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Determining the function of zebrafish epithalamic asymmetry

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As in many fishes, amphibians and reptiles, the epithalamus of the zebrafish, *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 *nodal*-related gene *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.

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 mediate shoaling behaviour in social fish species (Bisazza *et al.* 2000).

Fishes 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, *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 (Miklósi *et al.* 1997, 2001; Heuts 1999; Miklósi & 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 (Miklósi *et al.* 2001; Miklósi & 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 (Miklósi *et al.* 1997; Miklósi & Andrew 2006). Adults also tend

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One contribution of 14 to a Theme Issue 'Mechanisms and functions of brain and behavioural asymmetries'.

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

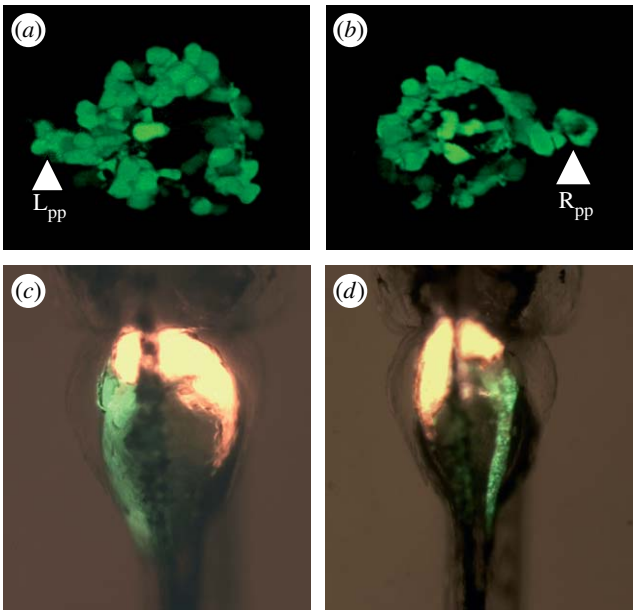
Recently, it has become possible to tackle the problem of how brain asymmetry arises developmentally using molecular genetic approaches afforded by the zebrafish model. Although there remains some controversy about the nature of the initial symmetry-breaking event in the early embryo, the ciliated Kupffer's 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 approximately 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.* 2007*a*,*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.* 2007*a*,*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.* 2007*a*,*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 lateralized 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 $L_{pp}R_{pa}$) that is found in more than 95 per cent of wild-type populations; those showing *situs inversus* or reversal of this pattern (designated $R_{pp}L_{pa}$); and two discordant classes with a right parapineal and right pancreas ($R_{pp}R_{pa}$) or a left parapineal and left pancreas ($L_{pp}L_{pa}$) (Gamse *et al.* 2005). Following this experimental manipulation, the four classes are not found in equal frequencies (figure 1*e*); however, a significantly greater number of larvae show reversed epithalamic and visceral asymmetry compared with wild-type strains. The MO is introduced into doubly transgenic progeny from matings between *Tg*(*foxd3*:*GFP*)^{fkg17} (Gilmour *et al.* 2002) and *Tg*(*ela3l:GFP*)^{gz2};*Tg*(*fabp 10:dsRed*)^{gz4} (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 1*a–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 1*e*). 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 $L_{pp}R_{pa}$ 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.



(*e*)

	% $L_{pp}R_{pa}$	% $L_{pp}L_{pa}$	% $R_{pp}R_{pa}$	% $R_{pp}L_{pa}$	total no. of larvae
injected *spaw* MO	43.9	10	25.2	20.9	540
mock-injected	100	0	0	0	50
uninjected	97.6	0	1.4	1.0	2743

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*)^{fkg17} (Gilmour *et al.* 2002) line. (*c*,*d*) Labelling of GFP in the pancreas and dsRed in the liver in 5 dpf *Tg*(*ela3l:GFP*)^{gz2};*Tg*(*fabp10:dsRed*)^{gz4} (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.

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 2007*a*). We first examined whether pre-sorted parapineal and pancreas reversed ($R_{pp}L_{pa}$) or discordant ($L_{pp}L_{pa}$ and $R_{pp}R_{pa}$) larvae showed differences from the $L_{pp}R_{pa}$ 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 $\mu W\ cm^{-2}$) 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 2007*b*). 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, $50.3\pm3.2\%$ 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 (2007*a*) 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 2007*a*). Both responses are followed by burst swimming movements, which rapidly propel larvae away from their initial position.

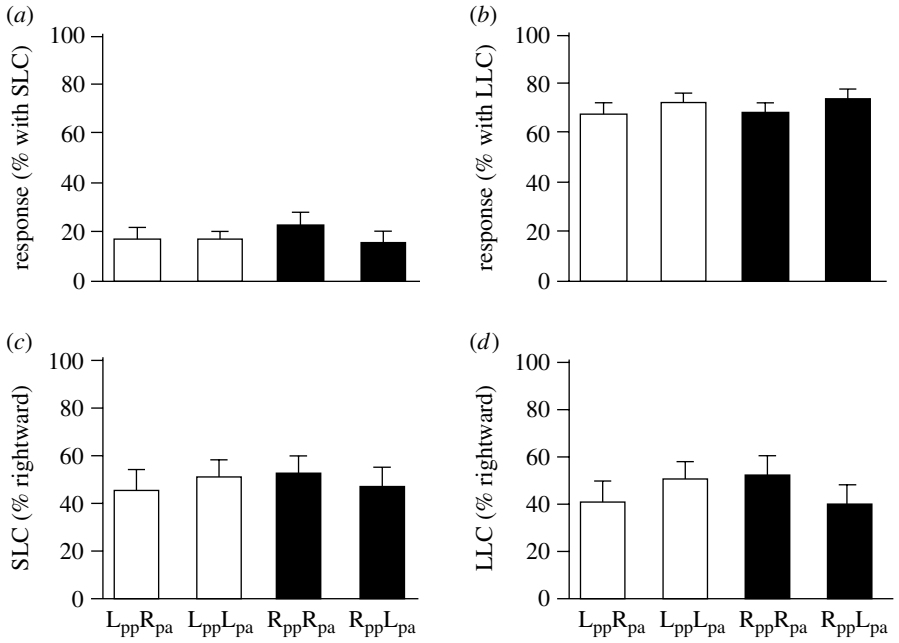


Figure 2. Equivalent startle responses in L–R reversed larvae. (*a*) Initiation frequencies for the short latency C-start (SLC) and (*b*) long latency C-start (LLC) responses. Movement initiation frequencies correspond to the percentage of trials in which SLC and LLC responses were observed. Larvae were tested in a 9-well grid and scored individually ($n=18$ per group). (*c*) Percentage of SLC and (*d*) LLC responses initiated in a rightward direction. A few larvae produced either no SLC ($n=7/72$) or LLC ($n=1/72$) responses and these were excluded from the analysis of directionality. Startle stimuli were generated and responses were recorded as previously described (Burgess & Granato 2007*a*) using a 1000 Hz horizontal vibrational stimulus of 3 ms duration and maximum acceleration 150 ms. Each set of larvae was tested with a series of 40 stimuli, presented at 15 s intervals. For these and all other assays, larvae were raised at a standard density of 30 larvae per 6 cm plastic Petri dish in E3 embryo media (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$ and 0.33 mM $MgSO_4$; Nüsslein-Volhard & Dahm 2002) and maintained at 27–28°C 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 2*a*) or LLC responses ($F_{3,68}=0.52$, $p=0.66$; figure 2*b*) 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 $L_{pp}R_{pa}$ 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 per cent of both SLC and LLC responses being initiated in a rightward direction (Burgess & Granato 2007*a*). Directional bias was also not observed in *spaw* MO-injected $L_{pp}R_{pa}$ larvae for either mode of startle response, with $44.9\pm8.5\%$ of SLC responses initiated in a rightward direction (one-sample *t*-test against 50%, $t_{14}=0.60$, $p=0.56$) and $45.3\pm6.4\%$ of LLC responses initiated rightward ($t_{16}=0.74$, $p=0.47$). 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 ($t_7=1.4$, $p=0.21$), allowing grouping of $L_{pp}R_{pa}$ with $L_{pp}L_{pa}$ and $R_{pp}L_{pa}$ with $R_{pp}R_{pa}$ 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 2007*b*). Because they tend to turn towards the extinguished light source (Burgess & Granato 2007*b*), 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, $t_{15}=0.16$, $p=0.87$; figure 3*a*) and O-bends were executed with equivalent kinematics in the two groups. For example, latency ($L_{pp}=458\pm22$ ms and $R_{pp}=458\pm20$ ms, $t_{15}=0.002$, $p=0.99$) and C-magnitude ($L_{pp}=141°\pm4°$and $R_{pp}=146°\pm4°$, $t_{15}=0.90$, $p=0.38$) were almost identical. The tendency of O-bends to be initiated towards the light

source ('bias', figure 3*b*) was significant (one-sample *t*-test against 0, for L_{pp} $t_8=3.9$, $p=0.005$ and for R_{pp} $t_7=4.3$, $p=0.004$) and of similar magnitude for the two groups ($t_{15}=0.12$, $p=0.91$).

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, $t_{4.3}=2.3$, $p=0.08$; figure 3*c*). Moreover, the two groups showed very similar movement kinematics, including latency to movement ($L_{pp}=412\pm28$ ms and $R_{pp}=395\pm25$ ms, $t_7=0.46$, $p=0.66$) and C-magnitude ($L_{pp}=97\pm4°$ and $R_{pp}=101\pm4°$, $t_7=0.56$, $p=0.59$). Thus, larvae with parapineal reversals both detect visual stimuli and have a normal magnitude of response. This assay also tests the directionality of response, as larvae show a strong bias to initiate turns away from the approaching 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 ($t_7=0.14$, $p=0.89$; figure 3*d*). 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 *et al.* (1999) for adult fish, and was similar to that described by 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 4*c*). Transgenic larvae injected with *spaw* MO ($n=200$, 50 for each anatomical class; figure 4*b*) also did not exhibit statistically significant differences in eye use upon mirror image viewing ($F_{3,199}=2.03$, $p=0.11$). To confirm this finding, we also examined uninjected transgenic larvae, screening through several thousands to identify the small number that showed spontaneous parapineal reversals (refer to figure 1*e*). 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 ($F_{2,117}=1.41$, $p=0.25$; figure 4*c*). In every control or experimental larval class, a subset did in fact show a left or right eye preference in mirror approaches (figure 4*d*,*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 4*f*; 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, $\chi^2_3=64.65$, $p<0.001$; figure 5*a*). 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 ± 9.2 and 54.9 ± 7.68 s, respectively, compared to 13.5 ± 2.5 s for $L_{pp}R_{pa}$, 14.9 ± 3.7 s for $L_{pp}L_{pa}$ and 4.67 ± 1.05 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 $R_{pp}L_{pa}$ and $R_{pp}R_{pa}$ larvae also showed a significant lag in the onset of navigation compared to their $L_{pp}R_{pa}$ siblings (Kruskal–Wallis test, $\chi^2_2=45.54$, $p<0.001$; figure 5*b*).

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 ($F_{3,117}=8.15$, $p\leq0.001$; figure 5*d*) and show a reduced average swimming speed ($F_{3,117}=8.18$, $p<0.001$; figure 5*e*). 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

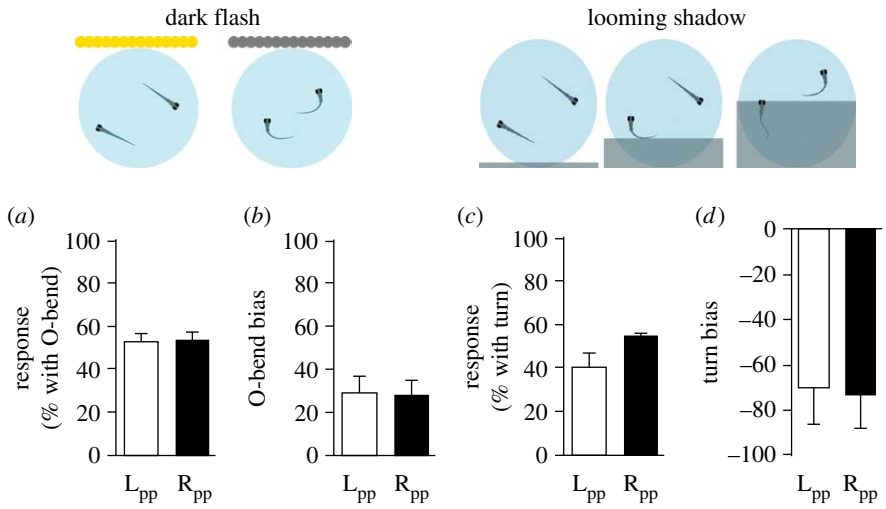


Figure 3. Directional behaviours are unaffected by epithalamic reversal. The (*a*) initiation frequency and (*b*) directionality of O-bend responses to dark flash stimuli were measured in L_{pp} and R_{pp} larvae (7 or 8 dpf). Dark flashes were generated as previously described (Burgess & Granato 2007*b*), 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 8–10 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 were concerned that altered visceral asymmetry might compromise swimming ability. However, opposite placement of the pancreas and liver (and presumably reversed directional coiling of the heart and intestines) in close to 50 per cent of larvae derived from *spaw* MO-injected embryos did not appear to modify spontaneous movements, the frequency or properties of C-bends during startle and escape responses, or the directional turning elicited by sudden changes in light. A probable reason for normal behavioural responses is that, even though the location and morphology of the heart and viscera are L–R reversed, the internal organs do not exhibit abnormal positioning with respect to one another (e.g. *situs ambiguous* or heterotaxia). For example, at 6 dpf, we never observed larvae that had their liver and pancreas positioned in the same orientation or both organs situated in the midline. In their initial description of *spaw*-depleted embryos, Long *et al.* (2003) found uncoupled defects in the directionality of the jogging and looping stages of heart tube morphogenesis, but 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.* 2007*a*,*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 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).

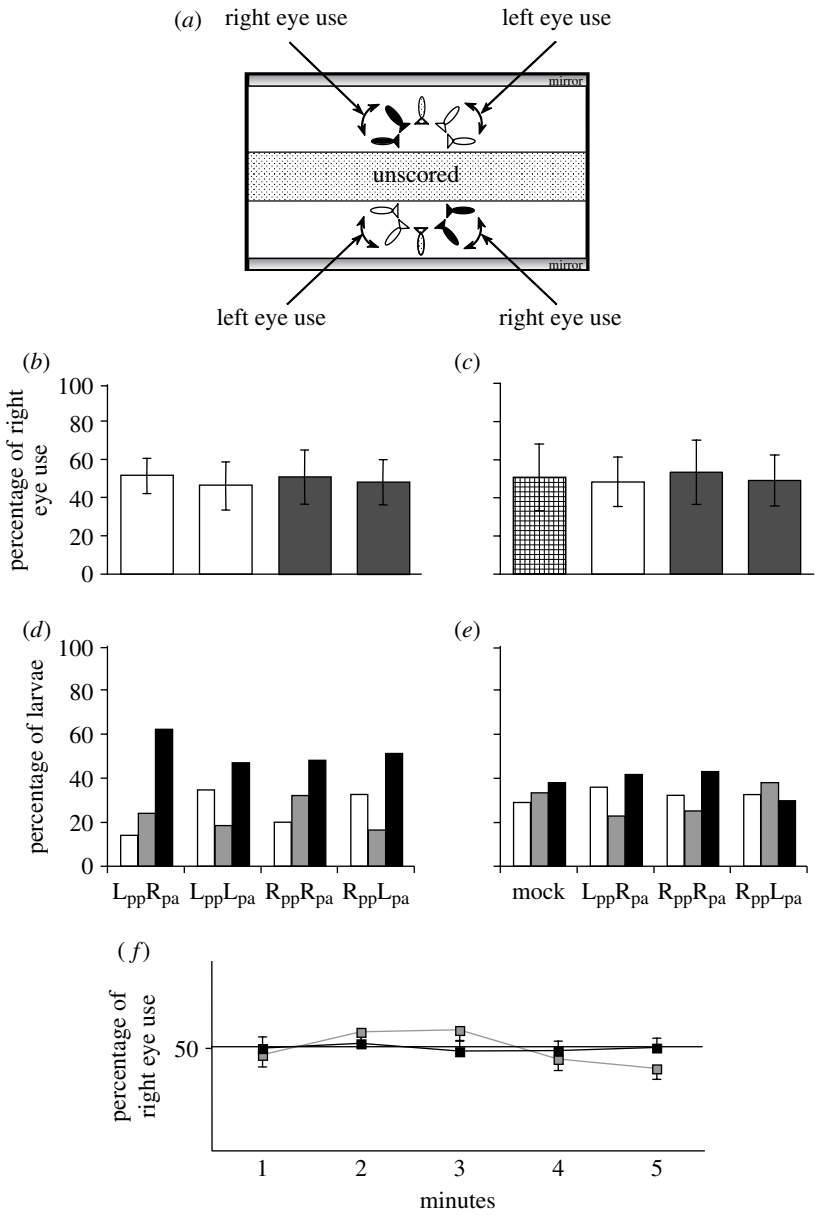


Figure 4. Larval populations do not show consistent eye preference in the mirror test. (*a*) The mirror test is conducted in a rectangular tank (10×4 cm) with two mirrors as the longer walls and two white screens as the shorter walls. The tank contains 28°C water at a depth of 3 cm, is evenly illuminated by overhanging 15 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 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)×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).

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

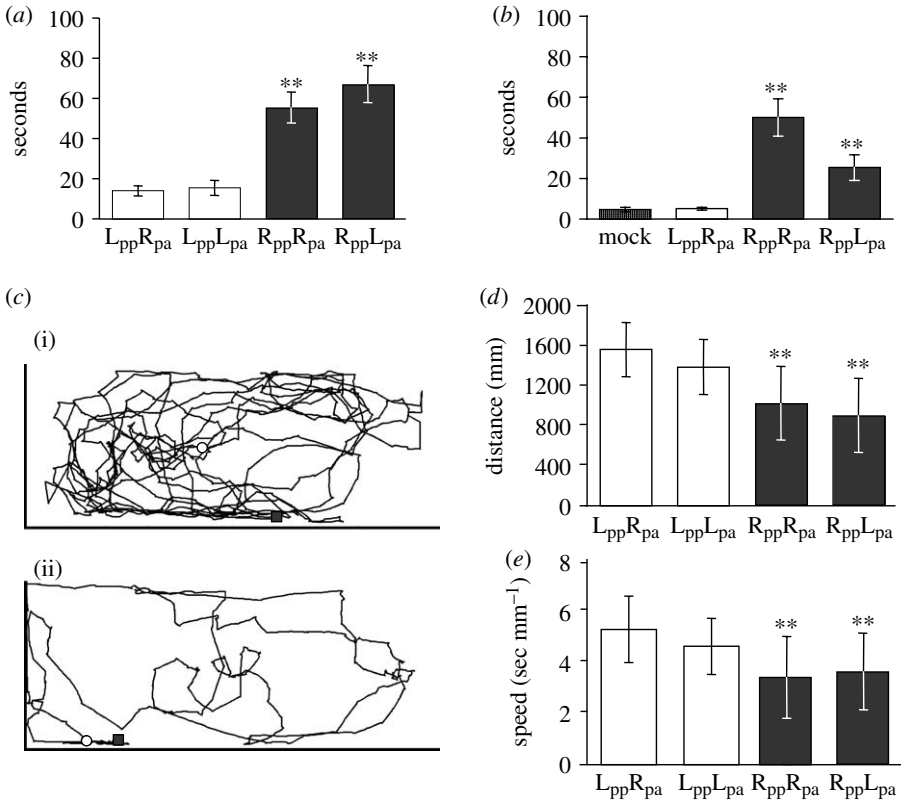


Figure 5. Larvae with reversed epithalamic asymmetry show altered navigational behaviour. (*a*) Mean and standard error of the elapsed time (in seconds) before a larva moves a distance equivalent to twice its body length in *spaw* MO-injected larvae. Differences between the four classes were calculated using the Kruskal–Wallis test ($^{**}p<0.001$). (*b*) Mean and standard error of the onset of navigation behaviour in mock-injected or uninjected $L_{pp}R_{pa}$ and uninjected $R_{pp}R_{pa}$ and $R_{pp}L_{pa}$ larvae. Spontaneous $L_{pp}L_{pa}$ 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 ($^{**}p<0.001$).

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 produced in undefined fish strains (Wan *et al.* 2006) or in the *golden* pigment mutant (Gilmour *et al.* 2002), and maintained in our aquatics facility through outcrosses to the Oregon AB line (Walker 1999), followed by intercrosses to preserve transgene homozygosity. Behavioural differences between strains of zebrafish have been previously observed in the mirror test (Sovrano & Andrew 2006; Andrew *et al.* in press), and could explain why we did not obtain evidence for consistent eye preference at the population level. The fact that some individuals in all laterality groups did demonstrate a left or right bias indicates that our testing paradigm for self-image viewing was a robust assay and was unlikely to be the source of the observed discrepancy between our results and prior work. Not only do strain differences exist in L–R eye preference, but it has also been suggested that single larvae modify their eye use for self-viewing during the course of a testing session, as their familiarity with the apparatus and visual stimuli increases. However, we did not find evidence for minute-by-minute changes in eye preference for any of the anatomical classes tested.

Another possible explanation for the differences observed between studies is that zebrafish larvae identified fortuitously in control populations, from strains with an enhanced predisposition for L–R reversals (Barth *et al.* 2005), or following genetic manipulations such as *spaw* MO injection, may not be morphologically identical. While this hypothesis cannot be ruled out, we do not favour it, as R_{pp} larvae showed very similar viewing behaviour irrespective of their derivation from injected or uninjected transgenic embryos. Moreover, *spaw* expression is restricted to the caudal region and left lateral plate mesoderm of developing embryos and has not been detected in the nervous system (Long *et al.* 2003). It is therefore unlikely that the *spaw* antisense MO would directly perturb brain development outside of its effect on L–R determination. Similarly, the *fsi* strain has only been described as increasing the frequency of concordant

visceral and epithalamic reversals and has not been associated with other developmental defects (Barth *et al.* 2005). The ability to generate large numbers of parapineal-reversed larvae using *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 *fsi* strain.

In our study, all larval groups displayed similar responsiveness and kinematics in tests for motor responses. Thus, it may appear contradictory that R_{pp} 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 R_{pp} 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 *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 *et al.* 2008). Moreover, there is recent evidence from rats that the medial habenula and IPN are also involved in modulating the dopaminergic pathway (Taraschenko *et al.* 2007*a*,*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 *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 *et al.* 2006; Boehmler *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-interpeduncular 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 *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 habenular 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 *et al.* 2007*a*,*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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