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The isthmic organizer and brain regionalization

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ABSTRACT Distinct neural identities are acquired through progressive restriction of developmental potential under the influence of local environmental signals. Evidence for the localization of such morphogenetic signals at specific locations of the developing neural primordium has suggested the concept of "secondary organizer regions", which regulate the identity and regional polarity of neighboring neuroepithelial areas one step further. In recent years, the most studied secondary organizer has been the isthmic organizer, which is localized at the hind-midbrain transition and controls anterior hindbrain and midbrain regionalization. *Otx2* and *Gbx2* expression is fundamental for positioning the organizer and for the establishment of molecular interactions that induce *Fgf8* expression and then, stabilize the autoregulative loop of *En1, Wnt1* and *Pax2* expression. Temporo-spatial patterns of such gene expressions are necessary for the correct development of the organizer which, by a planar mechanism of induction, controls the normal development of the rostral hindbrain from r2 to the midbrain-diencephalic boundary. *Fgf8* appears as the active diffusible molecule for isthmic morphogenetic activity and has been suggested to be the morphogenetic effector in other inductive activities revealed in other neuroepithelial regions.

KEY WORDS: *secondary organizer, planar induction, isthmic organizer, mid-hindbrain.*

In vertebrates, elaborate cellular interactions regulate the establishment of the complex structural pattern of the developing central nervous system. These interactions have a characteristic distribution in space and time with which they control the development of specific cell fates. Distinct neural identities are therefore acquired through progressive restriction of developmental potential under the influence of local environmental signals (Hemmati-Brivanlou and Melton, 1997; Jessel and Lumsden, 1998). The molecular and genetic aspects of such essential processes have been subject of much recent research.

Primary neural induction and fundamental antero-posterior or dorso-ventral regionalization of the early neural tube are due to the activity of the "primary organizer" (Spemann and Mangold, 1924). We shall focus our interest on recent studies centered on: i) inductive cellular interactions which generate novel localized sources of morphogens, ii) the identity of related molecular signals which control the fate of nearby cells and iii) the cell-intrinsic factors that commit neural cells to specific fates (Tessier-Lavigne and Goodman, 1996). Evidence for morphogenetic controlling processes at specific locations of the developing neural primordium has suggested the concept of "secondary organizer regions", which regulate the identity and regional polarity of neighboring neuroepithelial areas one step further (Ruiz i Altaba, 1998).

In recent years, the most studied secondary organizer is localized at the mid-hindbrain transition, the isthmus (Fig. 1A), and controls anterior hindbrain and midbrain regionalization (Martínez *et al.*, 1991, 1996, 1999; Marin and Puelles, 1995; Hidalgo-Sanchez *et al.*, 1999a; Irving and Mason, 2000; for review, see Alvarado-Mallart, 1993; Bally-Cuif and Wassef, 1995; Joyner, 1996; Puelles *et al.*, 1996). New exciting insights have been reported by numerous studies about the regulatory genetic mechanisms underlying the specification of the isthmic organizer at the mid-hindbrain transition (Broccoli *et al.*, 1999; Liu *et al.*, 1999; Martínez *et al.,* 1999; Millet *et al.*, 1999; Shamin *et al.*, 1999) and the molecular nature of its morphogenetic activity (Crosley *et al.*, 1996; Meyers, 1998; Reifers *et al.*, 1998; Martínez *et al.*, 1999;. Shamin *et al.,* 1999)

The following sections collect the most significant recent data on the isthmic organizer and the isthmic region and discuss the emerging models of molecular pattern specification in this neighborhood.

The isthmic organizer

The morphogenetic activity of the isthmic neuroepithelial region was first suggested by loss-of-function experiments centered on the Wnt-1 gene, whose expression domain includes a ventrally

Abbreviations used in this paper: AN, anterior pole of the brain; Di, diencephalon; I, isthmus; IsO, isthmic organizer; Mes, mesencephalon; MM, mammillary bodies; P1-6, prosomeres; r1-8, rhombomeres; Rh, rhombencephalon; T, telencephalon; ZL, zona limitans.

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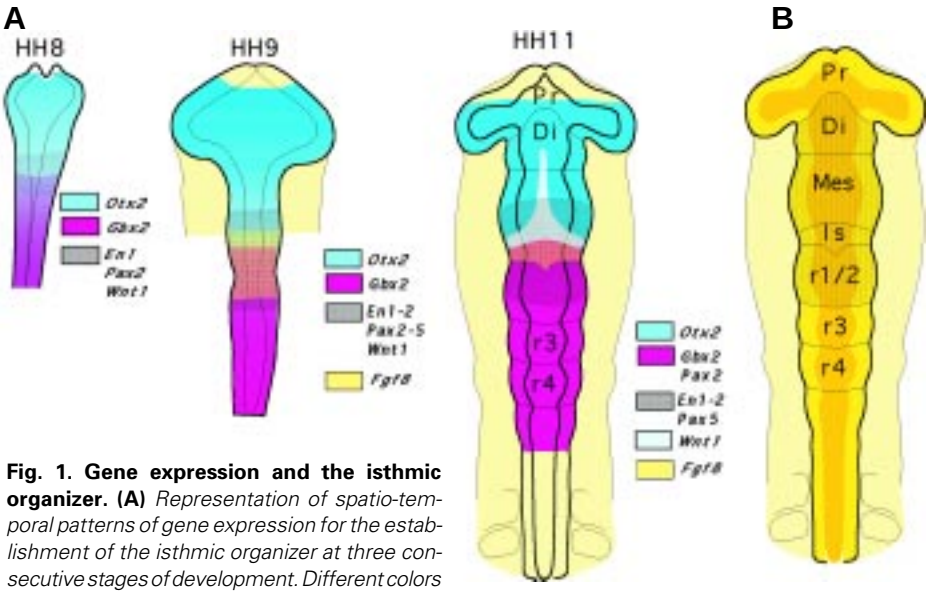

Fig. 1. Gene expression and the isthmic organizer. (A) *Representation of spatio-temporal patterns of gene expression for the establishment of the isthmic organizer at three consecutive stages of development. Different colors represent the expression of different genes. Changes in color codes are indicative of the overlapping of Gbx2/Fgf8/En1-2 expression domains.* **(B)** *Scheme of a chick embryo at stage HH11, showing the different segments of the anterior part of the neural tube. The shadowed area defines the large region which has been demonstrated to be sensitive to isthmic morphogenetic activity. Di, diencephalon; Is, isthmus; Mes, mesencephalon; Pr, prosencephalon; r1/2, 3, 4: rhombomeres.*

incomplete, transverse ring at the prospective isthmo-mesencephalic boundary. Knockouts of this gene produce partial or complete loss of midbrain, isthmic and cerebellar structures (McMahon and Bradley, 1990; Thomas and Capacchi, 1990). Moreover, isthmic tissue grafts in quail-chick chimeras have demonstrated its capacity to induce ectopically fully-differentiated midbrain, isthmus and cerebellum, depending on the recipient neuroepithelial environment (Martínez and Alvarado-Mallart, 1990; Nakamura, 1990; Martínez *et al.*, 1991; Gardner and Barald, 1991; Marín and Puelles, 1994; Martínez *et al.*, 1995; Hidalgo-Sanchez *et al.*, 2000 a; reviewed in Alvarado-Mallart, 1993; Joyner, 1996; Wassef and Joyner, 1997). Furthermore, such experimental designs have clearly demonstrated the unexpected existence of a large region of the neural tube that can be influenced by isthmic inductive signals, and which therefore has an occult histogenetic potentiality (Fig. 1B). The molecular and cellular aspects that control these phenomena have been illuminated by recent data, which are summarized below.

Molecular characteristics

A complex temporo-spatial pattern of gene expression has been described at the isthmic organizer (IsO; Fig 1A). We shall discuss here only those genes with an established role in local patterning. These data were reported by various groups working on different animal models; our description, then, should be largely valid for vertebrates in general, since most of the discussed processes have been detected in at least two different vertebrate models.

Otx-1 and *Otx-2* are widely expressed very early during development. Transcripts of these two genes become restricted at the gastrula stage to a large rostral neuroectodermal domain comprising the prospective prosencephalic and midbrain areas (Simeone *et al.*, 1992a,b). At the same stage, *Otx* genes abut caudally upon the rostralmost expression of another homeobox gene, *Gbx-2*, whose domain subsequently recedes caudalwards (Fig. 1A; Wassarman *et al.*, 1997; Hidalgo-Sanchez *et al.*, 1999b). At the end of gastrulation approximately, *Pax-2*, *Pax-5*, *En-1* and *Wnt-1* are each expressed in a transversal band, whose center co-localizes with the contact area between *Otx-2* and *Gbx-2* and subsequently with the IsO (Fig. 1A; Davis and Joyner, 1988; McMahon and Bradley, 1990; Rowitch and McMahon, 1995; Bally-Cuif and Wassef, 1995; Joyner, 1996; Hidalgo Sanchez *et al.*, 1999; Okafuji *et al.*, 1999). Finally, *Fgf-8*, another important gene, is expressed at late gastrulation stages at the hindbrain side of the organizer region (Fig. 1A; Heikinhenio *et al.*, 1994; Crossley and Martin, 1995; Crossley *et al.*, 1996).

FGF-8 has been postulated as one possible effector molecule for the morphogenetic activity of the IsO. It is expressed exactly at the most inductive region of the isthmic neuroepithelium, as can be empirically determined (Martínez *et al.*, 1991). The FGF-8 protein itself can induce IsO-characteristic fate alterations in the diencephalic caudal prosomeres, p1- p2, midbrain and hindbrain (Crossley *et al.*, 1996; Martínez *et al.*, 1999; Irving and Mason, 2000). Indeed, heparin beads soaked in recombinant FGF-8 protein, and implanted at these locations, induced an ectopic isthmic region in the host. This first reproduced the normal isthmic molecular pattern of nested gene expressions and later developed mirror-symmetric isthmo-cerebellar structures. In addition, the ectopic IsO also showed morphogenetic activity re-patterning the rostral neuroepithelium and inducing an orthogonal ectopic brain axis (Crossley *et al.*, 1996; Martínez *et al.*, 1999).

The morphogenetic activity of the IsO is clearly mediated by planar effects. Both grafting experiments and implants of FGF-8-loaded beads have demonstrated that the inductive effects require either integration of the graft into the host neuroepithelium or physical contact between the beads and the epithelium (Alvarado-Mallart, 1990; Crossley *et al.*, 1996). Resulting planar cell-communication effects are modulated nevertheless by differential permeability to morphogen diffusion of the interprosomeric boundaries (Martínez *et al.*, 1995, 1999; Bloch-Gallego *et al.*, 1996, Mellitzer, *et al.* 1999; Irving and Mason, 2000).

Loss-of-function studies of the genes expressed in the IsO have demonstrated that both the presence of their products and the normal combined pattern of expression are required for the morphogenetic process. Mutant mice or zebrafish lacking *Wnt-1*, *Pax-2*, *En-1*, *Gbx-2* or *Fgf-8* do not develop the isthmo-cerebellar complex and additional alterations in the midbrain were also described (McMahon and Bradley, 1990; Millen *et al.*, 1994; Wurst *et al.*, 1994; Brand *et al.*, 1996; Urbaneck *et al.*, 1997; Wassarman *et al.*, 1997; Meyers *et al.* 1998; Reifers, 1998). Moreover, genetic manipulations generating double mutations - *En-1/2* (Wurst, personal communication), *Pax-2/5* (Urbaneck *et al.*, 1997), *Otx-1/2* (Accampora *et al.*, 1997) and *Fgf-8* hypomorphic mice (Meyers *et al.*, 1998), showed that the observed anatomical malformations are dependent on mis-specification of the IsO.

More precisely, these data suggest that a minimal expression of *Gbx2* is required for the spatial establishment of a caudal limit of *Otx-2* expression, and that this limit then positions the IsO. Low dosage of *Gbx-2* produces a caudal extension of the *Otx-2* expression domain and a disruption of IsO-dependent structures (Wassarman *et al.*, 1997), while low dosage of *Otx-2* shifts the expression of *Fgf-8* rostrally (Accampora *et al.*, 1997). Transient expression of *Gbx2* in the caudal midbrain under the control of *Wnt1* enhancer shifts the organizer rostrally (Millet *et al.,* 1999), while caudal ectopic expression of *Otx2* in the rostral hindbrain under *En1* enhancer shifts the organizer caudally (Broccoli *et al.*, 1999). Retinoic acid is another factor which has been suggested as a possible factor for the blocking of the caudal extension of the *Otx-2* domain (Simeone *et al.*, 1995). Other recent experimental results are in agreement with the postulate that the transient zone of contact between *Otx-2* and *Gbx-2* domains initiates and positions the IsO. Quail/chick heterotopic grafts which generate ectopic contact areas between *Gbx-2* and *Otx-2* positive neuroepithelium induce *Fgf-8* expression at the graft/host boundary (Hidalgo-Sanchez *et al.*, 1999 a). In a recent work we observed that the sequence of an ectopic IsO induction, after Fgf8-bead insertion, is first initiated by *Gbx2* induction in the *Otx2* positive domain. *Otx2* is then repressed in this area and Fgf8 is induced at the new limit between the induced *Gbx2* and repressed *Otx2* domains, thus reproducing the molecular process of the IsO establishment in the isthmic region (Garda *et al.,* 2001).

The specification of the IsO at the isthmic region is regulated by inductive events, between *Gbx2* and *Otx2* products, which can have an intracellular (Garda*et al.,* 2001) and intercellular (Prochiantz, 1999) nature. Other molecules that mediate cell communication may also play a role in such regulation, although most of them are still unknown. These molecular interactions include the activation and regulation of *Pax-2*, *Pax-5*, *Wnt-1* and *En-1,2* expression, and seems to participate in a loop of mutual activation properties. This loop is maintained by the activation of *Fgf-8* (Wurst, Martin and Martínez personal observation).

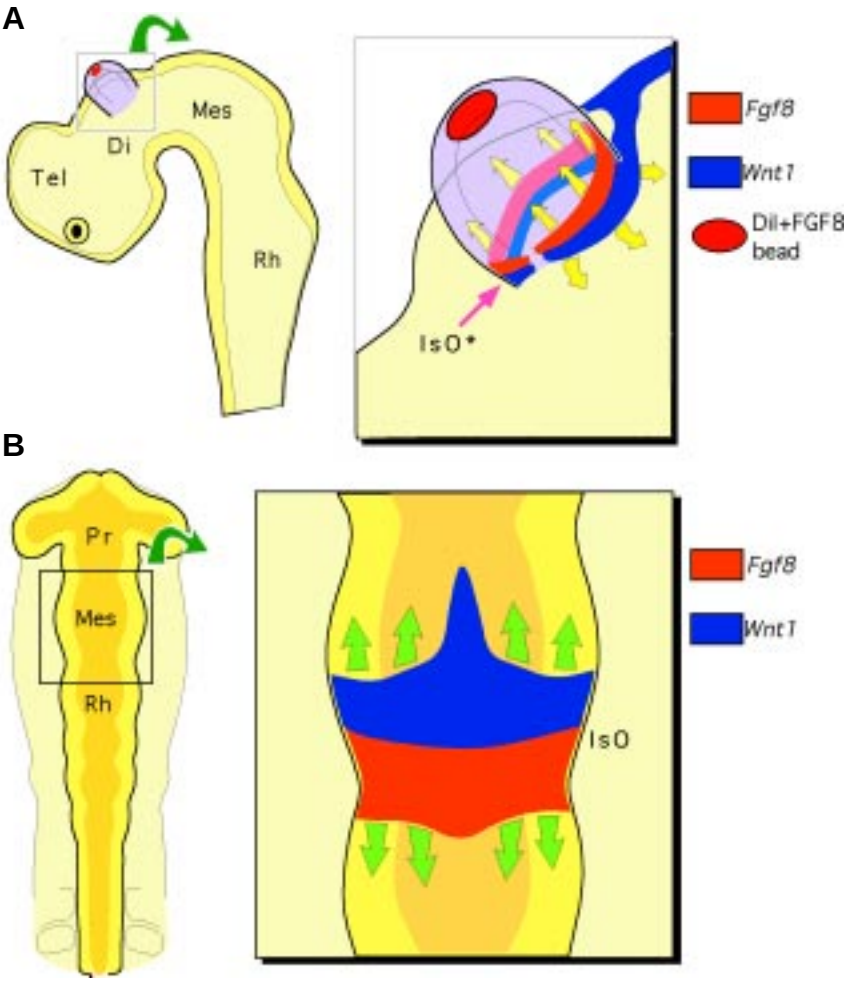

Fig. 2. Cell arrangement in the isthmic organizer. (A) *Scheme of the observed results in an experimental embryo after DiI-FGF8-bead insertion in the lateral diencephalic wall (described in Martinez et al., 1999). Gene expression domains are labeled by colors. Yellow arrows indicate cell movement in the neuroepithelium. The induced isthmic organizer generates a concentric domain of Fgf8 (central) and Wnt1(periphery) expression. The mitogenic activity of each of these genes, in two adjacent domains, determines the differential growth of the affected neuroepithelium, which expands around the ectopic isthmus and generates concentrically an ectopic vesicle. IsO*, ectopic isthmic organizer; Di, diencephalon; Mes, mesencephalon; Rh, rhombencephalon; Tel, telencephalon.* **(B)** *This experimental situation reproduces the normal pattern at the mid-hindbrain junction, where the two adjacent expressing domains of Fgf8 and Wnt1 control the morphogenetic movements of rostral hindbrain and mesencephalon. IsO, isthmic organizer; Pr, prosencephalon; Mes, mesencephalon; Rh, rhombencephalon.*

Cellular characteristics

Progress in understanding how the concerted action of a number of genes at the IsO produces such a complex morphogenesis, seems related to the analysis of the mitogenic effects elicited both at the *Otx-2/Wnt1*-positive midbrain side and at the *Gbx2/Fgf-8*-positive hindbrain side of the organizer (Fig. 2; Puelles *et al.*, 1996; Millet *et al.*, 1996; Hidalgo-Sanchez *et al.*, 1999b; Martínez *et al.*, 1999).

The WNT-1 secreted protein seems to have a short-range mitogenic effect (Balli-Cuif and Wassef, 1994; Wassef and Joyner, 1998) and the FGF-8 protein probably has a similar, longer-range effect (Martínez *et al.*, 1999; Shamin *et al.*, 1999). *Wnt-1* midbrain expression becomes progressively restricted to the dorsal midline and caudal boundary abutting the IsO. Neurogenetic data of both alar and basal midbrain structures correspondingly indicate an overall rostro-caudal proliferative gradient crossed with a ventro-dorsal gradient, and prolonged proliferation at the caudo-dorsal boundary of the isthmus (LaVail and Cowan, 1971). On the other hand, the growing isthmocerebellar dorsal midline largely lacks *Wnt-1* expression (present, nevertheless, more caudally at the rhombic lip). Available neurogenetic and clonal data on the cerebellum, isthmus and rostral hindbrain in general suggest an overall caudo-rostral proliferative gradient, crossed with the stand-

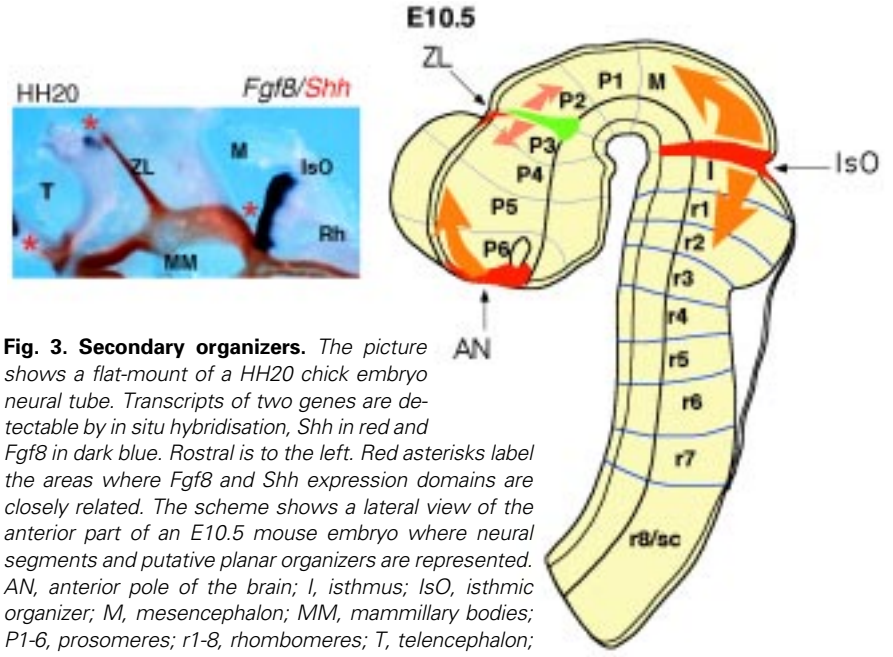

Fig. 3. Secondary organizers. *The picture shows a flat-mount of a HH20 chick embryo neural tube. Transcripts of two genes are detectable by in situ hybridisation, Shh in red and Fgf8 in dark blue. Rostral is to the left. Red asterisks label the areas where Fgf8 and Shh expression domains are closely related. The scheme shows a lateral view of the anterior part of an E10.5 mouse embryo where neural segments and putative planar organizers are represented. AN, anterior pole of the brain; I, isthmus; IsO, isthmic organizer; M, mesencephalon; MM, mammillary bodies; P1-6, prosomeres; r1-8, rhombomeres; T, telencephalon; ZL, zona limitans.*

ard ventrodorsal one (Puelles and Martínez-de-la-Torre, 1987; Puelles *et al*., 1992; Mathis *et al*., 1997). Prolonged proliferation thus occurs at the anterior part of the dorsal domain of the isthmus and the cerebellum.

The IsO lies at the all-round proliferative maximum. However, instead of growing itself, it apparently sheds its newborn neuroepithelial cells to either midbrain or hindbrain (preferentially to their dorsalmost areas; see Millet *et al*., 1996) and persists later on as a cell-poor histic domain at the isthmo-mesencephalic boundary (Puelles and Martínez-de-la-Torre, 1987). These properties contrast with those of inter-rhombomeric boundaries (Lumsden and Krumlauf, 1996) or the diencephalic zona limitans (Martínez *et al*., 1993), where proliferation is minimal compared to neighboring neuromeric domains. The notion of two proliferative regions appearing at both sides of a prospective boundary, which controls the size and shape of the respective fields, is found in the Entelechia model developed by García-Bellido and García-Bellido (1998) for *Drosophila* imaginal disks. An alternative model based on Meinhardt's (1982) thought, would suggest autocatalytic maintenance of mitogenesis and inhibition of differentiation at the IsO, perhaps by *Wnt-1/Fgf-8* interaction.

The IsO apparently influences differential proliferation and fate specification at the two slopes of the proliferative peak by means of the intrinsic rostrocaudal polarization implicit in the *Wnt-1/Fgf-8* tandem domains and the subjacent *Otx-2/Gbx-2* background difference (Fig. 2). It may be speculated that the nested expression domains of genes of the *Pax* and *En* families, present across the IsO (where their respective signals are maximal, too), may serve in part to regulate the size of the IsO and in part to participate combinatorially in the position-dependent, polarized specification of the diverse grisea produced within the two sub-fields. Indeed, experimental embryological data show that ectopic induction of an Engrailed gradient leads not only to ectopic midbrain or cerebellum fate induction, but also to re-polarizations, where rostral-typical structures coincide with the maximal Engrailed signal (Martínez *et al*., 1992, 1995; Marín and Puelles, 1994; Retaux and Harris, 1996).

The addition of newborn neuroepithelial cells at the IsO progressively causes differential growth of caudal midbrain and rostral hindbrain areas, but expression of the critical *Wnt-1* and *Fgf-8* genes is constantly restricted to bands only a few cell-diameters-wide at the IsO itself (Fig. 2). This means that expression of these genes is selectively down regulated in a position-dependent way. This interpretation has been recently corroborated by the analysis of differential neuroepithelial growth, ectopically induced by FGF-8-loaded beads (Fig. 2A; Martínez *et al*., 1999).

The possibility that Fgf-8 plays a morphogenetic role, interacting with other genes, to control local development at different levels of the neural tube, as well as the capacity of the IsO to induce effects in wide regions of the brain (albeit not in all of it; Martínez *et al*., 1992, 1995), suggest that the emerging model for detailed brain structural patterning at the midbrain-hindbrain transition may be useful for general reference. Double *in situ* hybridization for *Fgf8* and *Shh* showed interesting spatial relations between the expression domains of these two genes, which are actively involved in morphogenesis and regionalization of the vertebrate neural tube (Fig. 3). *Shh* shows abrupt spatial changes in its expression pattern, which is systematically shifted dorsally wherever *Fgf8* is expressed: in the isthmus (I), the zona limitans (ZLI) and, at the rostral pole of the brain, the commissural plate (Fig. 3). An inductive activity in the isthmus (isthmic organizer) and in the rostral pole of the brain (Shimamura and Rubestein, 1997; Houart, *et al*., 1998) has been demonstrated. The zona limitans is the region that now appears a suggestive area where a new organizer can display inductive and morphogenetic properties: controlling inductive opposite influences from the anterior pole of the brain and the isthmic organizer (Fig. 3).

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References

ACCAMPORA, D., AVANTAGGIATO, V., TUORTO, F. and SIMEONE, A. (1997) Genetic control of brain morphogenesis through Otx gene dosage requirement. *Development,* 124: 3639-3650.

ALVARADO MALLART, R.M. (1993) Fate and potentialities of the avian mesencephalic/metencephalic neuroepithelium. *J. Neurobiol.* 24: 1341-1355.

ALVAREZ-OTERO, R., SOTELO, C. and ALVARADO-MALLART, R.M. (1993) Chick/quail chimeras with partial cerebellar grafts: an analysis of the origin and migration of cerebellar cells. *J. Comp. Neurol.* 333: 597-615.

BALLY-CUIF, L. and WASSEF, M. (1994) Ectopic induction and reorganizations of *Wnt1* expression in quail/chick chimeras. *Development* 120: 3379-3394.

BALLY-CUIF, L. and WASSEF, M. (1995) Determination events in the nervous system of the vertebrate embryo. *Curr. Opin. Genet. Dev.* 5: 450-458.

BROCCOLI, V., BONCINELLI, E. and WURST, W. (1999) The caudal limit of *Otx2* expression positions the isthmic organizer. *Nature* 40: 164-168.

CROSSLEY, P.H. and MARTIN, G.M. (1995) The mouse *Fgf8* gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. *Development,* 121: 439-451.

CROSSLEY, P.H., MARTINEZ, S. and MARTIN, G.M. (1996) Midbrain development induced by FGF8 in the chick embryo. *Nature,* 380: 66-68.

GARCIA-BELLIDO, A.C. and GARCIA-BELLIDO, A. (1998) Cell proliferation in the attainment of constant sizes and shapes: the Entelechia model. *Int. J. Dev. Biol.* 42: 353-362.

GARDA, A.-L., ECHEVARRIA, D AND MARTÍNEZ, S. (2001) Neuroepithelial co-expression of *Gbx-2* and *Otx-2* precedes *Fgf-8* expression in the isthmic organizer. *Mech. Dev.* (in press).

HALLONET, M. R.E., TEILLET, M.A. and LE DOUARIN, N. (1990) A new approach to the development of cerebellum provided by chick/quail marker system. *Development* 108: 19-31.

HEMMATI-BRIVANLOU, A. and MELTON, D. (1997) Vertebrate neural induction. *Annu. Rev. Neurosci.* 20: 43-60.

-HIDALGO-SANCHEZ, M., SIMEONE, A. and ALVARADO-MALLART, R.M. (1999 a) Fgf8 and Gbx2 induction concomitant with Otx2 repression is correlated with the midbrain-hindbrain fate of caudal prosencephalon. *Development*, 126: 3191-3202.

HIDALGO-SANCHEZ, M., MILLET, S., SIMEONE, A. and ALVARADO-MALLART, RM. (1999b) Comparative analysis of Otx2, Gbx2, Pax2, Fgf8 and Wnt1 gene expressions during the formation of the chick midbrain/hindbrain domain. *Mech. Dev.* 80: 175-178.

HIS, W. (1893) Vorschlage zur Einteilung des Gehirns. *Arch. Anat. Physiol. Leipzig.* III/ IV: 172-179.

HOUART, C., WESTERFIELD, M. and WILSON, S.W. (1998) A small population of anterior cells patterns the forebrain during zebrafish gastrulation. *Nature,* 391: 788-792.

IRVING, C. and MASSON, I. (2000) Signaling FGF8 from the isthmus patterns anterior hindbrain and establishes the anterior limit of Hox gene expression. *Development,* 127: 177-186.

JESSEL, T.M. and LUMSDEN, A. (1998) Inductive signals and the assignment of cell fate in the spinal cord and hindbrain. In *Molecular and cellular approaches to neural development* (Ad. W. Maxwel Cowan, T.M. Jessel, S. Lawrence Zipursky). Oxford University Press, Oxford, pp. 290-333.

JOYNER, A.L (1996) *Engrailed, Wnt* and *Pax* genes regulate midbrain-hindbrain development. *Trends Genet.* 12: 15-20.

LUMSDEN, A. (1990) The cellular basis of the segmentation in the developing hindbrain. *Trends Neurosci.* 13: 329-334.

LaVAIL, J.H. and COWAN, W.M. (1971) The development of the chick optic tectum. II. Autoradiographic studies. *Brain Res.* 28: 391-419.

MARIN, F. and PUELLES, L. (1994) Patterning of the embryonic avian midbrain after experimental inversions: a polarizing activity from the isthmus. *Dev. Biol.* 163: 19-37.

MARTINEZ, S and ALVARADO-MALLART, R.M (1987) Rostral cerebellum originates from the caudal portion of the so-called «mesencephalic vesicle: A study using chick-quail chimeras. *European Journal of Neuroscience*, 1: 549-560

MARTINEZ, S., WASSEF, M. and ALVARADO-MALLART, R.M. (1991) Induction of a mesencephalic phenotype in the 2-day-old chick prosencephalon is preceded by the early expression of the homeobox gene En. *Neuron*, 6*:* 971-981.

MARTINEZ, S., GEIJO, E., SANCHEZ-VIVES, M.V., PUELLES, L. and GALLEGO, R. (1992) Reduced junctional permeability at interrhombomeric boundaries. *Development,* 116: 1069-1076.

MARTINEZ, S., GEIJO, E., SANCHEZ-VIVES, M.V., PUELLES, L. and GALLEGO, R. (1993) Diencephalic intersegmental boundaries in the chick: Boundary cells and junctional properties. *16th Annual ENA Meeting,* Madrid. pp. 112.

MARTINEZ, S., MARIN, F., NIETO, M.A. and PUELLES, L. (1995) Induction of ectopic engrailed expression and fate change in avian rhombomeres: intersegmental boundaries as barriers. *Mechanisms of Development*, 51: 289-303

MARTINEZ, S., CROSSLEY, P.H., COBOS, I., RUBENSTEIN, J.L.R and MARTIN, G.R. (1999) FGF-8 induces an isthmic organizer and isthmocerebellar development in the caudal forebrain via a repressive effect on Otx2 expression. *Development* 126: 1189-1200.

MATHIS, L., BONNEROT, C., PUELLES, L. and NICOLAS, J.F. (1997) Retrospective clonal analysis of the cerebellum using genetic *lacz/lacz* mouse mosaics. *Development* 124: 4089-4104.

McMAHON A.P. and BRADLEY, A. (1990) The *Wnt1* (*int-1*) proto-oncogene is required for development of a large region of the mouse brain. *Cell* 69: 581-595.

MEINHARDT, H. (1982) *Models of biological pattern formation.* Academic Press, London.

MELLITZER, G., XU, Q. and WILKINSON, D.G. (1999) Eph receptors and ephrins restricted cell intermingling and communication. *Nature,* 400: 77-81.

MEYERS, E.N., LEWANDOSKI, M. and MARTIN, G. (1998) An Fgf8 mutant allelic series generated by Cre- and Flp-mediated recombination. *Nat. Genet.* 18: 136-141.

MILLEN, K.J., WURST, W., HERRUP, K. and JOYNER, A.L. (1994) Abnormal embryonic cerebellar development and patterning of postnatal foliation in two mouse Engrailed-2 mutants. *Development,* 120: 695-706.

MILLET, S., BLOCH-GALLEGO, E., SIMEONE, A. and ALVARADO-MALLRT, R.M. (1996) The caudal limit of *Otx2* gene expression as a marker of the midbrain-hindbrain boundary: a study using in situ hybridisation and chick/quail homotopic grafts. *Development* 122: 3785-3797.

MILLET, S., CAMPBELL, K., EPSTEIN, D. J., LOSOS, K., HARRIS, E. and JOYNER, A.L. (1999) A role for *Gbx2* in repression of *Otx2* and positioning the mid/hindbrain organizer. *Nature* 40: 161-164.

PALMGREN, A. (1921) Embryological and morphological studies on the midbrain and cerebellum of vertebrates. *Acta Zool. Stockholm.* 2: 1-94.

PROCHIANTZ, A. (1999) Homeodomain-derived peptides. In and out of the cells. *Ann. N.Y. Acad. Sci.* 886: 172-9.

PUELLES, L. and MARTINEZ DE LA TORRE, M. (1987) Autoradiographic and Golgi study on the early development of n. Isthmi principalis and adjacent grisea in chick embryo: a tridimensional view point. *Anat. Embryol.* 176: 19-34.

PUELLES, L., MARIN, F., MARTINEZ DE LA TORRE and MARTINEZ, S. (1997). The midbrain-hindbrain junction: a model system for brain regionalization through morphogenetic neuroepithelial interactions. In *Mammalian Development* (De. P. Lonai). Harwod Academic Publishers, Chur. Switzerland , pp. 173-197.

PUELLES, L. and RUBENSTEIN, J.L.R. (1993) Expression patterns of homeobox and other putative regulatory genes in the embryonic mouse forebrain suggest a neuromeric organization. *Trends Neurosci.* 16: 472-479.

-RUBENSTEIN, J.L.R., SHIMAMURA, K., MARTINEZ, S. and PUELLES, L. (1998) Regionalization of the prosencephalic neural plate. *An. Rev. Neurosci.* 21: 445-477.

RUIZ I ALTABA, A. (1998) Deconstructing the organizer. *Nature* 391: 348-349.

SHIMAMURA, K. and RUBENSTEIN, J.L.R. (1997) Inductive interactions direct early regionalization of the mouse forebrain. *Development* 124: 2709-2718.

SPEMANN, H. and MANGOLD, H. (1924) Über induktion von Embryonanlagen durch implantation artfremder Organisatoren. *Wilh. Roux Arch. EntwMech, Organ.* 100: 599-638.

TESSIER-LAVIGNE, M. and GOODMAN, C.S. (1996) The molecular biology of axon guidance. *Science* 274*:* 1123-1133.

THOMAS, K.R. and CAPECCHI, M.R. (1990) Targeted disruption of the murine *int-1* proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development. *Nature* 346*:* 847-850.

URBANEK, P., FETKA, I., MEISLER, M.H. and BUSSLINGER, M. (1997) Cooperation ox Pax2 and Pax5 in midbrain and cerebellum development. *Proc. Natl. Acad. Sci. USA*. 94: 5703-5708.

VAAGE, S. (1969) The segmentation of the primitive neural tube in chick embryos *(Gallus domesticus). Adv. Anat. Embryol. Cell Biol.* 41: 1-87.

VAAGE, S. (1973) The histogenesis of the isthmic nuclei in the chick embryo (*Gallus domesticus)* . Y. A morphological study. *Z. Anat. Entwickl. Gesch.* 142: 283-314.

WASSARMAN, K.M., LEWANDOSKI, M., CAMPBELL, K., JOYNER, A.L., RUBENSTEIN, J.L.R., MARTINEZ, S. and MARTIN, G. (1997) Specification of the anterior hindbrain and establishment of a normal mid/hindbrain organizer is dependent on Gbx2 gene function. *Development* 124: 2923-2934 (1997).

WURST, W., AUERBACH, A.B. and JOYNER, A.L. (1994) Multiple developmental defects in Engrailed-1 mutant mice: an early mid-hindbrain deletion and patterning defects in forelimbs and sternum. *Development* 120: 2065-2075.