Shh en la ZLI en límite entre p2 y p3, mediando la formación de una espiga única de Shh determinante en la ZLI.

6. Bmp4 induce la expresión de Wnt1, regulando la correcta formación del eje dorso-ventral diencefálico. La sobreexpresión de Bmp4 muestra el mismo fenotipo que la sobreexpresión de Wnt1, lo cual demuestra que ambas moléculas están implicadas en la regionalización dorso-ventral de los territorios diencefálicos.

7. Wnt1 no induce la expresión de Bmp4. Sin embargo, es importante para el mantenimiento de su expresión en la placa del techo diencefálica.

Chapter VII

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