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Divide et Impera – the midbrain–hindbrain boundary and its organizer

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The midbrain–hindbrain organizer (MHO) is a signalling centre that orchestrates development of the mesencephalic and anterior metencephalic primordia. In recent years, details have been revealed about the molecular nature of these signals, their transmission and reception, and the regulatory processes associated with MHO function. This article reviews recent progress in understanding the genetic and molecular components of the MHO, and how they synergize to control brain development.

Despite its complexity, the vertebrate brain originates from a rather simple structure: the neural plate. The establishment of brain complexity is governed by two developmental mechanisms that are reminiscent of the emperors' principle *Divide et Impera* (divide and rule): under the influence of early signals [1,2], the neural plate is progressively subdivided into distinct areas, the development of which is then separately controlled to achieve further morphological and molecular specialization of these regions. As a result, the gastrula-stage neural plate is already molecularly subdivided into regions that prefigure the major parts of the embryonic brain (Figure 1a,c). One of the first molecular subdivisions separates the neural plate into a rostral and caudal territory, prefiguring the boundary between the prospective midbrain and hindbrain [the 'midbrain–hindbrain boundary' (MHB)]. The cells at this boundary act as an organizer that is therefore called the 'midbrain–hindbrain organizer' (MHO) or 'isthmic organizer' (because the *isthmus rhombencephali* will later form in this area). The term 'organizer' implies that these cells send instructive signals to the neighbouring tissues and thereby direct their developmental fates (Box 1). Indeed, the MHO has a crucial role in the development of the adjacent territories: on its rostral side, it acts to polarize the dorsal mesencephalon (the tectum), which processes visual and acoustic sensory information; on the caudal side, the MHO elaborates the cerebellum, which arises from the anterior hindbrain and serves as an important centre for motor control (reviewed in Refs [3–5]). This review will focus on recent progress in MHO research that highlights novel and interesting aspects of both boundary formation and organizer function.

Genetic architecture of the organizer

The organizer potential of the MHO was first demonstrated in classical grafting experiments in chick showing that MHB tissue, when transplanted to more anterior (diencephalic) or posterior (rhombencephalic) positions, could stimulate the development of ectopic midbrain or cerebellum structures, respectively [6]. Subsequently, molecular and genetic analyses began to uncover the genetic framework underlying formation of the MHB and its organizer potential. Early neural patterning leads to the adjacent expression of two homeodomain transcription factors of the Otx and Gbx class, which mark, respectively, the anterior and posterior compartments of the brain, and correlate with MHB positioning [7–9]. Subsequently, a series of transcription factors and signalling molecules that are crucial for MHB development become expressed at this junction [3–5,10–19] (Figure 1b provides a synopsis of the most prominent factors identified in chicken, mouse and zebrafish). The nature of this correlation, however, remains unclear. Are Otx and Gbx direct activators of these MHB-specific genes, or are the connections less direct? Recent evidence argues for the second possibility. First, fusion-protein studies in *Xenopus* suggest that the activity of Otx and Gbx needed for the positioning process is transcriptional repression rather than activation [20]. Second, transcription of murine *Fgf8* or *Pax2* can occur in the absence of both Otx2 and Gbx2 [21,22] (and references therein). These and other observations indicate that MHB initiation is independent of Gbx and Otx. Instead, these factors are likely to play a role in the spatial refinement of MHB gene expression, possibly by employing co-repressors such as the Groucho-related protein Grg4 [23–25] or homeodomain proteins of the Iroquois family [20,26]. In turn, expression of MHB-specific genes is also implicated in the refinement of the Otx–Gbx expression boundary, arguing for a dynamic process of boundary establishment [21,27].

Another area of ongoing study concerns the many cross-regulatory activities between different MHO- specific genes. Previous studies had already suggested two main phases of gene regulation at the MHB. In the establishment phase (Figure 1b,ii), MHB-specific genes

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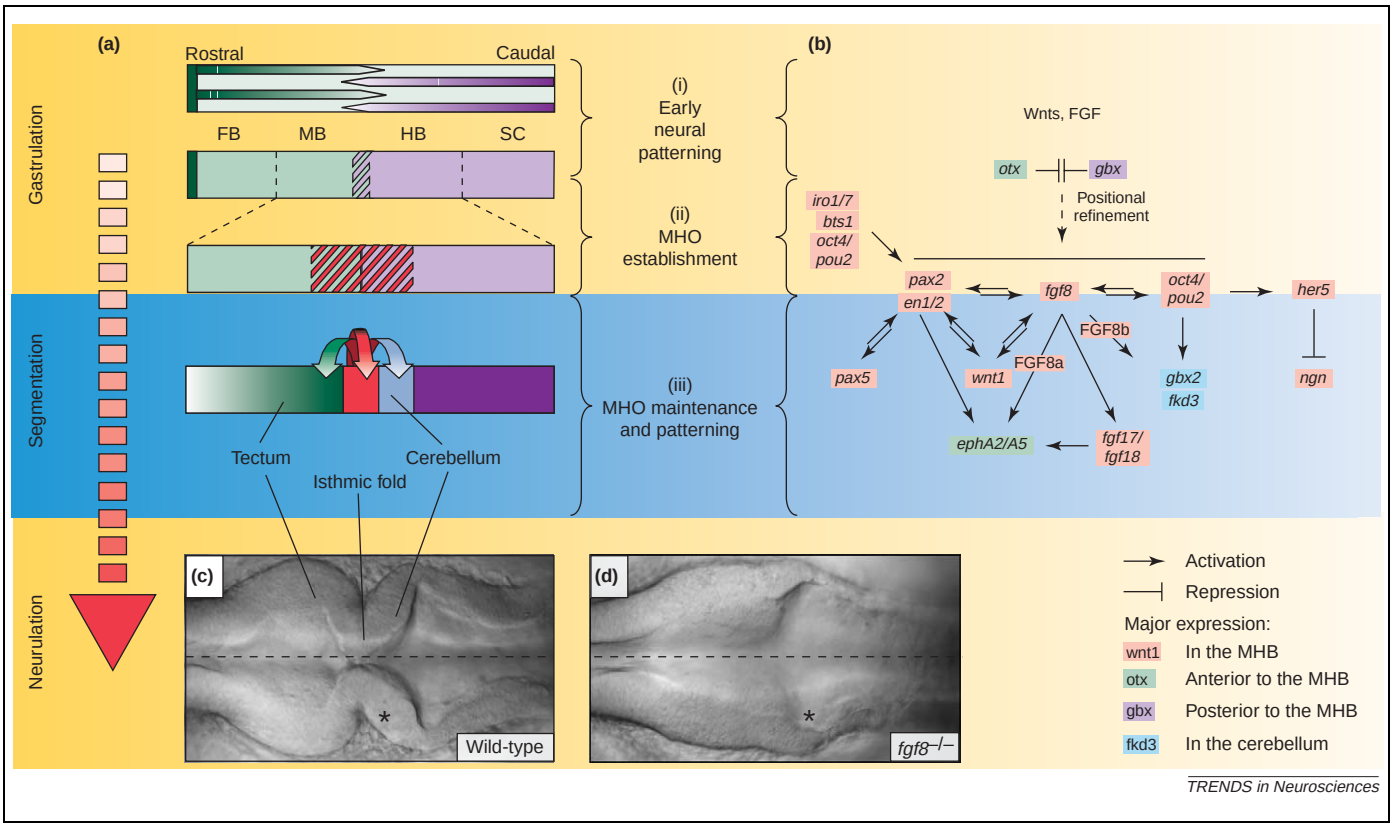

Figure 1. Important phases and regulators of midbrain–hindbrain boundary (MHB) and midbrain–hindbrain organizer (MHO) development. **(a)** Midbrain–hindbrain development progresses (left vertical arrow) through three main phases. (i) Primary patterning of the neural plate along the rostrocaudal axis leads to broad molecular domains prefiguring the later forebrain (FB), midbrain (MB), hindbrain (HB) and spinal cord (SC); this involves signalling centres such as the anterior neural boundary (dark green box). (ii) Expression of MHB-specific genes (hatched red domain) begins in the late gastrula, leading to the establishment of the MHO as a secondary organizer (red box). (iii) During early segmentation stages, the MHO displays distinct activities, including self-maintenance (red arrow), polarization of the midbrain tectum (green arrow) and elaboration of the cerebellum (blue arrow). **(b)** Some important regulators of, and their main actions during, the phases of MHB development. The panel integrates primary data from different vertebrate organisms (mouse, chicken and zebrafish). Colours indicate major expression sites in the MHB region, as explained below the panel; arrows indicate activating or repressive activities, which need not necessarily be direct. (i) During early neural patterning, the interface between *otx* and *gbx* expression domains is important for refinement of MHB positioning (broken arrow) but not for initiating expression of crucial components, such as *pax2* or *fgf8*. (ii) During MHO establishment, MHB-specific genes are activated largely independently of one another. (iii) In subsequent stages of MHO maintenance and patterning, genes become interdependent in a regulatory network (double arrows). **(c,d)** The MHO directs later brain morphology. (c) A dorsal view of the pharyngula-stage zebrafish brain displays several key morphological features derived from the early molecular patterns shown in (a) [e.g. the tectal lobes of the midbrain, the cerebellar primordium (asterisk) and the isthmic fold]. (d) Loss of the MHO in *fgf8*$^{-/-}$ mutants results in loss of the isthmic fold and cerebellar primordium (asterisk). Broken line indicates the midline. Panels (c,d) modified, with permission, from Ref. [19].

are expressed largely independently of one other. During somitogenesis stages, genes become interdependent (Figure 1b,iii). Taking *pax2* as an example, recent promoter studies in mouse and zebrafish support these distinct phases of gene regulation [28,29]. In mouse, an early *pax2* control element has been identified that acts at the time of *pax2* initiation. This element is bound by POU homeodomain proteins [28], consistent with the requirement of Pou2/Oct4 for correct *pax2.1* initiation in zebrafish [11–13]. Additionally, *pax2.1* appears to regulate its own early expression in a positive feedback mechanism that relies on activity of engrailed (En) proteins [29]. Indeed, a separate mouse enhancer element supports a direct role for members of the Pax2/5/8 family in control of late *pax2* expression [28].

Orchestrating brain development: signals and cues

How can cells of the MHO influence the surrounding tissues? Several secreted proteins are expressed at the midbrain–hindbrain junction. These include members of the wingless/Wnt (Wnt1, Wnt8b) and fibroblast growth factor (FGF8, FGF17, FGF18) families, as well as other secreted molecules such as isthmin [30] (F. Raible, I. Araki and M.Brand, unpublished). Out of this cocktail, Wnt proteins have been associated mostly with proliferative control (as will be discussed later in this review), whereas FGFs, and particularly FGF8, appear to be the best candidates for single molecules that exert most of the organizer functions. They not only are crucial for MHB development but also mimic several aspects of MHO core activities when provided ectopically, such as the molecular polarization of the tectum and the triggering of a cerebellar gene program, including the repression of anterior-most *hoxa2* expression [10,16,31].

As FGF8 also elicits the expression of other MHO genes, an important issue is whether endogenous FGF8 is the only mediator of the different organizer effects, or whether there are subordinate factors involved. One possibility that has gained support is that different FGF ligand types are responsible for mediating different effects. Consistent with that, two major splice variants of FGF8 – FGF8a and FGF8b – differ significantly in their respective organizer potential. Importantly, in both mouse and chicken, only FGF8b can ectopically induce

Box 1. The concept of embryonic organizers

The concept of the 'embryonic organizer' dates back to the times of classical embryology. Although initially used for the Spemann–Mangold organizer, the term generally describes a specific group of cells that can direct development of the surrounding tissue. Three main criteria define an organizing centre:

- The organizer activity resides in a defined population of cells, the removal of which leads to the lack of a specific structure during development
- When an organizer is grafted into ectopic locations, it can induce the formation of an ectopic structure that normally does not form at this location
- The term 'induction' implies that at least part of that structure is formed by the tissue around the organizer and not only by the organizer tissue itself. In addition, the response of the target tissue is not a default program, but depends on the signals emitted by the organizer.

The known examples of organizers seem to validate a classical concept (Figure I). According to this [74], 'secondary field organizers' (red in Figure I) occur and function at the border between two juxtaposed territories of different identity (primary embryonic fields; 'a' and 'b') that arose from earlier patterning steps. In this way, an organizer refines a simple primary spatial organization into a more elaborate pattern of secondary fields (a1–a3 and b1–b3), which serve as the building blocks for many diversified organs, including the brain.

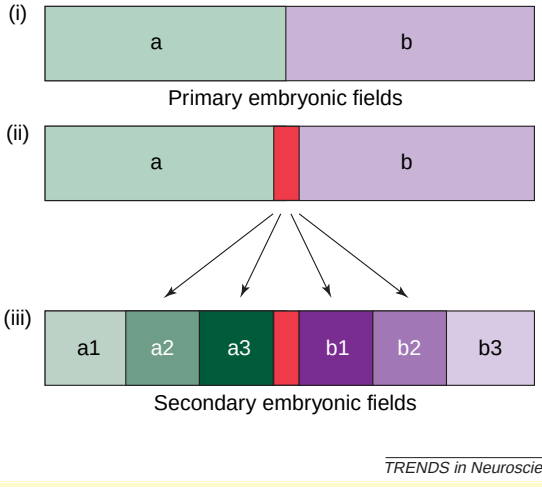

Figure I. The classical principle of a secondary field organizer. Primary embryonic fields (i) are divided by a secondary field organizer (red; ii) that organizes generation of secondary embryonic fields (iii).

'cerebellar' genes such as *Gbx2* in the midbrain region [32,33], whereas FGF8a regulates En2, as would be expected for an endogenous midbrain polarizing signal [33]. It will be interesting to determine whether this reflects mere differences in the effective levels of the two isoforms of FGF8 or whether qualitative differences are responsible. Besides FGF8a, two other FGF ligands – FGF17b and FGF18 – are likely to be involved in the midbrain-directed signal. Both behave like FGF8a when overexpressed in the midbrain, and none can elicit ectopic cerebellar development in the same way as FGF8b [34]. Because FGF8 is involved in maintenance of FGF17 and FGF18 in the MHB [18,35], FGF8 could therefore influence midbrain development both directly and through the activity of subordinate FGF ligands.

Shaping territories: linking molecular patterns and morphogenesis

The mechanisms that generate distinct gene expression patterns in the midbrain–hindbrain area do not necessarily explain how these 'blueprints' are translated into the elaborate morphological structures that arise from the alar and roof plate of this region, such as the isthmic constriction, the tectal lobes of the midbrain or, posteriorly, the cerebellar hemispheres and vermis. At the cellular level, selected regulation of proliferation, cell death, adhesion or mobility must account for these morphogenetic processes. So far, distinct molecular machineries that control these processes have not been identified. One reason for this might be that early patterning and later morphogenesis actually share several molecules and are thus difficult to separate. Indeed, Wnt1 might have such a dual function: mutant and knockout mice manifest a crucial role for Wnt1 in the MHB primordium, consistent with a role in the patterning phase, whereas gain-of-function studies in mouse and chicken implicate Wnt1 as a regulator of the proliferative state of the cells that influences either bulk growth of brain parts or of specific cell types (reviewed in Ref. [5] and discussed later in this review). One interesting hypothesis that awaits further experimental proof remains: that proliferative control is indeed the main function of Wnt1, and that its influence in the patterning phase needs to be interpreted as the control of cell populations that receive other instructive signals [5].

Further insight into the connection between cell regulation and morphogenesis comes from analysis of cell proliferation and cell death after genetic ablations of *fgf8*, in both the zebrafish [19] and the mouse [18] MHB. Conditional elimination of murine *Fgf8* during early somitogenesis results in the progressive loss of the midbrain. Further analysis showed that this loss is the result of massive cell death in the area corresponding to the midbrain and, subsequently, the anterior hindbrain, suggesting that FGF8 might be required for early cell survival in both areas, possibly in concert with Wnt1 [18]. In fish, loss of *fgf8* leads to milder defects, including loss of a prominent growth zone in the posterior tectum. Although this would be consistent with a role for FGF8 in proliferation control, a closer analysis of the *fgf8* mutant phenotype conflicts with the results obtained in mice: fish mutants for the *fgf8* gene display a loss of the cerebellum, whereas the tectum is actually enlarged [16,17,19]). This is unlikely to be due to redundancy in the function of zebrafish FGFs, because not even pharmacological or genetic interruption of all FGF signalling leads to an ablation of the fish midbrain [36–39]. Indeed, even loss of the cerebellum in *fgf8* mutant fish cannot be attributed to cell death, because tissue labelling and marker analyses show that cells in the corresponding region survive but are transformed to a more rostral identity, so that they differentiate as midbrain cells [19,40]. One way to reconcile the findings in both organisms would be to assume that the differences are merely due to species-specific

redundancies in factors other than FGFs, such as the zebrafish Wnts [41]. An alternative reason could lie in the different experimental approaches. Because the conditional removal of FGF8 in mouse employs an *En1* promoter–Cre fusion, the MHB cells must already be committed to parts of the MHB program by the time the removal of FGF8 becomes effective. Owing to this partially committed status, cells might undergo apoptosis rather than adopting a more rostral identity, as they do in fish mutants. Clearly, this controversial issue awaits further examination.

Besides proliferation, cell adhesion and coordinated cell movements contribute to the shape of the brain in many areas. From the early neural plate stage onwards, relative cell positions along the anteroposterior axis of the MHB remain mostly stable, suggesting that random mixing of cells is restricted (T. Langenberg and M. Brand, unpublished) [40] (but see Ref. [42]). Two recent studies have employed cell labelling, transplantation and genetic techniques to dissect later morphogenesis in the avian isthmic primordium [43,44]. These studies found that the dorsal isthmic area plays a crucial role in establishment of the roof plate in adjacent territories. Cells labelled at the dorsal isthmus populated either the midbrain roof or the cerebellar midline. Consistent with this, ectopic MHO structures that are induced by FGF8b-soaked bead implants in the chick diencephalon also trigger the migration of cells and the expression of roof-plate markers, indicating that this property is linked to the organizer program itself [44]. Clearly, studies such as these call for a closer re-investigation of cell fates within the organizer region, to distinguish the contribution of organizer tissue itself from its inductive action on the surrounding tissue, which is used to define the organizer activity (Box 1).

Checks and balances: controlling the organizer activity

An emerging topic of MHO research has been to understand how the powerful inducing ability of the MHO is properly tuned in time and space. At the cell biological level, one important line of research focuses on regulation of the FGF-dependent mitogen-activated protein kinase (MAPK) pathway. Factors that affect this pathway are likely to play a role in modulating midbrain–hindbrain development. Spatial and temporal co-expression of genes ('synexpression') is an indicator of functional correlation. Indeed, members of the 'FGF synexpression group' [45] provide an interesting collection of such modulators (Figure 2).

Sprouty (Spry) was originally identified in *Drosophila* as an inhibitor of growth factor signalling, and several vertebrate Spry homologues were subsequently found to be 'synexpressed' with FGF ligands [38,46]. Functional experiments have confirmed that the vertebrate genes not only are downstream targets of functional FGF signalling in the MHB [38,46] but also are important modulators of the pathway itself. In accordance with biochemical evidence that implicates Spry proteins as feedback inhibitors of the MAPK pathway [47], zebrafish Spry4 injections can prevent MHB formation *in vivo* [38]. Besides this inhibitory role of Spry proteins, experiments in cell culture and in *Xenopus* gastrulae suggest that they could even be more diverse modulators of intracellular signal flow (reviewed in Ref. [48]). Therefore, further analysis of Spry promises to be more revealing about the fine-tuning of signalling events at the MHB.

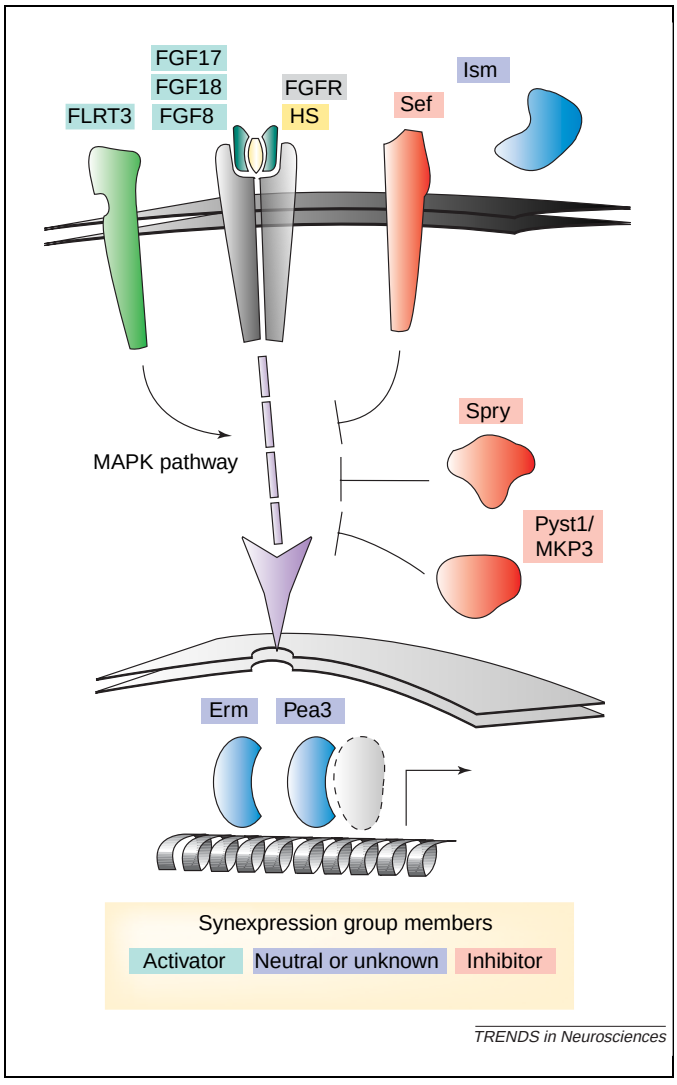

Figure 2. Transmitters and modulators of fibroblast growth factor (FGF) signalling at the midbrain–hindbrain boundary. A simplified scheme of the FGF signalling pathway, which is crucial for the function of the midbrain–hindbrain organizer. The focus is on components that are expressed in similar spatiotemporal fashion and thus constitute members of the 'FGF synexpression group' [45]. Proteins are highlighted in green or red to indicate a confirmed role in activation or inhibition, respectively. Proteins in blue belong to the FGF synexpression group but have not been unequivocally determined to be activators or repressors. Dimeric ligands (of FGF8, FGF17 and/or FGF18) activate their cognate FGF receptors (FGFR) in the presence of heparan sulfate chains (HS). Receptor *trans*-phosphorylation triggers various intracellular signalling pathways, of which the mitogen-activated protein kinase (MAPK) pathway (purple) is assumed to play a major role in midbrain–hindbrain development. Erm and Pea3 are downstream targets and possible effectors of FGF signaling; the function of isthmin (Ism) in FGF signaling is still unclear. Sprouty (Spry) proteins are recruited to the plasma membrane and are feedback inhibitors of the MAPK pathway. MAPK phosphatase 3 (Pyst1/MKP3) acts as a cytoplasmic inhibitor of the MAPK pathway. Sef ('similar expression to FGF') is a transmembrane protein and negative regulator of FGF signalling. FLRT3 can bind FGF receptors and stimulate downstream signalling.

Recently, three additional modulators of FGF signalling have been identified that are also coexpressed with Fgf8 at the MHB. These are the transmembrane proteins Sef ('similar expression to FGF' [49,50]), XFLRT3 [51] and the cytoplasmic MAPK phosphatase Pyst1/MKP3 [52,53] (and references therein). Functional studies in zebrafish

suggest that Sef acts as an inhibitor of FGF signalling *in vivo*, at least in situations where FGF signalling is partially reduced [49,50]. *In vitro* studies show that Sef can bind FGF receptor 1 (FGFR1) and prevent it from autophosphorylating, providing a biochemical explanation for the proposed action of Sef as a feedback inhibitor of FGF signalling [54]. XFLRT3 interacts with FGFR1 and activates FGF signalling *in vitro*, thereby constituting a potential positive regulator of the pathway. A challenge for the future will be to understand the sequence in which these potential feedback regulators act and to fit them into a coherent picture of MHB development (Figure 2).

Transcription factors of the ETS class are another group of molecules 'synexpressed' with FGFs. ETS transcription factors are known to be involved in mediating tyrosine kinase signalling in flies. Work on zebrafish revealed that two ETS transcription factors, Erm and Pea3, are expressed together with FGF8 and FGF3 during development [55–57], and that FGF signaling tightly regulates their expression [56,57]. A likely hypothesis is that Erm and Pea3 are immediate downstream targets of FGF signalling that could control secondary target genes. However, the functional redundancy of the two factors has so far precluded the analysis of specific downstream targets in MHB development.

FGF signalling is not only controlled by proteinaceous components: heparan sulfate chains are part of the FGF ligand–receptor complex. Consistently, enzymes involved in a crucial step of heparan sulfate biogenesis have been shown to be important for FGF signalling in flies [58]. In the murine brain, conditional knockout of Ext1, a glycosyltransferase involved in the chain elongation of heparan sulfate, causes defects in the inferior colliculi and the cerebellum, a phenotype reminiscent of a loss of the MHO [59]. Notably, expression of MHB-specific genes, such as *En1, En2, Wnt1* and *Fgf8*, is not lost, but expands along the anteroposterior axis. The correct distribution of FGF8 in the tissue might be affected in the mutants, but the correlation to the later morphological phenotypes still awaits further analysis.

Besides the modulation of FGF signalling components, another level of organizer control concerns the competence of the target cells to respond to the signal. A specific response might require the concerted action of constitutive FGF targets such as Erm and Pea3 with another factor that is not ubiquitously expressed around the MHB and thus limits the possible range of activity. A candidate regulator is Pou2/Oct4, which is expressed in the early MHB anlage and is affected in the zebrafish MHB mutant *spiel ohne grenzen* (*spg*). Besides an early role for the regulation of MHB genes [11–13], Pou2 has a cell-autonomous, strictly permissive role for the ability, or 'competence', of hindbrain cells to respond to FGF8, thereby restricting the functional range and impact of the organizer signal [13] (Figure 1). Similarly, it has been suggested that Otx could act as a competence factor for mesencephalic development [33].

Impact of the MHO on neural organization

Functional specialization of midbrain and cerebellum requires the regulation of neural differentiation, migration and connectivity. One basic level of control revolves around the distinction between neurogenic and non-neurogenic brain areas. The isthmic primordium itself remains a zone of delayed differentiation (reviewed in Ref. [60]). The transcription factor Her5 is expressed in the zebrafish MHB in a similar way to, but independent of, Pax2 [61]. Interference experiments in suggest that Her5 inhibits expression of proneural genes, and also prevents cell-cycle exit. It can thus maintain cells in a proliferative state [62]. Like *pax2*, *her5* expression depends on maintenance of the MHO [61]; however, neither gain-of-function nor loss-of-function experiments with Her5 interfere with *pax2*, *engrailed* or *wnt1* expression, suggesting that molecular patterning and neurogenesis are molecularly distinct processes. Similarly, a mouse double knockout of the related genes *Hes1* and *Hes3* leads to premature neurodifferentiation at the MHB region. However, this knockout also shows a patterning defects caused by the loss of MHO-specific genes [63]. It therefore remains to be determined to what extent members of the hairy and enhancer-of-split superfamily such as Hes1, Hes3 and Her5 influence neurogenesis by regulating MHO-specific genes and how neurogenesis influences MHO gene expression.

Besides neurogenesis, previous studies have indicated that the MHO also influences characteristic neuronal structures such as brain nuclei in the midbrain tegmentum and the medulla oblongata [6,64,65]. This raises the issue of to what extent these effects are directly linked to late activity of the organizer signal, as opposed to consequences of the early patterning processes. Primary cell cultures provide a good assay system with which to address this. In a recent study on mammalian dopaminergic neuron development, this technique helped to dissect the influences of different Wnt ligands on either the proliferation of precursor cells or their transition to dopaminergic neurons [66]. Besides the medical relevance of culturing dopaminergic neurons, one interesting outcome is that different Wnt molecules – Wnt1, Wnt3a and Wnt5a – play distinct roles in this process, suggesting that specification of neurons in the MHB area could be fine-tuned by different ligand combinations.

In addition to the previous examples, the MHO might also have an impact on the guidance of cells and axons. The trochlear nerve has emerged as an interesting model system for axonal pathfinding in the brain because, on their path from the ventral area of the first rhombomere to the dorsal side of the brain, its fibres cross the brain exactly at the MHB. Repellent interactions between the semaphorin Sema3F and neuropilin 2 provide a mechanistic model of how the trajectory along the MHB itself is determined [67]. In addition to these repellent interactions, explant and electroporation studies in chicken have shown that isthmic tissue also produces a chemoattractant for the ingrowing trochlear axons, and that FGFs (FGF4 and FGF8b) can partially mimic this effect. Still, it remains unclear which of the FGFs expressed in the isthmus at this stage act, either alone or in combination, as the endogenous cues [68], and whether the same cues could also play a role in the migration of neuronal cell bodies in the MHB, as has been observed

in vivo in the zebrafish [69]. Finally, an interesting alternative could be that Wnt proteins act as additional cues in the MHB, reminiscent of their role as chemoattractants in the mammalian spinal cord [70].

The rise and change of MHO activity in development and evolution

The midbrain–hindbrain junction and, later, the isthmus have a prominent role in distinct processes over a long period of brain development. In many instances, FGFs have been associated with these functional effects, underscoring the potent and versatile function of this molecule class. In turn, the repetitive use of the same signalling molecules reflects the differences in the molecular 'interpretation' of this signal by the target tissue. Therefore, 'the organizer' can hardly be viewed in isolation from the surrounding tissue. Both form complementary parts of one functional circuit. For these reasons, it is difficult to determine when the MHO first acts during development. In fish, where FGF8 becomes activated at an earlier stage than in mammals, clear regulatory functions of FGF8 for gene expression in the hindbrain can already be discerned before the end of gastrulation [13,27], whereas self-maintenance starts only at mid-segmentation stages [16]. Thus, 'the organizer' activity can be separated into distinct phases, presumably reflecting the activity of distinct enhancer elements in MHO gene regulation. It might in fact be a better analogy to view the MHO as a 'succession of organizers' that just happen to share at least a subset of their molecular players, such as Wnts and FGFs.

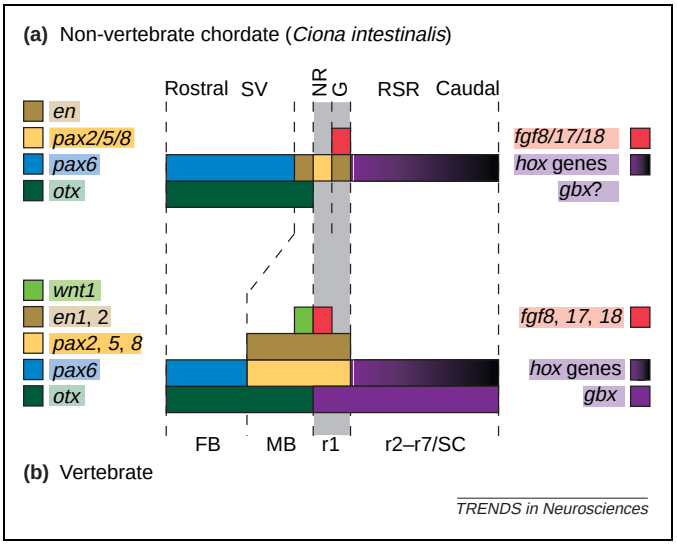

Figure 3. Molecular similarities between the CNS of basal chordates and vertebrates. Schematic expression of midbrain–hindbrain boundary genes and their orthologues along the anteroposterior axis of the embryonic CNS of **(a)** the basal chordate *Ciona intestinalis* or **(b)** a vertebrate (consensus from mouse, chicken and fish). Genes are colour-coded to indicate orthologous relationships across chordates. Gene families in vertebrates are typically expanded compared with their *Ciona* counterparts but the resulting paralogues are often similarly expressed and might share redundant functions. In both groups, an anterior, *otx*-positive territory is separated from a posterior, *hox*-positive territory by the presence of a *hox*-free domain (grey) that expresses members of the *fgf8/17/18* family. Although these similarities are consistent with the presence of a midbrain–hindbrain organizer-like activity in basal chordates, the equivalence of the investigated embryonic stages and the functional implications of these similarities remain to be determined. Abbreviations: FB, forebrain; G, visceral ganglion region; MB, midbrain; NR, neck region; r1, rhombomere 1 of the hindbrain; r2–r7, rhombomeres 2–7; RSR, rhombrospinal region; SC, spinal cord; SV, sensory vesicle.

The distinction between multiple steps of organization might reflect the separate steps in which the MHO arose during evolution. The MHB is located between an Otx-positive anterior territory and the anterior boundary of *Hox* expression, and therefore marks the middle region of a putative tripartite ancestral brain [71]. However, the MHO is assumed to be a vertebrate-specific phenomenon. Most support for this view comes from the non-vertebrate chordate *Amphioxus*, in which some genes that mediate organizer activity, such as *engrailed*, are not expressed at the intermediate part of the brain that – owing to its position – could correspond to the MHB [71]. This view is challenged by new expression analyses from a different non-vertebrate chordate, the ascidian *Ciona intestinalis*. Orthologues of Otx and Engrailed, as well as the Pax2/5/8 and FGF8/17/18 protein families, have been identified in *Ciona*, and studies on their expression reveal that they are activated in the ascidian CNS in a pattern reminiscent of that in vertebrates [72,73] (Figure 3). Therefore, expression of some crucial MHO determinants in the MHB might be more ancestral than previously assumed and could pre-date the branching of vertebrates off the chordate evolutionary lineage. The conserved expression of MHO genes in the medial region of the ascidian brain, however, is by itself no indication that this region has any of the organizer capabilities of vertebrates. Functional studies will have to address this issue and will, in turn, also shed more light on the specificities of vertebrate midbrain–hindbrain development, and how these evolved.

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