Cell. Mol. Life Sci. (2013) 70:3365–3374
DOI 10.1007/s00018-012-1240-x

Cellular and Molecular Life Sciences

REVIEW

Novel mechanisms that pattern and shape the midbrain-hindbrain boundary

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Received: 17 September 2012 / Revised: 18 November 2012 / Accepted: 10 December 2012 / Published online: 10 January 2013

Abstract The midbrain-hindbrain boundary (MHB) is a highly conserved vertebrate signalling centre, acting to pattern and establish neural identities within the brain. While the core signalling pathways regulating MHB formation have been well defined, novel genetic and mechanistic processes that interact with these core components are being uncovered, helping to further elucidate the complicated networks governing MHB specification, patterning and shaping. Although formation of the MHB organiser is traditionally thought of as comprising three stages, namely positioning, induction and maintenance, we propose that a fourth stage, morphogenesis, should be considered as an additional stage in MHB formation. This review will examine evidence for novel factors regulating the first three stages of MHB development and will explore the evidence for regulation of MHB morphogenesis by non-classical MHB-patterning genes.

Keywords Midbrain–hindbrain boundary · Isthmic organiser · Neural tube · Morphogenesis

Introduction

The vertebrate brain is the single most complex and dynamic organ to arise in the animal kingdom throughout evolutionary development. The brain originates from the neural ectoderm, formed during gastrulation, and begins its development as a flat sheet of neuroepithelial cells, termed the neural plate. Although some temporal, spatial and mechanistic differences exist, the formation and folding of the vertebrate brain is a remarkably well-conserved evolutionary process [1, 2]. The early neural plate undergoes a convergence and extension process (neurulation) to form the neural tube (embryonic brain and spinal cord). Patterning of this tube along the dorso-ventral axis is well understood and depends on the relative gradients of BMP4 (dorsalising) and Shh (ventralising) factors [3, 4]. Similarly, patterning along the anterior-posterior axis is also well defined, particularly with respect to the positioning, induction and maintenance of the most crucial neural developmental signalling centre, the midbrain-hindbrain boundary (MHB). Originally identified in chick [5–8], the isthmic organiser located at the MHB consists of cells that influence the fate of neighbouring cells to adopt either a mesencephalic (midbrain) or metancephalic (hindbrain) fate through expression of transcription factors and soluble signalling molecules [9]. This organiser is powerful enough to induce surrounding cells to re-fate to become ectopic midbrain and hindbrain if it is transplanted to other areas of the brain [10], indicating that correct positioning and patterning of organiser formation must be tightly and precisely regulated at an early stage in brain formation.

The subsequent use of a variety of developmental vertebrate models has helped to identify conserved patterning signals, which position and influence the formation of the MHB. Although the precise spatio-temporal expression of genes within the MHB cascade varies between vertebrate species [9], and different gene orthologues perform the same functions between species, broadly speaking, the conserved pathway operates as follows. The position of the prospective MHB is specified at the interface of the expression domains of the transcription factors *Otx2* and *Gbx1/2*

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within the neural plate [10–12]. A cascade of signalling (*FGF8/Wnt1*) and transcription (*Pax2/5/8*, *Eng1/2*) factors within the *Otx2/Gbx1/2* boundary induces formation of the MHB [9, 13–15], and subsequent interplay between these factors is critical for maintenance of the MHB. These factors (*Otx*, *Gbx*, *FGF8*, *Wnt, Pax*, and *Eng*) classically comprise the core MHB cascade, and disturbance of any of these factors leads to severe functional disruption in the formation of the isthmic organiser.

This core MHB cascade has been the subject of numerous excellent reviews [9, 10, 16–18] and will therefore not be the topic of this work. Rather, our focus will centre on novel factors and mechanisms that regulate the positioning, induction and maintenance of the MHB. This review will also examine the evidence that a fourth stage of MHB development, namely morphogenesis, is actively governed by a specific set of signalling cues and is not merely a passive consequence of correct patterning of the neural territory by genes of the core MHB cascade.

Novel factors regulating the stages of MHB development

Stage 1: Positioning and establishment

As the *Otx* and *Gbx* factors are critical for positioning the presumptive MHB within the neural territory, the spatiotemporal regulation of their expression is pivotal. Planar signals operating within the neural plate are central to multiple aspects of neurulation, from initial regionalisation of the neural plate to the ultimate morphogenesis of the neural tube [19]. Studies in zebrafish have shown that "posteriorisation" (conversion of part of the neuroectoderm to a more posterior fate) during early neurulation, mediated by *Wnt8*, is a critical requirement for the onset of *gbx1* and *otx2* expression [20]. The importance of the notochord in signalling to the neural plate to set up the *Otx2/Gbx1/2* boundary has been debated, although as the establishment of this interface in mice is unaffected in embryos lacking a (node-derived) notochord [21], the contribution of this signalling to the establishment of the MHB territory is now thought to be minor, and rather the non-axial mesendoderm underlying the neural plate appears to be a more critical source of ventral signalling to the neural plate [20].

A large body of work suggests that FGF8 is the critical organiser molecule (Fig. 1). FGF8 can regulate the expression of both *Otx2* and *Gbx2* by activating *Gbx2* and repressing *Otx2* within the isthmic region [22] and by suppressing *Otx2* expression in the hindbrain [23]. The expression domain of *FGF8* itself is in turn regulated by the boundaries of *Otx2/Gbx2* expression [24], indicating the presence of a feedback loop. Most interestingly, FGF8 is still

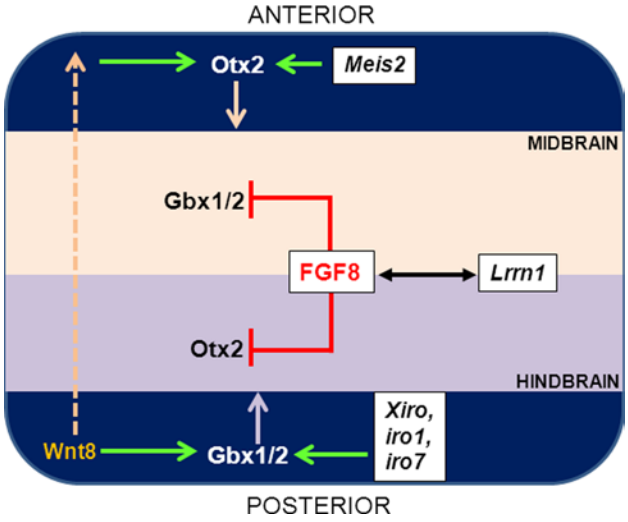

Fig. 1 Positioning of the presumptive MHB is regulated by the expression of *Otx* and *Gbx* genes. Following overall posteriorisation of the neuroectoderm by *Wnt8*, *Otx2* is expressed within the presumptive midbrain region, and *Gbx1/2* is expressed within the presumptive hindbrain territory. *Otx2* expression in the midbrain is regulated partially by *Meis2*, whereas *Gbx1/2* expression in the hindbrain is regulated by members of the *Iroquois* (*iro*) gene family. The tightly demarcated interface of *Otx/Gbx* gene expression is regulated by *FGF8*, which inhibits *Gbx1/2* expression in the midbrain and *Otx2* expression in the hindbrain, respectively, resulting in defined lineage restriction. *Lrrn1*-mediated suppression of *FGF8* results in defective boundary demarcation, lineage-mixing, and defective organiser formation, indicating that the establishment of a sharp boundary is critical for further MHB development

observed within the neuroectoderm in the absence of both these factors [24], indicating that *Otx2* and *Gbx2* may serve to refine rather than induce the expression of FGF8. Experiments in the chick model suggest that de-repression of *Otx2* transcriptional activity may be critical in the initial positioning of the midbrain-hindbrain domains. Overexpression of the midbrain-patterning gene *Meis2* [25] greatly increased the transactivation potential of *Otx2*, suggesting that in addition to specifying the midbrain domain, *Otx2* may also play an active process in gene induction of the core MHB cascade. Although *Meis2* clearly regulates the function of *Otx2*, it does not impact on the initiation of the MHB cascade, or possess MHB-organiser function itself [25], suggesting that despite regulating a critical early MHB-patterning gene, *Meis2* in itself is not an MHB-patterning factor.

Once established, the boundary demarcates the future midbrain ($Otx2^+$) and hindbrain ($Gbx1^+$) territories, generating an apparent lineage restriction (Fig. 1), whereby once fated, cells from the respective territories do not intermix [26, 27]. The expression of the bHLH gene *Her5* has been postulated to act as the earliest fate-determinant during gastrulation, acting to establish identities of future midbrain and hindbrain cells [28]. Although lineage restriction boundaries exist throughout the neural tube demarcating distinct regions

(neuromeres), the evidence for a lineage restriction at the MHB is not definitive. In vitro cell mixing and in vivo cell labelling studies in the chick MHB suggested that cells from both mesencephalic and metancephalic territories intermingled freely [26]. A subsequent study in the zebrafish MHB, tracking the fate of live cells in vivo, indicated that a tight lineage restriction was apparent [27]. While these observations may suggest that different lineage restriction mechanisms may operate within disparate species, this is unlikely given the overall conservation of MHB development within vertebrates. In support of this were further experiments in chick examining the role of a boundary-demarcating protein, *Lrrn1*, in MHB development. *Lrrn1* is a vertebrate orthologue of *Drosophila tartan/capricious*, transmembrane proteins that regulate cellular adhesion and are typically expressed in the midbrain, and not in the anterior hindbrain. Precisely how *Lrrn1* maintains lineage restriction is not known, but both loss of function in the midbrain, and overexpression of *Lrrn1* ectopically within the hindbrain resulted in the mixing of lineage-restricted cells [29]. Loss of *Lrrn1* led to the loss of isthmic organiser potential, whereas *Lrrn1* overexpression resulted in a loss of restriction of midbrain (*Otx2*) and hindbrain (*Gbx2*) markers, and subsequently of MHB organiser genes *Wnt1* and *Fgf8*. Whether the loss of isthmic organiser potential is specifically due to the loss of lineage restriction, or whether this observation is merely correlative, remains to be determined. However, it is known that *Lrrn1* interacts with FGF8 at the MHB [29], suggesting that irrespective of the importance of a lineage restriction boundary, this feedback loop may be an important element of MHB formation.

However, the establishment of a lineage-restricted boundary as well as expression of *Otx* and *Gbx* factors is not sufficient in itself to initiate the MHB programme or confer organiser activity onto the isthmic territory. For example, the primitive invertebrate lancelet, Amphioxus, shows expression of both *Otx* and *Gbx* factors in the presumptive MHB region, yet this region does not have organiser activity, and the MHB cascade is not activated over this boundary [30]. The converse experiments, examining the effects of the absence of *Otx* or *Gbx* gene expression on MHB formation, showed that mice lacking *Otx2* and/or *Gbx2* could initiate the MHB cascade, but the positioning of the MHB was located either anteriorly or posteriorly to its usual location [24]. These data clearly indicate that in addition to global positioning indicators within the neural plate, active instructional cues are also required to initiate the MHB cascade at the junction of the tightly patterned $Otx2^{+}$ and $Gbx1^{+}$ territories.

Stage 2: Induction

How then does the positional information conferred by *Otx2* and *Gbx1/2* expression translate to activation of the downstream MHB cascade? As both *Otx2* and *Gbx2* are thought to be transcriptional repressors [31], alleviation of repression may be a logical first step of MHB cascade induction. Induction of the MHB is characterised by the expression of members of the *Wnt*, *Pax*, *Engrailed* and *FGF* families [9, 10, 16, 17]. Seminal experiments in the chick indicated that FGF8 possesses inductive potential (Fig. 2), as overexpression in the forebrain could ectopically induce the MHB

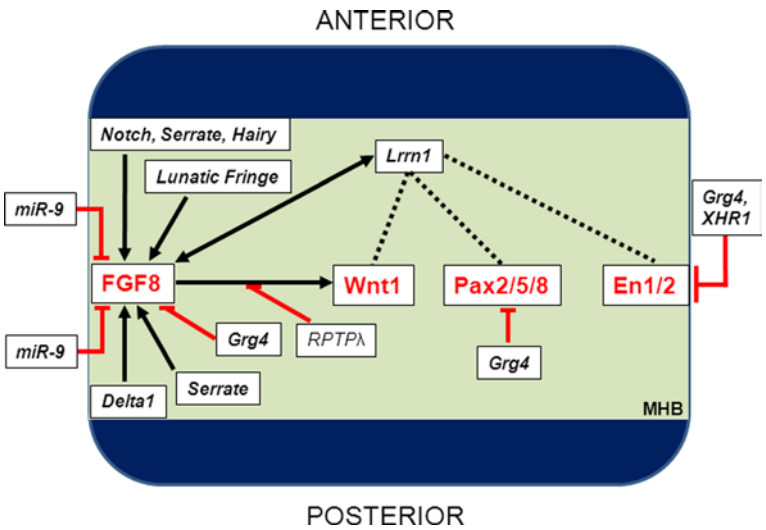

Fig. 2 Induction of the core-MHB cascade is regulated by several factors. Once the presumptive MHB territory has been established, genes of the *FGF*, *Wnt*, *Pax* and *Engrailed* gene families (the core-MHB cascade) are rapidly induced within this region. *FGF8* activity is critical, and expression and function of this molecule are regulated by *miR-9* and members of the *Notch* pathway (*Serrate*, *Hairy*, *Lunatic Fringe* and *Delta1*). The interplay between members of the core-MHB cascade begins, and this is regulated by *Lrrn1*, *Grg4* and *XHR1* at multiple levels within the cascade. Specific interactions between members have also been described, notably *RPTPλ*-mediated activation of *Wnt1* following *FGF8* stimulation and the transcription of *Engrailed 2a* by *FGF8*-mediated activation of *grhl2b*

cascade, leading to formation of both tectal and cerebellar tissues [32]. The formation of various isthmic proximal and distal neural structures is directly linked to the duration and extent of *FGF* activity [23], and vertebrate studies have shown that animals lacking *FGF8* do not form a cerebellum [33]. The critical importance of the *FGF8* pathway was again highlighted by novel work uncovering the role of the first known micro-RNA (miR) to regulate MHB patterning, miR-9 [34]. This study demonstrated that miR-9 in zebrafish was able to negatively regulate several members of the FGF pathway at the MHB, namely *fgf8*, *fgfr1* and *canopy1*, thereby suggesting a regulatory network operating at the level of the MHB. Supporting this theory was the finding that miR-9 could also directly inhibit a separate pathway regulating neurogenesis in and around the MHB via direct inhibition of another MHB marker gene, *her5*, a gene known to be critical in its own right for restricting neurogenesis at the MHB [35]. These data show that the gradients of gene expression, particularly FGF8, at the MHB must be critically regulated to ensure correct MHB development.

Although FGF8 may be thought of as the "master" MHB initiator, several lines of evidence indicate that it does not operate alone. The most convincing datum is that the MHB cascade is still initiated in animals lacking FGF8 [33, 36], although the expression of these genes is not subsequently maintained. Similarly, although the isthmus and cerebellum do not form, the tectum of zebrafish lacking FGF8 is largely unaffected [33], indicating that FGF8 is dispensable for at least some of the patterning functions attributed to the isthmic organiser. While the involvement of the *Pax*, *Eng* and *Wnt* families has been well described in the MHB cascade, it is clear that the transcriptional and signalling network that operates at the MHB comprises additional factors. For example, the *receptor protein tyrosine phosphatase* λ (RPTPλ) gene has been shown in the chick model to mediate the *FGF8*-induced activation of *Wnt1*, as overexpression of *RPTP*λ showed a decrease in *Wnt1* expression at the MHB, whereas RNAi-mediated knockdown of *RPTP*λ resulted in an expanded *Wnt1* domain [37]. Depending on the strength of FGF8 dosage, *RPTP*λ is either downregulated (at high doses) or upregulated (at low doses) by FGF8 [37], suggesting that this is a novel mechanism for the regulation of *Wnt1* expression in MHB development. Further work is necessary to determine the overall importance of this interaction to the MHB cascade, as FGF8-*RPTP*λ signalling appeared to be specific to the regulation of *Wnt1*; the expression of other MHB-markers including *En1*, *Pax2* or *Pax5* was not differentially regulated.

Studies in *Xenopus* have shown that *Xiro*, a member of the Iroquois family that is co-expressed with both *Otx2* and *Gbx2* before MHB induction, activates *Gbx2* expression in the hindbrain and appears to be critical for subsequent induction of FGF8 at the isthmic organiser [31], suggesting this gene family may be responsible for mediating the inductive organiser signal. Like *Xenopus Iro*, two members of the Iroquois family in zebrafish, *iro1* and *iro7*, are temporally expressed before either *gbx1* or *otx2*, and loss of function of these genes shows a marked reduction in downstream genes of the core-MHB cascade [38]. Other studies suggest that the *Notch* signalling pathway may be a crucial signalling cascade that regulates induction of the MHB programme [39]. The *Notch*-pathway genes *Serrate 1* (midbrain and hindbrain), *Serrate 2* (MHB), *Delta 1* (hindbrain), *Lunatic Fringe* (midbrain) and *Hairy1/2* (MHB) are expressed in and around the MHB following formation of the *Otx2*/*Gbx2* border, and critically, downregulation of *Notch* signalling at the MHB leads to a loss of *FGF8* and subsequent disrupted MHB formation [39], suggesting that Notch functions upstream of both *FGF*8 and the MHB cascade. Other pathways have also been discovered to operate upstream of induction of the MHB cascade. Additionally, transcriptional repression by *Grg4*, a vertebrate orthologue of *Drosophila groucho*, can negatively impact on expression of MHB genes [40], as does expression of dominant-negative forms of the HES-related gene *Xhr1* in *Xenopus* [41].

Experiments from the chick indicate that FGF8 can also regulate *Lrrn1*. Although *Lrrn1* is expressed within the neural tube during the onset of neurulation, its expression is strongly downregulated at the MHB as neurulation proceeds, although it continues to be expressed both anteriorly and posteriorly [29]. Downregulation of *Lrrn1* led to failure of MHB constriction formation, with concomitant loss of FGF8, suggesting that a feedback loop exists between these two molecules as MHB development proceeds. These data indicate that *Lrrn1* appears to be a novel gene required for both induction and maintenance of the MHB organiser. It will be interesting to see if in addition to its interaction with *FGF8*, *Lrrn1* can also directly interact with *Eng*, *Pax* or *Wnt* proteins in maintenance of the MHB (Fig. 2), and also whether its role is conserved in other vertebrates.

These data suggest that many of the genes outside the core MHB cascade that regulate induction of the MHB programme seem to do so via interaction with FGF8, consistent with the organiser function attributed to this molecule.

Stage 3: Maintenance

FGF8 is critical and indispensable for induction of the MHB genetic programme following establishment of the *Otx2*/*Gbx2* boundary, and this gene family continues to play a critical role as MHB development proceeds. The third phase of MHB development, that of maintenance, relies

largely on continued expression and interdependence of *FGF* with *Wnt*, *Eng* and *Pax* factors [42, 43]. Loss of any of these factors during the maintenance stage leads to a breakdown of the expression of the other factors, loss of isthmic stability and subsequent altered positional patterning (Fig. 3). A defining feature of genes operating as maintenance factors is the observation that loss of function does not compromise the initial induction of the MHB cascade, but the expression of the core MHB cascade genes is rapidly decreased as MHB formation proceeds. An example of failed maintenance comes from our laboratory, in experiments showing that zebrafish *grhl2b*, a vertebrate orthologue of Drosophila *grainy head*, acts downstream of *FGF8* to regulate the transcription of *eng2a* [44]. Although *eng2a* expression is reduced during the induction phase of MHB development, the expression of other core MHB patterning components, *pax2a*, *wnt1*, *her5* and *fgf8*, is unaffected during this stage. However, the expression of these genes was lost in *grhl2b* morphants during the maintenance phase, indicating that initial *eng2a* deficiency in this model impacts on MHB maintenance, putatively through loss of transcriptional interdependence. The activation of *grhl2b* by *FGF8* in contributing to the MHB cascade is putatively mediated by *Erk* signalling, as *FGF8* is responsible for phosphorylating *Erk* [45, 46], and studies in *Drosophila* have shown that *erk* activation phosphorylates *grainy head*, thereby influencing its expression and transcriptional activity [47].

Another example of disrupted maintenance is seen following the downregulation of *Wnt3a*, a factor not traditionally thought to be part of the core MHB cascade. *Wnt3a* loss does not impact significantly on the initial expression of the MHB factors, but these factors rapidly disappear because of failed maintenance [48]. Another critical factor for MHB maintenance appears to be *Lmx1b*, which is required for the continued maintenance of *Wnt1* during MHB ontogeny following induction by *FGF8* [49]; *En2* and *Pax2a* are also induced but rapidly lost following *Lmx1b* loss [50, 51]. Similarly, loss of the Hox and *Eng* co-factor gene *Pbx* leads to normal initiation of the MHB cascade, but complete failure to maintain signalling, leading to non-formation of the MHB constriction. [52]. A recent study has also suggested that *Autotaxin* (ATX) may be important in the maintenance of the MHB, as *ATX*-deficient mouse embryos exhibit aberrant cranial neurulation and MHB marker expression and increased apoptosis [53]. One caveat here is that *ATX* deficiency results in pleiotropic brain defects, and further conditional deletions may be required to independently assess the role that *ATX* plays in MHB maintenance specifically.

Another transcription factor that appears to be critical for maintenance of the MHB cascade is *pou2*. Zebrafish *spiel ohne grenzen* mutants, which lack this gene, exhibit loss of the isthmus in a manner consistent with defective

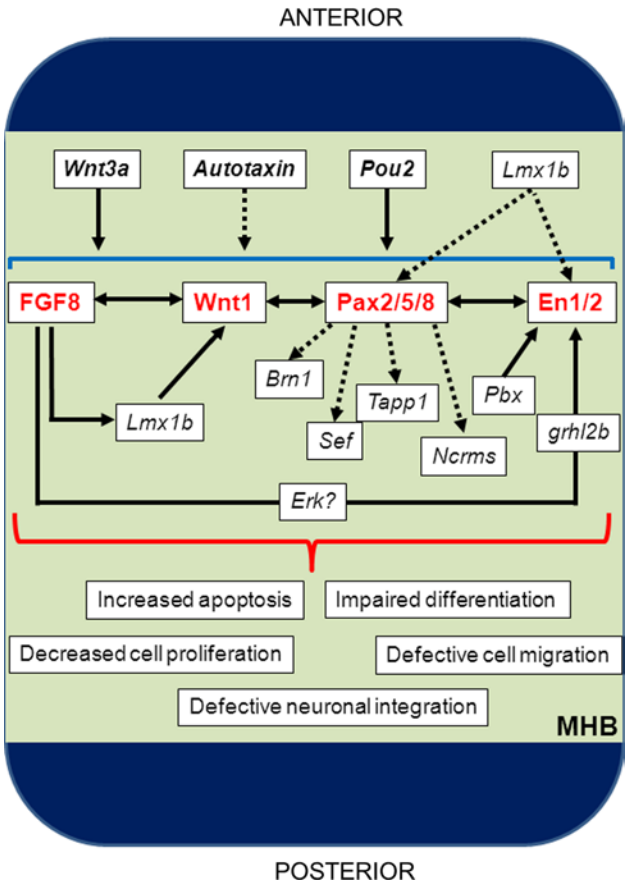

Fig. 3 Maintenance of MHB development relies on transcriptional interdependence between members of the core MHB cascade. During this stage of MHB development, functional interplay among *FGF8*, *Wnt1*, *Pax2/5/8* and *En1/2* is critical, as loss of any of these factors here leads to complete loss of isthmic stability. Several other factors are also postulated to regulate this cascade, namely *Wnt3a*, *Autotaxin* and *Pou2* which seem to globally regulate the cascade, as well as *Lmx1b*, *Pbx* and *grhl2b*, which exert their effects on specific genes within the cascade. In addition to regulation of *Wnt1*, *Lmx1b* may also regulate *Eng1/2* and *Pax2/5/8* in this context. Which pathways are activated by the interdependence of the core MHB cascade remain largely unknown, although some candidates (such as *Brn1*, *Sef*, *Tapp1* and *Ncrms*) are known to lie downstream of certain cascade members, in this case Pax2. Failure of MHB interdependence during this stage leads to a variety of pleiotropic defects with profound implications for organiser integrity, namely increased cell death, defective precursor cell proliferation, and impaired differentiation, migration and functional integration of more mature neurons within the midbrain and hindbrain territories

maintenance. The positioning of the *otx2*/*gbx1* boundary is not affected, and the initial expression of *fgf8* also does not differ from that of WT embryos [54]. These mutants also successfully initiate the expression of the other MHB cascade genes, albeit at somewhat reduced levels. However, the expression of all these markers, including *fgf8*, is not maintained, and therefore the MHB is not formed, suggesting a maintenance defect [54]. *Pou2* does not appear to be

an instructive signal for MHB formation, as fish mutants lacking either *fgf8* or *pax2* express *pou2* normally, yet still fail to form the MHB [54]. This suggests that the function of *pou2* may be to create a permissive environment for transcriptional MHB gene maintenance, either through direct regulation of MHB cascade genes or through maintenance of transcriptional interdependence via as yet unidentified factors.

While some of these novel genes required for MHB maintenance operate concurrently with the core MHB cascade to maintain the MHB, others undoubtedly lie downstream of this transcriptional network, as both *Pax2* and *Engrailed* are transcription factors. For example, microarray analysis of differentially regulated genes in the isthmus of *Pax2a*$^{-/-}$ mice revealed four novel factors—the transcription factors *Brn1* (*Pou3f3*), the intracellular signalling modifiers *Sef* and *Tapp1*, and the non-coding RNA *Ncrms* (as well as *En1*)—whose expression in the MHB was dependent on *Pax2a* [55]. However, the specific functions of these genes within the isthmic organiser (with the exception of *En1*) in maintaining the MHB remain unknown.

Maintenance of the MHB is also dependent on tightly regulated cell proliferation and migration, as well as differentiation into the functional neurons of both the midbrain and hindbrain [28]. Several genes within the MHB cascade, such as *FGF8* (as well as related family members FGF17 and FGF18) [56, 57], *Wnt1* [58] and *En1/2* [59], regulate cellular proliferation and/or survival independently of their patterning roles in the MHB cascade within the isthmic region; similarly, overexpression of *RPTP*λ also led to a reduction in progenitor proliferation [37]. Apoptosis undoubtedly also plays a role in failed maintenance of the MHB, as mouse deletion mutants lacking *Wnt1*, *FGF8* or *En1* display substantial apoptosis in and around the MHB, coinciding with failed or impaired development of either mid- or hindbrain structures [36]. In addition to disrupted MHB patterning, fish lacking *grhl2b* also displayed severe apoptosis in the MHB [44], consistent with studies that have shown that the *grhl2b* target, *eng2a*, is critical for the maintenance of neuronal survival [59, 60]. Taken together, data from these studies suggest that in addition to the critical role of the MHB genes in promoting correct fate acquisition, they may also be critical for ensuring cellular survival.

Stage 4: Morphogenesis?

Despite our knowledge of the specification and patterning of the neural tube, it is clear that the genes that control MHB positioning, induction and maintenance play only minimal roles in MHB morphogenesis. Although the genes of the core MHB cascade are critical for correct patterning and fating of the MHB, thereby allowing morphogenesis to proceed, we propose that morphogenesis should be thought of as a separate stage from that of MHB maintenance. Disruption of MHB morphogenesis in vertebrates with deficiencies in genes of the core MHB cascade is likely to be a secondary defect of impaired patterning. Rather than merely being a passive consequence of correct specification, however, recent data indicate that MHB morphogenesis is in fact actively regulated by genes that control cell shaping, cellular migration, cytoskeletal plasticity and cellular polarity (Fig. 4). In the zebrafish brain, the first morphological demarcation of the neural tube into a prospective folded structure appears at the 16 somite stage, where a small invagination between the midbrain and the hindbrain becomes apparent [27]. Defective polarity across the neuroepithelium (apico-basal polarity) still allows the correct neural boundaries to be specified (as seen by marker expression), but MHB folding and/or ventricle inflation is severely disrupted [2], indicating that morphogenesis in this context occurs independently from induction of the MHB cascade. Preliminary data from our laboratory suggest that *grhl2b* may interact with several members of the Wnt/PCP pathway in regulating MHB morphogenesis at this stage (Dworkin, unpublished), although the precise molecular mechanisms underpinning this failed folding are currently unknown.

Further data indicating that neural tube morphogenesis is actively regulated by non-neural patterning genes comes from early experiments examining deletion of the transmembrane adhesion molecule N-cadherin in mouse [61] and zebrafish [62]. The morphology of the entire neural tube is dismorphic and MHB formation in the fish is severely disrupted. Importantly, however, the expression of MHB genes such as *wnt1* or *pax2* is not affected [62], highlighting the presence of morphological defects in the context of correct MHB specification and patterning. A similar phenomenon is seen in zebrafish mis-expressing the transcription factor CREB [63], whereby overexpression of both a constitutively active (CREB-FY), or inactive (CREB-M1) form of CREB led to defects in MHB morphology, but no loss of the classical MHB markers, further supporting a separation of these processes.

Supporting data showing that the processes of MHB patterning and morphogenesis are dissociable came from investigation of deletion mutants of the miRNA processing molecule, Dicer. Maternal-zygotic (mz) Dicer-deleted zebrafish (which lack mature processed miRNAs) showed severe defects in neural morphology, particularly at the level of the MHB, without a disruption in expression of the MHB markers *pax2a* or *eng2* [64]. This group further analysed which specific miRNAs contributed to the morphology loss and found that the MHB defects in the mz-Dicer mutants could be rescued through injection of the *miR430* miRNAs. These data show that miRNAs can specifically regulate MHB morphology, and as miRNAs generally act to repress transcription or translation of targets, it suggests

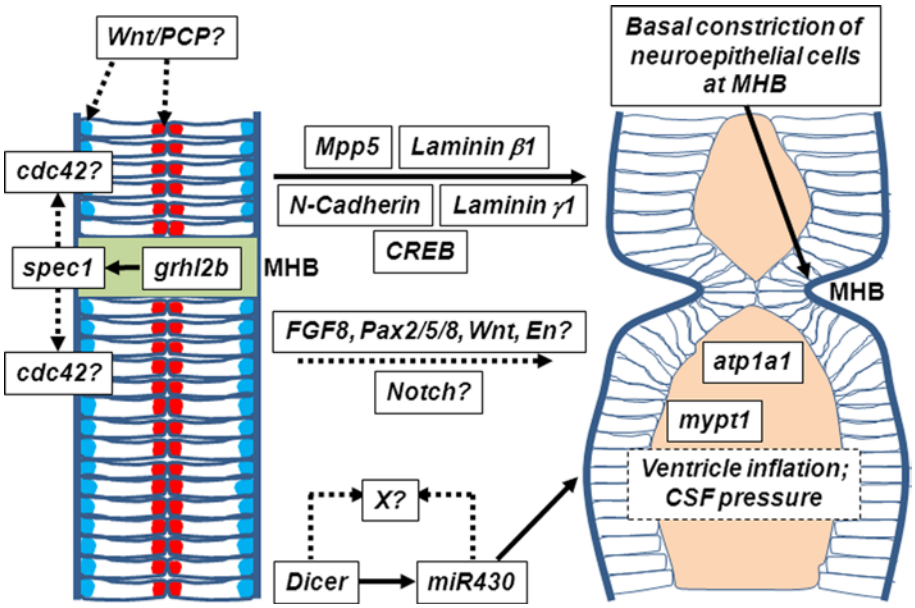

Fig. 4 MHB morphogenesis is genetically separable from the actions of genes of the core MHB cascade. The Wnt/PCP pathway plays critical roles in the establishment of planar polarity of the neural plate during neurulation and may also regulate correct apico-basal polarity of neuroepithelial cells during morphogenesis of the MHB. Formation of the MHB constriction is likely to involve regulation of cytoskeleton plasticity, regulated by *cdc42*, following *grhl2b*-mediated activation of *spec1*. Several classes of adhesion molecules, junctional complex and extracellular matrix proteins, such as laminin γ1, laminin β1, N-cadherin and Mpp5, as well as the transcription factor CREB, also play a role in shaping the MHB independently of the core-MHB cascade; precisely what active or instructive role the core-MHB cascade plays during this stage is not clear. Loss of Dicer leads to aberrant morphology of the neural tube, a defect that can be rescued by *miR430*. However, the identity of other mature miRNAs that are lost in Dicer mutants and direct genetic targets of *miR430* (both indicated by an "X?" on the figure) remain to be elucidated. Many factors, both genetic (e.g. *Atp1a1*, *mypt1*) and cell-intrinsic (cytoskeleton fidelity, cell polarity), are likely to play critical roles during basal constriction of MHB cells during morphogenesis, and extrinsic forces, such as ventricle inflation and cerebrospinal fluid (CSF) pressure, may play a role in allowing full ventricular expansion and MHB morphogenesis

that an over-abundance of some factors, or a disruption in the tightly controlled homeostatic regulatory network operating at the MHB, is responsible for the observable defects. Future work will undoubtedly focus on both the specific targets of miR430 and other miRNAs regulated by Dicer in this context.

Further work has come from the laboratory of Hazel Sive, who showed that during the morphogenesis phase of MHB development, cells at the MHB shorten to approximately 75 % of the length of their neighbours and concomitantly undergo a process termed "basal constriction", whereby these cells narrow at their basal side, closest to the developing ventricular lumen [65]. This appears to be an active process, rather than passive "pushing" from the cerebrospinal fluid (CSF) following ventricle formation, as ventricle-inflation-deficient mutants *snakehead* (*atp1a1*) and *nagie oko* (*Mpp5*) form a defined basement membrane constriction. However, presumptive folds are visible within the neural tube of snakehead, but no such morphological distinction appears in *nagie oko* [66], suggesting that mechanisms of tube morphology are distinct from those of ventricle development. In contrast to these two ventricle-inflation mutants, the laminin-deficient mutants *sleepy* (laminin γ1) and *grumpy* (laminin β1) do not form a tight constriction, thereby defining a novel role for the basement membrane, and particularly laminin proteins, in MHB morphogenesis. Furthermore, this process again appears to be independent of the first three stages of MHB patterning, as MHB-induction genes such as *eng2a* and *pax2a* are expressed normally in laminin mutants [65]. A requirement for other patterning genes such as *wnt1*, *fgf8* or *her5* has not been reported in these mutants; however, thereby not entirely excluding the MHB cascade from impacting on this process.

Work from our laboratory has shown that in addition to its transcriptional roles in the induction and maintenance of *eng2a*, loss of function of *grhl2b*, leads to a disruption in the characteristic shaping of the MHB folds [44]. Critically, it appears as though the folding defects occur independently of *eng2a* downregulation, as both our studies and previous data [52] did not provide evidence that *eng2a* regulates MHB morphogenesis. Rather, we identified a second *grhl2b* target gene, *spec1* (*small protein effector of cdc42*), which when downregulated phenocopied the non-folded MHB morphology seen in *grhl2b* morphants. These data were particularly interesting in light of the fact that expression of the core MHB

cascade in these morphants was not disrupted, and the *spec1*-morphants did not exhibit significantly increased apoptosis at the MHB region [44]. The folding defects following loss of *spec1* may be due to a defect in cell polarity caused by disruption of *cdc42*, a small molecule that influences actin accumulation in polarised T cells [67–69]. SPECs may generally be involved in *cdc42*-mediated polarity establishment in other cell types and may also regulate cell shape [67]. Interestingly, blocking of the Notch signalling cascade through electroporation of an inhibitor ligand into the MHB also resulted in a mis-folded neuroepithelium together with loss of MHB-gene expression, suggesting that like *grhl2b*, *Notch* signalling may also affect both MHB patterning and morphogenesis programmes via independent mechanisms [39]. Taken together, these studies demonstrate that specific defects in MHB morphogenesis can be caused through dysregulation of non-MHB cascade genes. Importantly, these defects are seen even in the context of correct specification and patterning by the MHB cascade, supporting the idea that MHB morphogenesis is in itself an active process, albeit one that requires that the neural territory be correctly patterned.

Conclusions

Despite these recent advances in the field, deciphering the genetic complexity of the regulatory network controlling MHB development remains a work in progress. In addition to a candidate gene approach, identification of factors that regulate any, some or all of the four stages of MHB development described in this review will most likely come from both forward genetic and mapping analyses of mutagenesis screens and reverse genetic studies of genes that are expressed at the MHB. The zebrafish forms a crucial model here, owing to the number of phenotypic mutants identified with MHB defects, where the responsible gene(s) remain unknown [70, 71], and conversely, also because of the large number of genes that have been shown to be expressed at the MHB (detected by high-throughput in situ hybridisation screening [72]), whose function in isthmic patterning remains unknown. These studies will further serve to shape our knowledge of how the MHB is formed and could provide valuable therapeutic insights for the multitude of human neural patterning defects.

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