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Molecular mechanisms controlling brain development: an overview of neuroepithelial secondary organizers

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ABSTRACT The vertebrate Central Nervous System (CNS) originates from the embryonic dorsal ectoderm. Differentiation of the neural epithelium from the ectoderm and the formation of the neural plate constitute the first phase of a complex process called neurulation which culminates in the formation of the neural tube, the anlage of the CNS in sauropsids and mammals (for review see Smith and Schoenwolf, 1997; Colas and Schoenwolf, 2001). At neural plate and neural tube stages, local signaling centers in the neuroepithelium, known as secondary organizers, refine the antero-posterior specification of different neural territories (for review see Echevarria *et al.*, 2003; Stern *et al.*, 2006; Woltering and Durston, 2008). In this review, we will describe the principle aspects of CNS development in birds and mammals, starting from early stages of embryogenesis (gastrulation and neurulation) and culminating with the formation of a variety of different regions which contribute to the structural complexity of the brain (regionalization and morphogenesis). We will pay special attention to the cellular and molecular mechanisms involved in neural tube regionalization and the key role played by localized secondary organizers in the patterning of neural primordia.

KEY WORDS: *patterning, neural plate, neural tube, gastrulation, neurulation, secondary organizer, anterior neural ridge, zona limitans intrathalamica, isthmic organizer*

Neural plate and neural tube formation

A fundamental early step in neural development is the allocation of a group of ectodermal cells as precursors of the entire nervous system (Hemmati-Brivanlou and Melton, 1997). This process involves an inductive interaction first demonstrated in amphibian embryos by Spemann and Mangold in the 1920's (see Spemann and Mangold, 2001). Their experiments which involved the grafting of differently pigmented species of newt established the concept of neural induction as an instructive interaction between the dorsal lip of the blastopore (the "organizer") and the neighboring ectoderm. The discovery of a neural organization center for the amphibian gastrula initiated a search for homologous structures in other vertebrates. Soon thereafter, the equivalent region was discovered in most vertebrate species, including the shield of teleosts. In birds and mammals, the region was named "Hensen's node" and "the node", respectively. When C.H. Waddington transplanted the Hensen node of a chick embryo, he observed the induction of an ectopic neural plate or the formation of a partial new embryonic axis containing neural tube, notochord and somites (Waddington, 1933; Waddington, 1936). This demonstration provided the first evidence that in chick embryos, the nervous system is induced by signals from non-neural cells. Recent works demonstrated that the capacity of ectodermal cells to undergo neural differentiation represents their default state. In fact, neural differentiation must be suppressed in the lateral ectoderm by signals transmitted between neighboring cells, in order to develop as epidermis. These molecular signals are members of the bone morphogenetic protein (BMP) subclass of transforming growth factor β (TGF-β)-related proteins (for review see Wittler and Kessel, 2004).

Abbreviations used in this paper: ANR, anterior neural ridge; AP, antero-posterior; BMP, bone morphogenetic protein; DV, dorso-ventral; FGF, fibroblast growth factor; IsO, Isthmic organizer; ML, medio-lateral; TGF, transforming growth factor; ZLI, zona limitans intrathalamica.

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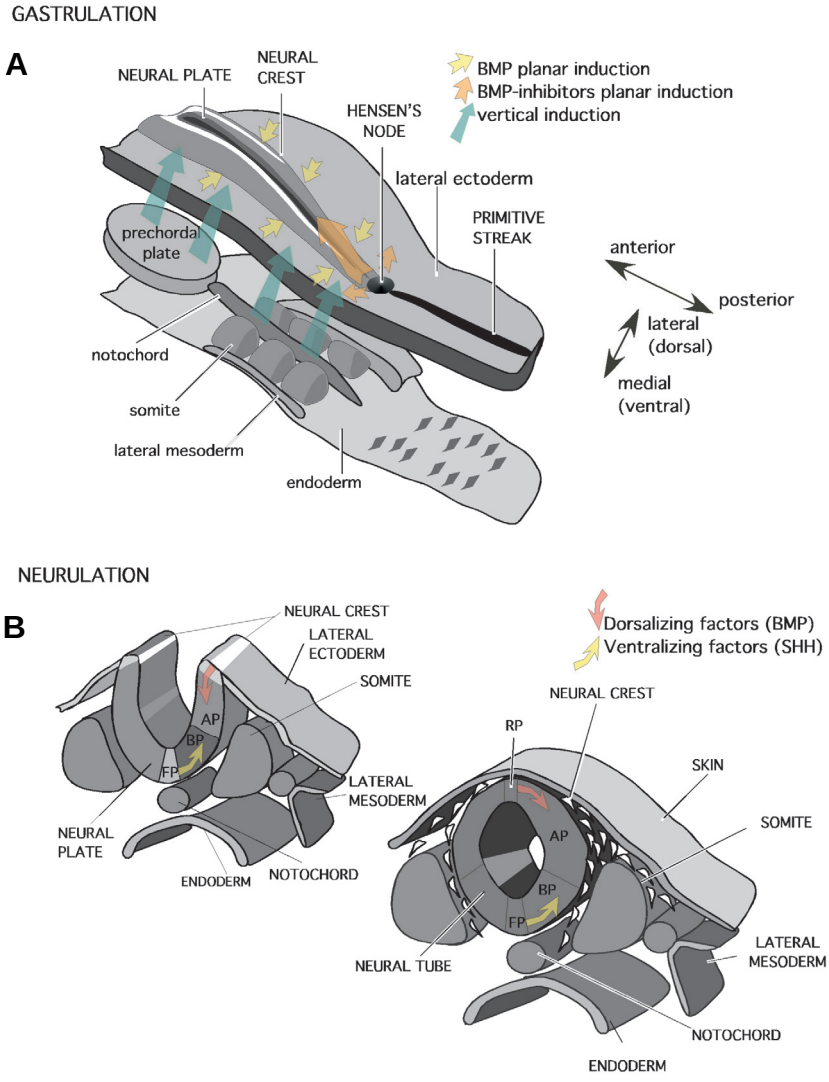

Fig. 1. **The neurulation process. (A)** *At neural plate stage, vertical induction (green arrows) from the underlying axial mesendoderm (notochord and prechordal plate), together with planar induction from Hensen's node (orange arrows) and ectoderm (yellow arrows) regulate dorsoventral polarity and the initial steps of antero-posterior regionalization in the neuroepithelium.* **(B)** *During neurulation, neural folds close at the dorsal midline. Neural crests cells delaminate and migrate from the neural folds before closure and the neural grove become the lumen of the neural tube. Planar information from the ventral midline (floor plate; FP; yellow arrow) and dorsal midline (roof plate; RP; red arrow) plays a fundamental role in the establishment of definitive dorsoventral regionalization, using sonic hedgehog (SHH) and bone morphogenetic proteins (BMP) as signaling molecules. As a consequence of these inductive events, the lateral wall of the neural tube is subdivided into two columnar domains: the basal plate (close to the floor plate) and the alar plate (close to the roof plate). AP, alar plate; BP, basal plate.*

Recent studies using chick embryos have shown that neural induction really begins prior to the formation of the organizer region and thus must be initiated by signals derived from other cellular areas. Members of other families of signaling molecules, notably the fibroblast growth factors (FGFs), have now been proposed as early-acting factors, which initiate neural induction by a progressive sequence of molecular interactions. First, the presumptive neural plate area is established by Fgf8 activity coming from the primary endoderm. Subsequently, the suppression of BMP signaling maintains rather than initiates the process of neural differentiation (Linker *et al*. 2009; for review see Stern, 2005).

These molecular interactions together with the participation of Hox genes (Woltering and Durston, 2008; Hooiveld *et al*., 1999) during the process of gastrulation regulate cellular inductive events leading to the definition of the antero-posterior and dorso-ventral axes of the embryo and to the generation of the three blastodermal layers: ectoderm, mesoderm and endoderm (Stern *et al*.,2006). Thus, in the central area of the embryo (at its prospective dorsal region), ectoderm cells are induced to develop as neural plate cells as a result of these progressive cellular and molecular interactions, acting via planar and vertical induction (Fig. 1A). Indeed, formation of the neural plate involves apicobasal thickening and pseudostratification of the ectoderm, resulting in the formation of a flat but thickened epithelial region which expresses a unique pattern of molecular markers (Smith and Schoenwolf, 1989; Keller *et al*., 1992).

Subsequently, the process of neurulation involves cell shape changes and epithelial rearrangement which result in the bending of the neural plate and apposition of its latent edges to form the neural tube. The neural plate lengthens along the antero-posterior axis and becomes narrower, so that subsequent bending will form a tube. Full antero-posterior formation and extension of the neural tube requires normal gastrulation movements and in particular, regression of the primitive streak (Voiculescu *et al*., 2007).

Bending of the neural plate involves the formation of hinge regions where the neural tube contacts surrounding tissues (for review see Colas and Schoenwolf, 2001). Elevation of the neural folds establishes a trough-like space called the neural groove, which becomes the lumen of the primitive neural tube after closure of the neural groove. In addition, the neural folds will generate the specialized cells of the neural crest. The neural tube closes as the paired neural folds are brought together at the dorsal midline (Fig. 1B). During this stage, the epidermal ectoderm from each fold detaches from its ipsilateral neuroepithelial partner and fuses with the epidermal ectoderm of the contralateral neural fold, contributing to the dorsal skin of the embryo. Similarly the detached neuroepithelial layers from both sides fuse together below the epidermal ectoderm, establishing the roof plate of the neural tube.

Cellular patterns and molecular regionalization of the neural plate

Experiments involving the labeling of individual cells or small groups of them, and analysis of their fate during gastrulation and neurulation have been performed in different species. The resulting fate maps showed that the generation of the neural plate and tube involves similar morphogenetic programs in

vertebrates (reviewed by Rubenstein *et al*., 1998; Cobos *et al*., 2001). During gastrulation, the bending of the neural plate, together with the intercalation of neuroectodermal precursors and with regional differences in proliferation, transform the initial medio-lateral arrangement of cells in the neural plate into the dorso-ventral organization of the neural tube (Fig. 1; Leikola 1976; Stern *et al*., 2006).

Gene expression patterns provide insights into the location, onset and developmental consequences of inductive processes, which generate regional specification within the developing brain. They also help to identify the candidate molecules which regulate these processes. During the past two decades, researchers have identified many regulatory genes whose patterns of expression in the embryonic neural plate and tube have yielded important insights into brain regionalization (Shimamura *et al*., 1995; 1997; Rubenstein *et al*. 1998; Crossley *et al*. 2001; Puelles and Rubenstein 2003; Echevarria *et al*. 2003; Aroca and Puelles, 2005). Interpretation of the expression patterns in terms of the topology of the neural plate axes provides a clearer picture of its molecular regionalization and also contributes to our understanding of how the expression of specific sets of genes in the neuroepithelium is related to brain histogenesis. Thus, longitudinal patterns reflect expression which extends along part or the entire antero-posterior axis and may mark the three primordia of the floor, basal and alar plates (Fig. 2). At the anterior pole of the neural plate, there is evidence indicating that medial (basal plate primordium) and lateral (alar plate primordium) domains form nested arcs which are concentric with the anterior neural ridge (Shimamura *et al*., 1995; Cobos *et al*., 2001; Echevarria *et al*., 2003).

Other genes have expression domains which are restricted to transverse regions of the neural plate, showing molecular discontinuities along the antero-posterior axis (Fig. 2). Interestingly some transcription factors are expressed in nested domains of the neural plate, playing a fundamental role in the establishment of particular transverse territories which actively regulate the development of the neighboring neuroepithelium. These nested domains have been termed secondary organizers (Fig. 2; Martinez, 2001; Echevarria *et al*., 2003; Aroca and Puelles 2005).

Regionalization of the neural tube

The early neural tube is, in most vertebrates, a straight structure. However, even before the posterior portion of the tube has formed, the most anterior portion of the neural tube is undergoing drastic changes. In this region, the tube balloons into three primary vesicles: the forebrain (prosencephalon), midbrain (mesencephalon) and hindbrain (rhombencephalon) (Fig. 3; reviewed in Martinez and Puelles, 2000). By the time the posterior end of the neural tube closes, secondary bulges – the optic vesicles – have extended laterally from each side of the developing forebrain. At this early stage of development (three vesicle stage), the bending of the long axis, already observed at the late neural plate stages, increases considerably after neurulation, leading to the cephalic and cervical flexures of the neural tube (Fig. 3). Then, the prosencephalon becomes subdivided into the anterior secondary prosencephalon (telencephalon and hypothalamus) and the more caudal diencephalon (Pombero and Martinez, 2009).

The discovery that putative regulatory genes are expressed in regionally restricted patterns in the developing forebrain has

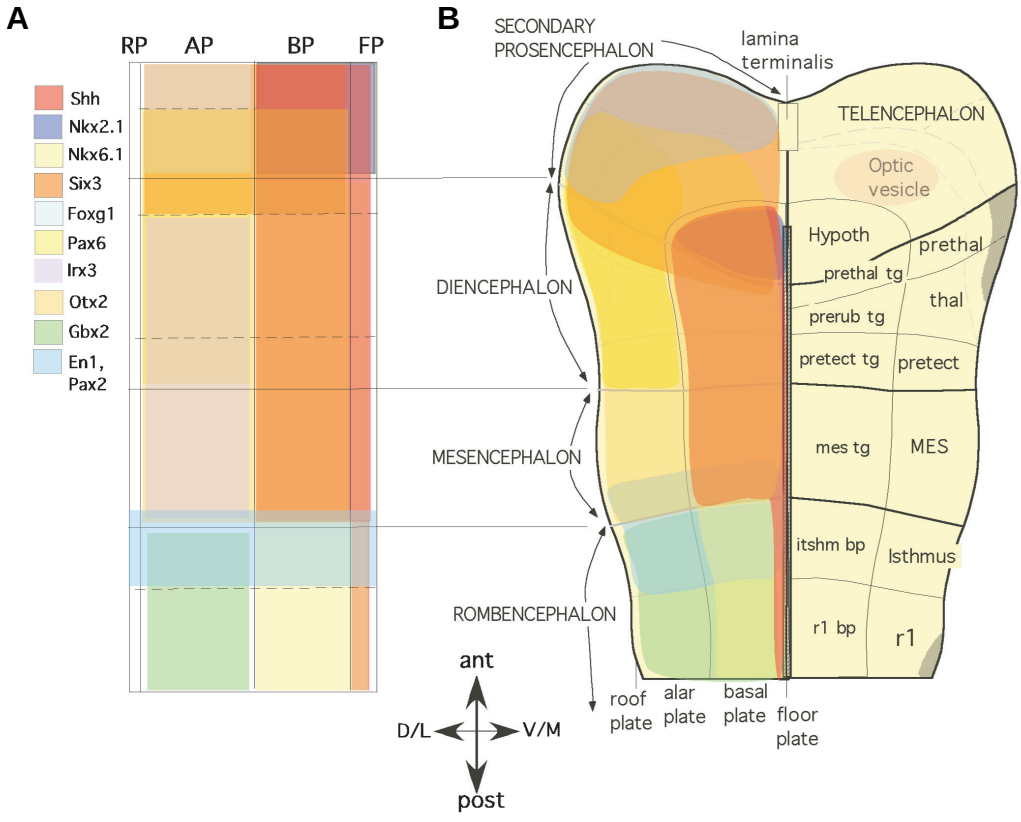

Fig. 2. Molecular regionalization of the neural plate. *Schematic* **(A)** *and realistic* **(B)** *representations of gene expression domains at the neural plate stage. Different colors represent different genes (gene symbol and color codes are identified in the schematic diagram). Medio-lateral (dorso-ventral) and antero-posterior (rostro-caudal) regionalization are identifiable by the limits between expression domains. The presumptive epithelia of different brain regions have been identified, as well as the presumptive localization of the secondary organizers, in relation to precise boundaries between gene expression domains. Secondary organizers: ANR: anterior neural ridge; ZLI: zona limitans intrathalamica; IsO: isthmic organizer. Longitudinal domains: RP, roof plate; AP, alar plate: BP (bp), basal plate; FP, floor plate. Abbreviations: Hypoth, hypothalamus; prethal, prethalamus tg; thal, thalamus; prerub, prerubral; pretect, pretectum; MES, mesencephalon; mes tg, mesencephalic tegmentum; r1, rhombomere 1.*

provided new tools for defining histogenic domains and their boundaries at higher resolution. Based on gene expression patterns as well as morphological information, two models have been used to interpret neural plate and tube regionalization: a regional-topographic model, largely aimed at saving the classic concept of sulcal division of the diencephalon into the 4 longitudinal columnar zones of Herrick (Alvarez-Bolado *et al*. 1995) and a segmental-topological model called the "prosomeric model" (Puelles and Rubenstein, 1993; Rubenstein *et al*., 1994). The latter is more consistent with emergent morphological, molecular and experimental data on the bent longitudinal forebrain axis, which cannot be satisfactorily rationalized in terms of the zones of Herrick. Although today there are still some authors who do not use the segmental paradigm to interpret gene expression patterns in the developing neural tube, most current studies do follow this prosomeric model, since it has demonstrated more topographic and anatomic accuracy, easily lends itself to comparative analysis and clearly displays higher predictive capacity than other alternatives.

The prosomeric model hypothesizes that the embryonic forebrain is a neuromeric structure subdivided into a grid-like pattern of histogenic domains by longitudinal (columnar) and transverse (segmental) boundaries, as a result of the evolution of the Cartesian organization of the neural plate (Puelles *et al*., 1987; Bulfone *et al*., 1993; Puelles and Rubenstein, 1993; Rubenstein and Puelles, 1994; Rubenstein *et al*., 1994; Shimamura *et al*., 1995; Puelles, 1995). The longitudinal boundaries segregate columns of cells with similar properties. These are specified by dorso-ventral (DV) patterning mechanisms, which are equivalent to the latero-medial patterning mechanisms of the neural plate. All these interactions, which occur during DV patterning, give rise to four longitudinal columnar territories, which from ventral to dorsal are the floor plate, basal plate, alar plate and roof plate (Fig. 1B).

Transverse boundaries subdivide the brain into segments (neuromeres). In the prosencephalon, these segments are called prosomeres (p1-6; Puelles and Rubenstein, 1993, or in a simplified schema, p1-p3 plus the secondary prosencephalon, Puelles and Rubenstein, 2003). In the rhombencephalon, the segments are termed rhombomeres (r1-r7) and pseudorhombomeres (r8-r11) (Marin and Puelles, 1995; Cambronero and Puelles, 2000) (Fig. 3). The mesencephalon has not been subdivided into internal sub-segments, and consequently is considered as single segmental unit.

The prosomeric model has unveiled the morphological significance of numerous gene expression patterns in the forebrain, suggesting the existence of additional molecular subdivisions of the main AP and DV zones, representing histogenetically specified domains of neural precursors (Fig. 3; Puelles *et al*., 1987; Bulfone *et al*., 1993; Puelles and Rubenstein, 1993; Rubenstein and Puelles, 1994; Rubenstein *et al*., 1994; Shimamura *et al*., 1995; Puelles, 1995). Examination of this molecular-structural association has shown how the prosencephalic expression of particular genes is directly related to specific morphogenetic and cytogenetic development. Thus, for instance *Gbx2* expression is associated with the generation of thalamic neurons which develop into the thalamo-cortical projection (Miyashita-Lin *et al*., 1999; Hasimoto-Torii *et al*., 2003), whereas *Nkx2.1* expression is associated with hypothalamic development (Sussel *et al*., 1999).

Medio-lateral (ML) / dorso-ventral (DV) patterning

The specification of longitudinally aligned regions within the

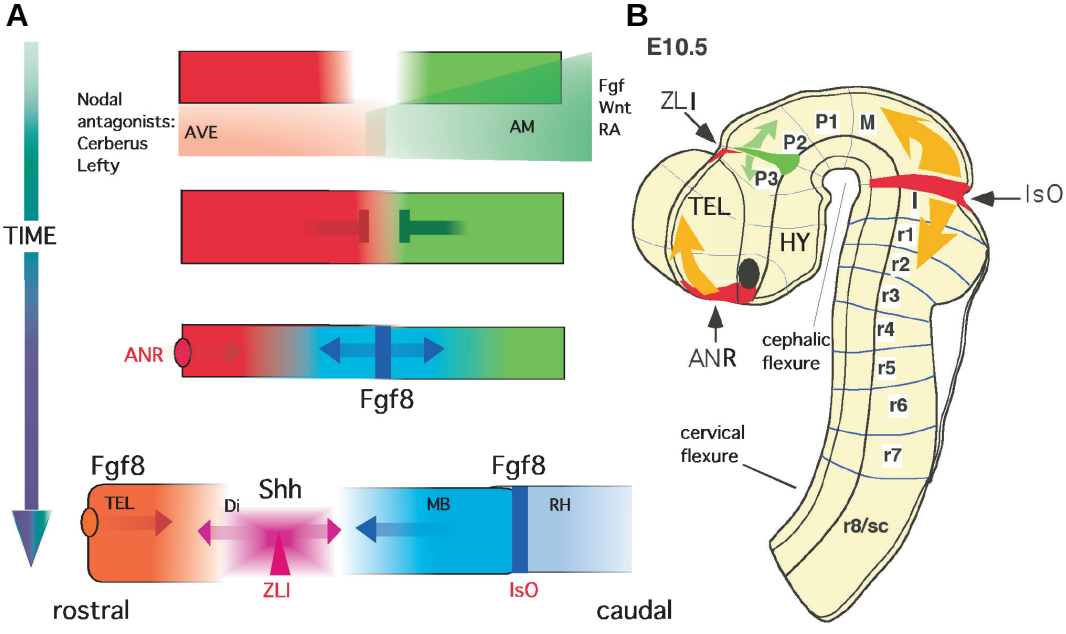

Fig. 3. Secondary organizer specification and neural tube regionalization. (A) *Schematic representation of secondary organizer specification in relation to gene expression patterns (color codes) and time (arrow). Morphogenetic signals from the anterior ventral endoderm (AVE) and axial mesendoderm (AM) determine the early establishment of anterior and posterior properties in the neuroepithelium and induce local domains of transcription factor expression in the overlying epithelium. Molecular interactions at the limits between these domains specify the development of morphogenetic organizers that generate a secondary wave of inductive signals (arrows) to regulate the development of structural properties in the surrounding neural tube regions.* **(B)** *Representation of a lateral view of an E10.5 mouse neural tube showing the main neuronal regions and the transverse segments of the neural tube in relation to the secondary organizers. Abbreviations: ANR, anterior neural ridge; AVE, anterior ventral endoderm; AM, axial mesoderm; DI, diencephalon; M, MB, midbrain; HY, hypothalamus; I, isthmus; IsO, isthmic organizer; RH, rhombencephalon; TEL, telencephalon; ZLI, zona limitans intrathalamica; p1-p3, prosomeres; r1-r8, rhombomeres; sc, spinal cord.*

CNS involves patterning along the ML dimension of the neural plate. This ML patterning in the neural plate is topologically equivalent to DV patterning in the neural tube (Figs. 1 and 2).

It has been well established that within the posterior neural plate, ML regional identities are specified in part by molecules produced by adjacent non-neural tissues. Both gain- and loss-of-function experiments have demonstrated that medial signaling is regulated by Sonic Hedgehog (Shh) produced by the axial mesendoderm (Echelard *et al*., 1993; Roelink *et al*., 1994, 1995; Hynes *et al*., 1995a; Marti *et al*., 1995; Tanabe *et al*., 1995; Chiang *et al*., 1996; Ericson *et al*., 1996; Shimamura and Rubenstein, 1997; for review see Tanabe and Jessel, 1996). Shh is first produced by the notochord, and later its expression is induced in the overlying medial neural plate (Fig. 1A). Moreover, gain-of-function experiments and gene expression data support the idea that lateral signaling is regulated by members of the TGF-β superfamily, such as BMP4 and BMP7 produced by non-neural ectoderm (Basler *et al*., 1993; Dickinson *et al*., 1995; Liem *et al.,* 1995; Shimamura and Rubenstein, 1997). Because the notochord does not underlie the anterior forebrain (the anterior end of the notochord ends at the level of the anterior diencephalon at the prethalamic basal plate), it is unclear if patterning of the medial (ventral) forebrain is regulated by mechanisms distinct from those which occur in more posterior regions. Anterior to the notochord, an axial mesendodermal structure named the prechordal plate underlies the anterior part of the floor plate. Several lines of molecular and genetic evidence now suggest that medial/ventral specification of the forebrain is regulated by the prechordal plate and involves molecular mechanisms similar to those in more posterior CNS regions. In fact, analysis of mice lacking a functional *Shh* gene has demonstrated that Shh is essential for medial patterning of the entire CNS (Chiang *et al*., 1996). In a study reported by Rubenstein and Shimamura (1997) using a neural plate explant method, the authors directly show that the prechordal plate (and the dorsal foregut) induces medial properties and represses lateral properties in prosencephalic explants (Shimamura and Rubenstein, 1997). Furthermore, they showed that the prechordal plate functions alone in the initial specification of the medial prosencephalon.

Thus, the prechordal plate and notochord share similar roles in medial neural plate specification and longitudinal neural patterning. However, they also exhibit distinct molecular properties which may endow them with specific inductive abilities (Placzek *et al*., 1993) which contribute to different properties in the overlying prechordal and epichordal basal plates. In fact, we recently demonstrated that diencephalic epichordal and prechordal basal plates have different inductive properties when ectopically grafted into the thalamus or telencephalon (Vieira *et al*. 2006).

Antero-posterior (AP) patterning

AP patterning is the process that leads to the generation of distinct transverse domains at different axial positions in the CNS. There is evidence that AP patterning begins during early gastrulation.

We have previously described how several experiments suggest that vertical signals from underlying tissues (mesoderm and endoderm) to the overlying dorsal ectoderm, and perhaps planar signals from the organizer (Hensen's node) through the plane of the ectodermal epithelium, contribute to the specification of AP regional differences (Fig. 1; for review see Doniach 1993; Ruiz i Altaba, 1994). The initial AP pattern is induced by the combined action of two signals produced by the dorsal mesoderm (for review see Doniach, 1993). The first signal initiates neural development and induces the neuroectoderm, which has an anterior neural fate (forebrain and midbrain). Candidate molecules to regulate this signal are *Lim1* and *Otx2,* while a candidate signal is the protein *Cerberus* (see below). A graded second signal then posteriorizes the neural plate, inducing hindbrain and spinal cord development. Candidate signals for the posteriorizing signal include retinoic acid (Durston *et al*., 1989; Papalopulu *et al*., 1991; Ruiz I Altaba and Jessell, 1991a, b; Blumberg *et al*., 1997) basic FGF (FGF2; Cox and Hemmati-Brivanlou, 1995; Kelly and Melton, 1995; Lamb and Harland, 1995; Hemmati-Brivanlou and Melton, 1997) and Hox genes. In mammals, this latter gene family comprises 39 closely related genes for homeodomain transcription factors, organized in 4 homologous clusters (A, B, C, D) (Pearson *et al*., 2005).The Hox genes have sharply defined anterior expression boundaries but their posterior boundaries are typically less clear and overlap with the expression of more posterior Hox genes (Hooiveld *et al.,* 1999).

In addition to signals acting during gastrulation, several groups including those of Janet Rossant and Siew-Lan Ang found that mesendodermal tissues underlying the anterior neural plate could regulate regional patterns of gene expression (e.g. Orthodenticle (*Otx2)* and *Engrailed (En1)*) within the rostral brain (Ang and Rossant, 1993; Ang *et al*., 1994). Moreover Darnell and Schoenwolf (1997) showed that regionally restricted vertical signals are capable of inducing neuroectoderm from naive tissue, and of patterning epiblast to express some mesencephalic/rhombencephalic markers. Recently we have demonstrated the requirent of prechordal mesoderm to develop normal regionalization of the ventral prosencephalon (Garcia-Calero *et al*., 2008). One protein that may regulate this process is named Cerberus. This secreted protein is expressed in a broad anterior domain flanking the expression of *Chordin* and *Lim1* in the prechordal plate. Thus, Cerberus may specify antero-lateral structures, such as anterior axial mesendoderm (prechordal plate) and would then regulate medial specification within the prosencephalic neural plate. When Cerberus is ectopically expressed in *Xenopus* embryos, it induces nearly complete head structures (Bouwmeester *et al*., 1996). Other proteins such as Noggin, Follistatin, Cripto and Chordin also induce anterior neural tissues, but these genes may not be essential for AP patterning (Liguori *et al*., 2003 and 2009; Lamb *et al*., 1993; Hemmati-Brivanlou *et al*., 1994; Lamb and Harland, 1995; for review see Doniach, 1993).

Two homeodomain transcription factors, *Lim1* and *Otx2*, are expressed in the tissues underlying the anterior neural plate and seem to be essential for the development of anterior CNS structures. Loss-of-function mutants result in mouse embryos lacking forebrain and midbrain, suggesting that *Lim1* and *Otx2* have a role in early AP patterning (Acampora *et al*., 1995; Matsuo *et al*., 1995; Shawlot and Behringer, 1995; Ang *et al*., 1996). *Lim1* is expressed in the primitive streak and prechordal

mesoderm. Because expression is not detected in the neural plate, the lack of forebrain and midbrain in *Lim1* mutants is evidence for an essential role of this mesoderm in anterior CNS development (Shawlot and Behringer, 1995). Understanding the mechanisms underlying the *Otx2* phenotype is more difficult because of the dynamics of its expression pattern and the complexity of its molecular interactions.

A variety of evidence indicates that AP regionalization can generate transverse blocks of neuroepithelium which have distinct competence to respond to the same inductive signal (Ericson *et al*., 1995; Hynes *et al*., 1995b; Simon *et al*., 1995; Shimamura and Rubenstein, 1997). This phenomenon is clearly illustrated by inductive responses to Shh. *Shh* is expressed along the entire AP extent of the prechordal plate and the notochord. Whereas Shh induces the expression of some genes (e.g. *Shh, HNF3β, Nkx2.2*) in all regions of the medial neural plate/ventral neural tube, other genes are induced within particular intervals along the AP axis. For instance, whereas *Nkx2.1* is expressed only in the prosencephalic neural plate, *Nkx6.1* is expressed in more posterior locations (Qiu *et al*., 1998). Thus, available evidence suggests that in some cases, distinct gene expression programs at different AP positions within the medial neural plate are due to intrinsic differences in competence to respond to a common signal.

Fgf8 is another example of an inductive signal which generates distinct molecular responses at different axial levels. When Fgf8 is applied to prosencephalic and mesencephalic domains of neural plate explants, it induces distinct genes: anteriorly, it induces *FoxG1 (Bf1)*, whereas posteriorly, it induces *En2* (Shimamura and Rubenstein, 1997).

Regionalization of the rostral brain involves signaling from secondary organizing centers

Regionalization of the anterior neural plate appears to result from the superposition of multiple distinct patterning mechanisms. AP patterning creates transverse zones, each with a distinct histogenic competence, while patterning along the ML axis generates longitudinally aligned domains. The combination of ML and AP patterning then generates a grid-like organization of distinct brain region primordia (see Fig. 2). Therefore, neural progenitors in the epithelium will establish their differen-

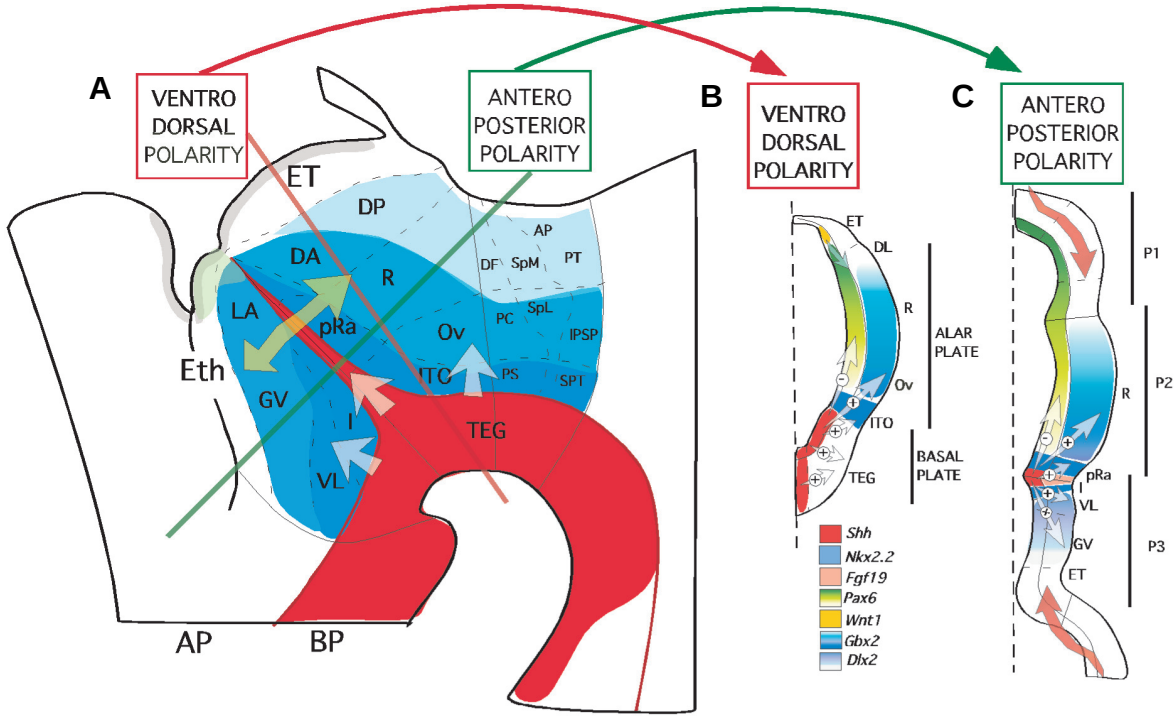

Fig. 4. Shh signal activity and diencephalic regionalization. *Schematic representation illustrating the diencephalic regionalization and its relation with thalamic and prethalamic gene markers. Shh morphogenetic activity has a gradient effect in the diencephalic alar plate that could influence ventro-dorsal and antero-posterior regionalization. Shh morphogenetic gradient from the basal plate and from the ZLI, represented by graded blue colors, control the expression of diencephalic selector genes (represented by color fields) and is fundamental for the correct specification of the diencephalic regions. The arrows are showing inductive interactions of the morphogenetic signals. Diagrams of transversal (dorso-ventral polarity) and horizontal (antero-posterior polarity) section planes in the diencephalon are represented to the right of the figure. White arrows represent Shh inductive (+) and repressive (-) effects, light blue arrow represent Wnt1 activity from dorsal midline, and light red arrows represent Fgf8 signals from the Isthmus and the anterior neural ridge. Abbreviations: AP, area pretectalis; DA, dorsomedial thalamic complex; DF, dorsofrontal nucleus; DP, dorsolateral thalamic complex; ET, epithalamus; Eth, eminentia thalamica; GV, lateral geniculate nucleus; I, nucleus intercalatus; IPSP, interstitial pretectal-subpretectal nucleus; ITO, interstitial nucleus of the optic tract; LA, anterior lateral nucleus; Ov, nucleus ovoidalis; P1, prosomer 1; P2, prosomer 2; P3, prosomer 3; PC, principal precommissural nucleus; pRA, perirotundic area; PS, superficial precommissural nucleus; PT, principal pretectalis nucleus; R, rotundus nucleus; SpL, lateral spiriformis nucleus; SpM, medial spiriformis nucleus; SPT, subpretectal nucleus; TEG, diencephalic tegmentum; VL, ventrolateralis nucleus.*

tiation program under the control of positional information (as defined in a Cartesian-type map) transmitted by molecular signals.

Distinct neural and glial identities are acquired by neuroepithelial cells through progressive restriction of histogenetic potential under the influence of local environmental signals. Evidence for morphogenetic controlling processes at specific locations of the developing neural primordium has led to the concept of secondary organizers, which regulate the identity and regional polarity of neighboring neuroepithelial regions (Fig. 3, Ruiz i Altaba, 1998; for review see Martinez, 2001; Echevarria *et al*., 2003). Thus, these organizers, secondary to those that operate throughout the embryo during gastrulation, usually develop within the previously broadly regionalized neuroectoderm at given genetic boundaries (frequently where cells expressing different transcription factors are juxtaposed) and their subsequent activity refines local neural identities along the AP or DV axes, patterning the anterior neural plate and neural tube, giving rise to the forebrain, midbrain and hindbrain vesicles (Meinhardt, 1983; Figdor and Stern, 1993; Wassef and Joyner, 1997; Rubenstein *et al*., 1998; Joyner *et al*., 2000).

Three regions in the neural plate and tube have been identified as putative secondary organizers (Fig. 3): the anterior neural ridge (ANR) at the anterior end of the neural plate/tube, the zona limitans intrathalamica (ZLI) in the middle of the diencephalon and the isthmic organizer (IsO) at the mid-hindbrain boundary.

Anterior neural ridge (ANR)

The anterior secondary organizer, the ANR, was first described by Houart el al. (1998) in zebrafish at the junction between the most rostral part of the neural plate, the anlage of the anterior commissure and non-neural ectoderm. Genes expressed in this region control others necessary for telencephalic regionalization (Shimamura and Rubenstein, 1997; Ye *et al*., 1998). In particular, the *Fgf8* gene is expressed very early in ANR cells and has been shown to be crucial for the specification of the anterior areas of the forebrain and telencephalon. *Fgf8* expression in the ANR is necessary for the induction and/or maintenance of *FoxG1* (*Bf1)* expression, which in turn is essential for telencephalic precursor proliferation (Xuan *et al*., 1995; Shimamura and Rubenstein, 1997). In addition, implantation of Fgf8 protein into the prospective area of the telencephalon in chick embryos generates changes in the patterns of gene expression and consequently a redistribution of telencephalic and optic derivatives (Crossley *et al*., 2001). Ectopic expression of *Fgf8* in the caudal telencephalon of mouse embryos produces duplication of functional areas of the cortex (Fukuchi-Shimogori and Grove, 2001). Fgf8 regulates prosencephalic regionalization, at least in part, through inhibition of Otx2 and Emx2 expression and cooperation with Bmp4, Wnt and Shh (Fig. 5; Crossley *et al*., 2001; Garel *et al*., 1997; Storm *et al*., 2006). Recently, other member of the FGF family, such as Fgf15, is expressed in the ANR. Its expression domain is closely related with that of *Fgf8* and its expression seems to be induced by Fgf8 protein (Gimeno *et al*., 2002, 2003).

Another signaling protein secreted near the ANR is Shh. Considerable evidence suggests that Shh is both necessary and sufficient for the specification of ventrality throughout the nervous system, including the telencephalon (Echelard *et al*., 1993; Chiang *et al*., 1996; Hammerschmidt *et al*., 1997; Wijgerde *et al*., 2002). Moreover, normal patterning in the telencephalon depends on the ventral repression of *Gli3* function by *Shh* and, conversely on the dorsal repression of *Shh* signaling by *Gli3* (Rallu *et al*., 2002). Finally, the activity of *Nkx2.1*, a homeodomain gene required for the development of the hypothalamus and ventral forebrain (Ericson *et al*., 1995; Brand *et al*., 1996) is also regulated by *Shh*.

Zona limitans intrathalamica (ZLI)

The diencephalon is a cerebral region, which develops from the caudal prosencephalon. The diencephalic territory is subdivided into three segments, which are transverse domains defined on the basis of morphological and molecular criteria. These are, from caudal to rostral, prosomeres 1 to 3 (Puelles and Rubenstein, 2003; Garcia-Lopez *et al*. 2004). The alar plate of the first diencephalic prosomere (p1) contains the presumptive pretectal region. Prosomere 2 alar plate develops into the thalamus, while p3 alar plate develops into the prethalamus. The limit between p2 and p3 is called the zona limitans intrathalamica (ZLI; reviewed in Martinez and Puelles, 2000). This intrathalamic limit appears early on in neural tube development and exhibits a unique pattern of molecular expression, which suggests an important role for this area as a secondary morphogenetic organizer in diencephalic histogenesis (Kiecker and Lumsden, 2004; for review see: Echevarria *et al*., 2003).

The cellular and molecular mechanisms which regulate positioning and specification of the ZLI could be explained in terms of the interaction between prechordal and epichordal neuroepithelia (Figs. 2 and 3). This pre-epichordal planar interaction in the alar plate would induce the conditions which permit the expression of *Shh* in the ZLI and would activate the morphogenetic properties of this organizer, specifying in turn the compartmentalization and cell fate of the different diencephalic prosomeres, through the control of specific gene expression (Fig. 4; Kobayashi *et al*., 2002; Vieira *et al*., 2005). The pattern of *Shh* expression in the ZLI is very dynamic in both mouse and chick embryos (Echelard *et al*., 1993; Shimamura *et al*., 1995; Fig. 3A in Echevarria *et al.,* 2001). It starts by being limited to the diencephalic basal plate and then extends dorsally into the basal part of the presumptive ZLI epithelium, by a process of homogenetic induction. The importance of *Shh* expression in the ZLI is supported by the fact that mice, which bear mutant *Shh,* show defects in early development, with an important reduction in size of the diencephalic vesicle (Chiang *et al*., 1996; Ishibashi and McMahon, 2002).

Cellular identity in the diencephalon may be under the control of genes whose expression is regulated by signaling cascades activated by ZLI-derived morphogens. Nested within the *Shh* expression domain, ZLI cells express the transcription factor *Sim1* (Fan *et al*., 1999). At both rostral and caudal sides of the ZLI, *Nkx2.2* and *Fgf15* are expressed (Fig. 4B and C; Price *et al*., 1992; Shimamura *et al*., 1995; Gimeno *et al*., 2002). The *Gbx2* transcription factor is expressed caudal to the ZLI and serves as a marker for the thalamus (Martinez-de-la-Torre *et al*., 2002). *Dlx2* and *Nkx2.1* are expressed in the alar and basal plate, respectively, just rostral to the ZLI (Gonzalez *et al*.,

2002). Also, the dorsal end of *Shh* expression in the ZLI is flanked by *Wnt1* caudally (McMahon and Bradley, 1990; Thomas and Capecchi, 1990) and *Fgf8* rostrally (Crossley and Martin, 1995; Marti *et al*., 1995; Crossley *et al*., 1996).

Recent experimental data from our group demonstrated that Shh signal from both the ZLI and the basal plate play an important role in the molecular patterning of the diencephalic alar plate (Fig. 4A; Vieira *et al*., 2006). Basal plate signals could play an initial role in the specification of longitudinal territories in the thalamic area (ventro-dorsal patterning; Fig. 4B), while posterior development of the ZLI represents an additional source of morphogenetic signals which superimpose antero-posterior information over thalamic epithelium (Fiure 4C). The combinatory effects of these two types of information could contribute to the complexity of thalamic molecular regionalization and as a consequence, to its complex anatomy.

The Isthmic organizer (IsO)

The IsO is localized at the mid-hindbrain transition and controls midbrain and anterior hindbrain regionalization (Fig. 3 and 5; Martinez and Alvarado-Mallart, 1989; for review see Martinez, 2001; Echevarria *et al*., 2003; Hidalgo-Sanchez *et al*., 2005; Aroca and Puelles 2005, Partanen 2007). Numerous experimental studies have demonstrated the morphogenetic properties of this region and its role in the specification and normal development of cerebellar, isthmic and mesencephalic territories. The isthmic organizer is critical for the development of adjacent regions (Martinez and Alvarado-Mallart, 1990; Alvarado-Mallart, 1993; Marin and Puelles, 1994), even when transplanted ectopically to rostral regions of the neural tube (Marin and Puelles, 1994; Martinez *et al*., 1991; Martinez *et al*., 1995).

Analysis of *Fgf8* expression in the mid-hindbrain region allows one to follow the development of the IsO, which occurs at the limit

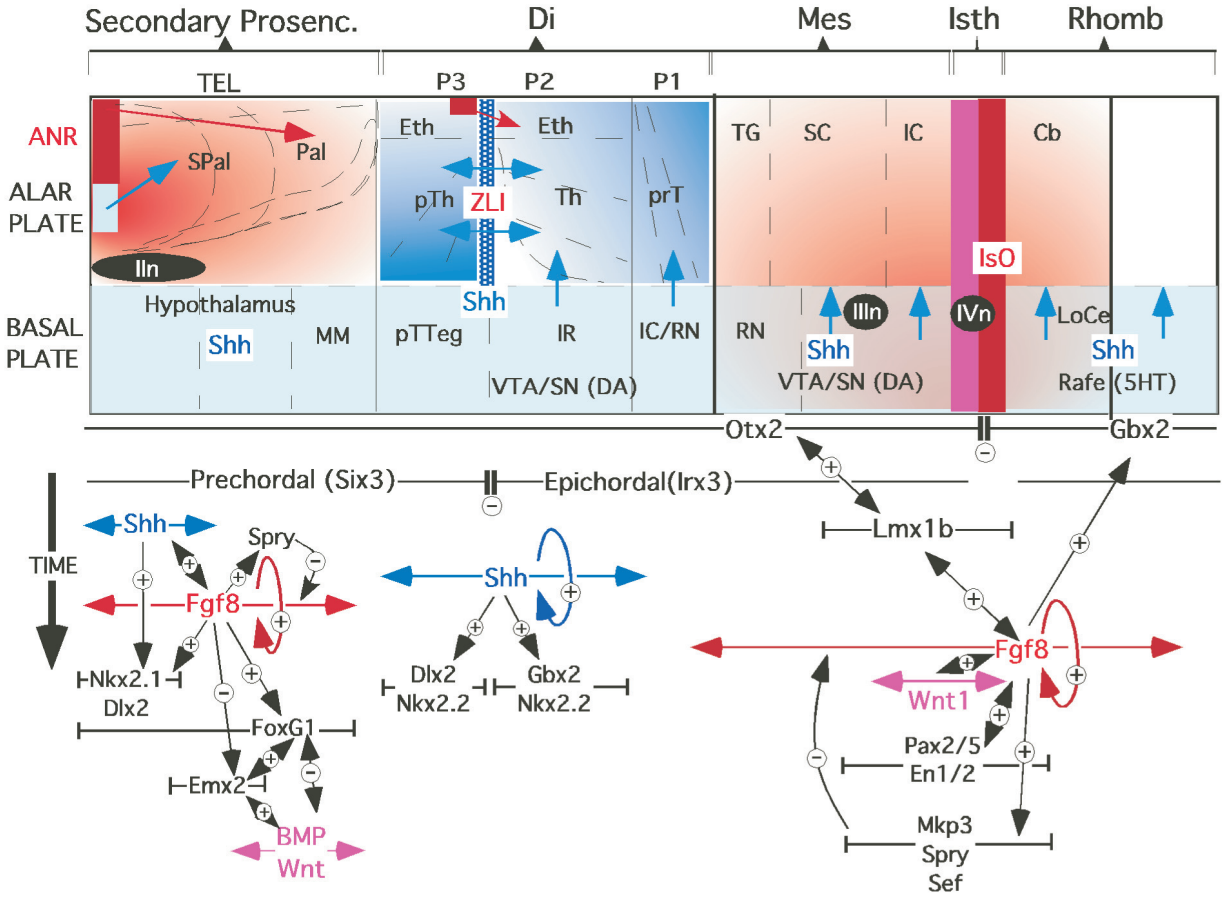

Fig. 5. Molecular pathways controlling brain regionalization. *A schematic diagram showing the neural territories influenced by the morphogenetic activity of secondary organizers (ANR, left; ZLI, middle and IsO, right; anterior is to the right). The molecular pathways regulating organizer specification are also shown, together with specific local activity associated with the gradient of the signaling molecules (color gradients). Genetic patterns are represented by their respective symbols inside a lineal sector. This sector ends in a vertical line when the gene is a transcription factor or in an arrowhead when the gene encodes a secreted molecule (signaling molecule). Gene interactions are represented by arrows showing the direction of the interaction and inductive (+) or repressive (-) effects. Abbreviations: ANR, anterior neural ridge; Cb, cerebellum; DI, diencephalon; Eth, eminentia thalami; IC, inferior colliculus; IC/RN, Interstitial of Cajal and parvocellular-red nuclei; IR, interstitial rostral nucleus; Is, isthmus; IsO, isthmic organizer; LCe, locus coeruleus; MES, mesencephalon (midbrain); MM, mammillary region; Pal, pallium; pT, prethalamus; pTTeg, prethalamic tegmentum; rafe, rafe nuclei (5HT, serotoninergic cells); RHOMB, rhombencephalon; RN, magnocellular-red nucleus; SC, superior colliculus; Sec. PROS, secondary prosencephalon; SPal, subpallium; T, thalamus; TEL, telencephalon; TG, tectal grey nucleus; VTA/SN, ventral tegmental area/substantia nigra (DA, dopaminergic cells); ZLI, zona limitans intrathalamica; IIIn, 3rd oculomotor nucleus; IVn, 4th trochlear nucleus.*

of expression of *Otx2* and *Gbx2* domains (Broccoli *et al*., 1999; Millet *et al*., 1999; Katahira *et al*., 2000; Garda *et al*., 2001; Li and Joyner, 2001; Martinez-Barbera *et al*., 2001). At early neural stages, *Otx2* and *Gbx2* are, for a short period of time, co-expressed in a domain at their respective interfaces of expression, having an intracellular or intercellular (Fig. 3; Prochiantz, 1999) molecular repressive interaction. *Otx2* is quickly down-regulated, being limited to the posterior part of the mesencephalon, whereas the expression of *Gbx2* is restricted to the isthmus and rhombencephalon (Millet *et al*., 1999; Garda *et al*., 2001). The isthmic organizer develops exactly at the limit between *Otx2* and *Gbx2* expression domains, suggesting that this cellular interaction at the expression boundary could be essential to specify or stabilize the position of the organizer. Loss of *Gbx2* function (Wassarman *et al*., 1997; Broccoli *et al*., 1999) or reduction of *Otx2* (Acampora *et al*., 1997; Millet *et al*., 1999) in mice shows a re-patterning phenotype in the mid-hindbrain regions.

Between the caudal and rostral limits of *Otx2* and *Gbx2*, respectively, *Fgf8* expression is induced (Fig. 3 and 5). This neuroepithelial region also dynamically expresses *Lmxb1* which is required for *Fgf8* expression and thus for the development of the isthmic organizer (Guo *et al*., 2007). Moreover, approximately at the end of gastrulation, *Pax2, Pax5, En1* and *Wnt1* are each expressed in a transverse domain which coincides with the contact area between *Otx2* and *Gbx2*, and subsequently with the IsO, but in a domain which is broader than that of *Fgf8* (Fig. 5; Davis and Joyner, 1988; Bally-Cuif *et al.,* 1999; Joyner, 1996; Urbanek *et al*., 1997; Funashashi *et al*., 1999; Hidalgo-Sanchez *et al.,* 2000; Rowitch *et al*., 1999; Hidalgo-Sanchez and Alvarado-Mallart, 2002).

The morphogenetic activity of the isthmic neuroepithelial region was first suggested by loss-of-function experiments centered on the *Wnt1* gene (McMahon and Bradley, 1990; Thomas and Capecchi, 1990) and was experimentally demonstrated by using quail-chick grafts of the isthmic neuroepithelium in anterior mesencephalic and diencephalic regions (Martinez *et al*., 1991). Fgf8 was later reported to be the signal molecule associated with isthmic activity (Crossley *et al*., 1996). Ectopic Fgf8 protein can induce IsO-characteristic and structural alterations in the diencephalic caudal prosomeres (p1-p2), the midbrain and the hindbrain (Fig. 5; Crossley *et al*., 1996; Martinez *et al*., 1999; Irving and Mason, 2000).

Experimental manipulations of these genes expressed in the IsO have demonstrated that both the presence of their protein products and their normal combined patterns of expression are required for the normal morphogenetic process. Mutant mice lacking *Wnt1, Pax2, En1, Gbx2* or *Fgf8* do not develop isthmocerebellar structures (McMahon and Bradley, 1990; Millen *et al*., 1994; Wurst *et al*., 1994; Urbaneck *et al*., 1997; Wassarman *et al*., 1997; Meyers *et al*., 1998). In addition, experiments involving mutations of *En1/2* (Liu and Joyner, 2001), *Pax2/5* (Joyner, 1996; Urbanek *et al*., 1997), *Otx1/2* (Matsuo *et al*., 1995; Suda *et al*., 1996; Acampora *et al*., 1997) and a hypomorphic allele for the *Fgf8* gene (Meyers *et al*., 1998; Chi *et al*., 2003), showed that the observed anatomical malformations in these models are due to the mis-specification of the IsO.

Shh signaling pathway

Since their isolation in the early 1990's (Lee *et al*., 1992; Echelard *et al*., 1993; Tashiro *et al*., 1993) members of the Hedgehog family of intercellular signaling proteins have come to be recognized as key mediators of many fundamental processes in embryonic development. Their activities are central to the growth, patterning and morphogenesis of many different regions within the body plans of vertebrates and insects. Sonic Hedgehog (Shh) is well known as the molecule responsible for the induction and maintenance of ventral neural tube structures. Recent data have shown that ventral neuronal populations react differentially to the amount of this morphogen not only in the spinal cord, but also in more rostral parts of the brain, in particular the development of the different mesencephalic basal nuclei in the absence of Shh (Perez-Balaguer *et al*. 2009).

Shh is proteolytically cleaved to produce two secreted proteins, a 19 kDa N-terminal protein (N-Shh) that mediates all signaling activities in vertebrates and invertebrates (for review see Hammerschmidt *et al*., 1996, 1997) and a 25 kDa C-terminal protein (C-Shh) which possesses protease activity (Porter *et al*., 1995; Porter *et al*., 1996). N-Shh is further modified by addition of a cholesterol moiety to the C-terminal amino acid and a palmitoyl group to the N-terminal of the processed N-Shh (Chamoun *et al*., 2001).

At the cell surface, Shh binds with high affinity to patched (Ptc), a 12-transmembrane protein. Binding of Shh to Ptc prevents the normal inhibition of smoothened (Smo), a 7-transmembrane protein (Alcedo *et al*., 1996; Van den Heuvel and Ingham, 1996; Hammerschmidt and McMahon, 1998; Hynes *et al*., 2000). Further regulators of the pathway, which act at the surface of cells responding to Shh, have been identified in the vertebrate CNS. Hedgehog-interacting protein (Hip) is a type I transmembrane protein which attenuates Shh signaling by binding N-Shh with an affinity similar to that of Ptc1 (Chuang and McMahon, 1999), whereas vitronectin, an extracellular matrix glycoprotein, enhances Shh activity during motor-neuron differentiation, by also binding Shh directly (Pons and Marti, 2000).

Within the nucleus of the responding cell, zinc-finger transcription factors of the Ci/Gli family act at the last known step of the Shh signal transduction pathway (Hynes *et al*., 1997; Ruiz i Altaba, 1998). A region specific combinatory effect of Gli2 activation and Gli3 repression by Shh in different areas of the neural tube determines regional activity of Shh signal in neural patterning and growth (Blaess *et al*., 2006; Zervas *et al*. 2004).

FGF signaling pathway

We have already mentioned that at early stages of development, Fgf8 is required to maintain the expression of genes which play a role in neural tube patterning. Moreover, it is essential for cell survival, as evidenced by the finding that inactivation of Fgf8 in the early neural plate causes extensive cell death throughout the mesencephalon and rostral hindbrain, resulting in full deletion of the midbrain and cerebellum. Interestingly, when Fgf8 expression is modestly reduced, rather than eliminated, the rostralmost portion of the midbrain is spared and appears normal, whereas the remaining dorsal midbrain, isthmus and cerebellum are absent (Chi *et al*., 2003; Partanen, 2007). This suggests that there are regional differences in sensitivity to FGF signaling within the mesencephalon and/or rhombencephalon.

FGF signaling is mediated via receptor tyrosine-kinases(RTKs). These transmembrane FGF receptors (FGFRs)

activate signaling cascades including the phosphatidylinositol-3 kinase (PI3K) and Ras-ERK pathways (MAPK) (for review see Martin, 1998; Niehrs and Meinhardt, 2002; Tsang and Dawid, 2004). The expression of molecules such as Mkp3/Sef and those belonging to the sprouty (Spry) family are induced by Fgf8 expression in the organizers (Fig. 5) and may determine the spatial reduction of Fgf8 activity in a gradient manner by interaction with the intracellular mechanism of the MAP kinase cascade (Furthauer *et al*., 2002; Tsang and Dawid, 2004; Echevarria *et al*., 2005a; Vieira *et al*., 2005; for review see Echevarria *et al*., 2005b). In particular, the negative feedback modulator of Fgf8 signaling, Mkp3, selectively inactivates the ERK1/2 class of MAP kinases by dephosphorylation leading to catalytic inactivation. It thus prevents translocation into the nucleus, resulting in inhibition of ERK1/2-dependent transcription (Camps *et al*., 1998; Groom *et al*., 1996; Muda *et al*., 1996).

Thus, FGFs activate signal from the organizer regions and present a gradient-like distribution in the extracellular compartment. This gradient, acting trough FGF receptors, activates intracellular transduction pathways which are required for cell-autonomous control of Fgf8 expression and for the activation of expression of multiple genes necessary for a variety of developmental processes, including cell fate decisions, determination of axial polarity and promotion of cell survival.

The morphogenetic activity of the IsO is then a consequence of specific molecular expression patterns, which regulate the differential specification of neuroepithelial territories. The decreasing gradient of Fgf8 concentration in the alar plate is fundamental for cell survival and the differential development of cerebellar, isthmic and mesencephalic regions (Basson *et al*., 2008; Chi *et al*., 2003; Nakamura *et al*., 2005). In the basal plate, Fgf8 concentration gradients are the key players in cell survival and, together with Shh, regulate caudal serotoninergic and rostral dopaminergic fates in cellular progenitors (Fig. 5), as well as the localization and development of other basal derivatives, such as noradrenergic cells in the locus coeruleus (in the rhombencephalon) and the red nucleus (in the mesencephalic tegmentum) (Chi *et al*., 2003; Puelles and Rubenstein, 2003,2004; Prakash *et al*., 2006; Prakash and Wurst, 2006). Finally, mesencephalic and diencephalic epithelia are receptive to Fgf8 signal (Martinez *et al*., 1991 and 1999; Crossley *et al*., 1996), which may regulate gene expression and neuroepithelial polarity in the alar plate of these territories (Vieira *et al*., 2006).

Conclusion

The overall organization of the vertebrate CNS is largely due to set down by the concerted action of morphogenetic signals acting during the early gastrula stage of embryonic development. Primary neural induction and fundamental antero-posterior or dorso-ventral regionalization of the early neural tube is due to the activity of the “primary organizer”. Slightly later in development, local signaling centers in the neuroepithelium, known as “secondary organizers”, refine the antero-posterior specification of the three main domains in the brain primordium: forebrain, midbrain and hindbrain. Additionally, the morphogenetic activity of these secondary organizers controls the polarity and the generation of neural sub-regions inside these main regions. Morphogenetic organizers developed in specific domains of the neuroepithelium as a consequence of interactions between two differently pre-specified zones. They confer positional identity by secreting a graded concentration of signal, which triggers concentration-specific genetic cascades. The fact that Gbx2 and Otx2, as well as prechordal and epichordal genes are expressed prior to Fgf8 and Shh in the Isthmic and ZLI organizers, respectively, would support this postulate.

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