Zebrafish and medaka: model organisms for a comparative developmental approach of brain asymmetry

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Comparison between related species is a successful approach to uncover conserved and divergent principles of development. Here, we studied the pattern of epithalamic asymmetry in zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*), two related teleost species with 115–200 Myr of independent evolution. We found that these species share a strikingly conserved overall pattern of asymmetry in the parapineal–habenular–interpeduncular system. Nodal signalling exhibits comparable spatial and temporal asymmetric expressions in the presumptive epithalamus preceding the development of morphological asymmetries. Neuroanatomical asymmetries consist of left-sided asymmetric positioning and connectivity of the parapineal organ, enlargement of neuropil in the left habenula compared with the right habenula and segregation of left–right habenular efferents along the dorsoventral axis of the interpeduncular nucleus. Despite the overall conservation of asymmetry, we observed heterotopic changes in the topology of parapineal efferent connectivity, heterochronic shifts in the timing of developmental events underlying the establishment of asymmetry and divergent degrees of canalization of embryo laterality. We offer new tools for developmental time comparison among species and propose, for each of these transformations, novel hypotheses of ontogenic mechanisms that explain interspecies variations that can be tested experimentally. Together, these findings highlight the usefulness of zebrafish and medaka as comparative models to study the developmental mechanisms of epithalamic asymmetry in vertebrates.

Keywords: brain asymmetry; development; teleosts; laterality; epithalamus; heterochrony

1. INTRODUCTION

Asymmetry is a fundamental and conserved feature of the brain, which is thought to enhance information processing and task performance in behaviours central for species survival, such as feeding, predator detection and memory (Güntürkün *et al.* 2000; Rogers 2000; Pascual *et al.* 2004; Vallortigara & Rogers 2005; Rogers & Vallortigara 2008). In addition, asymmetry has been proposed as the basis of speech and other behavioural traits (Sherman *et al.* 1982; Rogers & Andrew 2002; Hutsler & Galuske 2003; Toga & Thompson 2003) and abnormal asymmetry appears to associate with several neuropathologies including schizophrenia (Li *et al.* 2007), autism (Escalante-Mead *et al.* 2003) and neuronal degenerative diseases (Toth *et al.* 2004). In the last decade, experimental studies have provided valuable insights into the developmental basis of brain asymmetry. Particularly helpful have been genetic model organisms that allow a comprehensive

bottom-up (gene to behaviour) study of this phenomenon (Concha 2004). For example, recent work in the teleost zebrafish has unveiled genetic mechanisms that control the development of neuroanatomical asymmetries (reviewed in Halpern *et al.* 2003; Concha 2004) and established the first operational links between genetics, asymmetric morphology and lateralized behaviours (Barth *et al.* 2005).

One of the best-studied cases of brain asymmetry is observed in the epithalamus of vertebrates (Concha & Wilson 2001; Bianco & Wilson 2009). In zebrafish, epithalamic asymmetry is established through a sequence of developmental modules. Initially, *asymmetry* (structural differences between left and right sides at the individual level) and *laterality* (directionality of asymmetry at a population level) are determined by the coordinated activity of members of the fibroblast growth factor (J. Regan, M. Concha, M. Roussigne, C. Russell and S. Wilson 2007, unpublished data) and nodal (Concha *et al.* 2000) signalling pathways, respectively. Then, a sequential programme of asymmetric morphogenesis generates neuroanatomical asymmetries in the epithalamic pineal complex and

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Figure 1. (a) Zebrafish and (b) medaka share an overall pattern of molecular and morphological epithalamic asymmetry. ((i),(ii)) Asymmetric expression of components of the nodal signalling pathway in the presumptive epithalamus. mRNA expression of orthologue genes was detected by whole-mount *in situ* hybridization (arrows) at (i) normalized STU 35 (*Dr-lefty1*, *Ol-lefty*) and (ii) 43 (*Dr-pitx2c*, *Ol-pitx2c*) (table 2). The lateral flexure of the third ventricle is indicated by arrowheads. (iii) Left-sided positioning and efferent projection of the parapineal organ. Expression of GFP was detected in medaka *Tg(Fox2::GFP)* and zebrafish *Tg(FoxD3::GFP)* and pseudo-coloured in blue and green to label pineal and parapineal organs, respectively. The red arrowhead points to the terminal dandelion seed-head-shaped domain of parapineal efferent connectivity. (iv) Asymmetric organization of neuropil in the habenulae. Arrows indicate the regions of neuropil, which exhibit dissimilar growth between the left and right habenulae, as detected by immunostaining against acetylated α -tubulin. The red arrowhead points to a neuropil domain that is detected exclusively in the left habenula of medaka. (v) Asymmetric organization of neuronal cell bodies in the habenulae. Asterisks indicate equivalent regions of the left and right habenulae. The red arrowhead points to a domain devoid of fluorescent Nissl staining that is detected exclusively in the left habenula of medaka. ((vi),(vii)) Dorsoventral segregation of left–right habenular efferents in the IPN. Left and right habenular projections were labelled with DiD (red) and DiO (green), respectively. Images correspond to ((i)–(vi)) dorsal and (vii) lateral views with anterior and dorsal to the top, respectively. Stages of development correspond to 120 HPF (zebrafish, at 26°C) and Iwamatsu's stage 39 (medaka), unless otherwise stated. ((iii)–(vii)) Three-dimensional projections from confocal z-stacks. L, left; R, right; Lh, left habenula; Rh, right habenula; hc, habenular commissure; Lfr, left fasciculus retroflexus; Rfr, right fasciculus retroflexus; dIPN, dorsal domain of the IPN; vIPN, ventral domain of the IPN. Scale bars, ((i)–(v)) 20 μ m, ((vi),(vii)) 30 μ m.

habenulae. Early asymmetries of the pineal complex involve the asymmetric migration of the photoreceptive parapineal organ to the left side (Concha *et al.* 2003). Subsequent habenular asymmetries are characterized by differential growth of sub-nuclei (Aizawa *et al.* 2005,

2007; Gamse *et al.* 2005) and neuropil domains (Concha *et al.* 2000) between the left and right sides. Finally, asymmetries in the ratios of different subtypes of habenular projection neurons result in asymmetric target connectivity wherein left and right habenular

Figure 2. Heterotopic parapineal efferent connectivity in the left habenula of (a) zebrafish and (b) medaka. (a(i)–(vi)) Parapineal efferents blend into a dorsomedial neuropil domain of the left habenula in zebrafish whereas in (b(i)–(vi)) they segregate from other sources of habenular neuropil to form a distinct dorso-anteromedial domain in the left habenula of medaka. Images correspond to dorsal views of the left habenula with anterior to the top. Images were obtained after three-dimensional maximum projections from confocal *z*-stacks. The parapineal organ was pseudo-coloured in blue (parapineal body) and green (parapineal efferents) after immunostaining against GFP in (a(i),(iv)) 120 HPF zebrafish *Tg(FoxD3::GFP)* and (b(i),(iv)) St.39 medaka *Tg(fRx2::GFP)*. Distribution of neuropil and nuclei in the left habenula were detected by (ii) immunostaining against acetylated α -tubulin and (v) fluorescent To-pro staining, respectively. Merged images of double labelling are shown in the bottom panels ((iii),(vi)). Asterisks indicate nuclei-free domains of the left habenula where parapineal connectivity is distributed in zebrafish. Arrowheads point to the terminal dandelion seed-head-shaped domain of parapineal efferent connectivity in medaka. (vii) Summary model of parapineal efferent connectivity in zebrafish and medaka. (a(vii)) In zebrafish, parapineal efferents distribute broadly within a large dorsomedial neuropil domain of the left habenula situated immediately anterior to the habenular commissure. (b(vii)) In medaka, parapineal efferents form a thick bundle of axons, which after entering the left habenula, make a turn towards the midline to end in a well-defined dandelion seed-head-shaped neuropil domain situated in the most dorso-anteromedial aspect of the left habenula. All images correspond to dorsal views, with anterior to the top. The body of the parapineal organ and its efferent connectivity are shown in black, the habenular commissure in grey and neuropil domains in yellow. L, left; R, right; Lh, left habenula; Rh, right habenula; hc, habenular commissure. Scale bars, 20 μ m.

efferents are segregated along the dorsoventral axis of the interpeduncular nucleus (IPN) in the ventral midbrain (Aizawa *et al.* 2005; Gamse *et al.* 2005; Bianco *et al.* 2008).

Three main aspects are important to highlight about the development of epithalamic asymmetries. First, genetic pathways that establish *asymmetry* are autonomous from those that control *laterality* (Concha *et al.* 2000). Such independence in the developmental control of asymmetry and laterality makes the epithalamus of zebrafish an attractive vertebrate model to study the ontogenic (genetic and epigenetic) mechanisms that underlie directional asymmetries, in which most individuals are asymmetrical in the same

direction within the population (Van Valen 1962). Second, laterality of epithalamic asymmetry is coupled to laterality of visceral asymmetry (Concha *et al.* 2000; Long *et al.* 2003; Carl *et al.* 2007) in contrast to other structural and functional asymmetries of the vertebrate brain, e.g. asymmetries associated to speech and handedness (Torgersen 1950; Kennedy *et al.* 1999; Tanaka *et al.* 1999). This indicates that asymmetries controlled by independent mechanisms coexist in the vertebrate brain. Finally, epithalamic asymmetries are immersed in an evolutionarily conserved circuit involved in limbic-system-related responses (Sutherland 1982; Klemm 2004; Bianco & Wilson 2009), which has been implicated in the origin of neuropsychiatric disorders

(Sandyk 1991; Ellison 1994). Altogether, these observations underscore the relevance of understanding the evolutionary origin, genetic control, circuit configuration and behavioural correlates of epithalamic asymmetry to begin dissecting general principles of directional asymmetries and the specific role of the epithalamus, and its associated asymmetric circuit in normality and pathology.

Recent comparative surveys have revealed a striking conservation of epithalamic asymmetry among a wide range of vertebrate species (Concha & Wilson 2001; Guglielmotti & Cristino 2006). However, the lack of systematic comparative analyses addressing the genetic and developmental bases hampers the examination of general principles of epithalamic asymmetry development. In this context, the emergence of zebrafish and medaka as complementary model organisms suitable for comparative developmental approaches (Furutani-Seiki & Wittbrodt 2004) offers a unique opportunity. As lineages of zebrafish (*Danio rerio*, Order Cypriniformes) and medaka (*Oryzias latipes*, Order Beloniformes) diverged 115–200 Myr ago, comparison has the potential to unveil those aspects that represent the backbone of epithalamic asymmetry and those that are subjected to evolutionary variation.

In this study, we carried out a first systematic inter-species comparison of brain asymmetry development in teleosts. We analysed the morpho-topological organization of epithalamic asymmetry and studied the temporal organization of developmental modules using a novel method for time normalization based on the rate of somitogenesis. We found a strikingly conserved overall pattern of asymmetry in the parapineal–habenular–interpeduncular system. In spite of this, we observed heterotopic changes in the organization of parapineal efferent connectivity, heterochronic shifts in the timing of developmental events underlying the establishment of asymmetry and divergent degrees of population-level laterality. Altogether, these findings highlight the usefulness of zebrafish and medaka as comparative tools to study the developmental mechanisms of epithalamic asymmetry in vertebrates.

2. MATERIAL AND METHODS

(a) *Fish lines*

Zebrafish (*D. rerio*) lines used in this work were wild-type Tübingen and *Tg(foxD3::GFP)* (Gilmour *et al.* 2002). Medaka (*O. latipes*) lines were wild-type Cab, *Tg(fRx2::GFP)* and *Tg(fRx2/DE::GFP)* (Wittbrodt *et al.* 2002). Embryos and fry were obtained by natural spawning, raised at 26–28°C in standard embryo medium, and staged according to morphology (Kimmel *et al.* 1995; Iwamatsu 2004) and age (hours post fertilization, HPF).

(b) *Whole-mount in situ hybridization, immunohistochemistry, fluorescent Nissl and To-Pro staining*

In situ hybridization was performed according to standard protocols for medaka (Loosli *et al.* 1998) and zebrafish (Westerfield 1996). RNA antisense probes targeted *Dr-lefty1* (Bisgrove *et al.* 1999; Thisse & Thisse 1999), *Dr-pitx2c* (Essner *et al.* 2000), *Ol-lefty* (Soroldoni *et al.* 2007) and *Ol-pitx2* (Jaszczyszyn *et al.* 2007). Tubulin immunostaining was performed in larvae fixed in 2 per cent TCA/PBS at room temperature for 1.5 (medaka) and 3 (zebrafish) hours, as

described earlier (Concha *et al.* 2003). Mouse anti-acetylated α -tubulin (Sigma, 1 : 1000), rabbit-anti-green fluorescent protein (GFP) (Abcam, 1 : 1000) and Alexa-488/647 conjugated secondary (Molecular Probes, 1 : 200) antibodies were used. Fluorescent Nissl staining comprised an overnight incubation with NeuroTrace 530/615 red Nissl (Molecular Probes, 1 : 200) at 4°C. Incubation with To-Pro-3 iodide stain (642/661) (Molecular Probes, 1 : 1000) for 1 hour was used for nuclear counterstaining. Embryos were mounted in glycerol for microscopic observation and photography.

(c) *Labelling of habenular efferent projections*

For the labelling of habenular projections, larvae and embryos were immersed in fixative (4% PFA/PBS) and the skin covering the dorsal diencephalon and eyes removed. Crystals of lipophilic dyes DiD and DiO (Molecular Probes) were applied in left and right habenulae using tungsten needles connected to a micromanipulator (Aizawa *et al.* 2005). Labelled larvae were incubated in 0.5 per cent PFA/PBS at 4°C for 2 days in darkness, to allow lipophilic dyes reach the IPN.

(d) *Image acquisition, processing and three-dimensional reconstruction*

Fluorescent samples were imaged on either Zeiss LSM 5 Pascal confocal or UltraView RS spinning disc (Perkin Elmer) microscopes using an Achroplan 40 $\times$ /0.8 W dipping objective or a Plan-Apochromat 40 $\times$ /1.2 W objective. Images were deconvolved to reduce blurring and noise using Huygens Professional and Scripting DECONVOLUTION softwares. Three-dimensional image projections were obtained using the opacity reconstruction model in VOLOCITY software (Improvision).

(e) *Rationale and methodology for normalization of developmental time*

According to a hypothetical model of developmental time control, the overall rate of embryo development depends on both intrinsic clock and temperature-sensitive mass-specific metabolic rate signals (Reiss 2003). Zebrafish and medaka exhibit similar size of embryos, larvae and adults and probably share comparable mass-specific metabolic rates. To avoid the influence of temperature upon this variable, we considered the timing of onset and offset of developmental events at a single constant temperature (26°C). In zebrafish, developmental events were determined as HPF at 28°C and then scaled to HPF at 26°C according to Kimmel *et al.* (1995). In medaka, timings of developmental events were expressed as HPF at 26°C using the Iwamatsu developmental stage table (Iwamatsu 2004). To scale the influence of the internal clock, we normalized absolute times based on the rate of somitogenesis. This periodic segmentation process is known to be controlled by a molecular clock linked to oscillatory gene expression (Saga & Takeda 2001; Freitas *et al.* 2005) that depends on the rates of transcription and translation (Giudicelli & Lewis 2004). We considered the time needed for making a single somite during the linear phase of somitogenesis as a time-normalizing factor, and expressed the newly calculated normalized times in somite time units (STU). The calculation method used available data on the rate of somitogenesis at 26°C in zebrafish (Kimmel *et al.* 1995) and medaka (Iwamatsu 2004). Somite number versus time was plotted using ORIGINPRO v. 7.0220. The linear phase of somitogenesis extended between 4 and 30 somites for both species, and the total number of somites formed was 34 and 35 for zebrafish and medaka, respectively. Linear regression of the data revealed that zebrafish and medaka form 1.7 and 0.797 somites per hour, respectively. The reciprocal of the slope

values indicated the time needed for making a single somite (*t-1som*) in both species. Normalized times of development were obtained by dividing absolute time by *t-1som*.

3. RESULTS

(a) Morphological and topological organization of epithalamic asymmetries

(i) Asymmetric expression of nodal signalling genes in the embryonic epithalamus

In zebrafish, several components of the nodal signalling pathway are asymmetrically expressed in the epithalamus preceding the onset of asymmetric morphogenesis (Concha *et al.* 2000; Liang *et al.* 2000). For example, the nodal inhibitor *lefty1* (*Dr-lefty1*) and the downstream transcriptional effector *pitx2c* (*Dr-pitx2c*) define restricted dorsal domains of expression in the left side of the neural tube, posterior to the lateral flexure of the diencephalic ventricle (figure 1*a*(i),(ii)). Recent reports in medaka have shown that *Ol-lefty* (Carl *et al.* 2007; Soroldoni *et al.* 2007) and *Ol-pitx2* (Jaszczyszyn *et al.* 2007) also display asymmetric expression in the dorsal diencephalon, and a close examination indicates that the extent and topology of expression of these genes are similar to zebrafish (compare figure 1*a*(i),*b*(i) and *a*(ii),*b*(ii)).

(ii) Left-sided asymmetric positioning and connectivity of the embryonic parapineal organ

In zebrafish, asymmetric morphogenesis of the parapineal organ involves an initial phase of migration from the dorsal midline to the left side of the brain followed by the development of efferent connectivity directed to the left habenula (Concha *et al.* 2003). Confocal imaging of transgenic *Tg(foxD3::GFP)* zebrafish embryos reveals that the parapineal organ is located on the left and ventral sides of the pineal organ, and sends axonal projections that distribute broadly in the left habenula (figure 1*a*(iii)). In medaka, the parapineal organ of *Tg(fRx2::GFP)* embryos is also observed on the left side and develops efferent connectivity directed to the left habenula (figure 1*b*(iii)). However, the volume of the parapineal organ compared with the pineal organ is considerably larger in medaka (ratio of $0.6/1 \pm 0.13$, $n=3$ embryos, mean $\pm$ s.d.) than zebrafish (ratio of $0.1/1 \pm 0.02$, $n=3$) (compare figure 1*a*(iii),*b*(iii)). In addition, parapineal efferents form a thick and long bundle of axons that make an orthogonal turn towards the anterior, dorsal and the midline to end in a well-defined neuropil domain with the shape of a dandelion seed head (figure 1*b*(iii)).

(iii) Asymmetric cytoarchitectonic organization of the larval habenulae

In zebrafish, left and right habenular nuclei undergo distinct patterns of neurogenesis (Aizawa *et al.* 2007) and display asymmetric growth of neuropil domains (Concha *et al.* 2003; Gamse *et al.* 2003). We performed immunostaining against acetylated α -tubulin to reveal the distribution of neuropil (figure 1*a*(iv)) and fluorescent-Nissl staining to expose the spatial organization of neuronal cell bodies (figure 1*a*(v)). We confirmed that neuropil asymmetries in zebrafish are limited to a dorsomedial region of the left habenula

located in the vicinity of the habenular commissure (arrows in figure 1*a*(iv)). In medaka, neuropil asymmetries define a compact and Nissl well-delimited neuropil domain situated in the most dorso-anteromedial aspect of the left habenula (arrowheads in figure 1*b*(iv),(v)). Such a singular domain is not observed in the right habenula of medaka and is absent from both left and right habenulae of zebrafish (figure 1*a*(iv),(v),*b*(iv),(v)).

(iv) Contribution of parapineal connectivity to habenular asymmetry

Double immunostaining against acetylated α -tubulin (neuropil) and GFP (parapineal organ) in transgenic embryos reveals that parapineal efferents make a hidden morphological contribution to habenular asymmetry in zebrafish. Parapineal efferent connectivity blends into a neuropil domain situated immediately anterior to the habenular commissure, which becomes asymmetrically enlarged in the left habenula compared with the right counterpart (asterisks in figure 2*a*(i)–(vii); Concha *et al.* 2003). By contrast, parapineal efferents make a more explicit contribution to morphological asymmetry in medaka. Most parapineal axonal terminals segregate from other sources of habenular neuropil to form a distinct dorso-anteromedial domain situated distant from the habenular commissure, which corresponds to the singular left-sided habenular neuropil domain defined by acetylated α -tubulin and Nissl staining (arrowheads in figure 2*b*(i)–(vii); see also arrowheads in figure 1*b*(iv),(v)).

(v) Dorsoventral segregation of left–right habenular efferents in the larval midbrain

The target regions of habenular neurons can be determined by labelling left and right habenular nuclei with the lipophilic dyes DiD and DiO, respectively (Aizawa *et al.* 2005). In zebrafish larvae, efferent connectivity from left and right habenular nuclei forms distinct and segregated ring-shaped domains within dorsal and ventral regions of the IPN, respectively (figure 1*a*(vi),(vii); Aizawa *et al.* 2005; Gamse *et al.* 2005; Bianco *et al.* 2008). A similar pattern of habenular efferent connectivity is observed in the larval IPN of medaka (see figure 1*b* (vi),(vii); Carl *et al.* 2007). However, the cross-sectional area of the central fibre-free region of IPN rings, compared with the cross-sectional area of the entire IPN appears relatively larger in medaka ($26.7 \pm 3\%$ of total IPN, $n=3$ embryos, mean $\pm$ s.d.) than in zebrafish ($8.8 \pm 4.6\%$ of total IPN, $n=3$).

(vi) Laterality of epithalamic asymmetries and its correspondence to organ laterality

In zebrafish, the development of parapineal and habenular asymmetries is interdependent and result in larvae showing concordant laterality of epithalamic asymmetries (Concha *et al.* 2003; Gamse *et al.* 2003). In addition, laterality of epithalamic and visceral asymmetries are coupled as both depend on left-sided nodal signalling emerging from a common symmetry-breaking event (Concha 2004; Levin 2005). In medaka, we scored the laterality of parapineal (GFP),

Table 1. Concordant laterality of epithalamic and heart asymmetries in zebrafish and medaka.

parapineal laterality	habenular laterality ^a			heart jog/loop laterality ^b		
	left (%)	right (%)	no.	normal (%)	reversed (%)	no.
zebrafish (<i>Danio rerio</i>)						
left	100	0	48	98	0	306
right	0	0	0	0	2	6
total	100	0	48	98	2	312
medaka (<i>Oryzias latipes</i>)						
left	100	0	98	100	0	470
right	0	0	0	0	0	0
total	100	0	98	100	0	470

^aConcordant laterality of parapineal and habenular asymmetries was analysed by immunostaining against acetylated α -tubulin (habenulae) and GFP (parapineal) in *Tg(Foxd3::GFP)* (zebrafish, 140 HPF at 26°C) and *Tg(fRx2::GFP)* (medaka Iwamatsu St.39, 216 HPF at 26°C).

^bConcordant laterality of epithalamic and heart asymmetries was analysed in living embryos by scoring the orientation of heart jog/looping, and the position of the parapineal organ in *Tg(Foxd3::GFP)* (zebrafish, 56 HPF at 26°C), *Tg(fRx2::GFP)* and *Tg(fRx2/DE::GFP)* (medaka, Iwamatsu St.27, 58 HPF at 26°C).

habenular (acetylated α -tubulin) and organ (heart looping) asymmetries and found a similar concordant laterality pattern (table 1). Surprisingly, we were unable to find a single case of reversal from normal heart laterality in three different strains of medaka (*Tg(fRx2::GFP)*, $n=317$ embryos; *Tg(fRx2/DE::GFP)*, $n=153$; and *cab*, $n=148$), a situation that is common (5% of individuals on average) in other teleost species (Palmer 2004).

(b) Temporal analysis of epithalamic asymmetry development

To analyse how developmental time of epithalamic asymmetry has changed during the evolution of zebrafish and medaka lineages, we compared three main aspects: *sequence* (temporal arrangement of developmental modules); *relative timing* (onset/offset of developmental events with respect to some intrinsic time scale); and *duration* (overall rate of development). We found that all main developmental modules of epithalamic asymmetry were present in both species and temporally arranged in a similar *sequential* manner. For example, asymmetric nodal expression preceded left-sided positioning of the parapineal organ, which in turn was followed by the establishment of habenular asymmetry and segregation of habenular efferents in the IPN (figure 3a).

To perform a meaningful comparison of *relative timing* and *duration*, we scaled the absolute time of onset/offset of homologous developmental events to the duration of a conserved periodic process that depends on intrinsic embryo dynamics (e.g. somitogenesis), and produced a normalized time scale that could be compared among taxa (figure 3b and table 2; see rationale and description of methodology in §2). Comparison of normalized developmental times between zebrafish and medaka uncovered three main groups of events that reveal unexpected similarities/differences in the *relative timing*. A first group comprised early embryonic processes whose *timing* of onset became highly coordinated in both species after time normalization. Within this group, we found epiboly, gastrulation (shield formation), onset of expression of hatching enzymes genes (Inohaya *et al.* 1995, 1997) and somitogenesis (figure 3b). A second

group included developmental events whose absolute differences in *timing* become inverted after time normalization. Important examples within this group were the onset of asymmetric epithalamic nodal signalling, onset of parapineal axonal projection and the initiation of habenula–IPN connectivity (figure 3b). The onset of asymmetric epithalamic nodal expression exhibited a delay of approximately 6 STU towards later developmental times after normalization, when compared with medaka (table 2; figure 3b). Interestingly, the magnitude of this delay was comparable with the delay in the onset of heart beating (13 STU) but was considerably smaller than the temporal shift in the initiation of both parapineal axonal projection (50 STU) and habenula–IPN connectivity (40 STU; figure 3b). Finally, a third group included developmental events whose differences in *timing* were conserved after time normalization. The single example of this group corresponded to hatching, which occurred at an earlier developmental time in zebrafish than medaka (figure 3b).

A last step of comparison concerned the *duration* of developmental events. We focused our analysis on the expression of nodal signalling genes, as they were transient and could be determined with accuracy. Absolute duration of expression of *Ol-lefty* and *Ol-pitx2* doubled that of *Dr-lefty1* and *Dr-pitx2c*, respectively (table 2; figure 3a). However, the ratio between the lengths of *lefty* and *pitx2* expressions was equivalent in both species (zebrafish=0.25; medaka=0.24) suggesting that the differences in the absolute length of gene expression could result from variations in the intrinsic speed of embryo development. To test this hypothesis, we compared normalized lengths of gene expression and found them strikingly similar for each pair of orthologue genes: differences represented less than 15 per cent for *lefty* and 10 per cent for *pitx2* when calculating the ratio zebrafish/medaka (figure 3b).

4. DISCUSSION

(a) Overall conservation of asymmetry in the parapineal–habenular–IPN system of teleosts

In this study, we compared the main developmental modules of epithalamic asymmetry in two related teleost species with 115–200 Myr of independent

Figure 3. Comparison of *sequence*, *relative timing* and *duration* of developmental events during the establishment of epithalamic asymmetry in zebrafish and medaka. The diagrams show the temporal occurrence of key steps of asymmetric brain morphogenesis in zebrafish and medaka, expressed in (a) absolute and (b) normalized times. To provide a contextual view, the timing of main embryonic events is also included. The colour codes shown at the bottom of the figure indicate different developmental events (lines) and periods (boxes or bars) analysed in the temporal plots of (a,b). For clarity, equivalent events in medaka and zebrafish are joined. Diagrams of developmental stages were obtained from the literature (Kimmel *et al.* 1995; Iwamatsu 2004). Schematic of epithalamic asymmetry events (bottom right) correspond, from top to bottom, to: a frontal view of the epithalamus depicting left-sided asymmetric nodal expression, a dorsal view of the pineal complex showing the initiation of left-sided parapineal axonal projection and a dorsal view of the IPN (white circle) revealing habenular efferent connectivity reaching dorsal and ventral regions of the IPN. The scale was maintained in (a,b) to emphasize the effect of time normalization. Zebrafish and medaka show a conserved sequence of developmental events of epithalamic asymmetry although they exhibit distinct relative timing (heterochrony).

evolution. Our findings reveal a striking conservation of both the overall spatial organization of brain asymmetry and the temporal sequential arrangement of developmental modules underlying the formation of the parapineal–habenular–IPN system. Such conservative ontogenetic trajectory suggests a causal dependency between the different asymmetry modules. This idea is supported by recent experimental evidence showing that habenular asymmetry is affected by physical removal of the parapineal organ (Concha *et al.* 2003; Gamse *et al.* 2003; Bianco *et al.* 2008). In addition, segregation of habenular efferents in the IPN

depends on the proper development of asymmetry in the habenulae (Aizawa *et al.* 2005; Gamse *et al.* 2005; Carl *et al.* 2007; Kuan *et al.* 2007; Bianco *et al.* 2008). Evolutionary conservation also suggests that the overall pattern of asymmetry in the parapineal–habenular–IPN axis is plesiomorphic to teleosts. Indeed, habenular and parapineal asymmetries are described in a number of teleost species (Concha & Wilson 2001) and recent observations extend these findings to the IPN of the southern flounder (*Paralichthys lethostigma*; Kuan *et al.* 2007) and guppy (*Poecilia reticulata*; A. Villalón & M. L. Concha 2007, unpublished data). Interestingly, despite

Table 2. Comparison of developmental events of brain asymmetry between zebrafish and medaka.

developmental event	zebrafish (<i>Danio rerio</i>)			medaka (<i>Oryzias latipes</i>)		
	time (HPF) ^a		STU ^b	time (HPF) ^b		STU ^b
epithalamic nodal expression						
<i>lefty</i>						
onset	19.8 ± 1	(17 ± 1)	34.4 ± 2	36 ± 2	(St.21–22)	28.7 ± 2
offset	28.5 ± 1	(24.5 ± 1)	49.4 ± 2	52 ± 2	(St.25–26)	41.4 ± 2
duration	8.7 ± 1	(6.5 ± 1)	13.4 ± 2	16 ± 2		12.7 ± 2
<i>pitx2</i>						
onset	19.8 ± 1	(17 ± 1)	34.4 ± 2	36 ± 2	(St.21–22)	28.7 ± 2
offset	54.7 ± 1	(47 ± 1)	94.4 ± 2	103.5 ± 3	(St.32–33)	82.5 ± 3
duration	34.9 ± 1	(21.5 ± 1)	58.4 ± 2	67.5 ± 3		53.8 ± 3
parapineal asymmetry						
onset of axonal projection	57 ± 1	(49 ± 1) ^c	98.2 ± 2	66 ± 8	(St.27–29)	52.6 ± 7
habenular efferents in IPN						
initiation of connectivity ^d	64 ± 6	(74 ± 7)	128 ± 12	111 ± 10	(St.32–33)	88.5 ± 8

^aStaging is expressed in hours post-fertilization (HPF) at 26°C (Kimmel *et al.* 1995; Iwamatsu 2004). Corresponding times at 28°C (zebrafish) and Iwamatsu stages (St) (medaka) are indicated in brackets. Timing of onset/offset was calculated as the midpoint between the stage when the developmental event is first observed and the preceding/following stage, respectively. Variability corresponds to half the duration of the interval between these stages.

^bNormalized times are expressed in STU (see §2).

^cTaken from Concha *et al.* (2000, 2003).

^dInitiation of connectivity between the habenula and IPN is defined by the initial axonal branching of left and right fasciculi retroflexus within the IPN, prior to the establishment of dorsal and ventral ring-shaped domains.

the overall conservation of habenular asymmetry among a wide range of vertebrate groups (Concha & Wilson 2001) the segregation of left–right habenular efferents along the dorsoventral axis of the IPN appears unique to teleosts as it is absent in frogs (*Rana clamitans*), salamanders (*Ambystoma maculatum*) and mice (Kuan *et al.* 2007). Whether or not this peculiar asymmetry trait represents a variation of form evolved exclusively by the teleost lineage will need further experimental testing.

(b) *Heterotopic parapineal efferent connectivity suggests divergent principles of development between zebrafish and medaka*

Our results support the notion that left-sided positioning of the parapineal organ is a shared feature of asymmetric brain morphogenesis within the teleost group (Borg *et al.* 1983; Concha & Wilson 2001). However, the relative size of the parapineal organ (compared with the pineal organ) and its pattern of efferent connectivity greatly differ between zebrafish and medaka. In zebrafish, the body of the parapineal organ is relatively small in size ($\pm 10\%$ of the pineal) and its efferent connectivity distribute broadly in the left habenula. By contrast, the parapineal organ of medaka is larger ($\pm 60\%$ of the pineal) and its efferent connectivity forms a large and well-defined antero-dorsomedial neuropil domain within the left habenula (figure 2). Although the number and nature of parapineal–habenular synapses remains unknown, our results suggest that divergent principles of development and circuit configuration emerged during the independent evolution of zebrafish and medaka lineages. Such a variation in the relative size of pineal and parapineal organs is not exclusive to teleosts as it is also observed among species of reptiles developing a parietal eye

(the homologous structure to the parapineal organ; Concha & Wilson 2001).

Previous results suggest that the spatial organization of parapineal efferents depends on a bidirectional interaction established between the parapineal organ and habenulae during development (figure 4). Initially, early asymmetry in the presumptive habenular region is thought to guide asymmetric parapineal migration (Concha *et al.* 2003). Subsequent left-sided positioning of the parapineal organ is required for the amplification (and perhaps the topological setting) of distinct differentiation programmes in the left and right habenulae (Gamse *et al.* 2003; Bianco *et al.* 2008). Finally, parapineal axons distribute in regions of the left habenula, which exhibit enlarged neuropil (Concha *et al.* 2003) and asymmetric *leftover* expression (Gamse *et al.* 2003), therefore linking the topology of parapineal efferent connectivity to the underlying organization of differentiation domains within the left habenula.

Based on these observations, we propose two developmental models to explain the different topologies of parapineal efferent connectivity observed in zebrafish and medaka (figure 4). In the first model, the molecular/connectional identity of parapineal target cells is conserved in the two species, but the topological organization has diverged owing to changes in the spatial and/or temporal organization of a shared set of signals that pattern the habenulae (model 1; figure 4b). In the second model, the molecular/connectional identity of parapineal target cells has diverged as a result of divergent signalling mechanisms involved in either guiding parapineal connectivity or patterning the habenulae (model 2; figure 4c).

The proposed models have potential dissimilar implications in the function of the parapineal–habenular–IPN system. Whereas solitary changes in

the topology of parapineal target cells probably represent no major functional modification of the system (model 1; figure 4b), transformations in the identity of parapineal target cells might result in distinct neurotransmitter and/or connectional influences of the habenulae upon the IPN (model 2; figure 4c). Given the overall morphological and connectional conservation of the parapineal–habenular–IPN circuit, it seems reasonable to expect a conservation of parapineal functionality in the circuit (model 1). Nevertheless, it is possible that the parapineal organ plays no major role in this asymmetric circuit and that the observed phenotypic variation in the topology of parapineal efferent connectivity is a direct consequence of this feature (Hallgrímsson 2003). To date, we have no sufficient data to either sustain or discard this possibility. As the parapineal organ contains both photoneuroendocrine cells and projection neurons, it is possible that circadian variations of light influence the neuroendocrine activity of the parapineal organ and consequently the function of the habenular–IPN system (Concha & Wilson 2001). However, it has also been reported that parapineal photoreceptors are rather rudimentary (Rudeberg 1969; Van Veen 1982; Ekstrom *et al.* 1983) and that in many species the parapineal organ appears to have regressed in adulthood (Borg *et al.* 1983).

(c) *Heterochronic shifts and the ontogeny of epithalamic asymmetry*

The dimension of time is critical for development and a key factor in the generation of evolutionary diversity (Gould 1977). The examination of the temporal dimension of development among species allows the study of developmental trajectories, the detection of heterochronies (shifts in timing), the making of inferences about the coupling/uncoupling of developmental modules and the reconstruction of the ancestral sequence of developmental events (Reiss 2003; Zelditch 2003). In the present study, we searched for events of conservation and variation in each of the three main aspects of time underlying the development of epithalamic asymmetries. We found a major conservation in the *sequence* of developmental modules of brain asymmetry (see above). For a proper examination of *relative timing* and *duration*, we developed a method to normalize the intrinsic time scale of zebrafish and medaka development based on the clock properties of somitogenesis. Using this normalization method, we could synchronize the *relative timing* of early embryonic events. In addition, we found that *duration* of the expression of genes involved in the control of brain laterality matched after time normalization. This finding provides support to the usefulness of this normalization method for the comparison of developmental time among related species, compared with that of alternative methods (Dettlaff & Dettlaff 1961; Reiss 1989; Chipman *et al.* 2000; Clancy *et al.* 2001). Moreover, this observation suggests that both species share a similar *tempo* of nodal-dependent laterality determination, and that absolute differences in the duration of nodal signalling depend primarily on the intrinsic rate of embryo development of each species.

The normalization method also allowed the distinction of interspecies changes in the *relative timing* of

epithalamic asymmetry events. Three main heterochronic shifts involved the onset of epithalamic nodal signalling, the onset of parapineal axonal efferent projection and the initiation of habenula–IPN connectivity expressed as the initial branching of left and right axons emerging from the fasciculus retroflexus within the IPN (figure 3). The direction of these shifts is consistent with previous reports suggesting that brain development is delayed relative to somitogenesis in zebrafish compared with medaka (Wittbrodt *et al.* 2002). More recent data add extra support to this general concept as it reveals a reversal in the *relative timing* of expression of specific components of the nodal signalling pathway in the brain with respect to the lateral plate mesoderm (LPM) in the two species, e.g. in medaka mRNA of *nodal-related 2*, *lefty* and *pitx2* are detected earlier in the brain than that in the LPM while the opposite is observed in zebrafish (figure 3b; Rebagliati *et al.* 1998; Bisgrove *et al.* 2000; Soroldoni *et al.* 2007). Unexpectedly, the onset of parapineal axonal projection and the initiation of habenula–IPN connectivity exhibited a pronounced heterochronic shift with respect to the onset of nodal signalling, being largely delayed in zebrafish with respect to medaka (figure 3b). As parapineal connectivity appears to be linked to the programme of habenular differentiation, it is possible that the latter is delayed in zebrafish, and that the more dispersed distribution of parapineal target cells of the zebrafish larvae represents a transitional state towards a more segregated distribution reached in the habenulae at post-larval stages. Consistent with the idea of a shift in the timing of habenular differentiation, we observed that the onset of axonal branching of habenular efferents within the IPN is also delayed in zebrafish compared with medaka (figure 3b). Further experimental testing of this hypothesis might provide a causal link between the heterotopic and heterochronic changes described in this study.

It is important to note that aspects of organogenesis such as the onset of heart beating are shifted in the same temporal direction as shifts in brain development. This observation opens the possibility that organogenesis as a whole has undergone a heterochronic shift during the evolution of medaka and zebrafish lineages. In this respect, it is intriguing that hatching shows a reversed heterochronic shift to that observed for organogenesis, e.g. it is delayed in medaka compared with zebrafish. As the onset of expression of hatching enzyme genes is comparable in zebrafish and medaka (figure 3b; Inohaya *et al.* 1995, 1997), it is likely that the differences in hatching time are a result of dissimilar chorion composition and thickness between the two species (Hart *et al.* 1984; Hart & Donovan 2005). Regardless of the underlying developmental mechanism, a main consequence of the heterochronic shift in hatching is the definition of zebrafish as altricial (immature) and medaka as precocial (more developed) species (MacArthur & Wilson 1967).

(d) *Is the laterality of asymmetry canalized in medaka?*

Although left-sided laterality of heart asymmetry is a well-conserved trait of vertebrates, a small percentage of individuals in the population show spontaneous

Figure 4. Developmental models of heterotopic parapineal efferent connectivity. (a) Zebrafish: asymmetries of the parapineal organ and habenulae interact in three consecutive steps during development. (i) Initial left–right biases in the presumptive habenular region guide parapineal migration to the left side. (ii) Subsequent left-sided positioning of the parapineal organ is involved in the induction/propagation of a distinct spatial pattern of habenular differentiation. (iii) Finally, the topology of subdomains arising after habenular differentiation determines the position of habenular neurons receiving parapineal efferent connectivity. (b) Medaka (model 1): heterotopic parapineal efferent connectivity arises from a different topological organization of habenular subdomains. In this model, spatial differences in the location of the parapineal organ at the time of habenular differentiation and/or underlying differences in habenular (i) pre-patterning lead to (ii) distinct topological programmes of habenular subdomain differentiation and (iii) subsequent positioning of parapineal target cells within the habenulae. (c) Medaka (model 2): heterotopic parapineal efferent connectivity arises from different selection of parapineal target cells within the habenulae. In this model, parapineal migration and habenular differentiation are equivalent in both species. However, parapineal projections reach different target neurons in the habenula of both species owing to the differences in axon guidance cues. (i) Shaded green regions depict molecular left–right biases within the presumptive habenulae. The movement of the parapineal organ from the midline to the left side (arrows) is represented as partially overlapping drawings of parapineal outlines. (ii) White and red regions illustrate putative subdomains of the habenulae. Arrows illustrate the direction of the inductive properties of the parapineal organ. (iii) The topological pattern of parapineal efferent connectivity (black) and the location of parapineal target cells within the habenula (colours) are shown (see also figure 2). Colours represent equivalent cellular identities. All diagrams correspond to dorsal views, with anterior to the top. For clarity, only the left habenula is illustrated, and the right habenula is depicted with dotted lines. L, left; R, right; Lh, left habenula; Rh, right habenula; hc, habenular commissure.

reversal of this asymmetry. Incidence of heart reversals have declined during vertebrate evolution from fishes (approx. 5%) through amphibians and birds (1–2%) to mammals (less than 0.1%), indicating a canalization of heart laterality during vertebrate evolution (Palmer 2004). Our finding that medaka showed 0 per cent of heart reversals indicates that this species deviates from the expected teleost pattern (e.g. zebrafish, trout and salmon). Although we cannot discard the notion that the inbreeding nature of the medaka strains (Wittbrodt *et al.* 2002) reduces the normal fluctuation of individual laterality, it is possible that symmetry-breaking mechanisms are more robust and resistant to genetic and environmental perturbations in medaka than in the other teleosts that have been analysed. A major mechanism of vertebrate laterality determination involves the generation of extracellular leftward fluid flow (the so-called nodal flow) within the ventral node of mice (Hirokawa *et al.* 2006) and the Kupffer's vesicle (KV) of teleosts (Essner *et al.* 2005; Kramer-Zucker *et al.* 2005; Okada *et al.* 2005). Recent reports have revealed that the KV of medaka shares more similarities to the mammalian node than to the zebrafish KV, when

considering the cytoarchitectonic organization of ciliated cells and the robustness of the nodal flow (Essner *et al.* 2005; Kramer-Zucker *et al.* 2005; Okada *et al.* 2005; Hirokawa *et al.* 2006; Oteiza *et al.* 2008). Hence, we propose that the canalization of embryo laterality may be linked to the morphology of laterality organs and consequently the nature of the nodal flow they produce. In this context, other developmental conditions that have been proposed to make laterality decisions more predictable (e.g. placental environments; Palmer 2004) would play only additive roles.

5. CONCLUSIONS: ZEBRAFISH AND MEDAKA AS MODELS FOR COMPARATIVE DEVELOPMENTAL BIOLOGY OF VERTEBRATE BRAIN ASYMMETRY

Since the initial proposal of medaka and zebrafish as complementary model organisms suitable for comparative developmental biology (Furutani-Seiki & Wittbrodt 2004), several reports have made use of the experimental and evolutionary advantages of these genetic organisms to start revealing conserved and species-specific principles of vertebrate development

(e.g. Lynn Lamoreux *et al.* 2005; Gajewski *et al.* 2006; Carl *et al.* 2007). The present study brings additional support to this notion, offers new tools for time comparison between these species and provides novel comparative data and hypotheses to start addressing the ontogenic mechanisms that explain interspecies variations of epithalamic asymmetry. Together, these findings highlight the usefulness of zebrafish and medaka as comparative models of brain asymmetry development and function.

All procedures of animal care and management conformed to high standards in agreement with the revised Council of Europe guidelines (ETS123) on housing, and were approved by a local Committee of Bioethics on Animal Experimentation at the Faculty of Medicine, University of Chile.

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