Determining the function of zebrafish epithalamic asymmetry

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As in many fishes, amphibians and reptiles, the epithalamus of the zebrafish, \textit{Danio rerio}, develops with pronounced left–right (L–R) asymmetry. For example, in more than 95 per cent of zebrafish larvae, the parapineal, an accessory to the pineal organ, forms on the left side of the brain and the adjacent left habenular nucleus is larger than the right. Disruption of Nodal signalling affects this bias, producing equal numbers of larvae with the parapineal on the left or the right side and corresponding habenular reversals. Pre-selection of live larvae using fluorescent transgenic reporters provides a useful substrate for studying the effects of neuroanatomical asymmetry on behaviour. Previous studies had suggested that epithalamic directionality is correlated with lateralized behaviours such as L–R eye preference. We find that the randomization of epithalamic asymmetry, through perturbation of the \textit{nodal}-related gene \textit{southpaw}, does not alter a variety of motor behaviours, including responses to lateralized stimuli. However, we discovered significant deficits in swimming initiation and in the total distance navigated by larvae with parapineal reversals. We discuss these findings with respect to previous studies and recent work linking the habenular region with control of the motivation/reward pathway of the vertebrate brain.

\textbf{Keywords:} habenula; brain asymmetry; behaviour

1. INTRODUCTION

The functional significance of brain laterality has been a long-debated topic in cognitive neuroscience. Theories abound as to the advantages of the left–right (L–R) specialization of the nervous system and as to why directional biases in neuroanatomy and behaviour are found throughout the animal kingdom (Vallortigara & Rogers 2005). For example, light-induced neuroanatomical asymmetry in the visual system of developing birds correlates with some enhanced visual behaviours in adulthood (Güntürkün et al. 2000; Rogers 2008), and preferential eye use has been argued to mediateshoaling behaviour in social fish species (Bisazza et al. 2000).

Fish species are a valuable system for examining functional lateralization at the individual and population level (Bisazza et al. 1998). Because the eyes are positioned laterally on the head and each is exposed to a different visual landscape, left or right eye use upon viewing familiar or novel objects, or when self-viewing (‘mirror test’) provides a simple assay to detect biases (Facchin et al. 1999; Sovrano et al. 1999; De Santi et al. 2001; Sovrano et al. 2001). Systematic preferences in eye use are proposed to be a behavioural manifestation of specialization of the two sides of the brain in processing incoming visual information, since each eye predominately projects to the contralateral side of the brain (Vallortigara 2000). Turning to avoid barriers or to navigate complex environments, prey capture and aggressive behaviours also have been found to have a preferred directional component in some fish species (e.g. Heuts 1999; Bisazza et al. 2000, 2001; Bisazza & de Santi 2003; Reddon & Hurd 2008 and refer to Vallortigara & Bisazza 2002).

The zebrafish, \textit{Danio rerio}, has obvious benefits in exploring behavioural laterality, as a well-studied developmental model amenable to genetic manipulations. Functional lateralization in this species has been previously documented for a number of behavioural tests both in adults (Miklósí et al. 1997, 2001; Heuts 1999; Miklósí & Andrew 1999) and in young fry (Watkins et al. 2004; Barth et al. 2005; Sovrano & Andrew 2006).

Adult zebrafish show a right eye preference when first exposed to new objects or complex scenes that require immediate monitoring and response (Miklósí et al. 2001; Miklósí & Andrew 2006). However, the left eye is preferentially used on subsequent trials, for visual inspection of familiar stimuli or those with moderate novelty and, presumably, comparisons with the memory of similar stimuli. Thus, left eye viewing appears to be better equipped for comprehensive assessment of familiarity, while the right eye system has been proposed to be more resistant to distraction and to mediate decision-making responses (Miklósí et al. 1997; Miklósí & Andrew 2006). Adults also tend

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to use the right eye when approaching an object to bite; however, no bias in eye use is found when a familiar object is investigated and not bitten (Miklósi & Andrew 1999). When faced with a barrier blocking access to a perceived predator, adult zebrafish show a detour response that is biased for left eye inspection and turning to the right (Bisazza et al. 2000).

Larval zebrafish as young as 8 days post-fertilization (dpf) appear to exhibit behavioural biases. Watkins et al. (2004) described biases in the directionality of turning, which were correlated with changes in light intensity that an 8-day-old larva experienced while navigating through a multicompartment swimway. They also found preferential left eye inspection and less avoidance behaviour in larvae exposed to a dark stripe that had previously been presented in the left visual field. Their findings were consistent with the left eye bias described for adult zebrafish in assessing stimuli with respect to prior experiences (Miklósi et al. 1997). Sovrano & Andrew (2006) modified the mirror test to study the development of visual lateralization in zebrafish larvae and also found a preference for left eye viewing. However, left eye bias was strain, age and distance dependent and was sustained for varying periods within the testing window. A more recent study (Andrew et al. in press) also suggests that, as in developing chicks (refer to Rogers 2008), early exposure to light may influence bias in L–R eye use.

2. THE ZEBRAFISH AS A MODEL OF EPITHALAMIC L–R ASYMMETRY

Recently, it has become possible to tackle the problem of how brain asymmetry arises developmentally using molecular genetic approaches afforded by the zebrafish model. Although there remains some controversy about the nature of the initial symmetry-breaking event in the early embryo, the ciliated Kupffer’s vesicle present in the caudal midline at somitogenesis (Bisgrove et al. 2005; Essner et al. 2005) and Wnt signalling (Carl et al. 2007; Inbal et al. 2007) have been implicated in the determination of L–R differences. Components of the Nodal signalling pathway involved in specifying the L–R axis across vertebrates also show a conserved function in the establishment of zebrafish visceral asymmetry (refer to Liang & Rubinstein 2003; Schier 2003).

However, only in fishes have Nodal-related TGF-β family members been shown to influence L–R determination in the brain, specifically in the epithalamic region of the dorsal diencephalon (Concha et al. 2000; Liang et al. 2000). Loss of Nodal-related signals (cyclops/nodal-related 2 or southpaw/nodal-related 3) does not disrupt L–R asymmetry, but rather results in a randomization in directional asymmetry across the population. For example, more than 95 per cent of all wild-type zebrafish embryos form a parapineal organ on the left side of the brain (Concha et al. 2000; Gamse et al. 2002). The parapineal is closely associated with the pineal organ and arises from cells in a shared pineal complex anlage (Concha et al. 2003; Snelson et al. 2008). In approxi-mately 50 per cent of embryos with Nodal signalling blocked or that lack southpaw (spaw) function, the parapineal develops to the left of the pineal, while the other 50 per cent form the parapineal on the right.

While this might seem like a minor disruption, the position of the parapineal has striking consequences on the development of the epithalamic region flanking the pineal complex, the bilateral habenular nuclei, and their connectivity with a shared midbrain target. In the vast majority of larvae, the left habenula is in close apposition to the parapineal and is larger, exhibits more dense neuropil and a different gene expression profile than the right habenula (Concha et al. 2003; Gamse et al. 2003, 2005; Kuan et al. 2007a,b). L–R patterns of gene expression appear to correlate with differences in subnuclear organization and proliferation of habenular neurons (Gamse et al. 2003; Aizawa et al. 2007). The right habenula may be a default state because, when the parapineal is destroyed, the left habenular nucleus develops with properties more similar to the right habenula (Concha et al. 2003; Gamse et al. 2003). However, an exception is that distinct left and right neuronal morphologies appear to still be maintained (Bianco et al. 2008).

Neurons from the left habenula normally project their axons to dorsal and ventral regions of the interpeduncular nucleus (IPN) in the ventral midbrain, whereas projections from the right habenular neurons are confined ventrally (Gamse et al. 2005). Expression of the gene encoding the axon guidance receptor Neuropilin-1 (Nrp1) is restricted to the left habenula, which most probably accounts for the L–R difference in target connectivity (Kuan et al. 2007a,b). Morpholino-mediated disruption of Nrp1 or parapineal ablation leads to a similar outcome, with both left and right habenular efferents primarily innervating the ventral target. Larvae with the parapineal on the right side of the brain not only show a L–R reversal in habenular identity as assessed by differences in size, amount of dense neuropil and gene expression (including right habenular expression of nrp), but they also exhibit a corresponding reversal in the IPN innervation pattern (Gamse et al. 2005; Kuan et al. 2007a,b). Because neither the distinct functions of the dorsal and ventral IPN nor their post-synaptic partners have yet been determined in zebrafish, it is unknown what effect parapineal and, hence, habenular L–R reversal would have on neural pathways influenced by the habenular-IPN connection.

Mutations in a variety of developmentally important genes disrupt directional asymmetry in zebrafish embryos, and L–R randomization in mutants can uncouple visceral and brain asymmetries (Sampath et al. 1998; Essner et al. 2000). A zebrafish line, frequent-situs-inversus (fsi), that has a tendency to produce a higher than usual frequency of larvae with concordant heart, gut, pancreas and parapineal L–R reversals has also been described (Barth et al. 2005). This trait does not segregate as a simple single-gene mutation, but intercrosses within the fsi line variably increase the rate of situs inversus from 5 to 25 per cent in a single clutch. Analyses of fsi individuals with L–R reversed epithalamic neuroanatomy indicated a corresponding reversal in the directionality of some later-alized behaviours (Barth et al. 2005). The ability to alter the L–R orientation of the brain in a predictable manner by genetic manipulations is a valuable feature of the zebrafish system for studies on the behavioural consequences of an asymmetric nervous system.
Using an antisense morpholino (MO) against the spaw gene (Long et al. 2003) injected into one-cell stage embryos, we can reliably generate four distinct classes of zebrafish larvae: those with the typical pattern of left parapineal and right pancreas (designated L.ppRpa) that is found in more than 95 per cent of wild-type populations; those showing situs inversus or reversal of this pattern (designated R.ppLpa); and two discordant classes with a right parapineal and right pancreas (R.ppRpa) or a left parapineal and left pancreas (L.ppLpa) (Gamse et al. 2005). Following this experimental manipulation, the four classes are not found in equal frequencies (figure 1c); however, a significantly greater number of larvae show reversed epistaphalic and visceral asymmetry compared with wild-type strains. The MO is introduced into doubly transgenic progeny from matings between Tg(foxd3:GFP)$^{het17}$ (Gilmour et al. 2002) and Tg(ela3l:GFP)$^{e22}$;Tg(fabp10:dsRed)$^{e44}$ (Dong et al. 2007) adults, in which the pineal complex and pancreas, and the liver, are labelled with green fluorescent protein (GFP) and red fluorescent protein (RFP), respectively (figure 1a–d). The resultant larvae can be unambiguously sorted at 3 dpf on the basis of the position of the GFP$^+$ parapineal to the left or right of the pineal organ, and at 5 dpf for the location of the GFP$^+$ pancreas on the left or the right side of the body (figure 1e). This approach allows larvae (and adults) to be maintained in four discrete anatomical classes and ensures the availability of large numbers for behavioural analyses. The L.ppRpa group, bearing the configuration of the majority of wild-type or transgenic larvae, also serves as an internal control for potential artefacts associated with MO injection.

### 3. EPITHALAMIC REVERSAL DOES NOT AFFECT MOTOR RESPONSES

To test whether sensory and motor responses differ between the four anatomical groups, we took advantage of the Flote automated system for high-speed video recording and analysis. Flote was designed to measure the detailed kinematics of individual motor behaviours simultaneously in groups of larvae, in an observer-independent manner (Burgess & Granato 2007a). We first examined whether pre-sorted parapineal and pancreas reversed (R.ppLpa) or discordant (L.ppLpa and R.ppRpa) larvae showed differences from the L.ppRpa group in the directionality of their spontaneous movements. To assess spontaneous movements, groups of 7 dpf larvae (8–10 per group) were pre-adapted to a set level of light (170 μW cm$^{-2}$) consistent with the intensity of illumination in the testing arena. After dishes were transferred to the testing arena, larvae were given 3 min to stabilize the levels of locomotor activity prior to video recording. Under unperturbed conditions, larvae typically swim in bouts of forward-directed movements termed ‘slow swims’ or ‘scoots’ and also execute reorienting movements referred to as ‘routine turns’ (R-turns; Budick & O’Malley 2000; Burgess & Granato 2007b). For each anatomical group tested, the kinematics of turning were normal (data not shown) and there was no difference between the groups in the percentage of R-turns executed in a rightward direction (no effect of parapineal laterality ($F_{1,4} = 0.39$, $p = 0.56$) or visceral laterality ($F_{1,4} = 0.003$, $p = 0.96$) using two-way ANOVA). Combining all groups, 50.3±3.2% of R-turns were initiated in a rightward direction (one-sample $t$-test for 50%; $t_7 = 0.11$, $p = 0.93$), indicating that there was no intrinsic L–R bias in turning behaviour under baseline conditions.

We measured the responsiveness and kinematics of larval startle responses following exposure to an intense acoustic/vibrational stimulus (refer to Burgess & Granato (2007a) for details of the startle paradigm). Zebrafish larvae have two primary stereotyped response modes to an acoustic startle stimulus, an explosive C-bend with a short latency (4–8 ms, short latency C-start or SLC) and a second type of C-bend initiated with slower and prolonged duration and with a much longer latency (20–50 ms, long-latency C-start or LLC) (Kimmel et al. 1974; Burgess & Granato 2007a). Both responses are followed by burst swimming movements, which rapidly propel larvae away from their initial position.

**Figure 1.** L–R reversal of anatomical asymmetry in larval zebrafish. (a,b) Dorsal views of the pineal and asymmetrically positioned parapineal (arrowhead) at 3 dpf, following injection of the southpaw MO into the Tg(foxd3:GFP)$^{het17}$ (Gilmour et al. 2002) line. (c,d) Labelling of GFP in the pancreas and dsRed in the liver in 5 dpf Tg(ela3l:GFP)$^{e22}$;Tg(fabp10:dsRed)$^{e44}$ (Wan et al. 2006; Dong et al. 2007) larvae viewed ventrally (c) right pancreas and (d) left pancreas. (e) Frequencies of the four asymmetric configurations in spaw MO-injected, mock-injected and uninjected larvae.
under uniform lighting in a 14 L : 10 D cycle.

In **spaw** MO-injected larvae, no differences were found in the initiation frequency for either SLC responses ($F_{3,68} = 0.80, p = 0.50$; figure 2a) or LLC responses ($F_{3,68} = 0.52, p = 0.66$; figure 2b) between the four anatomical classes. The kinematics of the SLC and LLC responses were also indistinguishable. For example, for the first C-bend of the LLC responses, the latency ($F_{3,67} = 0.30, p = 0.83$), magnitude ($F_{3,67} = 1.13, p = 0.34$), duration ($F_{3,67} = 1.69, p = 0.18$) and angular velocity ($F_{3,67} = 0.57, p = 0.63$) showed no group effect, nor was any group significantly different by t-test from the LppRpa group. These results indicate that all larvae, regardless of their anatomical laterality, sense the startle stimulus normally and respond with a stereotypic C-bend and characteristic succession of movements.

As a population, wild-type zebrafish larvae do not show an intrinsic directional bias in the acoustic startle assay, with 50% of both SLC and LLC responses being initiated in a rightward direction (Burgess & Granato 2007a). Directional bias was also not observed in **spaw** MO-injected LppRpa larvae for either mode of startle response, with 44.9±8.5% of SLC responses initiated in a rightward direction (one-sample t-test against 50%, $t_{14} = 0.60, p = 0.56$) and 45.3±6.4% of LLC responses initiated rightward ($t_{14} = 0.74, p = 0.47$). Moreover, there were no significant differences between the four anatomical groups for directionality of either SLC ($F_{3,61} = 0.17, p = 0.91$) or LLC ($F_{3,67} = 1.6, p = 0.19$) responses. Thus, parapineal or visceral asymmetry was not associated with a L–R bias in C-bends during the startle response.

**4. MOTOR RESPONSES TO DIRECTIONAL STIMULI**

Next, we employed two tests in which motor responses of zebrafish larvae were directionally modulated by an asymmetrically presented stimulus, in the expectation that epithalamic reversal would disrupt lateralization of behavioural activity. For both assays, statistical analyses confirmed that visceral sidedness had no measurable effect, e.g. directionality of responses were not significantly different in either the dark flash test (independent samples t-test, $t_{15} = 0.45, p = 0.66$) or the looming escape response ($t_{20} = 1.4, p = 0.21$), allowing grouping of LppRpa with LppLpa and RppRpa with RppLpa into two datasets (refer to figure 3).

The first test used an abrupt reduction in illumination from an asymmetrically positioned light source (‘dark flash’). Wild-type larvae respond to a dark flash with a stereotyped movement initiated with a large amplitude C-bend (termed ‘O-bend’; Burgess & Granato 2007b). Because they tend to turn towards the extinguished light source (Burgess & Granato 2007b), directionality of an O-bend depends on which side of the larva initially faced the light.

Larvae with a left or right parapineal showed a similar level of responsiveness to a dark flash (independent samples t-test, $t_{14} = 0.16, p = 0.87$; figure 3a) and O-bends were executed with equivalent kinematics in the two groups. For example, latency (Lpp = 458±22 ms and Rpp = 458±20 ms, $t_{15} = 0.002, p = 0.99$) and C-magnitude (Lpp = 141°±4° and Rpp = 146°±4°, $t_{15} = 0.90, p = 0.38$) were almost identical. The tendency of O-bends to be initiated towards the light

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**Phil. Trans. R. Soc. B** (2009)
source (‘bias’, figure 3b) was significant (one-sample t-test against 0, for \( L_{pp} \) \( t_s = 3.9, p = 0.005 \) and for \( R_{pp} \) \( t_s = 4.3, p = 0.004 \)) and of similar magnitude for the two groups (\( t_s = 0.12, p = 0.91 \)).

The second test is based on the observation that many species of fishes, including adult zebrafish, are known to swim away from a looming object by reorienting in the same direction as the moving shadow, and then swimming vigorously forward (Dill 1974; Li & Dowling 1997). To assess the looming escape response, free-swimming larvae in a 6 cm dish were exposed to a moving shadow sweeping across the testing area at a constant rate. For each group of \( L_{pp} \) and \( R_{pp} \) larvae, eight repetitions of the looming stimulus were presented at 60 s intervals in alternating directions.

In this assay, larvae initiate turning manoeuvres to reorient away from the looming shadow, and then perform bouts of forward swimming in the same direction the shadow moves (H. Burgess & M. Granato 2007, unpublished observations). No significant difference in the frequency of turn initiations was detected between \( L_{pp} \) and \( R_{pp} \) larvae (independent samples t-test with unequal variance, \( t_s = 3.3, p = 0.08 \); figure 3c). Moreover, the two groups showed very similar movement kinematics, including latency to movement (\( L_{pp} = 412 \pm 28 \) ms and \( R_{pp} = 395 \pm 25 \) ms, \( t_s = 0.46, p = 0.66 \)) and C-magnitude (\( L_{pp} = 97 \pm 4 \) and \( R_{pp} = 101 \pm 4 \), \( t_s = 0.56, p = 0.59 \)). Thus, larvae with parapineal reversals both detect visual stimuli and have a normal magnitude of response. This assay also tests the directional bias of turn movements away from the shadow. Thus, larvae facing the shadow with their left side tend to turn rightward, whereas those facing the shadow with their right side primarily turn leftward. The directional bias of turn movements away from the shadow was almost identical in \( L_{pp} \) and \( R_{pp} \) larvae (\( t_s = 0.14, p = 0.89 \); figure 3d). These experiments demonstrate that sensory acuity for acoustic and visual stimuli, movement kinematics and levels of responsiveness are all normal in larvae with parapineal reversals.

5. LARVAL POPULATIONS DO NOT SHOW CONSISTENT EYE PREFERENCE

A behavioural test with inherent directionality is the choice of left or right eye used by a larva to view its mirror image. The procedure used to measure eye preference in zebrafish larva was adapted from the mirror test of Sovrano & Andrew (2006). At 8 dpf, each larva was tested individually by gently placing it in the middle of a tank lined with mirrors and recording over a 5 min period its self-viewing approaches towards the mirrors using the left or right eye. Mock-injected larvae showed no population bias in eye use (\( n = 50 \); one-sample t-test against 50%, \( t_{49} = 0.277, p = 0.78 \); figure 4c). Transgenic larvae injected with \( spatz \) MO (\( n = 200, 50 \) for each anatomical class; figure 4b) also did not exhibit statistically significant differences in eye use upon mirror viewing (\( F_{2,199} = 2.03, p = 0.11 \)). To confirm this finding, we also examined un injected transgenic larvae, screening through several thousands to identify the small number that showed spontaneous parapineal reversals (refer to figure 1c). As a group, neither \( R_{pp}L_{pa} (n = 28) \) nor \( R_{pp}R_{pa} (n = 37) \) larvae showed an eye preference in the mirror test and their viewing behaviour was indistinguishable from transgenic siblings with normal \( L_{pp}R_{pa} (n = 53) \) orientation (\( F_{2,111} = 1.41, p = 0.25 \); figure 4c). In every control or experimental larval class, a subset did in fact show a left or right eye preference in mirror approaches (figure 4d,e); however, there was no consistent bias at the population level. While L–R eye use was measured over the entire 5 min period, larval viewing behaviours were also quantified during each 1 min interval, as previous work had suggested that larvae can shift their L–R preference over the course of testing (Barth et al. 2005; Sovrano & Andrew 2006). In a minute-by-minute analysis, \( L_{pp} \) and \( R_{pp} \) larvae also failed to exhibit a significant difference in L–R eye preference (figure 4f; interaction between time in minutes and laterality, \( F_{4,724} = 1.23, p = 0.3 \)).

6. PARAPINEAL REVERSED LARVAE EXHIBIT NAVIGATIONAL DELAY AND REDUCED EXPLORATION

In the course of executing the mirror test, we observed that larvae with the right parapineal configuration showed a significant lag in the onset of navigation (Kruskal–Wallis test, \( \chi^2 = 64.65, p < 0.001 \); figure 5a). The onset was defined as the time that elapsed between the introduction of a larva into the testing chamber and its swimming a distance comparable to twice its body length. Swimming delay was unrelated to positioning of the viscera, as both \( R_{pp}L_{pa} \) and \( R_{pp}R_{pa} \) larvae had a pronounced lag of 66.6 \pm 9.2 and 54.9 \pm 7.68 s, respectively, compared to 13.5 \pm 2.5 s for \( L_{pp}R_{pa} \). 14.9 \pm 3.7 s for \( L_{pp}L_{pa} \) and 4.67 \pm 1.05 s for the mock injected \( L_{pp}R_{pa} \) group. Analyses of transgenic larvae with spontaneous parapineal reversals provided further support for a correlation with delayed navigational behaviour. Spontaneous \( L_{pp}L_{pa} \) and \( R_{pp}L_{pa} \) larvae also showed a significant lag in the onset of navigation compared to their \( L_{pp}R_{pa} \) siblings (Kruskal–Wallis test, \( \chi^2 = 45.54, p < 0.001 \); figure 5b).

By tracking movements over a 5 min period, we also measured the total distance covered by individual 8 dpf larvae (\( n = 118, 35 L_{pp}R_{pa}, 33 L_{pp}L_{pa}, 30 R_{pp}R_{pa}, 20 R_{pp}L_{pa} \)) and their average speed for all swimming episodes. Not only do larvae with parapineal reversals exhibit a navigational delay compared to their left parapineal siblings, but they also cover far less territory (\( F_{3,117} = 8.15, p < 0.001 \); figure 5d) and show a reduced average swimming speed (\( F_{3,117} = 8.18, p < 0.001 \); figure 5e). This finding was independent of visceral orientation (Scheffe post hoc test, \( p < 0.001 \)). A minute-by-minute analysis of the distance traversed (data not shown) indicates that the altered behaviour of \( R_{pp} \) larvae persists throughout the testing period (\( F_{19,569} = 9.89, p < 0.001 \)).

7. DISCUSSION

The results from a battery of behavioural tests indicate that the motor responses of larval zebrafish with reversed laterality of the epithalamus and viscera are largely indistinguishable from those of their siblings with the predominant \( L_{pp}R_{pa} \) anatomical configuration. Neither

Phylo. Trans. R. Soc. B (2009)
Figure 3. Directional behaviours are unaffected by epithalamic reversal. The (a) initiation frequency and (b) directionality of O-bend responses to dark flash stimuli were measured in L_{pp} and R_{pp} larvae (7 or 8 dpf). Dark flashes were generated as previously described (Burgess & Granato 2007b), by extinguishing an array of LEDs (800 μW cm^{-2}) positioned at one end of the dish. Each group (8–10 larvae) was tested with a series of 24 such stimuli, presented at 60 s intervals. Only larvae oriented within 45° of perpendicular to the light source were scored. Bias measures the directionality of responses, where a score of +100 means all O-bends are in the direction of the recently extinguished light source (bias = (% O-bends towards target) × 2–100). L_{pp} (n = 9 plates) and R_{pp} larvae (n = 8 plates) show very similar levels of dark flash responsiveness and directional bias (see text for statistics). The (c) initiation frequency and (d) directionality of turning manoeuvres in response to a looming shadow were measured in L_{pp} and R_{pp} (7 dpf) larvae. A projector was used to illuminate the testing arena (200 μW cm^{-2}) and to cast an area of darkness (4 μW cm^{-2}) expanding at 70 mm s^{-1} across the plate. Groups of 6–10 larvae were tested with eight repetitions of the looming stimulus, which was presented at 60 s intervals in alternating directions. Five groups of L_{pp} and four groups of R_{pp} larvae were tested. Only larvae oriented perpendicular to the direction of movement of the shadow were scored. Turn bias is calculated as for (b), but values are negative because larvae turn away from the approaching shadow. For both assays, 1000 ms recording windows were used to measure responses.

complete nor partial L–R reversals affect a larva’s ability to react appropriately to acoustic and light stimuli; therefore, modified swimming behaviours cannot be accounted for merely by deficits in sensory processing, motor control or muscle activity.

Because all four classes of MO-injected individuals are viable and develop into fertile adults (Long et al. 2003; Gamse et al. 2005), it is unlikely that they harbour severe malformations, such as the vascular abnormalities that are frequently associated with situs defects in mammals (Icardo & Colvee 2001; Peeters & Devriendt 2006). We and others had previously shown that the position of the parapineal represents directional asymmetry throughout the nervous system in either natural or genetically manipulated populations remains to be demonstrated. It may not be the case that a reversal in parapineal position is indicative of reversed asymmetry throughout the brain or predictive of a corresponding shift in lateralized behaviours. Indeed, L–R reversed fsi larvae also exhibited some lateralized behaviours with normal directionality (Barth et al. 2005; Andrew 2006).

they did not report whether these changes were concordant with L–R positioning of the pancreas or other visceral organs. An unaccounted for observation, however, is that the L_{pp}L_{pa} group was always significantly underrepresented following MO injection. L_{pp}L_{pa} larvae have also not been spontaneously recovered from wild-type populations. There may be an early developmental disadvantage for this configuration compared to the other groups, although this has not been directly determined.

We and others had previously shown that the position of the parapineal is tightly coupled to the directional asymmetry of the paired habenular nuclei, including differences in their size, amount of dense neuropil, gene expression and innervation of their shared midbrain target, the IPN (Concha et al. 2000, 2003; Gamse et al. 2003, 2005; Aizawa et al. 2005; Kuan et al. 2007a,b). Thus, reversal of parapineal position, which is typically observed in 2–3% of larvae from wild-type strains, is a readily scored indicator of more pronounced changes in the epithalamus and in epithalamic connectivity. However, whether the position of the parapineal represents directional asymmetry throughout the nervous system in either
Although previous studies have indicated that adult and larval zebrafish as well as many other teleost species exhibit a left eye bias upon self-viewing (Sovrano et al. 1999, 2001; De Santi et al. 2001; Watkins et al. 2004), we recorded no baseline difference in eye preference in the doubly transgenic larvae used in this study. Analyses of $L_{pp}$ and $R_{pp}$ larvae from the $fsi$ strain indicated that they exhibited opposite eye preference upon mirror viewing and an inverse shift in eye preference occurred over time in both groups (Barth et al. 2005). We did not find evidence for similar population biases in eye use for any of the $spaw$ MO-injected groups. Moreover, transgenic larvae we collected that showed spontaneous parapineal reversals

Figure 4. Larval populations do not show consistent eye preference in the mirror test. (a) The mirror test is conducted in a rectangular tank ($10 \times 4 \text{ cm}$) with two mirrors as the longer walls and two white screens as the shorter walls. The tank contains $28^\circ \text{C}$ water at a depth of $3 \text{ cm}$, is evenly illuminated by overhanging $15 \text{ W}$ fluorescent lamps and can be monitored in its entirety by a video camera suspended above the apparatus. Measurements of L–R eye use are confined to the lateral monocular visual field and scored by a larva’s body position with respect to the closest mirror at 1 s intervals. Larvae in the $10 \text{ mm}$ wide central area of the testing chamber (shaded in light grey) or at angles of either 0° or more than 90° with respect to the mirror are not scored. The frequency of right-eye use was calculated as (frequency of right-eye use)/(frequency of right-eye use + frequency of left-eye use) $\times 100$. Analysis of variance was carried out using SPSS v. 16.0 (SPSS Inc., Chicago, IL) to detect significant differences between anatomical classes. Mean and standard deviation of right eye use in (b) $spaw$ MO-injected, (c) mock-injected and spontaneous anatomical larval groups. $L_{pp}L_{pa}$ larvae were not found spontaneously from transgenic intercross progeny (refer to figure 1). (d) Percentage of $spaw$ MO-injected larvae showing a statistically significant bias (left or right) or no bias in eye use for each anatomical group. For every individual, the statistical significance of eye use was determined by a chi-squared test at a level of 5%. (e) Percentage of larvae showing a statistically significant bias (left or right) or no bias in eye use for mock-injected and uninjected spontaneous anatomical larval groups, calculated as in (d) (white bars, left bias; grey bars, right bias; black bars, no bias). (f) Mean and standard error of eye use during each minute of viewing by $spaw$ MO-injected larvae with a left ($n=65$) or right ($n=85$) positioned parapineal (grey squares, left parapineal; black squares, right parapineal).
also did not demonstrate a statistically significant bias in L–R eye preference. In addition, we have not observed other behavioural asymmetries during responses to a variety of directional and non-directional stimuli.

A simple explanation for these apparently conflicting results is the existence of variability between zebrafish strains. The transgenic lines used in this study have complex genetic backgrounds, as they were initially strains. The transgenic lines used in this study have complex genetic backgrounds, as they were initially

**Figure 5. Larvae with reversed epithalamic asymmetry show altered navigational behaviour.** (a) Mean and standard error of the elapsed time (in seconds) before a larva moves a distance equivalent to twice its body length in spaw MO-injected larvae. Differences between the four classes were calculated using the ANOVA test (**p < 0.001**). (b) Mean and standard error of the onset of navigation behaviour in mock-injected or uninjected LppRpa and uninjected RppRpa and RppLpa larvae. Spontaneous LppLpa larvae were not recovered. Differences between the three classes were calculated using the Kruskal–Wallis test (**p < 0.001**). (c) Representative swim paths of two spaw MO-injected larvae over 5 min. Swimming behaviour was recorded to videotape (30 frames s⁻¹) and was subsequently digitized. Video processing and analysis were performed using MATLAB (The MathWorks, Natick, MA). Larval position is indicated by an open circle at the start, and a black square at the end of recording (i) left parapineal and (ii) right parapineal. (d) Mean and standard deviation of the total distance covered (in mm) over a 5 min period starting from the first movement of individual spaw MO-injected larvae. Differences between the four classes were calculated using the ANOVA test (**p < 0.001**). (e) Mean and standard deviation of the average speed (mm s⁻¹) for the total swimming episodes of spaw MO-injected larvae. Differences between the four classes were calculated using the ANOVA test (**p < 0.001**).
visceral and epithalamic reversals and has not been associated with other developmental defects (Barth \textit{et al.} 2005). The ability to generate large numbers of parapineal-reversed larvae using \textit{spaw} MO should enable strain differences in mirror image viewing to be examined more rigorously and, perhaps, in parallel with tests on individuals from the \textit{fsl} strain.

In our study, all larval groups displayed similar responsiveness and kinematics in tests for motor responses. Thus, it may appear contradictory that \textit{R}_{sp} larvae showed a delay in the onset of movement and reduced overall swimming in the mirror test. However, there are important operational differences between these behavioural assays. Testing of rapid kinematic responses to acute stimuli is performed simultaneously on small groups of larvae in a pre-adapted environment. The mirror testing chamber provides an unfamiliar environment, one in which individually assayed larvae repeatedly encounter their reflection and have an increased area to explore.

We propose that these differences account for the behavioural response, in that a \textit{R}_{sp} larva, while possessing normal motor reactivity, appears less motivated or more fearful to initiate exploration in a novel environment. Recent work in mammals has uncovered an interesting link between the habenular region and control of the dopaminergic mesolimbic pathway that mediates fear, motivation and reward (Heldt \& Ressler 2006; Morissette \& Boye 2008). Specifically, the lateral habenula nucleus was found to provide inhibitory signals to dopaminergic neurons in the ventral midbrain (Matsumoto \& Hikosaka 2007). Midbrain dopaminergic neurons in turn send input to the limbic system and, notably, to the amygdala and nucleus accumbens, brain areas implicated in fear and reward (Di Chiara \& Bassareo 2007; LeDoux 2007).

The lateral habenular nuclei also receive substantial dopaminergic input, suggesting a further level of cross-regulation (Gruber \textit{et al.} 2007). Zebrafish seem to lack structures equivalent to the lateral habenula (Concha \& Wilson 2001); however, as in other recent studies, comparative gene expression analyses may identify brain regions that are functionally homologous with mammals (Wullimann \& Rink 2002; Mueller \textit{et al.} 2008). Moreover, there is recent evidence from rats that the medial habenula and IPN are also involved in modulating the dopaminergic pathway (Tarasenko \textit{et al.} 2007a,\textit{b}). Intriguingly, the firing rates of neurons in the medial and lateral habenulae, as well as the IPN, closely correspond with locomotor activity in rats (Sharp \textit{et al.} 2006).

In zebrafish, a mesolimbic-like circuit is present in larvae and adults, although there are some differences in the location of dopaminergic neurons (Rink \& Wullimann 2002). Pharmacological studies have also implicated dopamine in the control of larval locomotor activity (Giacomini \textit{et al.} 2006; Boehmler \textit{et al.} 2007; Thirumalai \& Cline 2008). It will be of great interest to examine whether the altered exploratory behaviour of parapineal-reversed larvae is caused by changes in the differentiation, connectivity or function of dopaminergic neurons. However, why L–R reversal of habenular identity and efferent projections to the dorsal and ventral IPN would disrupt this proposed modulatory function is unclear.

In addition to modulating the dopaminergic pathway, the habenulo-interpuduncular system has been implicated in regulating monoaminergic and cholinergic transmission in the mammalian brain, and in functions as diverse as olfaction, feeding, mating, nociception, attention, sleep/wake cycling, stress, fear and learning (reviewed in Sutherland 1982; Klemm 2004; LeCourtier \& Kelly 2007). To assess behavioural impact, lesioning of the habenulae in rats or mice is typically performed, but experimental approaches often do not discriminate between the medial and lateral habenular nuclei or take their complex subnuclear organization into account. Notwithstanding these caveats, impairments in attention, learning and memory have been widely documented. For instance, habenular-lesioned animals have difficulty in learning conditioned avoidance to aversive stimuli (Rausch \& Long 1974) and show a marked increase in premature responses (Sasaki \textit{et al.} 1990). Following habenular lesions, rats also respond prematurely in a spatial learning paradigm, suggesting that behaviour becomes more impulsive (LeCourtier \& Kelly 2005). In some cognitive assays, the effect of habenular loss is enhanced if stress levels are increased (Thornton \& Bradbury 1989; Heldt \& Ressler 2006). There is also evidence that habenular neurons respond to retinal illumination and may serve to link circadian and motivational pathways in the brain (Zhao \& Rusak 2005).

An essential goal for future studies in the zebrafish will be to learn more about the targets of the IPN and how L–R reversal of habenal connections with the IPN might influence neuronal activity elsewhere in the brain. Although the habenulo-IPN projection is highly conserved across vertebrates (Sutherland 1982; Concha \& Wilson 2001), knowledge of its integration with other conduction systems is lacking. Without this information, it will remain a challenge to understand why epithalamic laterality evolved and persisted in fishes, amphibians and reptiles. In addition, even though pineal-associated structures and the habenulae are asymmetric in these species (Concha \& Wilson 2001), only fishes seem to exhibit differential innervation of the dorsal and ventral IPN by left and right habenular neurons (Kuan \textit{et al.} 2007\textit{a,\textit{b}}). A further mystery is why morphological differences between the left and right habenular nuclei are rarely found in mammals (Sutherland 1982), suggesting that functional specialization of this part of the brain may be more important for aquatic species.

While the behaviours associated with epithalamic L–R asymmetry may prove more complicated and variable than previously appreciated, the zebrafish model has emerged as a valuable system for genetic manipulation of asymmetry, analyses of neuroanatomical development and connectivity and the application of diverse functional assays to tackle this exciting problem.

Protocols for use of zebrafish were approved by the Institutional Animal Care and Use Committee of the Carnegie Institution Department of Embryology.

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