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Nodal signalling and asymmetry of the nervous system

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The role of Nodal signalling in nervous system asymmetry is still poorly understood. Here, we review and discuss how asymmetric Nodal signalling controls the ontogeny of nervous system asymmetry using a comparative developmental perspective. A detailed analysis of asymmetry in ascidians and fishes reveals a critical context-dependency of Nodal function and emphasizes that bilaterally paired and midline-unpaired structures/organs behave as different entities. We propose a conceptual framework to dissect the developmental function of Nodal as asymmetry inducer and laterality modulator in the nervous system, which can be used to study other types of body and visceral organ asymmetries. Using insights from developmental biology, we also present novel evolutionary hypotheses on how Nodal led the evolution of directional asymmetry in the brain, with a particular focus on the epithalamus. We intend this paper to provide a synthesis on how Nodal signalling controls left–right asymmetry of the nervous system.

This article is part of the themed issue 'Provocative questions in left–right asymmetry'.

1. Introduction

Nodal is a secreted ligand belonging to the transforming growth factor-ß (TGF-ß) superfamily that fulfils many essential functions during embryonic development. It acts as a morphogen during body axis determination, controls the specification and patterning of the germ layers, regulates pluripotency in embryonic stem cells and plays a pivotal role in left–right (L–R) axis determination (reviewed in [1–4]). Among these functions, the role in L–R patterning has attracted the attention of both developmental and evolutionary biologists as asymmetric Nodal expression is widespread and linked to the ontogeny of various types of structural asymmetries. In recent years, efforts have been made to elucidate the evolutionary and developmental functions of Nodal in the establishment of body and visceral organ asymmetries. However, comparably less information and discussion are available about its role in nervous system asymmetry. This case is striking because Nodal and nervous system asymmetry are both evolutionarily conserved and serve critical functions in metazoans. In this paper, we review and discuss how Nodal controls the ontogeny of nervous system asymmetry using a comparative developmental approach. By focusing on the nervous system of ascidians and fishes, we construct a conceptual framework to approach the study of Nodal function as asymmetry inducer and laterality modulator. We also present evolutionary hypotheses on how directional asymmetry emerged in the brain under the control of asymmetric Nodal signalling, with a particular focus on the epithalamus.

2. Nodal and nervous system asymmetry across metazoans

Since the initial description of brain asymmetry in humans [5], it has become clear that anatomical and functional differences between the left and right sides of the nervous system are distributed widely among animals. Also, nervous system asymmetry is proposed to provide advantages for neural processing and

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behavioural performance (for recent reviews, see [6–8]). Remarkably, several cases of nervous system asymmetry are linked with the asymmetric expression of Nodal during the development of deuterostomes and protostomes (figure 1). In cephalochordates, besides the prominent left-sided asymmetric locations of the mouth and entire pharyngeal region, the arrangement of the bilaterally paired somatic neurons and the peripheral nerves associated with the somites are also asymmetric. Importantly, these asymmetries are preceded and controlled by asymmetric Nodal expression (figure 1*a*) [10]. In the same animals, an asymmetric funnel-shaped lobe of the right ventral margin of the brain connects with the right-located Hatschek's pit through the infundibulum that descends along the right side of the notochord [11]. However, the involvement of Nodal in this asymmetry of the adult brain is still unknown (figure 1*a*, #). In urochordates, the lumen of the sensory vesicle (SV) and the location of the photoreceptor cell associated with the ocellus are both on the right side and are controlled by an early asymmetric expression of Nodal in the left epidermis and SV (figure 1*b*) [12,13]. In craniates, the dorsal diencephalic epithalamic region is asymmetric in a wide range of species, including fishes (agnathans, chondrichthyans and teleosts), amphibians and amniotes (e.g. lizards, chick and rodents) (reviewed in [14]), and recent reports also extend this asymmetric feature to humans [15,16]. Asymmetries comprise the bilaterally paired habenular nuclei (Hb) and some midline components of the pineal complex, and show significant variations in morphology, and in the extent and sidedness of L–R differences among species [14,17–19]. Importantly, developmental studies in fishes reveal that epithalamic asymmetry is preceded and controlled by the asymmetric expression of Nodal (figure 1*c–e*) [20–22] (see details below in §4). In echinoderms, Nodal is expressed in the right ciliary band, specifically in a cluster of prospective neurons located on the right side of the apical tuft (figure 1*f*) [23]. However, the presence of asymmetry in this neural structure is yet to be determined. In the hemichordate *Saccoglossus kowalevskii*, Nodal is asymmetrically expressed in a right ectodermal domain along the entire dorsoventral axis (figure 1*g*), in the area separating the putative proboscis from the collar [24]. This large domain of Nodal expression is consistent with the diffuse pattern of neurogenesis observed in the ectoderm of this animal [25]. Although nervous system asymmetries have not been described, it is important to note that hemichordates have a nervous collar cord formed by neurulation [26] and that the proboscis expresses neural patterning genes also expressed in the chordate forebrain [27]. Finally, in snails (*Lymnea stagnalis*), the parietal and visceral ganglia are fused only on one side of the nervous system, leaving the contralateral visceral ganglion unpaired (figure 1*h*). The side of fusion corresponds to the chirality of the animal shell such as that in dextral individuals only the right parietal and visceral ganglia are fused [28]. Although further studies in snails are needed to determine a possible direct control of Nodal signalling on nervous system asymmetry, it has been shown that the chirality of the shell is dependent on the side of asymmetric Nodal expression [29,30]. It is particularly interesting that asymmetries of the nervous system and chirality of the shell also match a behavioural asymmetry in snails. The mating behaviour of this animal involves a circle made by the male snail over the shell of the female: dextral individuals make an anti-clockwise circle while sinistral snails turn in the opposite direction [28]. Such association in the direction of molecular, morphological and behavioural asymmetries described in snails is remarkable, and shares similarities with the lateral asymmetries described in the teleost zebrafish. In this species, larvae with a complete reversal of molecular (Nodal) and morphological (visceral and brain) asymmetries also show a reversal in the turning preference in a mirror-view behavioural test [31].

3. Context-dependency of Nodal functions in nervous system asymmetry

While comparative studies highlight the evolutionarily conserved role of Nodal in L–R patterning, they also unveil the diversity of mechanisms used by Nodal and its effector Pitx2 to control the development of L–R asymmetry in different organs and the same organ among species. One possible reason for such complexity resides in the recognized context-dependency of Nodal function (reviewed in [1,3]). It is known, for example, that Nodal signalling can maintain pluripotency and prevent differentiation to neuroectoderm in human embryonic stem cells (hESC) and mouse epiblast-derived stems cells (mEpiSC) [32] while it is necessary for endoderm differentiation in pluripotent cells and the mouse gastrula embryo [3,33]. Similarly, Nodal can inhibit cell migration, invasion and proliferation in human trophoblasts [34,35] while it promotes the opposite in human glioma and breast cancer cells [36,37]. Such divergent mechanisms create the necessity of developing a conceptual framework to help recognize L–R patterns and assess the phenomenon in a meaningful manner. For this, it is mandatory to categorize not only the Nodal function itself but also the context (tissue/organ) in which Nodal exerts its asymmetric function.

It has been proposed that Nodal acts as an instructive signal to produce asymmetry, i.e. it induces the acquisition of a particular identity in the zone in which it is active, in most cases left character owing to its common left-sided expression domain. Howard Holtzer [38] proposed the term *instructive* interaction in contrast with *permissive* interaction, being these two primary modes of cell/tissue induction. However, in the study of L–R asymmetry, the original sense of the term has historically changed its emphasis and become slightly confusing. Also, the term 'permissive interaction' is not used in works of asymmetry and it is possible to recognize that, at least conceptually, Nodal has to be instructive at some level of biological organization to exert its function. Thus, we propose explicitly to leave aside the instructive definition of Nodal function in favour of another conceptual dichotomy: Nodal as an inducer of asymmetry *per se* versus Nodal as a modulator of asymmetry laterality. In our opinion, this categorization has a higher explanatory power because the two different conditions can be distinguished in a sharper manner through the analysis of experimental data using loss (Nodal absent) and gain (Nodal bilateral) of function approaches.

Loss and gain of Nodal function have been described in the nervous system of animal models such as ascidians and fishes. In ascidian embryos, the L–R patterning of the SV and the two sensory organs it contains (ocellus and otolith) is dependent on asymmetric Nodal expression (figure 2). In *Ciona intestinalis*, the ocellus is surrounded on the right side by a photoreceptor cell expressing opsin and arrestin [13]. It has been shown that Nodal expression inhibits photoreceptor fate for the cells on the left side through repression of the *Rx* gene [13]. Accordingly, the abrogation of Nodal function (Nodal absent) results in loss of

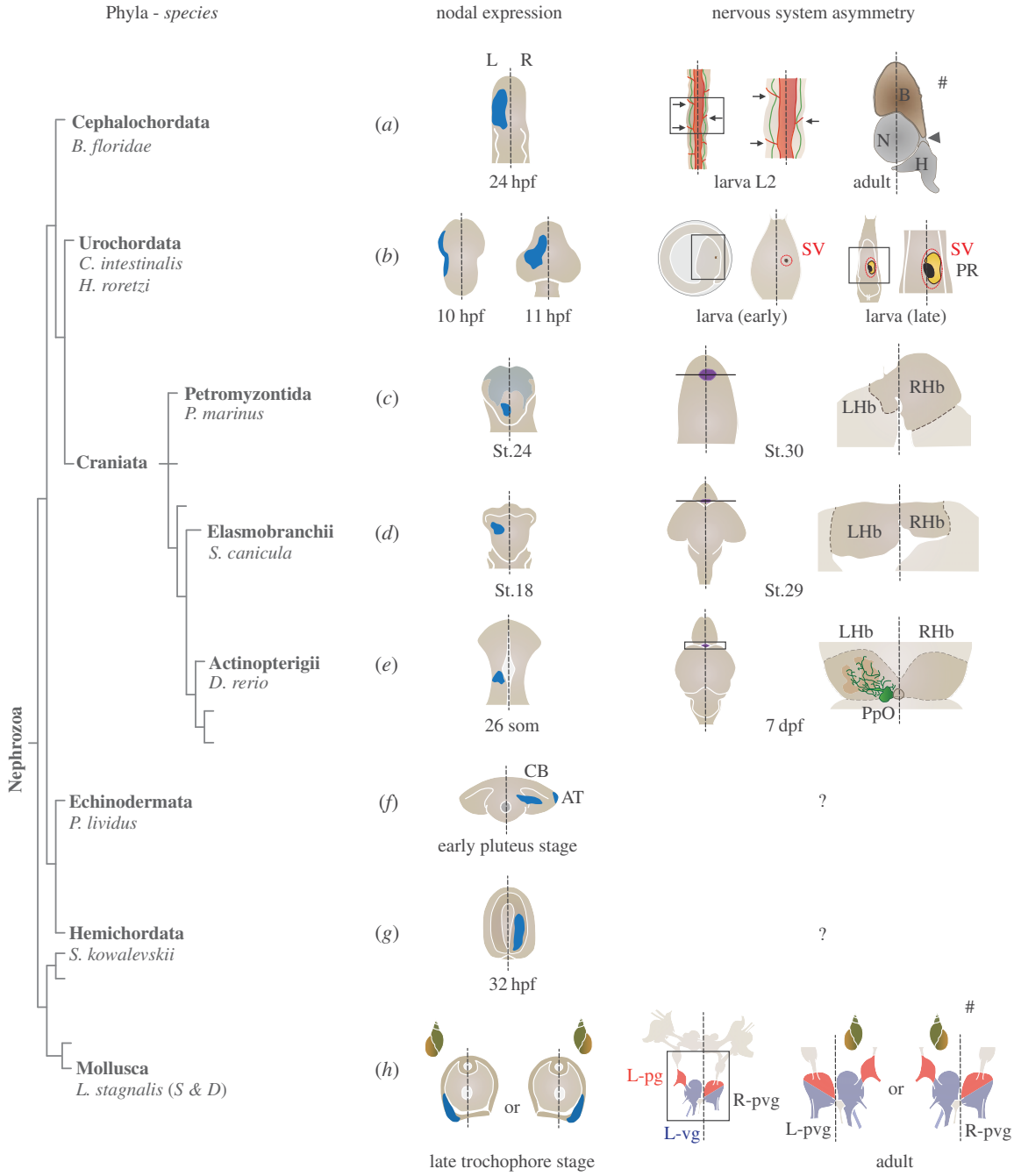

Figure 1. Phylogenetic distribution of asymmetric Nodal expression associated with the development of nervous system asymmetries across metazoans. For each phylum and species, the asymmetric pattern of Nodal expression is shown on the left while the observed morphological asymmetry of the nervous system is on the right. (*a*) Nodal expression in the left side of the future head of the cephalochordate *Branchiostoma floridae* precedes and controls the development of asymmetries in the bilaterally paired somatic neurons and peripheral nerves (arrows) associated with the somites. The role of Nodal in the asymmetry of the adult brain involving the right infundibulum (arrowhead) and related brain lobe (B) is yet to be determined (#). Notochord (N), Hatcheck's pit (H), hours post-fertilization (hpf). (*b*) Nodal expression in the left epidermis and sensory vesicle (SV) of the ascidian embryo precedes and controls the right-sided asymmetric positioning of the SV lumen and photoreceptor cell (PR, yellow). *C. intestinalis, Ciona intestinalis*. (*c–e*) Nodal expression in the left epithalamus of different craniate species precedes and controls the development of asymmetries in the bilaterally paired habenular nuclei (Hb) and the midline-unpaired parapineal organ (PpO). The right Hb (RHb) is larger than the left Hb (LHb) in the lamprey *Petromyzon marinus* (*c*) while the opposite is seen in the elasmobranch *Scyliorhinus canicula* (*d*) and in the teleost zebrafish (*Danio rerio*) (*e*). In zebrafish, the PpO also locates on the left side of the epithalamus. Somites (som). (*f*) In the sea urchin *Paracentrotus lividus*, Nodal is expressed in the right ciliary band (CB), in a cluster of prospective neurons located on the right side of the apical tuft (AT). However, this expression has not been yet related to nervous system asymmetry. (*g*) In the hemichordate *Saccoglossus kowalevskii*, Nodal is asymmetrically expressed in the right ectoderm along the entire dorsoventral axis, a site where diffuse neurogenesis occurs. However, asymmetries in this region have not been described. (*h*) In snails, sided expression of Nodal in either the left or right ectoderm controls the chirality of shell rotation. Chirality of shell rotation, in turn, associates with the side where the parietal and visceral ganglia will fuse in the nervous system of *Lymnaea stagnalis*. Direct control of Nodal in this type of nervous system asymmetry has yet to be examined (#). Dextral (*D*), left parietal ganglia (L-pg), right parietal ganglia (R-pg), left visceral ganglia (L-vg), right visceral ganglia (R-vg), left parietal-visceral ganglia (L-pvg), right parietal-visceral ganglia (R-pvg), sinistral (*S*). Figures correspond to dorsal views with anterior to the top and left to the left, with the exception of the Hb of *P. marinus* and *S. canicula*, where the Hb is shown in cross-sections of the brain, with dorsal to the top and left to the left (right panels of (*c*) and (*d*), respectively). The stage of analysis is shown at the bottom of each panel. '?' represents an unknown feature (no information available). The animal phylogeny of this figure is based on [9]. See the text for additional details.

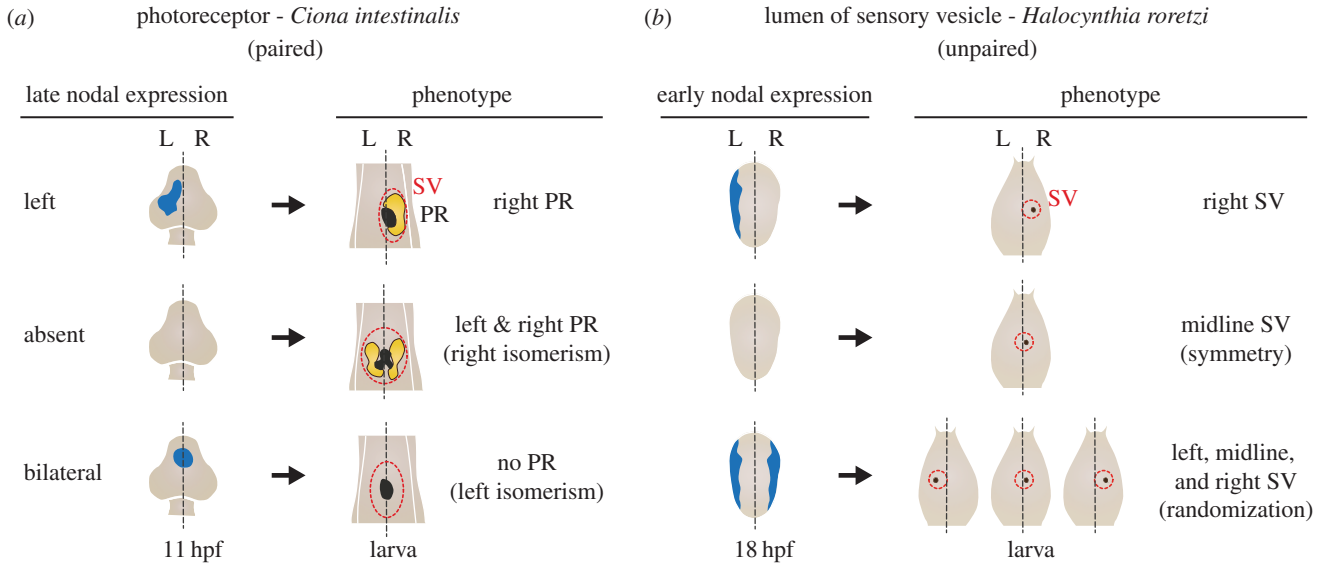

Figure 2. Nodal and nervous system asymmetries in ascidians. Two different types of asymmetry in the ascidian nervous system where Nodal acts as asymmetry inducer. For each panel, the pattern of Nodal expression in the left sensory vesicle (SV) and epidermis is shown on the left, while the resulting asymmetric phenotype is on the right. The results of three experimental conditions are compared: wild-type left-sided Nodal expression (top), pharmacological inhibition of Nodal function that is equivalent to absent Nodal (middle) and gain of Nodal function that induces bilateral Nodal expression (bottom). (*a*) Asymmetry of the photoreceptor cell (PR, yellow) surrounding the ocellus (black), in the SV (red circle). The PR domain can be regarded as a bilaterally paired structure concerning the bilateral competence to form PR cells. Asymmetry involves L–R differences in PR differentiation. Nodal inhibits photoreceptor cell fate; therefore the PR in the wild-type is located on the right side. Loss and gain of Nodal function result in right and left isomerism, respectively. Hours post-fertilization (hpf). (*b*) Asymmetry of the lumen of the SV. The lumen of the SV (red circle) containing the ocellus (black dot) can be regarded as a midline-unpaired structure of initial midline position. Asymmetry of the SV involves clockwise rotation of the neural tube in which Nodal is presumably involved. Asymmetric Nodal expression probably promotes morphogenetic transformations that result in neural tube rotation. Therefore, loss of Nodal function generates a midline-positioned SV (symmetry). By contrast, differences in Nodal expression between left and right sides, which are normally observed in bilateral Nodal expression, are presumably responsible for generating a randomized phenotype that combines left-, midline- and right-positioned SV. See §§3 and 4 for additional details.

asymmetry in the form of bilateral presence of photoreceptors while bilateral Nodal in the SV leads to the absence of photoreceptors [13]. Thus, loss and gain of Nodal function both induce isomerism (right and left, respectively) of photoreceptor differentiation revealing an asymmetry inducer role of Nodal signalling (figure 2*a*).

Another observed asymmetry of the ascidian nervous system is the right-sided position of the lumen of the SV (figure 2*b*). Pharmacological inhibition of Nodal signalling results in a midline-positioned SV, thus supporting an asymmetry inducer role of Nodal [13,39]. However, bilateral Nodal expression in *Halocynthia roretzi* leads to randomization in the positioning of the SV (figure 2*b*) [39]. At first sight, this finding appears inconsistent with an asymmetry inducer role of Nodal and rather suggests a role as laterality modulator. However, such apparent contradiction, and the divergent results induced by the gain of Nodal function in photoreceptor and SV lumen asymmetries, can be resolved if we take into account the nature of the tissue/organ where Nodal exerts its function.

4. Nodal as asymmetry inducer in bilaterally paired and midline-unpaired neural structures

A straightforward macroscopic categorization can distinguish two main types of structures/organs involved in L–R patterning: bilaterally paired and midline-unpaired [6]. In bilaterally paired organs and circuits, the same components are present on both sides of the midline and do not fuse or directly connect. Even if long-range or indirect supra-organ interactions are not excluded and can operate during embryonic development, left and right portions of a bilaterally paired structure are not mutually dependent. Thus, they can develop their genetic and cellular programmes autonomously and undergo differential or similar development and morphogenesis. The induction of isomerism in these structures in contexts of symmetric Nodal activity (absent or bilateral) is thus the expression of this underlying developmental independence, which allows mirror-image duplication of structures that usually diverge under the modulation of asymmetric Nodal signalling. Such independence of left and right sides also implies that Nodal has no other possibility than to function as asymmetry inducer in bilaterally paired structures, at least when acting directly on one side and not through the mediation of an additional midline-unpaired structure (see §5). We have already mentioned the example of the ascidian ocellus photoreceptor, which can be regarded as a bilaterally paired structure concerning the bilateral position of competent cells sensitive to *Rx* gene function [13]. Another example of a bilaterally paired structure that displays isomerism when Nodal becomes absent or bilateral is the Hb of lampreys and catsharks (figure 3*a*,*b*) [22], and for some types of asymmetries the Hb of zebrafish (i.e. parapineal-independent asymmetries; see §5 and figures 3*c* and 4*a*) [52]. Also, symmetric Nodal signalling can induce isomerism in non-neural structures such as the adult rudiment of the sea urchin [53], the gonads of the female chick [54] and the murine lungs [55,56]. For the Hb of lampreys and catshark, it is remarkable that left-sided Nodal expression associates with contrasting morphological phenotypes, i.e. a smaller size of the left Hb compared with the right Hb in lampreys, and the opposite phenotype in catshark (figure 3*a*,*b*) [22]. The mechanisms responsible for this different behaviour are unclear. However,

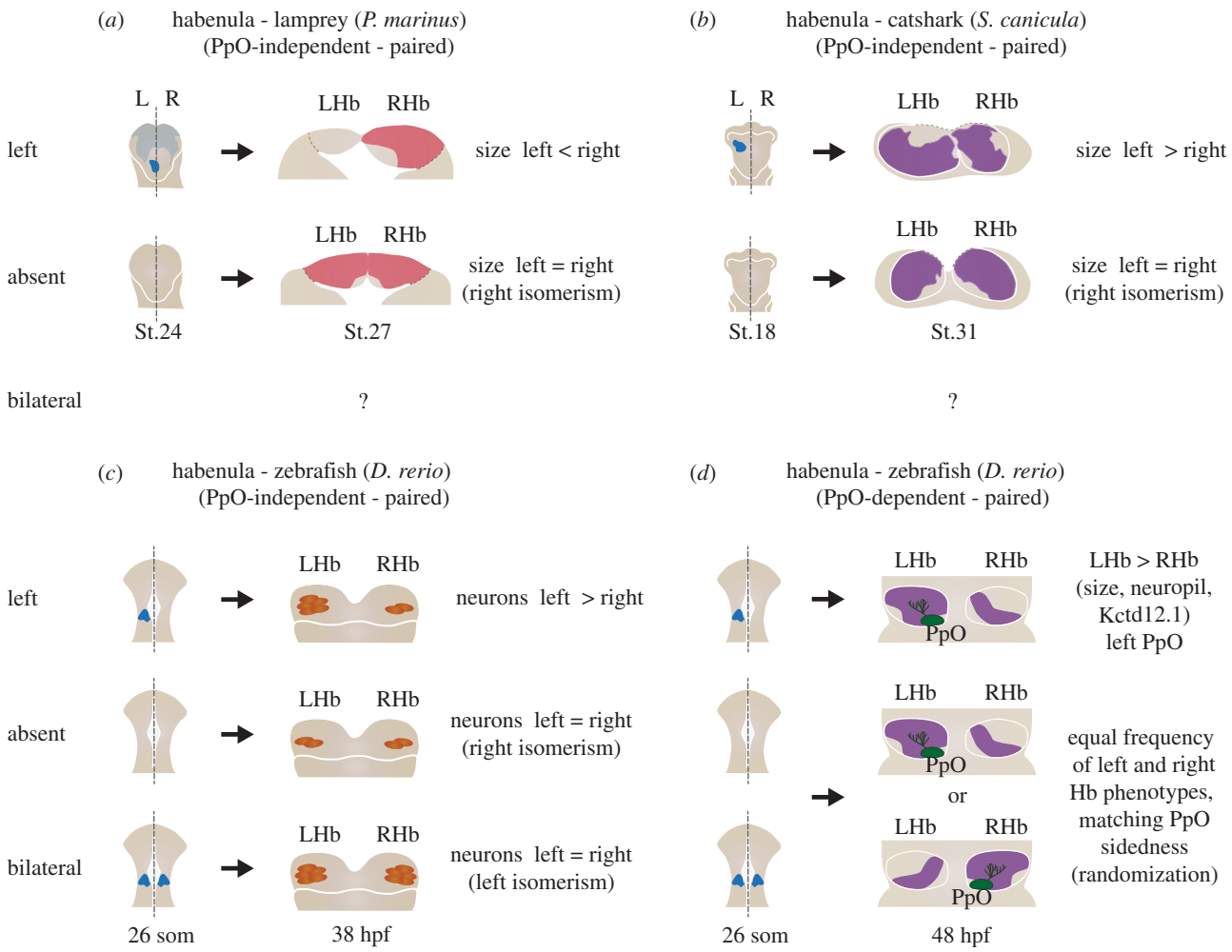

Figure 3. Nodal and nervous system asymmetry in the epithalamus of fishes. Asymmetries in the epithalamic bilaterally paired habenular nuclei (Hb) in different species of fishes in which Nodal acts as asymmetry inducer and/or laterality modulator. For each panel, the pattern of Nodal expression in the prospective epithalamus is shown on the left, while the resulting habenular phenotype is on the right. The results of three experimental conditions are shown: wild-type left-sided Nodal expression (top), genetically induced or pharmacologically mediated inhibition of Nodal function (absent Nodal) (middle), and bilateral Nodal expression (bottom). Asymmetries of the Hb can be classified into two types according to their dependency on the midline-unpaired parapineal organ (PpO). In zebrafish, the PpO can direct the development of a sub-type of habenular asymmetries (PpO-dependent asymmetries) (*d*). Other subtypes of habenular asymmetries in zebrafish (*c*), and the asymmetries described in the Hb of lampreys and catshark (*a*,*b*) are independent of the PpO (PpO-independent asymmetries). (*a*) Asymmetry in the Hb of the lamprey *Petromyzon marinus*. The right Hb is usually larger than the left Hb and expresses phospho-ERK (red). Nodal functions as asymmetry inducer. Therefore, loss of Nodal signalling (Nodal absent) induces right isomerism with both sides of the Hb showing a large size and expressing phospho-ERK. Gain of function experiments (Nodal bilateral) have not been performed in this species. (*b*) Asymmetry in the Hb of the catshark *S. canicula*. The left Hb is usually larger and shows a more-extended pattern of Kct12b expression (purple) compared to the right Hb. Nodal functions as asymmetry inducer. Therefore, absence of Nodal results in right isomerism, with both sides of the Hb showing a small size and a right-type pattern of Kct12b expression. Gain of function experiments (Nodal bilateral) have not been performed in this species. (*c*) Parapineal-independent asymmetry of the Hb in zebrafish (*Danio rerio*). At early stages of development, the left Hb contains more cells expressing elav3/HuC (orange) than the right Hb. Nodal functions as asymmetry inducer. Therefore, loss and gain of Nodal function induce right and left isomerism, with both sides of the Hb showing a symmetric pattern of elav3 expression of right and left characteristics, respectively. Hours post-fertilization (hpf), somites (som). (*d*) Parapineal-dependent asymmetry of the Hb in zebrafish. The parapineal organ (PpO, green) typically locates on the left side and induces the elaboration of asymmetry in the Hb. This sub-type of habenular asymmetry is characterized (among other features) by a larger left Hb with an extended Kctd12.1 expression domain (purple) compared with the right Hb. Nodal functions as laterality modulator in this type of habenular asymmetry, although indirectly, through modulating the laterality of PpO asymmetric migration. Therefore, loss (Nodal absent) and gain (Nodal bilateral) of function approaches both unmask an antisymmetry of the PpO (and as a consequence induce antisymmetry of the Hb), with equal frequencies of left (50%) and right (50%) asymmetry phenotypes in the population. See §4 for additional details.

this finding highlights the context-dependency of Nodal function in L–R asymmetry of the nervous system. A possible direct role of Nodal in other asymmetries of bilaterally paired neural structures such as the cephalochordate brain lobe associated with the infundibulum and the asymmetrically fused parietal/visceral ganglia of snails have yet to be determined (figure 1*a*,*h*). Should this be the case, we predict that conditions of absence and bilateral Nodal signalling should result in right and left isomerism, respectively.

In contrast with bilaterally paired organs, midline-unpaired structures are characterized by direct contact and interdependence between left and right sides. Midline-unpaired organs usually form after fusion of bilateral precursors around the midline where they finally give rise to a coherent unity of directly interacting cells/domains. In this type of structure, the identification of isomerism after loss or gain of Nodal function is feasible if the asymmetry consists of a sided regionalization related to differential cell fate. However,

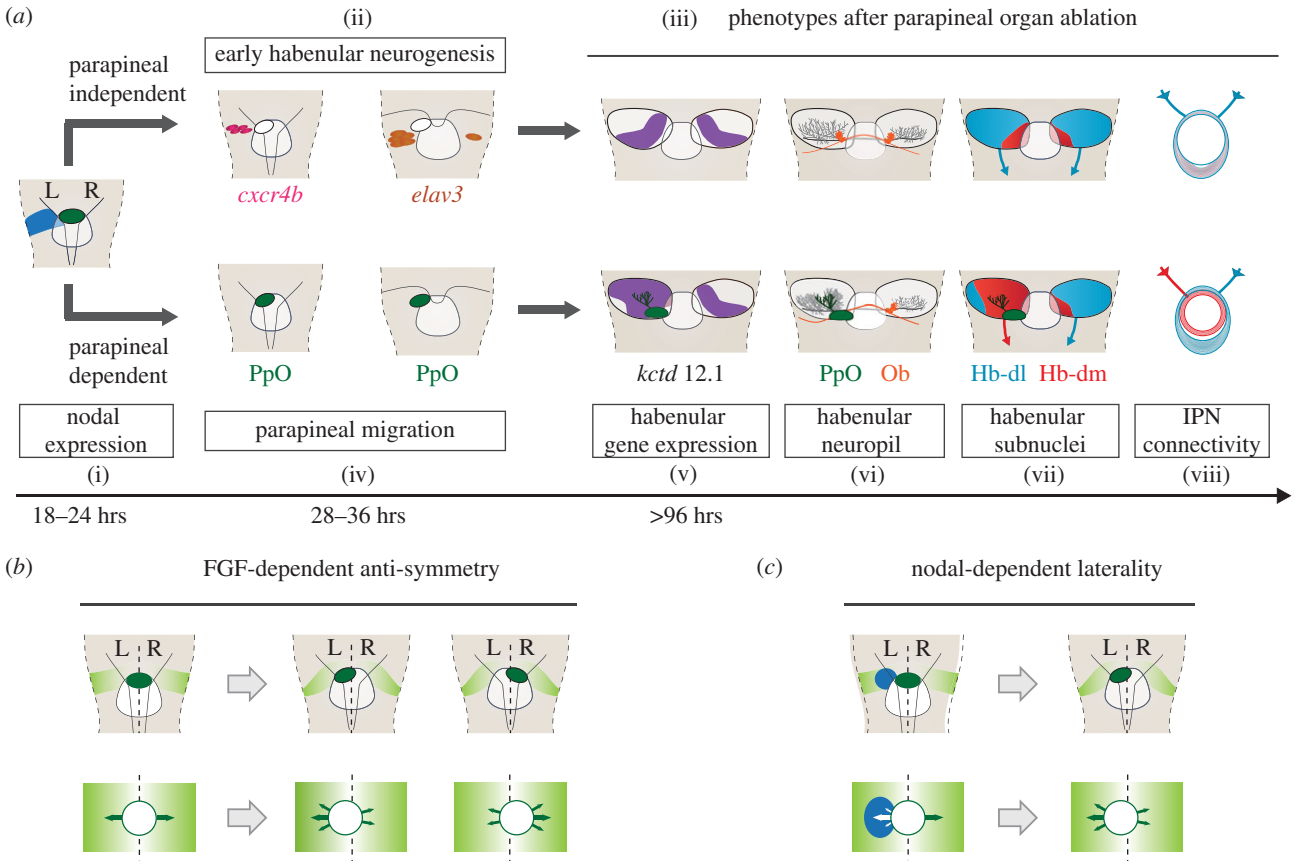

Figure 4. Ontogeny of epithalamic asymmetry in zebrafish. (*a*) Developmental paths leading to the development of asymmetries in the Hb. Two distinct paths classified according to their dependence on the parapineal organ (PpO) develop in parallel, under the control of asymmetric left-sided Nodal signalling (i). In the PpO-independent path (top), Nodal functions as asymmetry inducer and generates an enhanced level of neurogenesis in the left Hb, with increased number of *elav3/HuC* and *cxcr4b* positive cells compared with the right Hb (ii). At later stages, subtle asymmetries develop in the habenular neuropil and axonal terminal morphology in the interpeduncular nucleus (IPN), which become evident after ablation of the PpO (iii). In the PpO-dependent path (bottom), Nodal functions as laterality modulator directing PpO migration to the side of Nodal expression (iv). As a consequence of PpO asymmetric positioning, the Hb then develops striking structural and functional differences between the left and right sides. These asymmetries involve: gene expression (v); morphology (size and neuropil content); afferent connectivity from the olfactory bulb (ob) to the right Hb and from the PpO to the left Hb (vi); sub-nuclear organization, with enlarged dorsolateral (Hb-dl) and dorsomedial (Hb-dm) sub nuclei in the left and right Hb, respectively (vii); and efferent connectivity towards the midbrain IPN, with left and right habenular neurons projecting primarily to dorsal and ventral domains of the IPN, respectively (viii). Also, activation of habenular neurons to visual and olfactory stimuli are asymmetric and mostly involve the left and right sides of the Hb, respectively (not shown). Schemes are based on references [40–49] and correspond to dorsal views of the epithalamus, with anterior to the top. Time is in hours post fertilization. (*b*) Model of PpO antisymmetric migration. In the absence of Nodal signalling, a bi-stable cell migratory event dependent on fibroblast growth factor (FGF) signalling establishes PpO antisymmetry, with equal left- (50%) and right- (50%) sided migration [50]. PpO cells (dark green) express the FGF receptor *fgfr4*, while cells along the path of PpO migration express the ligand *fgf8* (light green). The migratory state of the PpO is unstable at the midline. Small differences in FGF signalling between left and right sides, probably owing to stochastic lateral differences in the level of FGF8 protein, break this unstable state and induce the PpO to migrate to either the left or right side with a random frequency (green arrows). Autocatalytic events then amplify the initial differences in PpO asymmetric migration. (*c*) In the presence of asymmetric Nodal signalling (blue), the FGF-dependent PpO antisymmetry becomes biased towards the side of Nodal expression [50,51]. Once the PpO adopts an initial left-sided position, autocatalytic events then reinforce the lateral migration as in (*b*).

it becomes challenging if the asymmetry involves lateral differences in morphogenesis or deformation of the structure like swelling, bending or looping. In these cases, the existence of functional cross-talks between left and right portions of the structure hampers the duplication and coexistence of left and right developmental programmes. Therefore, what one side does has a direct impact on the contralateral side. Such interaction/interdependence between parts-of-a-whole, some of which express (or receive the influence of) Nodal while others do not, opens a full spectrum of possibilities mediated through genetic and cellular mechanisms of different nature such as inhibition, competition, induction and mechanical interactions, among others. As a consequence, when Nodal is acting as asymmetry inducer in a midline-unpaired structure, the functional modulation of Nodal signalling can lead to phenotypes other than simple isomerism. This result depends on the nature of the mechanisms involved in building the asymmetry (cell fate versus morphogenesis) and the type of modulation of Nodal signalling (absent versus bilateral Nodal). We can expect that loss of Nodal function results in loss of asymmetry in a midline-unpaired structure, independently of whether Nodal mediates processes of cell fate or morphogenesis. However, the bilateral activation of Nodal can in principle lead more or less efficiently to the generation of asymmetry. This possibility is more likely to occur if asymmetry relates to a morphogenetic process, and depends on how crosstalk functions between left and right domains and how sensitive the mechanism of asymmetry is to lateral differences in Nodal activation.

Re-examining the case of ascidians, the SV is an initially midline-unpaired structure, and its morphological asymmetry is the

result of clockwise rotation of the neural tube [12]. Nodal signalling appears to play an asymmetry inducer role in this process as in its absence the rotation does not occur, and the SV remains at the midline [39]. However, the bilateral activation of Nodal does not lead to a simple midline-positioned SV (symmetry) but instead it randomizes the asymmetry with equal frequencies of left, midline and right phenotypes (figure 2*b*). If Nodal activation generates biochemical or mechanical processes on the side of expression to induce neural tube rotation, then a randomized laterality of this process can be explained by considering that bilateral expression does not necessarily mean identical levels of Nodal signalling on both sides. Indeed, it has been shown that the intensity of the induced bilateral Nodal expression preceding randomized SV asymmetry is not equal in left and right sides within the population of ascidian embryos [12]. Thus, we propose that the observed combination of corresponding left, midline and right SV phenotypes reflect the effect of small stochastic L–R differences in Nodal signalling working on a midline-unpaired structure.

5. Nodal as laterality modulator in midline-unpaired structures

If Nodal works as laterality modulator, it should exert its function over an asymmetry generated by other mechanisms, which should not be disrupted by loss or gain of Nodal function. This underlying asymmetry can be totally non-directional (or antisymmetric) and thus exhibit equal frequencies of left (50%) and right (50%) phenotypes, or show some degree of directionality at a population level. In this context, unilateral Nodal signalling works to induce a bias that directs the asymmetry to either the same or contralateral side of Nodal expression, thus converting an underlying antisymmetry into a directional asymmetry, or reinforcing an already existing directional asymmetry. Consequently, both loss (Nodal absent) and gain (Nodal bilateral) of function approaches result in the uncovering of the underlying Nodal-independent asymmetry. The zebrafish epithalamus contains the best (and perhaps the only) example to date of asymmetric Nodal signalling working as a true laterality modulator [6,7,51,57]. Asymmetry in this brain region comprises the bilaterally paired Hb and the midline-unpaired parapineal organ (PpO), a pineal complex-derived structure [40]. In zebrafish, Nodal is expressed in a restricted region of the left epithalamus that includes cells of the prospective Hb and PpO (figure 4*a*(i)) [20,40]. From this expression domain, Nodal controls the development of asymmetries of the Hb in two divergent ways according to their dependency on the PpO (figure 4). In the previous section, we already mentioned the example of habenular asymmetries of lampreys, catshark and zebrafish in which Nodal works as asymmetry inducer (figure 3*a–c*). These asymmetries are PpO-independent in the three species, although by different ontogenic mechanisms: (i) catsharks do not form a recognizable PpO [22], (ii) lampreys do have a midline PpO, but habenular asymmetries develop before the PpO is formed [22] and (iii) zebrafish have an asymmetrically positioned PpO but a sub-type of habenular asymmetries develops even in the absence of PpO, i.e. when the PpO is physically ablated [40,41,52]. In zebrafish, PpO-independent asymmetries of the Hb are very subtle and are frequently masked by the second type of epithalamic asymmetry, which is more conspicuous and dependent on the PpO (figure 4). In the PpO-dependent asymmetries, Nodal functions as laterality modulator.

Developmental studies reveal that PpO precursors translocate from their site of origin at the dorsal midline towards the left side of the brain just after asymmetric Nodal expression (figure 4*a*(iv)) [40]. This first morphological asymmetry of the zebrafish epithalamus is followed by the development of prominent structural and functional asymmetries of the Hb (figure 4*a*(v–viii)) [40,42]. Experiments of PpO ablation and mutant conditions that delay the onset of PpO asymmetric translocation reveal a requirement of an asymmetrically positioned PpO for the subsequent development of a sub-type of asymmetries in the Hb (figure 4*a*(iii)) [40–42,58]. These asymmetries result from L–R differences in cell fate decisions towards two main neuronal identities, and it is possible that the PpO modulates these fate decisions as well as the timing of asymmetric habenular neurogenesis by acting on the Notch and Wnt signalling pathways [43,44].

The mechanisms involved in the early asymmetric positioning of the PpO are currently unknown, although evidence suggests that PpO asymmetric morphogenesis involves the movement of a midline-unpaired structure that organizes as a cohesive rosette-like structure [40,59] that can form a cellular stream [60]. From the genetic perspective, PpO asymmetric migration requires the concerted activity of fibroblast growth factor (FGF) and Nodal signalling pathways. Work in the zebrafish mutant *acerebellar/fgf8* (*ace*) and several mutant, morpholino-based knock-down and pharmacological conditions that result in either absence or bilateral epithalamic Nodal expression have provided insights into how these pathways may control the asymmetric positioning of the PpO. *fgf8* is expressed bilaterally in the Hb in a domain that coincides with the site where the PpO will migrate [50]. The PpO remains stationary at the dorsal midline in *ace/fgf8*-/- mutants and after pharmacological inhibition of FGF receptor signalling, suggesting a requirement of FGF signalling for PpO asymmetric movement [50]. Also, experiments that place an ectopic source of FGF protein in the epithalamus reveal that FGF signalling can direct PpO migration but only in the absence of epithalamic Nodal expression [50]. Importantly, conditions in which epithalamic Nodal expression becomes either absent or bilateral result in randomized positioning of the PpO with an equal frequency of left- (50%) and right-sided (50%) PpO at a population level [20,50]. Together, these findings suggest that FGF signalling controls a type of bi-stable cell migratory process of the PpO that in the absence of Nodal signalling leads to PpO antisymmetric migration (figure 4*b*). In this context, asymmetric Nodal expression introduces an additional asymmetry or generates a bias in the bi-stable process to direct PpO migration consistently towards the side of Nodal expression (figure 4*c*) [20,50,51]. Therefore, Nodal works as laterality modulator and directs the side of asymmetric migration of the PpO. As a consequence of this asymmetry, Nodal also directs the laterality of a sub-type of habenular asymmetries that depend on the asymmetric positioning of the PpO, but in an indirect manner.

6. Coexistence of different types of asymmetries: epithalamus and the heart

Unravelling the mechanisms controlling the development of asymmetry in the zebrafish epithalamus has been a challenge

Table 1. Comparison of asymmetries in the epithalamus and heart of zebrafish.

	epithalamus	heart
structure/organ	single midline-unpaired (PpO) bilaterally paired (Hb)	single midline-unpaired (heart disc/tube)
asymmetry type	sided PpO migration L–R differences in Hb neurogenesis and fate	sided heart disc migration, involution and rotation sided torsion of heart tube
asymmetry interaction	different asymmetries in different organs at the same time	different asymmetries in the same organ but at different times
asymmetry dependence	PpO directs Hb asymmetry, but asymmetry in the Hb can occur independently from the PpO	jog might direct loop, but loop can occur independently from jog
masking of asymmetry	Ppo-independent masked by PpO-dependent asymmetries of the Hb antisymmetry masked by directional asymmetry of the PpO	intrinsic directional heart loop masked by Nodal-mediated directional asymmetry
nodal-independent asymmetry	FGF-mediated antisymmetry of PpO migration (50% L, 50% R)	self-organized heart tube properties (directional, 70% D-loop)
nodal as asymmetry inducer	sided increase in Hb neurogenesis	sided increase of cell migration in heart cone sided increase of actin in heart tube?
nodal as laterality modulator	bias in PpO migration (direct) bias in PpO-dependent Hb asymmetries (indirect)	reinforcement of D-loop? (direct)

owing to the coexistence of different types of asymmetries. We have seen that epithalamic asymmetries involve cellular mechanisms of a different kind (cell fate versus morphogenesis) and structures of different nature (bilaterally paired versus midline-unpaired), which can also interact or not with each other. Epithalamic asymmetries also coexist in different structures at the same time, and the most conspicuous asymmetries can hide the most subtle ones. Furthermore, epithalamic asymmetries can be independent of Nodal or be controlled by asymmetric Nodal signalling in different manners, either by Nodal acting as asymmetry inducer or laterality modulator, and in the latter case through direct or indirect mechanisms. Such complexity of epithalamic asymmetry finds a parallel in the asymmetries developed by the zebrafish heart (table 1). The heart is a midline-unpaired structure of bilateral origin where the left and right progenitors fuse at the midline to form the cardiac cone that then transforms into the heart tube [61,62]. Asymmetries involve the left-sided positioning of the cardiac cone (left jog) followed by a dextral curvature of the heart tube (D-loop) [62,63]. Several cellular processes drive cardiac jogging including migration and involution, which result in a clockwise rotation and torsion of the heart disc, where left becomes dorsal and right becomes ventral, and that precedes the formation of the cardiac tube [62,64,65]. Notably, some of these processes are reminiscent of what is observed in the development of nervous system asymmetries. For example, the sided migration of the PpO in zebrafish [40,50], the clockwise rotation of the neural tube in ascidians [12,39] and the conversion of left–right into dorsoventral seen in the projections from the Hb to the interpeduncular nucleus of zebrafish [45]. Symmetrical Nodal (absent and bilateral) result in the lack of jog (symmetry) [65–67], thus suggesting a role of Nodal as asymmetry inducer in this particular aspect of heart asymmetry. Notably, symmetry in both cases is produced through different ontogenic pathways. Symmetrically decreased migration induces no jog in the absence of Nodal signalling, while the lack of jog in bilateral Nodal signalling results from symmetrically enhanced motility [66].

Rotation of the heart cone during jogging might be directing the later torsion of the heart tube leading to D-loop in normal conditions. However, this seems unnecessary as mutants with no jog retain their ability to loop [62,63,68–70]. Indeed, the heart tube in the absence of Nodal signalling shows D-loop in about 70% of embryos and this is maintained even after the heart tube is cultured *ex vivo* [67]. Therefore, looping of the heart is controlled by a Nodal-independent mechanism that seems to rely on intrinsic mechanical properties dependent on the actin cytoskeleton [67]. Interestingly, this asymmetry is reminiscent of the bi-stable system driving asymmetric PpO migration although, in the case of the cardiac tube, the asymmetry is directional and not antisymmetric as observed in the PpO. Asymmetric Nodal provides robustness to the intrinsic D-loop but whether it works as a true laterality modulator is still unclear. Results of loss (Nodal absent) and gain (Nodal bilateral) of function are confusing: the former increases the number of reversals (L-loop) while the latter induces both reversed (L-loop) and symmetric (no loop) phenotypes [63]. This discrepancy is intriguing and will have to be resolved in the future by experiments in which the effect of ectopic Nodal signalling during looping is controlled both in time and space.

7. A conceptual framework to study the role of Nodal in asymmetry

Taken together, the analyses of epithalamic and heart asymmetry reveal that several types of asymmetries controlled or not by Nodal signalling can coexist in different structures at the same time and in the same structure at different times (table 1). Such complexity requires strategies to uncouple the different asymmetries. Among the approaches, experimental

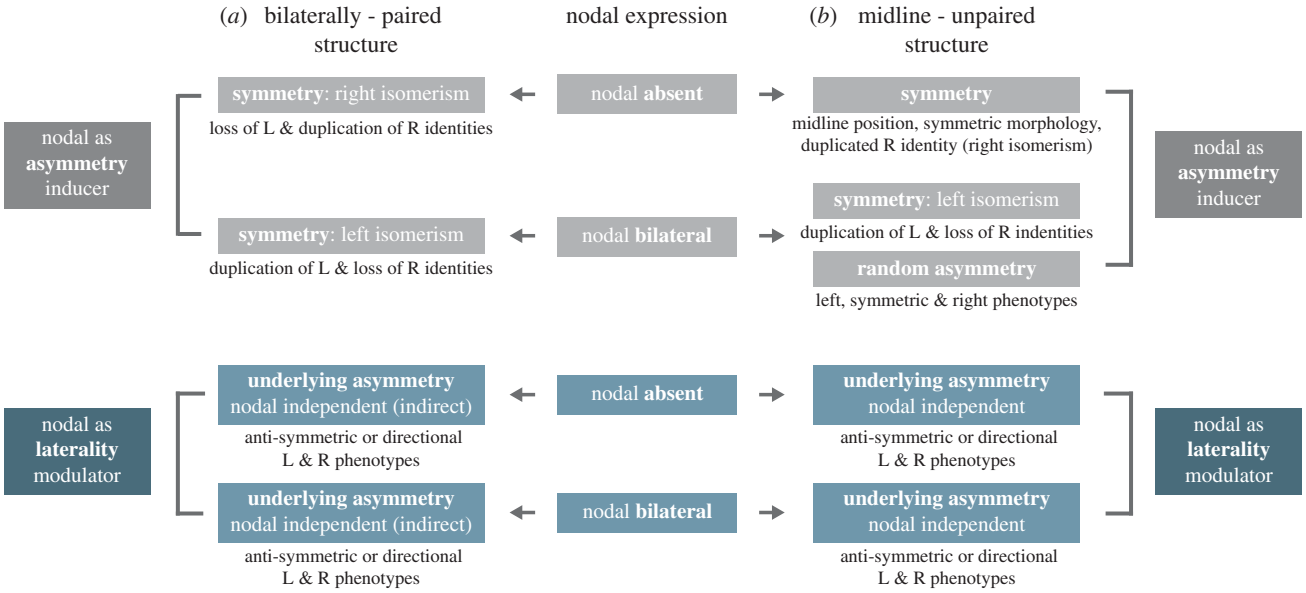

Figure 5. A conceptual framework to study the role of Nodal signalling in the development of asymmetry. For details, see the text in §7.

conditions of absent and bilateral Nodal are the best first approximation to provide evidence that supports a role of Nodal as asymmetry inducer or laterality modulator (figure 5).

We propose a conceptual framework for the analysis of Nodal function in L–R asymmetry that can be applied not only to nervous system asymmetry but also to cases of body and visceral organ asymmetry under the control of asymmetric Nodal signalling. We emphasize that bilaterally paired and midline-unpaired structures behave differently and thus that it is necessary to analyse them under different conceptual perspectives. In a bilaterally paired structure, the lack of communication between the two domains of the structure allows the duplication of phenotypes on both sides, thus right and left isomerisms are usually found in experimental conditions of absent and bilateral Nodal signalling, respectively (figure 5*a*). This finding implies that Nodal can only work as asymmetry inducer in a bilaterally paired structure. However, there are two possibilities in which Nodal can induce a phenotype that appears to resemble a role as laterality modulator in a bilaterally paired structure. These are when the two sides of the bilaterally paired organ interact (in this case the bilaterally paired structure 'behaves' as a midline-unpaired organ) and when Nodal modulates the laterality of a midline-unpaired organ, which then induces asymmetry in the bilaterally paired structure. PpO-dependent asymmetries of the zebrafish Hb is an example of the latter case.

Midline-unpaired structures, on the other hand, are characterized by communication between left and right portions, which together behave as a single unit. As a result, complex phenotypes can emerge in conditions of absent and bilateral Nodal signalling (figure 5*b*). In a midline-unpaired structure, Nodal can work in two possible ways: as asymmetry inducer or laterality modulator. When Nodal works as asymmetry inducer, the absence of Nodal should result in a symmetric phenotype. Symmetry can manifest as midline positioning or symmetric morphology if asymmetries are related to morphogenesis, or as double right-sided identity (right isomerism) if asymmetries are related to cell fate. However, bilateral Nodal can induce different asymmetry phenotypes depending on the cellular process involved in the generation of asymmetry and if the system is sensitive or not to small L–R differences in Nodal ligand/signalling. Such phenotypes can manifest as double left-sided identity (left isomerism) if asymmetries are related to cell fate, or can produce a type of randomization with coexisting left, symmetric and right phenotypes, especially when asymmetries are related to morphogenesis. Finally, if Nodal works as laterality modulator in a midline-unpaired structure, we should expect to uncover a Nodal-independent asymmetry (antisymmetric or directional) in both conditions, absent and bilateral Nodal signalling.

8. Hypothesis on the evolution of asymmetry in the epithalamus

L–R asymmetry is a trait easy to recognize even in very different animals, structures and cellular contexts, and thus is suitable for meaningful comparisons between species. Indeed, based on comparative studies Palmer proposed that the evolution of directional asymmetry from a hypothetical symmetric ancestor proceeded along two possible routes [71]. These correspond to a direct route in which asymmetry and laterality evolved simultaneously (figure 6*a*-1, red arrow) and an indirect route consisting of two steps, where antisymmetric phenotypes evolved first and were followed by the evolution of laterality mechanisms that direct the asymmetry (figure 6*a*-2, blue arrows) [71]. Even if it is not possible to consider that embryonic development recapitulates the evolutionary history of an animal species, studying the ontogenic mechanisms that produce and control asymmetry, and specially antisymmetry, can help to understand evolutionary processes [71]. In this context, epithalamic asymmetry has provided valuable information. The proposal of an antisymmetric control of PpO asymmetric migration mediated by FGF is an example of such mechanism, which provides support to the idea that epithalamic asymmetry evolved along the two-step route. However, recent developmental and evolutionary information revealing the ancestral condition of PpO-independent habenular asymmetries [22] prompts us to re-evaluate the existing hypotheses about the evolution of epithalamic asymmetry [14,51].

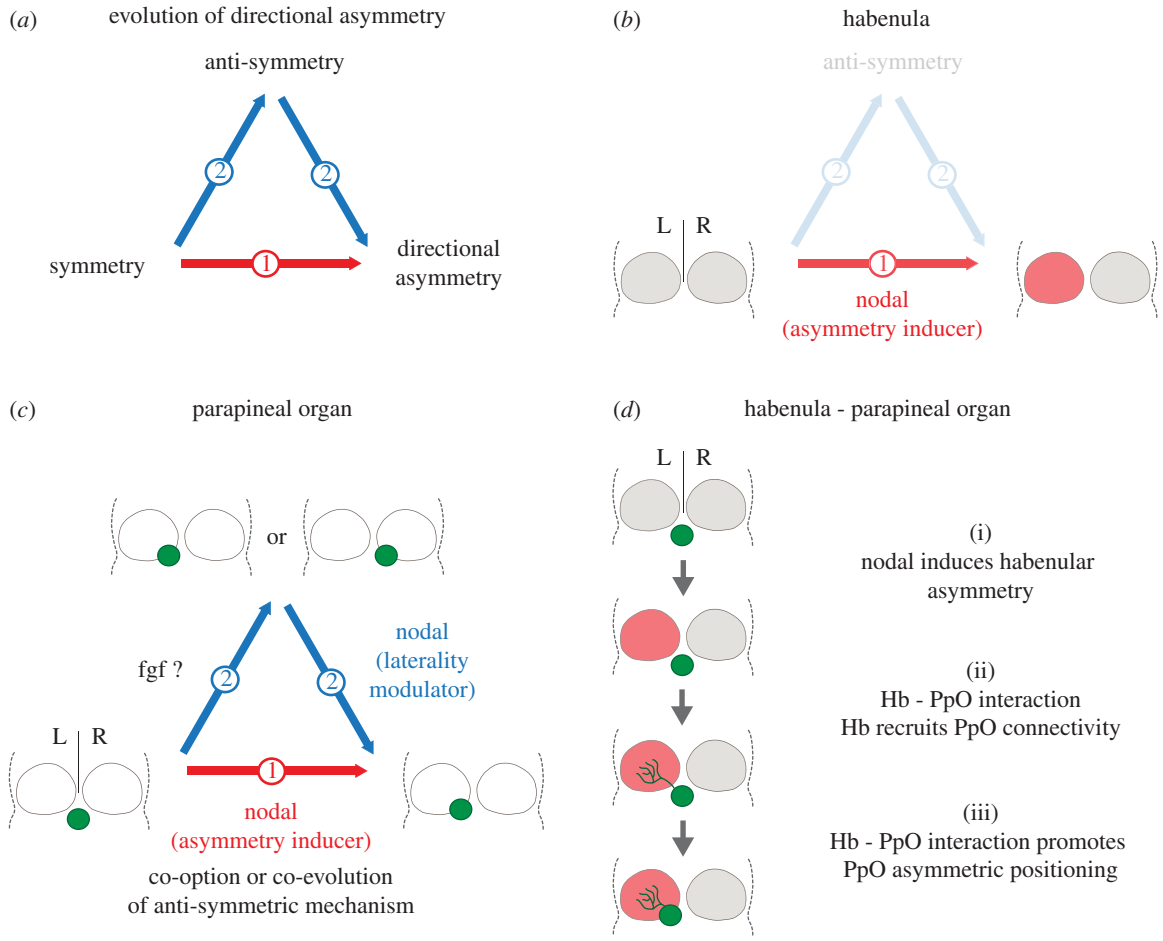

Figure 6. Evolutionary routes to directional asymmetry in the epithalamus. (*a*) Model of the evolution of directional asymmetry from a symmetric ancestral condition proposed by Palmer [71]. Two routes can be distinguished. In the direct route (red arrow, 1) both asymmetry and laterality of asymmetry evolve together in a single step likely by conventional evolution (genotype precedes phenotype). In the indirect or two-step route (blue arrows, 2), a non-inheritable antisymmetric phenotype evolves first by mutation or developmental mechanisms under the control of environmental and/or behavioural factors (left). Then, one of the asymmetric phenotypes is fixed by the appearance of a laterality mechanism (right), resulting in directional asymmetry. (*b*) Proposed evolutionary route leading to directional asymmetry of the habenula (Hb). As a bilaterally paired structure, directional asymmetry of the Hb probably evolved by a direct route through Nodal acting as asymmetry inducer (red arrow, 1). (*c*) Proposed models of evolutionary paths leading to directional asymmetry in the position of the parapineal organ (PpO). As the PpO is an initially midline-unpaired structure, both direct and indirect routes are equally possible. In the indirect two-step route (blue arrows, 2), an antisymmetric positioning of the PpO first evolved independently from Nodal (e.g. mediated by FGF signalling) (left). In a second step, asymmetric Nodal might have been co-opted to direct the antisymmetric mechanism of PpO migration to the side of Nodal expression, thus transforming antisymmetry into directional asymmetry (right). Alternatively, directional asymmetry of the PpO evolved along the direct route (red arrow, 1) by Nodal acting as asymmetry inducer. In this scenario, Nodal directly induced the asymmetric positioning of the PpO towards the left side. This initial asymmetry might have then co-opted antisymmetric mechanisms of PpO migration, or coexisted with them, with Nodal gaining a new function as laterality modulator. (*d*) Model for the evolution of epithalamic asymmetry based on Hb–PpO interactions. The hypothetical ancestor had a symmetric Hb and a medially positioned PpO with no left-sided projection to the Hb (or bilateral projection) (top). Left-sided Nodal then acted as asymmetry inducer to generate directional asymmetry of the Hb (i). Then, the establishment of interactions between the Hb and PpO led to the recruitment of afferent axonal connectivity from the PpO (ii). Finally, intimate Hb–PpO interactions promoted asymmetric positioning of the PpO, which became influenced by asymmetric Nodal signalling during ontogeny. See §8 for additional details.

Because PpO-independent asymmetries exist in the Hb, it is not possible to analyse the evolution of all epithalamic asymmetries as a single phenomenon. As we have seen, asymmetries of the Hb and PpO have different ontogenic routes and affect structures of different nature (bilaterally paired versus midline-unpaired), and thus it is plausible that they had different evolutionary histories. Also, the data we have analysed in previous sections show that asymmetries of related structures can evolve independently and be produced by various mechanisms in a context-dependent manner. Thus, it becomes relevant to identify the different types of asymmetry and correctly understand whether and how they are dependent on each other. Detailed functional and comparative studies are essential to accomplishing this task.

The Hb is a bilaterally paired structure and according to our proposal, its development is not compatible with a mechanism of laterality control. Accordingly, it is likely that habenular asymmetry evolved through a transition from symmetry to directional asymmetry under the control of Nodal acting as asymmetry inducer (figure 6*b*). We propose that asymmetric Nodal gained the ability to interact with the left side of the hypothetical ancestral Hb, which was symmetric, resulting in the acquisition of a unique trait with directional asymmetry in a single step. Manipulation of Nodal in the Hb of lampreys, catshark and zebrafish supports this proposal [22,52]. The fact that a type of PpO-dependent asymmetries of the Hb has an underlying anti-symmetry does not argue against this idea as this asymmetry of the Hb is a consequence of the asymmetric positioning of the PpO.

As the PpO is a midline-unpaired structure, both direct and indirect routes are equally possible during the evolution of directional asymmetry (figure 6*c*). Indeed, developmental data support the two-step route as the abrogation of directional asymmetry through loss and gain of Nodal function unveils a masked antisymmetric mechanism controlled by FGF signalling. Although it is tempting to speculate that this ontogenic mechanism resembles evolutionary steps and that FGF signalling directed this first step in evolution, it is necessary to bear in mind that this might not be the case as evolution and development act at very different levels of biological organization. If directional asymmetry of the PpO evolved through the two-step route, then the ancestral PpO should have been initially insensitive to Nodal. In a first step, the PpO gained the ability to display a lateral movement without a particular direction, resulting in an antisymmetric PpO localization (figure 6*c*-2, left). In a second step, Nodal might have been co-opted in the prospective field of the PpO to exert a novel function as laterality modulator and direct the side of PpO movement (figure 6*c*-2, right). If this is the case, and the evolution of species has left footprints of the intermediate evolutionary step in modern animals, future comparative studies should reveal the existence of a species with antisymmetric PpO positioning, irrespective of the left-sided expression of Nodal.

However, it is also possible that directional asymmetry of the PpO could have evolved along the direct route (figure 6*c*-1, red arrow). The results in lampreys and catshark suggest that asymmetric expression of Nodal in the epithalamus was probably present before the appearance of teleost radiation [22]. Thus, in the hypothetical ancestral condition, a direct transition from symmetry to directional asymmetry could have been promoted by Nodal acting as asymmetry inducer in the PpO. As a result, a first directional asymmetry of the PpO could have been created, generating new types of genetic/tissue interactions in the epithalamus. In the lineage of teleosts, the Nodal-induced directional asymmetry of the PpO could have promoted the emergence of an antisymmetric inductor mechanism (for example the one mediated by FGF) followed by the appearance of a new function of Nodal as laterality modulator of PpO migration. In this context, the first type of directional asymmetry of the PpO mediated by Nodal could either have been lost or still be present but masked by the newer antisymmetry/laterality mechanism. If a modern species that conserves this entire pathway exists, then the abrogation of Nodal function in this species should result in loss of asymmetry with a medially positioned PpO.

Even if the indirect route is the correct representation of the evolutionary process of PpO asymmetry, it is important to note that other directional asymmetries in the epithalamus—probably induced by Nodal and especially involving the Hb—could have evolved in parallel and be independent of the PpO. Lampreys provide an example that allows us to make new hypotheses on the evolution of epithalamic asymmetry. In these animals, Nodal acts as asymmetry inducer in the Hb while the PpO has a symmetric position at the midline. Importantly, the medially located PpO develops asymmetric projections to the left Hb [72]. Interestingly, this type of asymmetric connectivity is observed in all groups showing a PpO, including lampreys, teleosts and lizards [14]. Such configuration argues against the idea that asymmetry of PpO projection is mediated by the asymmetric positioning of the organ and instead suggests that this asymmetric projection could have evolutionarily preceded the asymmetric positioning of the PpO.

We thus propose a new model for the evolution of epithalamic asymmetry (figure 6*d*). The hypothetical ancestral condition had a symmetric Hb and a medially positioned PpO. During evolution, asymmetric Nodal acting as asymmetry inducer appeared on the left, making the Hb acquire a directional asymmetry (figure 6*d*(i)). A second step involved the establishment of new interactions between the asymmetric left Hb and the PpO, which resulted in the development of PpO connectivity to the left Hb while keeping the symmetric position of PpO (figure 6*d*(ii)). Such intimate interaction between the Hb and PpO could have brought the PpO into the field of influence of asymmetric Nodal, which already was acting on the Hb, either by moving the PpO to the left or by modifying spatial or temporal aspects of PpO ontogeny (figure 6*d*(iii)). Evidence that such a modification might have occurred comes from studies showing that the onset of PpO connectivity is heterochronic when comparing the ontogeny of epithalamic asymmetry among related teleost species [18,73]. The fact that in the lamprey the PpO only develops after asymmetries of the Hb are anatomically distinguishable [22] only argues against a possible direct control of Nodal on the asymmetric projection of the PpO in this species, but is coherent with the idea that this effect can be mediated by the asymmetry of the Hb. Therefore, we explicitly propose that Hb-dependent PpO asymmetries played a key role in the evolution of epithalamic asymmetries and that these types of asymmetries might be present in modern species. Future comparative studies will have to demonstrate this possibility.

9. Concluding remarks

In this paper, we show that asymmetries of the nervous system under the developmental control of asymmetric Nodal signalling are widespread across Bilateria. Using examples from ascidians and fishes, we propose a conceptual framework for the analysis of Nodal function in L–R asymmetry that can be applied not only to nervous system asymmetries but also to cases of body and visceral organ asymmetry under the control of asymmetric Nodal signalling. We emphasize that the function of Nodal signalling in the development of asymmetries is strongly context-dependent, and that bilaterally paired and midline-unpaired structures can behave differently under the influence of asymmetric Nodal signalling. A first experimental strategy to distinguish between asymmetry inducer and laterality modulator roles of Nodal is to perform loss (Nodal absent) and gain (Nodal bilateral) of function approaches. Right and left isomeric phenotypes are expected in bilaterally paired structures as Nodal can only work as asymmetry inducer owing to the developmental independence of left and right cellular domains. By contrast, the left and right sides of a midline-unpaired organ can communicate. The result of perturbing asymmetric Nodal signalling in this context depends on the mechanisms building asymmetry, how left and right sides interact, the sensitivity of the mechanism inducing asymmetry to small lateral variations of Nodal activity, and in particular on whether Nodal acts as asymmetry inducer or laterality modulator. In experimental conditions of bilateral Nodal signalling, the proportion of left, right and in particular symmetric phenotypes becomes relevant. If Nodal works as laterality modulator, we expect the unmasking of an underlying Nodal-independent antisymmetry or directional asymmetry, with no symmetric phenotypes.

Insights provided by comparative developmental studies have helped us to construct hypotheses on the origin and

evolution of Nodal-dependent asymmetries of the nervous system. In particular, we propose that directional asymmetry of the Hb evolved in a single step and that asymmetric connectivity of the PpO to the left Hb preceded and might have promoted the asymmetric positioning of the PpO observed in modern teleosts. Thus, the projection of the PpO to the left Hb might be an example of a Hb-dependent PpO asymmetry not yet recognized. Future detailed studies on the ontogenic path leading to epithalamic asymmetry in different species should resolve this proposal and provide new perspectives on the evolution of epithalamic asymmetry.

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