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Research report

Patterns of early embryonic light exposure determine behavioural asymmetries in zebrafish: A habenular hypothesis

Sergey Budaev, Richard John Andrew*

Biology and Environmental Sciences, School of Life Sciences, University of Sussex, Falmer, Brighton BN1 9QG, United Kingdom

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ABSTRACT

Releasers of innate responses are more effective in many vertebrates when seen by the left eye. In zebrafish *Brachydanio rerio*, this asymmetry is linked with neuroanatomical asymmetry of the habenular complex (enlarged left lateral and right medial habenular nuclei): if habenular asymmetry is reversed, reader response to releasers shifts to the right eye. Light exposure (schedule: 14/10 h, L/D) during the first few days of development post fertilisation (pf) controls the patterning of this asymmetry. We show here, using response to a model predator on day 7 pf, that absence of light on day 1 pf alone causes high responsiveness to shift from left to right eye and to intensify. Absence on day 2 pf or day 3 pf produces lesser shifts, whereas absence for all 3 days reduces responsiveness without any shift. Action on day 1 pf is likely to be due to modulation of gene expression. A known disturbance of gene (*nrp1a*) expression causes rerouting of the outflow of the left lateral habenula to the way station of the right medial habenula, providing a possible explanation of shifts. Variation in exposure of eggs to light is likely to produce inter-individual variation in the field.

1. Introduction

Modulation of brain and behavioural asymmetries by the action of light during development occurs in chick [22] and pigeon [19]. Two behavioural asymmetries, which are those relevant to the current study, were certainly affected. These were (1) facilitated response to releasers of species-specific behaviour (e.g., sex, attack), when the left eye is in use [23], and (2) enhanced ability to inhibit response to distractors (e.g., to pebbles, whilst feeding on food grains), when the right eye is in use [23].

Zebrafish show left eye use when viewing a social fellow and novel stimulus [7,26] and right eye use when approaching a selected target [7]. In both birds and zebrafish, these two abilities reverse together, suggesting that they depend on common mechanisms. In birds, such reversal was due to exposure of the left, rather than as normal the right eye to light late in development [23]. In zebrafish, it was associated with reversal of habenular asymmetry. Effects of light on behavioural asymmetry were therefore sought in the zebrafish.

In the domestic chick, a range of tests, which depend on the use of learned information, reveal asymmetries associated with eye use, and these differ between chicks exposed to light or not exposed to light late in development. This is clear for tests contrasting the use of spatial- or object-specific cues [12,21]. In the zebrafish too [7], there are behavioural asymmetries that are unaffected when reversal of habenular asymmetry results in reversal of the two abilities, with which we are here concerned. However, the patterns of lateralization of these latter abilities are likely to indirectly affect tests aimed at measuring asymmetries in abilities such as competence in the use of different types of information. Use of selected light regimes offers a way of teasing apart the roles of different lateralised mechanisms.

After absence of light for the first six days of development, the bias to left eye use when viewing a conspecific was greatly reduced, although the duration of viewing was unchanged [3], whilst the intensity of response during left eye viewing to a pattern with features (composition of separate subunits) characteristic of a potential refuge was also reduced [4].

We describe here strikingly different effects of absence of light for one day only, which differ not only from the effects of normal light/dark schedules throughout, but also from absence of light for the whole first 3 days. Absence of light on day 1 post fertilisation (pf) reverses the association of enhanced responsiveness to releasers from left to right eye, and greatly enhances it. This must involve a quite different route of action from birds, where light acts via a retinal route: normally the right eye looks outwards through the shell and so receives light; experimental reversal of illumination to the left eye reverses both asymmetries [23]. Specialised photosensitive mechanisms are entirely absent on day 1 pf in the zebrafish

* Corresponding author. Tel.: +44 1273 67 8504; fax: +44 1273 67 8535.
E-mail address: bafe8@sussex.ac.uk (R.J. Andrew).

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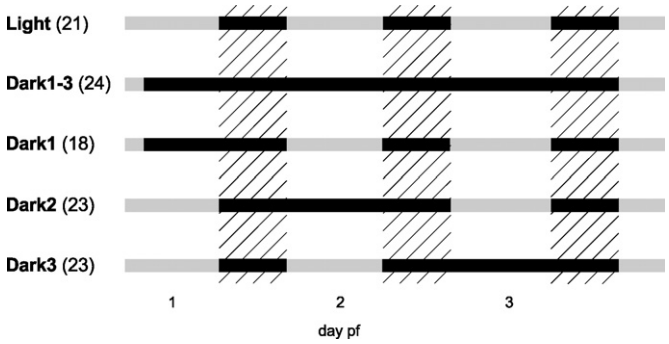

Fig. 1. Scheme of the light schedules used in the different experimental groups. Shaded areas represent darkness in all groups. Numbers in parentheses refer to the sample sizes of each group (total $n = 109$).

[14]. The most probable route of early action of light is on habenular asymmetries: reversal of habenular asymmetry is associated with reversal of the two behavioural asymmetries studied here [7], and there is widespread early photosensitivity in the epiphyseal area containing the developing habenulae.

2. Materials and methods

2.1. Light schedules and behavioural tests

Outbred zebrafish (*Brachydanio rerio*) from a local pet store (Brighton, UK) were used. Two to three hours after fertilization, eggs were removed from the parental aquarium, divided into five experimental groups (Fig. 1) and transferred to white plastic boxes (140 mm × 80 mm × 50 mm, 15–20 eggs per box, 28 °C). The baseline light:dark cycle (adult breeders and eggs on days of exposure to light) was 14:10 h. During the light phase, the illumination in aquaria, and for eggs exposed to light was about 100 lux, and during darkness, less than 0.01 lux (Extech EA30 digital light meter). Larvae hatched en mass during day 4 pf and were not fed prior to the behavioural experiments. After day 7 pf they were raised on a commercial dry food for zebrafish larvae). The test apparatus (Fig. 2(a)) had two compartments (50 mm × 40 mm), connected by a vertical 5 mm slit in the middle of the dividing wall. Each compartment was lit from above by two lamps, which could not be seen by the larvae, and could be dimmed slowly to full darkness to avoid startle. Fuller details are given elsewhere [10,29]. Testing was on day 7 pf. The water in the apparatus, which was at the home tank temperature, was changed after each test. At test, the larva was sucked into a large pipette (entrance diameter 6 mm) together with an adequate amount of water. It was then gently released into the first compartment (which was lit by the lamps whereas the second compartment was darkened), and left undisturbed for 4 min. Subsequently the light in the first compartment was dimmed to darkness over 20 s and then similarly raised in this second compartment. The larva entered the second compartment under positive phototaxis.

The predator model stimulus, a black oval (10 mm × 20 mm) with white eyes and mouth (Fig. 2(b)), was placed either on the left or on the right side wall of the test compartment. It was seen monocularly before entry, whilst the larva was within the slit connecting the two compartments. After the larva emerged in the test compartment it was video recorded for 5 min. Teleost fish usually respond to key stimuli of a predator with eyes and mouth as to a live fish predator [2].

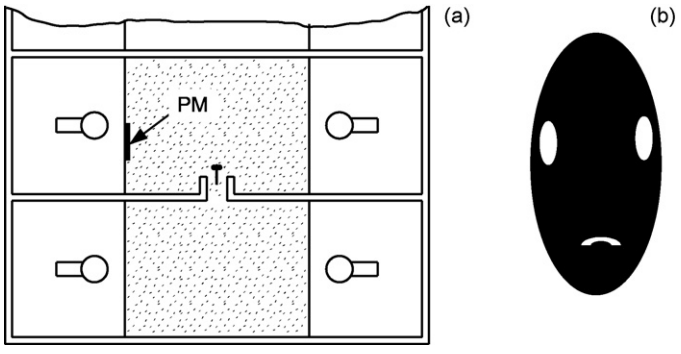

Fig. 2. A schematic view of the swim way used for testing (a) and the predator model stimulus (b). The area filled with water in (a) is dotted, the fry (in the test compartment immediately after emergence) and the predator model (black bar, PM) are also present.

2.2. Behavioural measures and data analysis

During analysis, a coordinate grid (1 cm × 1 cm) was imposed on the video image. Locomotion score was recorded as the number of crossings/s of the grid lines. The latency to emerge fully into the test compartment was recorded. Using an on-screen measurement software (http://www.markus-bader.de/MB-Ruler) we measured the distance between the fry and the stimulus every 5 s, and the minimum recorded distance was used in analysis.

The R software package was applied for the data analysis (http://www.r-project.org). All p values are two-tailed. We used ANOVA, with appropriate transformations when the scores deviated from the normal distribution. We also used the nonparametric Puri test for trend in one-way layouts, which is the most powerful among several other similar tests [8]. For group comparisons we used the Mann–Whitney test, with exact p values.

3. Results

Response to a predator in animals as vulnerable as zebrafish larvae would be expected to involve delay in leaving shelter (latency), reduction in locomotion and failure to go near (minimum distance). Latency to emerge, locomotion score, and minimum distance between the larva and the stimulus were significantly mutually intercorrelated (all Pearson $r > 0.4$; all p's < 0.0001), with high latency and high minimum distance going with low locomotion. Therefore, a principal component analysis was performed (supplementary material), yielding a single overall 'avoidance score' (68.3% of the total variance accounted, minimum loading 0.69). There were no significant effects shown by the three different measures, considered separately, which were not present in analysis of avoidance scores (supplementary material).

There was significant variation in avoidance scores between the five lighting regimes (Fig. 3; two-way ANOVA: $F_{4,99} = 5.23$, $p = 0.001$). The interaction between the left/right position of the predator model and the light regime was also significant ($F_{4,99} = 3.6$, $p = 0.008$; main effect of left/right position: $F_{1,99} = 0.56$, $p = 0.456$). A separate two-way ANOVA limited to groups **Light** and **Dark1–3** indicated that avoidance was significantly higher with a left stimulus ($F_{1,41} = 4.30$, $p = 0.045$), and in **Light** ($F_{1,41} = 6.91$, $p = 0.012$; interaction, $F_{1,41} = 0.39$, $p = 0.54$).

Absence of light on day 1 pf (**Dark1**) dramatically reversed the pattern characteristic of **Light** (Fig. 3). The right stimulus now produced an intense avoidance score, resulting in a significant left/right difference (Mann–Whitney test, $W = 14$, $p = 0.021$). The effect persisted after emergence (which showed strikingly high latency), with little locomotion and high minimum distance (supplementary material). The avoidance score for the right side tests was significantly higher in **Dark1** than in **Light** ($W = 17$, $p = 0.006$) or in all other groups combined ($W = 388$, $p = 0.0002$). Left stimulus scores showed no significant differences between **Light** and **Dark1**

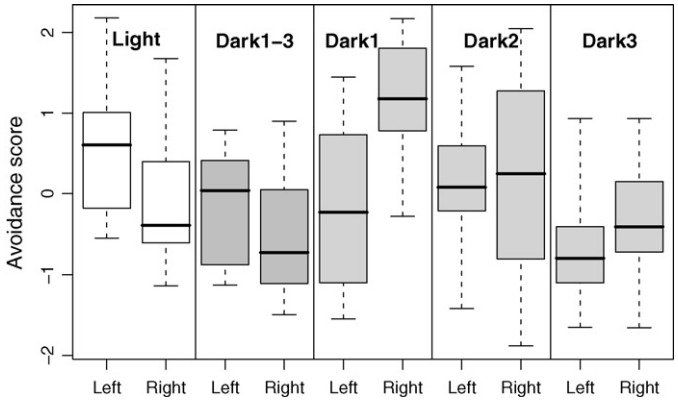

Fig. 3. The overall avoidance score (based on principal component analysis) for the different light groups with the stimulus presented on left and right. The median, quartiles (25–75%) and extremes (minimum–maximum) are shown.

($W = 56$, $p = 0.17$), nor in comparison of **Dark1** with all other groups combined ($W = 169$, $p = 0.73$).

There was a monotonic tendency for the avoidance score to decrease from **Dark1** through **Dark2** to **Dark3** for right side tests but not for left side tests (Fig. 3, Puri test for trend, respectively, $P = 139.68$, $p < 0.001$; $P = 65.96$, $p > 0.05$). Absence of light thus has much the largest effect on day 1. An additional effect was also found in **Dark3** in left side tests: here avoidance scores were significantly lower than in **Light** ($W = 106$, $p = 0.002$; right side tests: $W = 70$, $p = 0.56$, Fig. 3). **Dark3** and **Dark1–3** also differed, due to the lower avoidance in left side tests in **Dark3** (regime: $F_{1,38} = 14.44$, $p = 0.001$; side x regime: $F_{1,38} = 11.129$, $p = 0.002$).

4. Discussion

4.1. Behavioural asymmetries

The association of left eye use with intense response to species-specific releasers for behaviour such as attack, defence, sex and social responses is the most widely reported behavioural asymmetry in vertebrates [6]. In both zebrafish [3,4,7] and birds [16,24], it is coupled with a second asymmetry associated with right eye use: heightened ability to sustain an initiated response (such as selection of a target). In both birds [23,24] and zebrafish [7], these two asymmetries reverse together under experimental manipulation, so that each is now associated with the other eye.

Higher avoidance of an object with features of a predator is here added to a growing body of evidence that left eye use facilitates diverse responses, including viewing and approaching a social fellow [3], and approach to a pattern with features likely to be presented by a refuge [4]. The effects of left eye use thus may consist of a general enhancement of effectiveness of releasers of innate responses. The range of responses facilitated by left eye use in the zebrafish would be consistent with effects on many, if not all innately motivating stimuli, including conditioned reinforcers (although this latter remains to be established; below).

4.2. The habenular hypothesis

These behavioural asymmetries may be mediated by the habenula, a paired epithalamic structure, which in zebrafish and other lower vertebrates shows significant anatomical asymmetry [13]. As already noted, reversal of habenular asymmetry is accompanied by reversal of behavioural asymmetry in zebrafish [7]. The link between habenular and behavioural asymmetry is further supported by resemblances between the functions of the main divisions of the habenulae in zebrafish and in rats (even though it is difficult to establish homologies between habenular divisions in these distantly related species). In the rat, the medial habenulae are involved in effects of reward [28], which probably also explains their involvement in the effects of addictive drugs [17].

The right eye is normally used by zebrafish to select and approach a target [7,20], much as birds use the right eye to guide the bill to grasp a target [5]. In rats [25], lateral habenular units are active during targeting head movements in pursuit of a moving target. In zebrafish lateral habenular mechanisms are thus probably chiefly affected by right eye inputs, and medial by left eye inputs. In view of habenular asymmetry, right eye inputs are likely to act in the zebrafish via the enlarged left lateral habenula, and left eye via the enlarged right medial habenula (LlatHb, RmedHb).

Action of light on day 1 cannot involve specialised photoreceptors, since none exist. However, at this time, gene expression in the zebrafish CNS is affected by the action of light on undifferentiated cells [27]. The routing of the outflow from LlatHb to its normal main way station depends on expression of *nrp1a*, which provides the neuropilin signal guiding the axonal growth cones; in the absence of this signal [18], all of the outflow shifts to the way station of RmedHb: the ventral interpeduncular nucleus (vIPN). This would explain the complete shift of responsiveness to right eye use in **Dark1**.

The extreme behaviour shown by **Dark1** in right side tests suggests that the right eye input may activate a system normally affected by the left eye. Furthermore, it appears to do so more persistently and effectively than the left eye input. Habenular anatomy and function may again provide the explanation: given that LlatHb is involved in sustaining response, it is likely to show more persistent activation than RmedHb.

The very low responsiveness shown in left eye tests can be explained by properties of the way station now shared by LlatHb and RmedHb. The vIPN has a unique anatomy: inputs from either side affect both sides of the nucleus because axons repeatedly cross the midline [1]. As a result, after rerouting, inputs from LlatHb and RmedHb are likely to compete. In **Dark1** in left side tests, the right eye is unlikely to see the predator before emergence. Its input could sustain the response of emergence under positive phototaxis, which would compete with further examination of the stimulus by the left eye. **Dark3** differs from **Light** chiefly in showing reduced avoidance in left eye tests. This suggests that competition may still be present within vIPN.

Initial (day 1) action of light on gene expression is thus followed by later action, which helps to sustain normal routing. One likely route for such action is the parapineal (a structure of the left epithalamus), which establishes innervation of the LlatHb in the course of day 3 [15]. Parapineal ablation prevents establishment of neuropilin labelling and of normal routing of the LlatHb ouflow [9,18].

Finally, the low avoidance shown by **Dark1–3** relative to **Dark1**, coupled with complete absence of any shift of responsiveness to the right eye, suggests that in **Dark1–3** the main sources of asymmetry may disappear. It is possible that a failure of development of LlatHb asymmetry occurs and is associated with comparable effects on RmedHb. Our findings thus make specific and testable predictions about the effects of light on habenular anatomy.

4.3. Ecological implications

Exposure of eggs to light is likely to be common in fish like the zebrafish spawning in shallow well-lit waters [11]. At the same time, irregularities of the substrate presumably cause such exposure to vary between eggs. The various effects of light on development, which are described here, thus might generate individual differences that ensure that some proportion of offspring are suited to at least one of a range of conditions (e.g., arising from changes in predation pressures). We have already shown [10] that **Light** and **Dark** larvae are respectively bold and shy in a novel environment. The intense response to predators shown by **Dark1** suggests that further enhancement of response to potentially dangerous stimuli is possible by an appropriate light regime.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbr.2008.12.030.

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