The midbrain–hindbrain boundary organizer

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Cell fate in the cephalic neural primordium is controlled by an organizer located at the midbrain–hindbrain boundary. Studies in chick, mouse and zebrafish converge to show that mutually repressive interactions between homeodomain transcription factors of the *Otx* and *Gbx* class position this organizer in the neural primordium. Once positioned, independent signaling pathways converge in their activity to drive organizer function. Fibroblast growth factors secreted from the organizer are necessary for, and sufficient to mimic, organizer activity in patterning the midbrain and anterior hindbrain, and are tightly controlled by feedback inhibition.

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Abbreviations

ace	*acerebellar*
ANR	anterior neural ridge
E	embryonic day
FGF	fibroblast growth factor
MHB	midbrain–hindbrain boundary
noi	*no isthmus*
WT	wild-type

Introduction

The initial subdivision of the neural plate, or regionalization, is the first step towards generating cellular diversity in the vertebrate brain. The subdivision is reflected by gene expression in restricted domains along the length of the neural primordium. As development proceeds, this rough subdivision is further refined within each region, ultimately generating the multitude of cell types in the central nervous system (CNS). Both vertical signals from the mesoderm to the overlying ectoderm [1] and planar signals travelling in the plane of the ectodermal epithelium are thought to be involved in generating cell diversity [2–4].

Patterning of the neural primordium also involves neuroepithelial organizers — special groups of cells that produce secreted molecules and thus control the cell fate of the surrounding cells. The two best-studied organizers are the anterior neural ridge (ANR, or row 1 [the first row of cells in the zebrafish neural plate]) acting on the forebrain neural plate [5,6,7•]), and the midbrain–hindbrain boundary organizer (MHB organizer, or isthmic organizer) acting on the midbrain and hindbrain primordium [8–10].

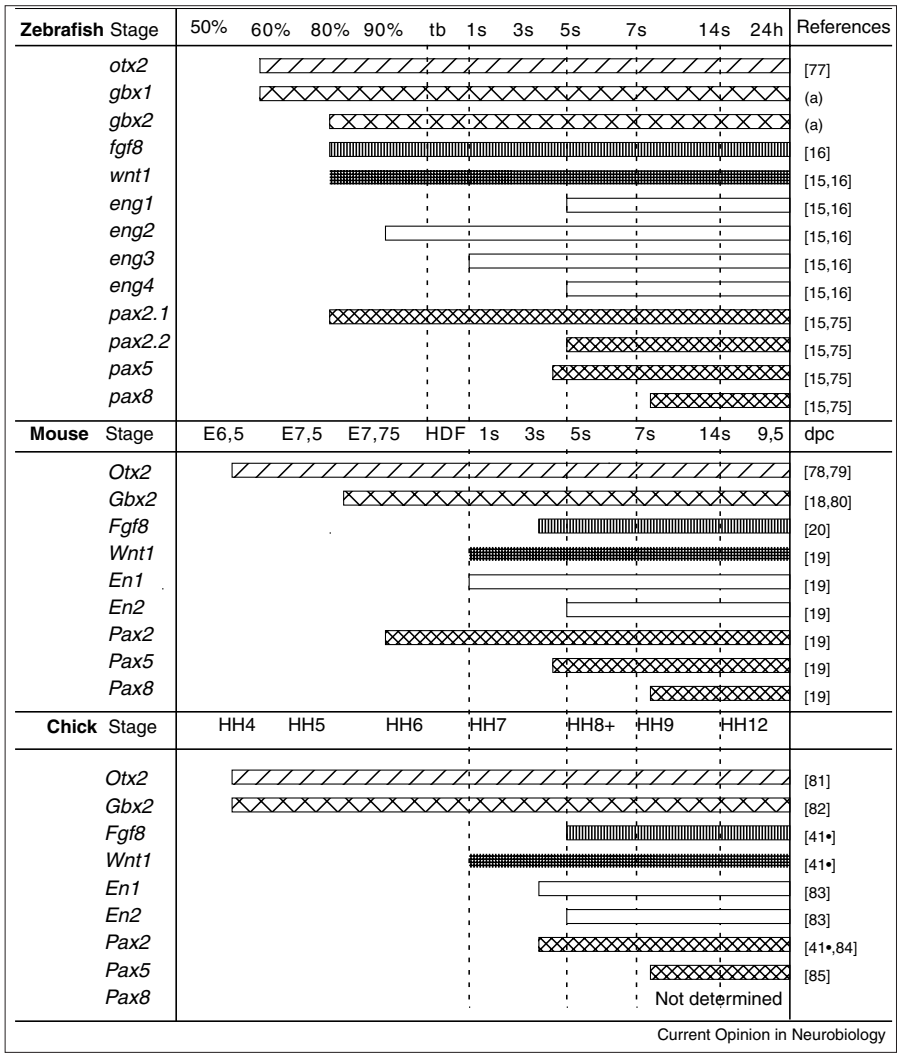
The MHB organizer was initially identified through transplantation experiments in chick embryos. When MHB tissue is transplanted into the caudal forebrain of chick embryos, the surrounding host tissue switches fate and adopts an isthmic or midbrain character [11,12]; in the rhombencephalon, MHB tissue induces cerebellar fate [13]. These experiments suggested that this tissue also acts as an organizing center in its normal location at the MHB. This review focuses on recent progress in understanding how the midbrain–hindbrain boundary organizer develops and functions.

Several genes, encoding either transcription factors *(Engrailed* [*En*], *Pax*, *Otx* and *Gbx* families) or secreted proteins (Wnt and Fgf [fibroblast growth factor] families), are expressed within the midbrain–hindbrain territory at early embryonic stages (Figure 1). Several groups have generated mutations in these genes in mice through gene targeting [9,10]. Mutagenesis screens in zebrafish have yielded *acerebellar* (*ace*), a probable null-allele of *fgf8*, an allelic series of *no isthmus* (*noi*) alleles in the *pax2.1* gene [14–16], and several mutants in which molecular identification is ongoing. The different mutants lack MHB structures and/or neighboring brain territories to varying degrees, as listed in Table 1. From the mutant analysis, several regulatory steps are distinguished in MHB development. During the establishment phase, a crucial first step is the subdivision into an *Otx2*- and a *Gbx2*-expressing domain (see below). At this interface between *Otx2* and *Gbx2*, at least three signaling pathways become activated independently of each other, as monitored by the expression of the *wnt1*, *pax2.1* and *fgf8* genes (Figure 2a) [15,16]. Establishment is followed by the maintenance phase, during which expression of the above genes comes to depend on each other. Perturbation of any one gene disrupts the continued development of the MHB. During this period, *Fgf8* expression is activated at the MHB, thus probably endowing these cells with organizing capacity (Figure 2b).

The *Otx–Gbx* interface and positioning of the isthmic organizer – or how much of a fly wing is the MHB?

The establishment of organizing centers is thought to require the prior specification of two distinct, adjacent cell populations. Local cellular interactions then result in the production of molecules with longer-range signaling properties [17]. This phenomenon has been studied extensively, for example, at the anterior–posterior compartment boundary of the fly wing. How are the two cell populations that generate the MHB organizer defined? During normal CNS development, one of the earliest events is the subdivision into an anterior *Otx2*-positive and a posterior *Gbx2*-positive domain. During late gastrulation/early neural plate stages, *Otx2* is expressed from the anterior limit of the neural plate to a posterior border at the presumptive MHB and *Gbx2* is expressed in a comple-

Figure 1



Comparison of the onset of expression of the different genes associated with midbrain–hindbrain organizing activity in three different species: mouse, zebrafish and chick. The mRNA expression patterns of the different genes (*Otx2, Gbx, Fgf8, Wnt1, En* and *Pax*) are shown schematically on the basis of the results of *in situ* hybridization analyses. (a) M Brand, unpublished data.

mentary fashion in the posterior embryo [18]. Subsequently, *Pax2* is activated, followed by *En1*, *Wnt1* [19] and *Fgf8* [16,20,21]. These genes are activated around the *Otx2–Gbx2* interface, consistent with the notion that the region where *Otx2* and *Gbx2* abut demarcates the primordium of the MHB. Furthermore, the MHB has the ability to regenerate after its removal, suggesting that it is normally generated and/or maintained by cell–cell interactions between *Otx2*- and *Gbx2*-expressing neuroepithelial cells [22,23••]. In addition, transplantations, co-cultures and electroporation experiments show that the confrontation of *Otx2*- and *Gbx2*-expressing territories activates expression of *Fgf8*, a key mediator of the MHB organizing activity [23••,24,25••,26].

The above data suggested that creating the *Otx2–Gbx2* border in the right place is important to position the MHB organizer, and genetic analysis of *Otx2* and *Gbx2* in mice provides evidence for this (Figure 3). *Otx2*-null mutants lack the brain rostral to rhombomere 3 ([27–29]; for a review, see [30]). Furthermore, in mutants with a reduced copy number of *Otx* genes, the caudal limit of *Otx2* expression, and the MHB organizer with it, are shifted anteriorly at early somite stages. Such embryos form neither midbrain nor caudal forebrain, and the anterior hindbrain is expanded rostrally [31]. Conversely, *Gbx2*-null mutants show a failure of anterior hindbrain development and display a caudal expansion of the midbrain and of *Otx2*, *Wnt1* and *Fgf8* expression, apparently due to a respecification of the hindbrain at early somite stages (six somites) [18,32•].

Evidence from misexpression experiments is complementary to that of the loss-of-function studies (Figure 3). When *Otx2* expression is forced in a more caudal position using an *Otx2* transgene driven by an *En1* promoter, *Gbx2* expression is repressed and the MHB is shifted posteriorly [33•]. Conversely, ectopic expression of *Gbx2* in the caudal midbrain, driven by a *Wnt1*–promoter–*Gbx2* transgene, represses *Otx2* and shifts the induction of MHB markers to the level of the newly created interface; surprisingly, this

Table 1

Phenotypes of embryos carrying a mutation in genes expressed at the MHB.

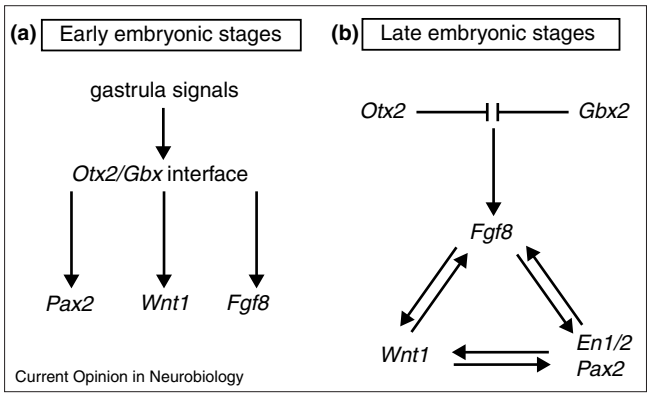
Gene	Species	MHB mutant phenotype	References
Otx1	Mouse	Homozygous *Otx1* mutant adult mice have cortical defects, an abnormal midbrain and abnormal cerebellar foliation. Cooperates with *Otx1* in MHB development; double mutants show an increase in strength of the embryonic MHB phenotype.	[30,31]
Otx2	Mouse	Homozygous *Otx2* mutant embryos lack the brain rostral to hindbrain rhombomere 3. Cooperates with *Otx1* in MHB development. In chimeric embryos that have only OTX protein in the visceral endoderm, the forebrain and midbrain induction is rescued. Absence of OTX protein in the neuroectoderm leads to incorrect regionalization.	[27–29,31,34,35,48]
Gbx2	Mouse	*Gbx2* mutant embryos lack anterior hindbrain and show a caudal expansion of the posterior midbrain. The *Otx2* expression domain is expanded posteriorly. Consequently, *Wnt1* and *Fgf8* expression domains are also shifted caudally.	[18,32•]
Pax2	Mouse	The effect of the *Pax2* mutation is influenced by the genetic background of the mouse strain analysed, ranging from deletion of the posterior midbrain and cerebellum or exencephaly to almost normal development of these structures.	[86,87,90]
Pax2.1 (*noi*)	Zebrafish	*No isthmus* (*noi*) mutants lack the midbrain, MHB and cerebellum. *eng3* activation is completely and *eng2* is strongly dependent on *noi* function. In contrast, onset of *wnt1* and *fgf8* occurs normally.	[14,15,75]
Pax5	Mouse	*Pax5* mutant embryos show defects in the inferior colliculi and anterior cerebellum. Deletion of the midbrain and cerebellum is consistently observed in *Pax2*/*Pax5* double mutants, suggesting a dose-dependent cooperation between these genes.	[88–90]
Pax8	Mouse	Homozygous *Pax8* mutant embryos show a hypoplasia of the thyroid gland.	[91]
En1	Mouse	*En1* mutant mice die shortly after birth. In the brains of newborn mutants, most of the colliculi and cerebellum are missing and the third and fourth cranial nerves are absent. A deletion of mid-hindbrain tissue was observed as early as E9.5, and the phenotype resembles that reported for *Wnt1* mutant mice.	[92]
En2	Mouse	Mice homozygous for a targeted deletion of the *En2* gene are viable but have an altered adult cerebellar foliation pattern.	[93]
Fgf8	Mouse	These embryos show gastrulation defects. Mesoderm and endoderm do not form, probably due to elimination of *Fgf4* expression in the mutants. Anterior markers are widely expressed due to mislocalisation of the visceral endoderm and/or absence of mesoderm, and posterior markers are not expressed. In mice carrying a hypomorphic *Fgf8* allele there is a deletion of the posterior midbrain and cerebellar tissue, similar to the phenotype observed in zebrafish *ace* mutants.	[59•,60]
Fgf8 (*ace*)	Zebrafish	Ace mutants lack the MHB and the cerebellum, and anterior–posterior polarity of the midbrain and projection of retinal ganglion cell axons to the midbrain and the retinotectal map is disturbed. *Fgf8* function is required to maintain, but not to initiate, expression of *pax2.1*, *wnt1* and *eng* genes. Further defects are in the commissural region of the forebrain and in the telencephalon.	[7•,14,16,56•]
Fgf17	Mouse	*Fgf17* mutants show a proliferation defect of precursors of the medial part of the cerebellum after E11.5, which increases in severity when heterozygous for *Fgf8*.	[70•]
Wnt1	Mouse	Homozygous mutant mice show a loss of the midbrain and adjacent cerebellar component of the metencephalon. By introducing a transgene expressing *En1* driven by *Wnt1* promoter into *Wnt1*$^{-/-}$ mutants, the phenotype is rescued, suggesting a role for Wnt1 in the maintenance of *En1* expression.	[52,81,94]
NI (*aus*)	Zebrafish	*aus* mutant embryos exhibit widespread up-regulation of *fgf8* and *pax2.1*. The mutant embryos show defects in the differentiation of the forebrain, midbrain and eyes.	[66]
NI (*spg*)	Zebrafish	*spiel-ohne-grenzen* (*spg*) mutants lack the MHB and the cerebellum, resembling the phenotype of *ace*.	[95]

NI, not identified.

shift appears to be only transient [32•]. These results together suggest that *Gbx2* directly or indirectly represses *Otx2*, and that *Gbx2* is required to maintain a sharp caudal border of the *Otx2* expression domain.

Similar results were obtained by misexpression experiments of *Otx2* and *Gbx2* in chick [26] and in zebrafish, but with an interesting twist. Zebrafish *gbx2* is expressed at the MHB only after *pax2.1* and *fgf8* (Figure 1), and thus apparently too late to fulfill the same function it has in mice

Figure 2



Stepwise development of the MHB. **(a)** During early embryonic stages (establishment phase), three parallel pathways (*Pax, Wnt and Fgf*) are activated around the *Otx–Gbx* interface in similar, but not identical domains in the primordia of the early midbrain, MHB and anterior hindbrain. The activating signals are unknown, but may derive from mesendoderm. **(b)** During later embryonic stages (maintenance phase), expression overlaps at the MHB organizer, which secretes Wnt1 and Fgf8 signaling molecules. At this stage, the pathways become mutually dependent.

[33•]. In contrast, zebrafish *gbx1* expression occurs early, complementary with *otx2* gene expression, and is able to shift MHB position when misexpressed (K Lun, M Rhinn, M Brand, unpublished data). This suggests that in zebrafish an evolutionary switch occurred, where *gbx1* instead of *gbx2* is required for the correct early specification of the MHB primordium.

Given the importance of the *Otx2–Gbx* interface, it will be of great interest to understand how it is set up during gastrulation. Like *Otx2*, *Gbx2* is already expressed during gastrulation (embryonic day [E]7.5–E8), and could therefore define the posterior *Otx2* border also during gastrulation. The *Gbx2* mutant mice will have to be examined during gastrulation stages to address this point; however, analysis of *Otx2* function suggests that in gastrulation, different rules may apply, in that the *Otx2* and *Gbx2* domains are set up independently of each other. Neural induction in *Otx2* mutants is compromised, but can be rescued by providing Otx protein to the visceral endoderm. Although such embryos lack Otx2 in the neural ectoderm, the anterior border of *Gbx2* expression is established correctly at gastrulation stages ([34]; A Simeone, personal communication). At later stages, however, MHB marker expression shifts anteriorly [34,35]. These findings suggest that initially the positioning of the anterior border of *Gbx2* expression is independent of *Otx2*, and only later comes to depend on *Otx2*.

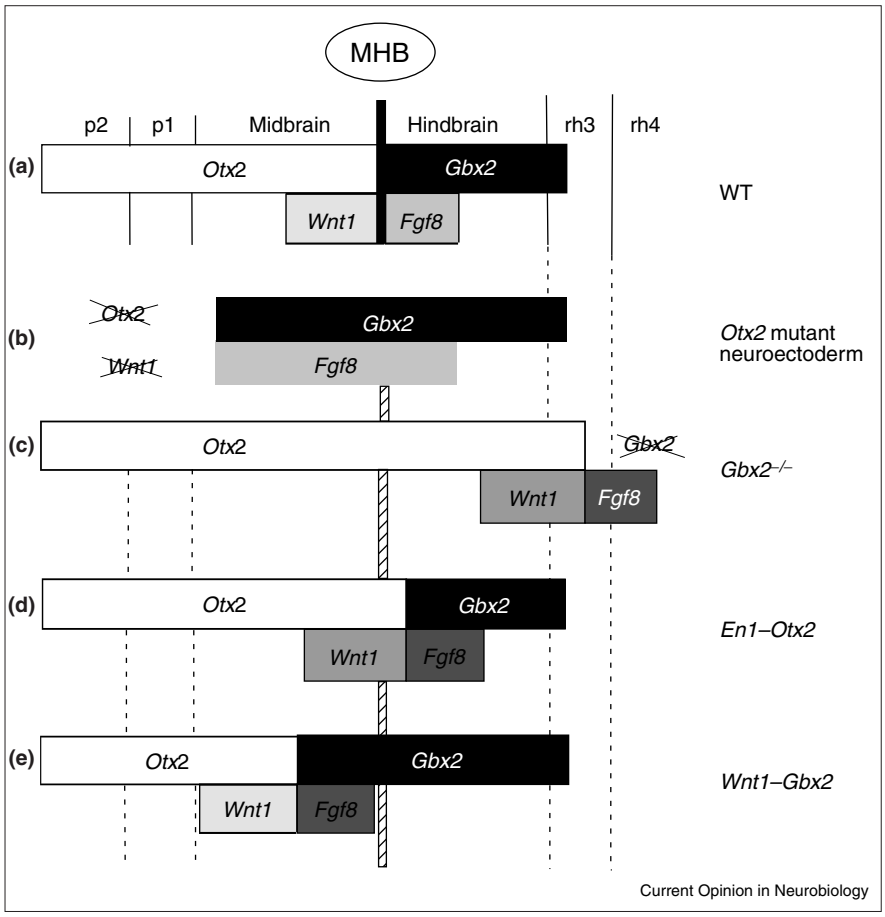
Several new questions are raised by these observations. First, what are the signals that, in turn, position the *Otx2* and *Gbx* interface in the neural plate? Studies in amphibian, chick and mouse embryos suggest that signals from anterior mesendoderm or notochord regulate expression of *En1* and *Otx2* [36–38]. Signals such as Wnts, Fgfs and retinoic acid are implicated but it is not known which exact molecule is involved and how direct its action is [39,40]. Secondly, in chick embryos, a candidate for a vertical signal involved in positioning the *Otx2–Gbx* interface may be *Fgf4* released from the anterior notochord. In explant assays, Fgf4 can activate *En1* expression in the neuroectoderm [41•]; however, expression of *Fgf4* has not been reported in the notochord of other species, although it is conceivable that a different Fgf performs this function in other species. On the other hand, in zebrafish and mouse mutants lacking notochord [42–45], anterior–posterior polarity and the MHB is correctly specified. This is also the case in zebrafish embryos depleted of mesendoderm by injection of the transforming growth factor-β (Tgf-β) inhibitor, antivin [46,47•]. Presumably, several pathways cooperate to position the *Otx2–Gbx* interface. Third, once the *Otx2–Gbx* border in the neural plate is generated, how does this molecular interface lead to restricted domains of gene expression, for instance of *Fgf8*, around it? The fly wing teaches us that this is a multistep process in itself. Finally, the morphogenetic behavior of cells is different on either side of the boundary, and it is unclear why. For instance, clones of *Otx2* mutant cells segregate from wild-type (WT) cells in the midbrain neuroepithelium, perhaps caused by the reduced expression of two molecules mediating cell adhesion, R-cadherin and the ephrin ligand ephrin-A2, in these cells ([48]; see also [49,50]).

Fgfs and their role at the MHB

Once the organizer is positioned properly, secreted Fgf8 and Wnt1 proteins from the organizer are thought to mediate its organizing influence on the surrounding neural tissue. Wnt1 functions as a mitogen and to maintain expression of En genes, but is unable to mimic the activity of the organizer when misexpressed [51,52]. *Fgf8* is expressed at the right time and place to mediate the organizing activity [16,20,53]. In contrast to Wnt1, the ectopic application of Fgf8 protein mimics the activity of the MHB organizer and induces isthmic-like structures and MHB-specific gene expression [25••,54,55] (M Brand, unpublished data). Because Fgfs can mimic each other's activity in gain-of-function experiments, loss-of-function mutants are important to support a function for Fgf8 in induction and/or patterning of the MHB region. The zebrafish mutant *ace* lacks functional Fgf8, the MHB organizer and a cerebellum [16,56•]. Fgf8 is required to maintain marker gene expression in the midbrain and isthmus, but not to induce midbrain [16]. Moreover, the analysis of the midbrain in *ace* mutants shows that the MHB is required for anterior–posterior polarization of the midbrain, including the graded expression of ephrin ligands in the midbrain neuroepithelium, and for proper retinotectal map formation [56•].

Fgf8 secreted from the MHB organizer is also involved in patterning the anterior hindbrain [57,58]. Rhombomere 1

Figure 3



Relative position of the MHB and associated genes in wild-type embryos and after manipulating the position of the *Otx2–Gbx2* interface. **(a)** Expression domains of *Otx2*, *Gbx2*, *Wnt1* and *Fgf8* in a WT mouse embryo at E9.5. *Otx2* is expressed in the midbrain with a sharp limit at the MHB, and *Gbx2* is expressed in the hindbrain with a sharp limit that abuts the *Otx2* expression domain. *Wnt1* is expressed in a stripe in the caudal midbrain and *Fgf8* is expressed in the rostral hindbrain. **(b)** Expression domains of the same genes in *Otx2* chimeric embryos at the six-somite stage. The visceral endoderm in these embryos is composed of WT cells that rescue the induction of the anterior neural plate. The neurectoderm is composed of *Otx2*$^{-/-}$ cells. Expression of *Gbx2* and *Fgf8* is expanded anteriorly and expression of *Wnt1* is abolished in the absence of *Otx2* [34,35]. **(c)** Expression domains of the same genes in a *Gbx2* homozygous mutant embryo at the six-somite stage. *Otx2* expression is expanded posteriorly, and *Wnt1* and *Fgf8* expression domains are shifted correspondingly [18,32•]. **(d)** Expression domains of the same genes in a transgenic mouse embryo at E9.5 that expresses *Otx2* under the *En1* promoter. The *Otx2* expression domain is extended further posteriorly. Endogenous *Gbx2* and *Fgf8* are repressed in this ectopic position, causing a shift of the *Otx2–Gbx2* interface and a repositioning of the MHB [33•]. **(e)** Expression domains of the same genes in a mutant mouse embryo at the six-somite stage that expresses *Gbx2* under the *Wnt1* promoter. *Gbx2* is now expressed ectopically in the midbrain. The caudal limit of the *Otx2* expression domain is shifted rostrally, and so are *Wnt1* and *Fgf8*, indicative of a more anterior position of the MHB [32•]. p1, prosomere 1; p2, prosomere 2; rh3, rhombomere 3; rh4, rhombomere 4.

lies closest to the MHB, and is the only rhombomere that does not express any *Hox* genes; however, after transplantation to an ectopic position, rhombomere 1 tissue expresses *Hox* genes. Both MHB tissue and Fgf8 can inhibit this expression [57]. Thus, *Fgf8* may define, directly or indirectly, the anterior limit of *Hox* gene expression. In a mouse null mutant of *Fgf8*, definitive endoderm and mesoderm are not formed, probably due to simultaneous lack of *Fgf4* (which is, however, present in *ace* mutants, explaining why the fish *fgf8* mutants gastrulate normally). This early phenotype has, thus far, precluded the analysis of *Fgf8* function in brain development [59•]; however, a weaker allele shows a morphologically similar phenotype to *ace* mutants [60].

Given its potency as a signaling molecule, the activity of *Fgf8* must be carefully controlled in the embryo. An emerging theme for several signaling pathways is that extracellular or intracellular inhibitors control their activity. *Drosophila sprouty* functions in development of the trachea and eye, as a target gene and feedback inhibitor for Fgf and epidermal growth factor (EGF) signaling [61]. Several studies reveal a surprisingly good correlation of the expression of vertebrate *sprouty* homologues with regions of ongoing Fgf signaling, including the MHB [62•,63,64•]. As in flies, vertebrate *sprouty* genes can be induced locally with recombinant Fgf8 protein [62•,63,64•]. In *ace* (*fgf8*) mutants, *sprouty4* is never activated at the MHB and anterior hindbrain, suggesting that Fgf8 regulates *sprouty4* expression. In addition, overexpression of *sprouty4* antagonizes the effects of both *fgf8* and *fgf3* injection [64•]. This suggests that zebrafish *sprouty4* is a component of an *Fgf8*-dependent inhibitory feedback loop at the MHB. Additional observations support the existence of such a feedback loop: *Fgf8* RNA is upregulated in *ace* mutants [7•,65] and in zebrafish *aussicht* (*aus*) mutants [66] — *aus* may therefore encode a component of the feedback loop. Possibly, the feedback loop could serve additional functions, for instance to maintain the MHB organizer itself, as this structure is missing in the zebrafish and mouse *Fgf8*

mutants [16,60]. The feedback loop also involves *Otx2* and *Gbx2*, because local expression of Fgf8 represses *Otx2* [25••,32•,55] and reduction of *Otx* copy number shifts *Fgf8* and *Gbx2* expression anteriorly [31,67]. The existence of the feedback loop may explain why *Fgf8*-bead implantations are able to reactivate the whole genetic cascade of MHB development; however, in some genetic combinations the players in the feedback loop can be spatially separated (A Simeone, personal communication), suggesting that the loop is not always functional.

Considering the potent abilities of *Fgf8*, it is notable that different Fgf8 isoforms [55] and additional *Fgfs* related to *Fgf8* are also expressed in the MHB organizer [65,68,69]. *Fgf17* and *Fgf18* are turned on at the MHB after the onset of *Fgf8* [65,70•], suggesting a role in maintaining the MHB organizing activity. Indeed, *Fgf17* injections have similar effects as *Fgf8* injections; Fgf17 acts downstream of *pax2.1* and *fgf8* [65], and both *Fgf17* and *Fgf18* can be induced ectopically in the forebrain by Fgf8 [65,71]. Mice carrying a null mutation in *Fgf17* have later defects in the cerebellar anlage, a phenotype that is more severe in a *Fgf8* heterozygous background [70•]. Thus, *Fgf8*, *Fgf17* and *Fgf18* may cooperate to maintain the organizing activity and each other's expression at the isthmus. Fgf8 is also a crucial component of the forebrain organizer located in the ANR/row 1 [5,7•] where it is coexpressed with at least one other *Fgf*, *fgf3* [64•,65], suggesting a similar functional redundancy of *Fgf* signals. Given these and other similarities, it is likely that the MHB organizer will continue to serve as a good model for understanding how brain organizers function in general.

Vertebrate brains are different

Studies in amphioxus indicate that the MHB organizer is probably a vertebrate-specific invention [72], although part of this genetic machinery (*Pax2* expression) may be conserved in ascidians [73]; hence, it is of particular interest to understand the actions and genetic regulation of this organizer and how this could generate the various brain morphologies in different species. From the available evidence so far, the genetic network controlling MHB development appears to be very similar in mouse, chick and zebrafish. There are, however, some interesting differences, even 'high up' in the genetic hierarchy. Several gene families including *Otx*, *Engrailed* and *Pax* genes are further diversified in zebrafish (Figure 1) as a result of a partial genome duplication in teleosts [74]. Relative temporal onset of expression can be different, for instance for *Fgf8* expression (Figure 1), and gene functions may be distributed differently among the members of a gene family, as may be the case for the *gbx* genes. A nice example of this phenomenon is provided by the *Pax2/5/8* genes, where such differences are linked to slight but telling alterations in function: in mice, inactivation of *Pax2* results in a very variable reduction of the MHB, depending on the genetic background. Full inactivation of both *Pax2* and *Pax5*, however, results in a reliably strong phenotype, suggesting that *Pax5* partially compensates for the absence of *Pax2*, and *vice versa*. In contrast, a null mutation in the zebrafish *noi* (*pax2.1*) shows a reliably strong phenotype. Moreover, *pax5* and *pax8* completely depend on *pax2.1* at the MHB, making the elimination of *pax2.1* equivalent to the (hypothetical) triple knockout in mice (see [15,75], and references therein). Interestingly, functional *Pax2* binding sites are nevertheless present in the murine *Pax5* promoter and *Pax2* partially regulates *Pax5* also in mice [76•]; the regulatory hierarchy found for zebrafish *Pax2/5/8* genes is therefore at least partially preserved in the mammalian lineage. It remains to be explored what consequences such differences in the genetic network driving MHB development have for the evolution of different brain morphologies.

Conclusions

Results discussed in this review suggest that two distinct phases in MHB development can be recognized. The first phase is a phase of establishment that involves the consecutive or parallel activation of different factors (*Otx2*, *Gbx2*, *Fgf*, *Wnt1*, *Pax*, *En*) at the *Otx–Gbx* interface. It remains to be determined which signal(s) creates the *Otx–Gbx* interface during gastrulation, and how this interface causes the ordered activation of MHB organizer genes around it. The second phase is a maintenance phase, in which expression of the above genes depends on each other; perturbance of any one gene disrupts the continued development of the MHB. Several Fgfs, in particular Fgf8, are the crucial molecular components active in the MHB organizer, and feedback inhibition mechanisms have evolved to control their activity. Organizer-derived signals are needed for the proper polarization of the midbrain retinotectal map to maintain its own integrity and that of the cerebellum, and to set the anterior limit of *Hox* gene expression in the hindbrain.

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The authors examined the role of *fgf8* in patterning the zebrafish forebrain through analysis of *ace* mutant fish. They show that a variety of defects are present in the rostral forebrain of *ace* embryos. For instance, major defects occur in commissural axon pathfinding, indicating that *ace* has a crucial role in patterning midline tissue in the commissural region of the forebrain. These defects are preceded by an early failure in anteromedial gene expression at the margin of the forebrain neural plate, which contains the row 1 organizer. Nevertheless, telencephalic and diencephalic territories are specified, arguing that *fgf8* activity is unlikely to induce the telencephalon or underlie all the activity of the ANR. These data suggest that *fgf8* is a component of the signal patterning the forebrain neural plate from the row 1 organizer.

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The authors show that FGF8 protein is able to mimic isthmic grafts into the hindbrain and can regulate gene expression in a manner appropriate to rhombomere 1. This suggests a difference in competence between midbrain and hindbrain in response to FGF8 signaling. By using a quail–chick heterotopic grafting strategy, the authors show that FGF8 at the isthmus provides a repressive signal that establishes the anterior limit of *Hox* gene expression and positions the rhombomere 1/2 boundary.

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The authors have implanted beads soaked in recombinant FGF8 in the caudal diencephalon or in the midbrain. This induces ectopic formation of mirror-image duplicated midbrains. They have observed that FGF8-bead implantation represses *Otx2* and activates *Wnt1*, *Fgf8* and *En1*. The authors suggest that there is a negative feedback loop in the MHB that involves the repression of *Otx2* by FGF8 and similarly, in the midbrain, a negative feedback loop in which OTX2 represses *Fgf8* .

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The authors have further analyzed the *Gbx2*$^{-/-}$ mutants and have observed that the earliest phenotype is a posterior expansion of the *Otx2* domain at early somite stages. They have observed that other genes expressed at the MHB are expressed at this shifted border of *Otx2* and in a normal spatial relationship. To check whether *Gbx2* is sufficient to position the MHB organizer, they transiently expressed *Gbx2* under the control of a Wnt1 enhancer in the caudal *Otx2* domain. They observed that the caudal border of *Otx2* was shifted rostrally and that the MHB organizer is established at the new border.

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The authors examine whether the caudal limit of *Otx2* expression is required to position the isthmic organizer. They have overexpressed *Otx2* in the presumptive anterior hindbrain using a knock-in strategy into the *En1* locus. They observe that the isthmic organizer and hindbrain markers are shifted caudally in the presumptive hindbrain territory. These data suggest that the caudal limit of *Otx2* is sufficient for positioning the isthmic organizer.

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The authors identified and characterized the zebrafish *dkk1* (*dickkopf*) gene, previously identified in *Xenopus* as a Wnt inhibitor with potent head-inducing activity. *Dkk1* is expressed in the prospective dorsoanterior mesendoderm and the dorsal yolk syncitial layer after mid-blastula transition, and in the anterior region of axial mesendoderm at later gastrulation. Misexpression of *dkk1* in WT embryos results in enlargement of the anterior nervous system. The authors also show that expression of *dkk1* in the dorsoanterior mesendoderm during gastrulation depends on *boz/dharma*, *sqt* (*squint*) and *oep* (*one-eyed pinhead*). Overexpression of *dkk1* promotes anterior neuroectoderm development in the absence of dorsoanterior mesendoderm. These results suggest that *dkk1* promotes the specification of anterior neural fates and the formation of axial mesendoderm, acting downstream of *boz/dharma* and *Nodal* signaling.

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The authors have investigated the requirement of the MHB organizer in *ace* mutants, which lack a MHB and cerebellum but retain a tectum. *Fgf8* is required for anterior–posterior polarization of the midbrain retinotectal map and for graded expression of ephrin ligands in the midbrain neuroepithelium. Some retinal ganglion cell axons overshoot beyond the mutant tectum, suggesting that the MHB also serves as a barrier for axonal growth. By transplanting eye primordia between wild-type and mutant embryos, they show that this defect depends on tectal but not retinal genotype.

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The authors analyze *Fgf8*$^{-/-}$ embryos and show that they fail to express *Fgf4* in the primitive streak. In the mutants, epiblast cells move into the streak and undergo an epithelial-to-mesenchymal transition, but most of the cells fail to move away from the streak. As a consequence, no embryonic mesoderm- or endoderm-derived tissues develop. Anterior neuroectoderm markers are widely expressed, at least in part because the anterior visceral endoderm is not displaced proximally. Posterior neuroectoderm markers are not expressed, presumably because of the absence of mesoderm. These data suggest that *Fgf8* is an essential gene for gastrulation.

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The authors have isolated a zebrafish *sprouty4* homologue that is expressed in a similar but slightly wider domain than *fgf8* and *fgf3*. By using gain- and loss-of-function injection experiments, and by studying *sprouty4* expression in *ace* mutants, they observe that *fgf8* and *fgf3* act to induce the expression of *sprouty4*, which in turn inhibits the activity of both of these factors. This suggests that *sprouty4* acts as a target gene and feedback inhibitor of FGF8 and FGF3 throughout zebrafish embryogenesis; furthermore, the authors demonstrate a functional requirement for *sprouty4* using antisense morpholino injections.

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