Deep-brain photoreception links luminance detection to motor output in Xenopus frog tadpoles

Deep-brain photoreception links luminance detection to motor output in pro-metamorphic *Xenopus* tadpoles.

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ABSTRACT

Non-visual photoreceptors are widely distributed in the retina and brain but their roles in animal behaviour remain poorly understood. Here we document a novel form of deep-brain photoreception in *Xenopus laevis* frog tadpoles. The isolated nervous system retains sensitivity to light even when devoid of input from classical eye and pineal photoreceptors. These preparations produce regular bouts of rhythmic swimming activity in ambient light but fall silent in the dark. This sensitivity is tuned to short wavelength UV light; illumination at 400nm initiates motor activity over a broad range of intensities while longer wavelengths do not cause a response. The photosensitive tissue is located in a small region of caudal diencephalon - this region is necessary to retain responses to illumination while its focal illumination is sufficient to drive them. We present evidence for photoreception via the UV-sensitive opsin protein OPN5 since a population of OPN5-positive neurons resides within the caudal diencephalon. This represents a hitherto undescribed vertebrate pathway that links luminance detection to motor output. The pathway provides a simple mechanism for light avoidance and/or it may reinforce classical circadian systems.

SIGNIGFICANCE STATEMENT

Detecting and responding to light is a basic requirement of nearly all life forms. Species from bacteria to man use light to regulate diverse behaviours from acute phototropism, and visual processing to seasonal breeding cycles. Here we describe a novel form of photoreception in the deep brain of frog tadpoles. The photoreceptors are preferentially activated by UV light and link ambient light levels to swimming activity. The pathway may be a simple method to optimise lighting conditions for feeding and avoiding predation or may overlay and reinforce classical circadian systems. Deep brain photoreception is of broad significance since the proteins involved are phylogenetically conserved.

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INTRODUCTION

Animals utilise spatiotemporally patterned light information for image formation via their eyes, while the crude changes in brightness that occur over the course of a day can be detected by additional photosensitive regions including the pineal organ. Both visual processing and luminance detection depend on specialised opsin proteins which are widely expressed in the animal kingdom including man and located in multiple tissues (1, 2). The idea that regions of the brain other than the pineal complex or retina are sensitive to light was proposed over a century ago when von Frisch demonstrated that blinded and pinealectomised European minnows (*Phoxinus phoxinus*) retained an ability to change their colour in response to light (3). In addition it was demonstrated that lesions to the diencephalon removed this response and thus it was concluded that the periventricular tissue of the brain was directly light-sensitive. Since then deep-brain photoreception, specifically in the hypothalamus, has been studied extensively in relation to its role in gonadal induction in birds (4–9). In adult amphibians evidence also exists for non-retinal, non-pineal photoreception (10, 11). In Ranid frogs, electrophysiological unit recordings were made from close to the 3rd ventricle in the rostral diencephalon and from the region of the deep tegmental commissure, where tectospinal pathways are located. The majority of units were activated by light and fell silent in the dark although a function for this sensitivity was not discussed.

Movement in response to light is potentially as ancient as photosensitivity itself. It is reasonable to assume that cyanobacteria, which have existed for around 2.8 billion years, were some of the first organisms to sense light (12). The bacteria, *Synechocystis*, exhibit positive phototaxis to light between 560nm (green) and 720nm (red) while they exhibit negative phototaxis to UV-A light (360nm). Moreover, they can distinguish between the quality of the light, avoiding blue (470nm) or red (600-700) light when it is at high intensity (13, 14). In vertebrates the first evidence for extra-retinal, extra-pineal 'photomotor' behaviour came from experiments on blinded and pinealectomised lampreys (15, 16). A similar study in blinded, pinealectomised eels (*Anguilla anguilla*) showed they too responded to illumination of the head with a change in motor behaviour (17) In zebrafish, both positive and negative phototaxis is known to occur (18, 19). The fish will swim away from a bright light and generally prefer dark conditions but in a dark environment they will swim towards a localised region of light. While the eyes are required for proper orientation towards a light stimulus, a general increase in motor activity upon loss of illumination, termed dark photokinesis, persists in enucleated fish (20). Using genetic manipulations, Fernandes et al.

(2012) were able to narrow the photosensitive region to a population of melanopsin-positive neurons of the anterior preoptic area. Another light-driven but non-visual, non-pineal motor behaviour displayed by larval zebrafish is the photomotor response (PMR (21)). The PMR is characterised by low-frequency, high-amplitude coiling and higher frequency, lower amplitude swimming behaviours, which are both increased in response to flashes of bright light. The response only occurs transiently during development and is mediated by cells within the caudal hindbrain, which are both necessary and sufficient for the behaviour (22).

Here we have studied the effects of ambient lighting conditions on the spontaneously generated fictive locomotion produced by the isolated nervous system of pro-metamorphic *Xenopus laevis* larvae (23). This preparation, devoid of all afferent inputs from the lateral eyes or pineal complex, retains photosensitivity, with episodes of spinal ventral root locomotor activity occurring spontaneously in the light but with preparations falling relatively quiescent or completely silent in the dark. When exposed to a range of wavelengths, the response is found to be tuned to short-wavelength (390-410 nm) UV illumination. The nervous system generates fictive motor output during relatively low intensity UV illumination while it fails to respond to longer wavelengths even at much higher intensity. Focal illumination experiments reveal that a confined region of caudal diencephalon is required to generate the response. Moreover, immunostaining for OPN5, a known UV-sensitive opsin (8, 9), reveals a cluster of neurons in this region of the tadpole diencephalon that express the protein. Cryptochrome 1 (24, 25), another photoreceptive protein with an appropriate spectral sensitivity is expressed intensely in cells of the hypothalamus and pituitary, but the locomotor response to UV light is retained even when these structures are surgically removed. Together these results suggest the *Xenopus* larvae are equipped with a set of short-wavelength sensitive neurons deep within the brain that link environmental luminance to motor output and may underlie a simple light avoidance response and/or potentially overlay classical circadian systems.

RESULTS

The isolated nervous system of pro-metamorphic (stage 53-62) *Xenopus la*evis tadpoles (Fig. 1Aii) generates periodic episodes of rhythmic locomotor-like activity (Fig. 1Bi; 23). As has been shown at embryonic and early larval stages of development (24), motor bursts recorded from spinal ventral roots display left-right alternation between opposing sides of the spinal cord and a brief rostro-caudal delay as activity propagates from head to tail (Fig. 1Bii). However, instead of requiring sensory stimulation to trigger locomotor activity, episodes at these later larval stages now occur spontaneously (23).

Despite being devoid of input from all known photoreceptive tissues including the lateral eyes and the pineal complex the preparations are sensitive to changes in ambient light. When illuminated with a broad-spectrum halogen light source, preparations produced periodic episodes of coordinated locomotor activity (Fig. 1B). However, when placed in the dark (Fig. 1Bi, grey box), the preparations generally fell silent. Data from 23 preparations where there were at least two 15 minute periods alternating between light and dark, reveal a significant increase in time spent active, from 1.39 ± 0.40% in the dark to 9.44 ± 2.29% in the light (Fig. 1Biii; $p<0.01$). This effect relates specifically to the probability of fictive locomotion occurring; other parameters of swimming were unaffected by the changing light conditions. Relative to the value in the dark the burst duration (BD) was 100.72 ± 3.37% (N = 18); the cycle period (CP) was 100.12 ± 2.60% (N = 16); and the episode duration (ED) was 112.75 ± 11.75% (N = 23). Following a period of darkness (Fig. 1Bv see grey box in inset), spontaneous, rhythmic locomotor-like activity was initiated with a short delay. The delay to activation was variable between preparations but was consistent within the same preparation (Fig. 1Bv). The shortest delay before activation of swimming was 3.94 ± 0.47s while the longest was 122.43 ± 37.51s (N = 9). Given the link between light and heat, and knowing that swimming in *Xenopus* is temperature sensitive (26), it was important to rule out a thermal contribution to the light sensitivity of these preparations. The experiments were therefore designed to minimise the effect of temperature in two ways: i) all experiments were carried out in bath controlled by a Peltier cooler, which maintained the saline at 16.5 ± 0.5°C; and ii), the cold light source used generated negligible amounts of heat from the distal end of the fibre optic light pipe which was positioned ~10cm from the recording bath.

Since classical light sensitivity in the nervous system is dependent on opsin proteins which have ‘stereotypical spectral fingerprints’ (1), a first step in exploring the phototransduction

mechanisms of the isolated nervous system was to test responsiveness to different wavelengths of light. The halogen light source used in the initial experiments (see Figure 1) emitted a broad spectrum of white light, so a series of relatively narrow wavelength LEDs were used instead to generate a basic action spectrum of the light sensitivity. Illumination of the nervous system (Fig. 2Ai) with short wavelength UV light (390-410nm – 39 lux) produced a robust locomotor response: the time spent active increased to 16.56 ± 6.76% compared with 1.24 ± 0.63% before illumination; and 1.68 ± 1.26% immediately after the lights-on period ($N = 7$; $p<0.05$; Fig. 2Aii & iii - purple). Illumination of the same area with Blue (468nm – 461 lux), Green (523nm – 136 lux) or Red (635nm – 36 lux) light did not increase activity above the value recorded in the dark (Fig. 2Aii & iii – colour corresponds to wavelength used).

The intensity of light used depended upon the specific LED used. Compared to the white light source (~13, 000 lux), UV light elicited a ventral root motor response even at 39 lux (the total time spent active increased to 11.09 ± 1.72% compared with 0.05 ± 0.05% before illumination and 0.30 ± 0.18% immediately after the lights-on period; $N = 4$; $p<0.01$) and 23 lux (the total time spent active increased to 3.89 ± 1.56% compared with 0.19 ± 0.19% before illumination and 0.28 ± 0.28% immediately after the lights-on period; $N = 4$; $p<0.05$ – Fig.2Bi &ii). In addition, 2/4 preparations tested showed activity in response to UV light at 10 lux and 5 lux (see Fig. 2Bi). In comparison, blue, green and red light failed to cause a response to light at their maximum intensity values of 461, 136 and 36 lux, respectively (Fig. 2Aii & iii). This tight spectral tuning is particularly clear when you compare the robust UV light responses to the next shortest wavelength, blue light, which did not elicit a response at ten times the light intensity.

As well as the total time spent active, the intensity of UV light also dictated the latency to the onset of first swimming episode when the illumination is turned on (Fig. 2Bi & iii). The mean latency to first activity was significantly shorter at 39 lux (32.63 ± 11.27s) than at 5 lux (121.50 ± 4.5s; $N = 4$, $p<0.01$). This graded response to the illumination intensity could be important behaviourally, allowing the animal to respond appropriately to the relative amount of light in the environment.

Having established that UV wavelengths produce a maximal response to illumination, the next step was to localise the sensitivity within the isolated nervous system. When light was shone on the spinal cord alone, no response could be elicited at any intensity or wavelength,

including broad spectrum white light, suggesting that light sensitivity resides within the brainstem. The standard dissection in these experiments involved making a cut level with the caudal extent of the 3rd ventricle (Fig. 3Ai). Shining UV light on these preparations produced a reliable, robust response (see Fig. 3Aii & iii and also Fig. 2A). When a more caudal cut was performed –flush with the optic tectum and removing the entire diencephalon (Fig. 3Bi) – the preparations became insensitive to light. In preparations that were spontaneously active (see the episode of activity in the dark period in Fig. 3Bii), illumination did not increase locomotor activity. The mean time spent active during the lights-on period was 2.10 ± 1.60% compared with 2.75 ± 2.23% before illumination and 2.95 ± 2.16% immediately after (Fig. 3Biii; N = 4).

In a parallel set of experiments, a smaller diameter light guide was used to focally illuminate three different areas of the light-sensitive, diencephalon-attached preparation. Illumination of area 1 (see Fig. 3Ci), the rostral extent of the preparation including the caudo-ventral diencephalic tissue, produced a significant increase in both the time spent active (Fig. 3Ciii) and the number of swim episodes (Fig. 3Civ; also see Fig. 3Cii). The time spent active increased to 18.37 ± 2.18% compared with 0.85 ± 0.80% before illumination and 1.31 ± 0.66% after the lights-on period (Fig. 3Ciii; N = 4, $p<0.05$). The total number of episodes increased to 10.07 ± 3.28 compared with 2.23 ± 2.11 before illumination and 2.00 ± 1.68 after the lights-on period (Fig. 3Civ; N = 4, $p<0.05$). Illumination of either area 2, mid-brainstem, or area 3, the caudal brainstem, did not elicit an increase in locomotor activity during illumination with UV light – the time spent active during illumination of area 2 was 4.75 ± 4.08% compared with no activity recorded before illumination and 11.88 ± 8.26% after the lights-on period; during these same conditions the mean number of episodes was zero before illumination, 2.5 ± 1.5 during UV illumination and 2.25 ± 0.72 after the light-on period (Fig. 3Cii-iv; N = 4).The time spent active was zero both during and before illumination of area 3 and 1.06 ± 0.86% after the lights-on period; the mean number of episodes during this condition was 2.0 ± 1.53 (Fig. 3Cii-iv; N = 4). Taken together these results strongly suggest that the light sensitivity of the isolated tadpole nervous system is dependent on the diencephalic tissue located between the caudal extent of the 3rd ventricle and the optic tectum. To provide further evidence for this we investigated the possible means of phototransduction in the tadpole diencephalon, paying particular attention to the region where the light sensitivity apparently resides.

Since all known phototransduction in the vertebrate nervous system is mediated by light-sensitive opsin proteins, the next step was to try and locate opsin-positive neurons within the tadpole caudal diencephalon. Evidence for a UV-specific opsin (OPN5) mediating seasonal reproduction in the quail (8, 9) rendered this protein a good candidate. The OPN5 is found within the peri-ventricular organ (PVO) of the quail hypothalamus close to the sensitive region in our experiments. Moreover, its peak sensitivity of 420nm is similar to the spectrally tuned response in the tadpole nervous system. Immuno-fluorescent labelling of OPN5 positive neurons was therefore performed. Both longitudinal (Fig. 4Bi) and coronal (Fig. 4Bii-iii) slices through the tadpole brain (see Fig. 4A; N = 13) revealed a bilateral cluster of OPN5-positive neurons within the candidate light-sensing region of the caudal diencephalon. The neurons had an average diameter of 8.28 ± 0.73μm (only clearly defined somata were measured, n = 30 neurons; N = 3 animals). The cluster was at the level of hypothalamic ventricle and extended approximately 150μm laterally from the ventricle and spanned a dorso-ventral region of approximately 200μm. This places a population of potentially light-sensitive OPN5 positive neurons in the region of the tadpole brain that mediates the photomotor response. Furthermore, the fact that OPN5 is particularly sensitive to short-wavelength UV light is a good match for the spectral sensitivity of the light-triggered locomotor behaviour.

In addition to OPN5, cryptochrome proteins have been reported as blue light sensors (24, 25) with a spectral sensitivity that closely matches the wavelengths responsible for the light activation of fictive swimming. To assess the possible contribution of cryptochrome proteins 1 and 2 (CRY1, CRY2) we performed immunohistochemistry on the isolated larval CNSs and report widespread, protein-specific expression. CRY2 expression is abundant only in non-neuronal cells (microvasculature; S5, N = 3) but is not regionally restricted with sporadic staining throughout the brainstem and spinal cord. Thus CRY2 is highly unlikely to be involved in the increases in fictive swimming induced by light. CRY1 expression on the other hand was distinctly different from CRY2. Within the isolated nervous system there was a background, low level of labelling that was widely distributed, including the OPN5 positive region of the diencephalon (Fig. 4E, N = 8). In contrast, we found intense CRY1 labelling in ventral diencephalic structures including the hypothalamus and pituitary, located ventral to the brainstem proper (Fig. 3Di, S4 A, Bii), suggesting that CRY1 could be responsible for or contribute to the light sensitivity we describe.

To test this idea we first recorded photic activation of swimming in control isolated CNSs (Fig. 3Di,ii, upper panels). Next we surgically removed the ventral diencephalon to dissect away the structures with strong CRY1 expression (but retaining the OPN5 neurons) and then we re-assessed the photic responsiveness of the preparation. In each case a robust light-on response was recorded from spinal ventral roots (Fig 4Di,ii lower panels, Diii; n=3). Taken together these data suggest CRY1 is unlikely to play a role in acute locomotor responses to light we have described. We propose that a group of OPN5-positive photosensitive neurons are essential to enable the isolated nervous system to link changes in luminance to motor behaviour. Nevertheless, it remains unknown precisely how the putative deep brain photoreceptors couple to the locomotor CPG.

In the zebrafish hypothalamus the non-retinal opsin, melanopsin (OPN4), is co-expressed with tyrosine hydroxylase (TH) within A-11 type dopaminergic neurons, and although their function is unknown it is presumed they may be important for light-mediated locomotor responses (20). We found no evidence that OPN5 was located within dopaminergic neurons (S5). However, we did identify a cluster of dopaminergic neurons in the same region of the hypothalamus, located just dorsal to the OPN5-positive cluster. These TH-positive neurons are the rostral-most members of a population of dopaminergic neurons that is contiguous with the dopaminergic neurons of the posterior tuberculum (PT), found more caudally in the hypothalamus (S6).

DISCUSSION

We have demonstrated that the brainstem of pro-metamorphic *Xenopus* frog tadpoles is sensitive to light via a mechanism that does not involve the classical photoreceptive tissues of the eyes or pineal gland. This photosensitivity has been localised to a small region of the caudal diencephalon and shown to be tuned to short-wavelength UV light. Two main candidates with appropriate spectral sensitivity to function as the photo-transducers in the lights on response are OPN5 and cryptochrome. We present evidence in favour of OPN5 as the major participant in the acute activation of swimming in response to light. Both OPN5 and CRY1 are expressed in a region that broadly matches the light sensitive part of the isolated CNS. At this stage we cannot completely rule out a contribution from CRY1, which is strongly expressed in the caudal diencephalon that lies ventral to the brainstem. However, in support of OPN5's important involvement, surgical removal of the only region with strong CRY1 expression, leaving the periventricular OPN5 neurons intact, does not eliminate light sensitivity. Nevertheless, strong CRY1 expression in the hypothalamus and pituitary suggests that it may play a role in light detection, but this could relate to slower, hormonal and/or diurnal changes in tadpole behaviour. Future approaches to tease apart the respective roles of CRY1 and OPN5 in photic control of behaviour could involve loss of function experiments following knockdown of the genes for these proteins, for example the CRISP/dCAS9 system. However, this approach is beyond the scope of the present study and would best be tackled in genetically more tractable model animal such as *Xenopus tropicalis*.

The discovery of neurons within this light-sensitive region of the tadpole brain that express the UV-specific opsin, OPN5, strongly suggests that this is the mediator of phototransduction. Since photosensitivity in vertebrates is thought to originate from periventricular neurons of the diencephalon, it seems plausible that this mechanism is phylogenetically conserved and may represent a light detecting component present in the brain of a primitive aquatic proto-vertebrate (27). An important facet of these experiments is that the light sensitivity only links directly to the probability of occurrence of spontaneous locomotor activity. Upon illumination, the isolated nervous system produced regular episodes of fictive locomotion, while in the dark the preparations were generally silent. There were no differences between the coordination or basic parameters of the locomotor rhythm in the

different light conditions, suggesting that the photic system of the brain controls merely how likely the animal is to swim.

The function of this deep brain light sensitivity could be a simple mechanism to maintain the tadpole in an optimal photic environment. It could, for example, help avoid exposure to UV radiation from the sun, which can cause DNA damage and which is a remarkably well conserved trait found even in bacteria (13, 14). In addition it may help to avoid the brightest lit areas of the environment where detection by predators is likely to be increased. This form of light avoidance strategy is found in many fish species where it is thought to be a specific advantage in the face of aerial predation (28). In embryonic *Xenopus* tadpoles light avoidance is achieved by a pineal driven motor response that causes upward swimming in response to shadows cast in the water (29, 30). While this behaviour is sufficient to maintain the relatively dormant embryos in an optimum environment for survival, the addition or predominance of other light sensitive systems during development may aid survival in highly active, free-feeding larvae. Another, non-mutually exclusive, possibility is that the deep-brain light sensitivity could overlay classical circadian control mechanisms, which regulate behaviour in response to predictable diurnal fluctuations in the environment. Given the tuning of this response to short wavelengths, it may be appropriate to detect subtle changes in the lighting conditions in an aquatic environment, where the influence of longer wavelengths is filtered out by the water. Indeed it has even been suggested that the evolution of circadian systems may have begun with primitive blue-light photoreceptors (31). In bacteria, DNA damage caused by UV radiation is repaired by a set of flavoproteins called photolyases (32). Their activity is dependent on UV light and they are closely related to cryptochromes. It is thought that an original need to avoid harmful UV radiation led the proteins involved in DNA repair to become specialised for short wavelength light detection, and that subsequently these proteins became an integral part of circadian control systems (31).

An important next step will be to determine which neuronal pathway links the photoreceptive neurons to the activation of the motor system. The OPN5-positive neurons were found in close proximity to a set of dopaminergic neurons potentially related to the A-11-type population, which are known to project to the spinal cord and control motor output in other species (33). However, it is also plausible that the OPN5 neurons activate other supra-spinal centres involved in vertebrate locomotion, such as the mesencephalic locomotor region (MLR) in the midbrain and/or reticulospinal nuclei in the hind brain (34). Both of these

possibilities could be involved simultaneously, since dopaminergic neurons within PT of the lamprey have been found to project to and excite the MLR directly (35).

In zebrafish, the photoreceptors underlying dark photokinesis have been localised to the anterior pre-optic area and they transduce light via the photopigment, melanopsin (20). The photosensitivity we report in *Xenopus* is not mediated by the equivalent region of the brain because the pre-optic area has been removed in these light-sensitive preparations. However, melanopsin was also found more caudally in zebrafish, in neurons of the PT (20), an area that is present in the light-sensitive *Xenopus* preparations. This is particularly relevant since the cells in question were A-11 type dopaminergic neurons which comprise a diencephalo-spinal population implicated in motor control (33). However, there are a number of reasons why they are unlikely to be the means of phototransduction documented here. Firstly, the original work that identified melanopsin as a photopigment was carried out in *Xenopus* and while it was found in both the pre-optic nucleus and the suprachiasmatic nucleus, there is no evidence for it being present in the caudal hypothalamus (36). Secondly, since the photomotor behaviour in *Xenopus* is tuned to short-wavelength UV light, it does not correspond to the profile of a melanopsin-mediated response, which should peak around 480nm (1, 37, 38).

Alternatively, OPN5 is a UV-specific opsin that has recently been shown to be a component of the photoperiodic response in quail (8, 9). In this case OPN5 was located within the quail PVO, a structure within the caudal hypothalamus that is present in the photosensitive tadpole preparation. Moreover, cells within the PVO of other species have been shown to contain DA, NA and / or 5-HT (39), which are all known modulators of locomotion in *Xenopus* (40–42). A particularly interesting example is the three-spined stickleback which has large dopaminergic neurons in the PVO forming a contiguous group with the dopaminergic neurons of the PT (43). This more caudal group are thought to be homologous to the dopaminergic neurons of the mammalian zona incerta, which makes up the sub-thalamic diencephalic locomotor region, an area important in the supraspinal control of locomotion (44, 45). The discovery of OPN5-positive neurons in close proximity to dopaminergic neurons that appear to form a continuous group with the dopaminergic neurons in the PT in *Xenopus* suggests they may be ideally positioned to influence the descending control of locomotion. While these experiments have found no evidence that OPN5 is expressed within dopaminergic neurons, as is the case with melanopsin in the zebrafish PT (20), it remains possible that there could be direct excitatory connections between these presumed

photosensitive neurons and those of the descending locomotor control centres located in this region of the tadpole nervous system.

What is the behavioural significance of this novel photomotor response in *Xenopus* tadpoles? The lighting conditions were at physiological levels for a species native to ponds in South Africa: the broad spectrum, white light was approximately 13,000 lux and so within the range of intensity you would expect to experience during the day while not in direct sunlight (10,000-25,000 lux; 44); the brightest LED (blue; 468nm) was approximately 460 lux and so similar to the light intensity experienced at sunrise or sun set; the UV LED (390-410nm) that elicited the maximal response to light only emitted 39 lux and occasionally elicited a response at as low as 5 lux. Negative phototaxis as a method of predator avoidance is a common behaviour in many species (47). In aquatic fish species this means avoiding the surface waters during the brightest parts of the day when predation, especially from aerial pescivores, is highest due to increased visibility. Additionally, many plankton species display similar daily migrations in the water column (50). In contrast to the fish, however, the plankton actually seeks out the bright surface water during the day, both to avoid predation and to maximise photosynthesis (51). There is therefore a trade-off between maximising feeding opportunities and minimising predation risks. In the larval tadpoles, which are obligate filter feeders, there may be a similar trade off whereby their feeding strategy must be adjusted over the course of the day to account for the lighting conditions, and the associated predation risk. Deep brain photoreception may promote light avoidance behaviour by increasing locomotor activity relative to light intensity, and so increasing the probability of navigating to dimly lit areas. A role for deep brain photoreception in negative phototaxis has already been shown in eels (17). This response involved the activation of a specialised, backwards swimming motor pattern. In contrast, the generalised increase in locomotor activity seen in the isolated *Xenopus* nervous system is more similar to the dark photokinesis behaviour displayed by larval zebrafish (20).

In the eel, deep brain photoreception was also shown to mediate photoentrainment to a circadian cycle of increased nocturnal activity (17). While there is no evidence for circadian variation in activity during larval life, adult *Xenopus* are nocturnal, being almost twice as active at night compared to during the day (48). Tadpoles of the American toad (*Bufo americanus*) display increased activity and feeding during the day and are generally inactive overnight (49). They also swim and feed less on cloudy days when light levels are lower. In tadpoles of *Xenopus laevis* we propose that deep brain photoreception serves the dual purpose

370 of reducing exposure to the damaging influences of both predation and UV on the one hand
371 and automatically adjusting energetically expensive bouts locomotor activity to diurnal
372 changes in light intensity on the other hand.

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FIGURE LEGENDS

Fig. 1. – **Fictive locomotion in pro-metamorphic *Xenopus laevis* larvae is sensitive to ambient lighting conditions.**

(Ai) Cartoon of a stage 56 larva including the approximate location of the nervous system within the intact animal. (Aii) Schematic depicting the preparation including the location of glass suction electrodes on ventral motor roots. (Bi) Extracellular record from three ventral motor roots showing spontaneous episodes of fictive locomotion. (Bii) On an expanded time base, the coordination of this spontaneous activity can be observed - on the same side of the cord, upper two traces, the activity propagates with a brief rostro-caudal delay while across the cord, lower two traces, the activity alternates in a left-right pattern. Various parameters of the activity are illustrated here, including burst duration (BD); cycle period (CP); and episode duration (ED). Spontaneous motor activity recorded is shown to be sensitive to ambient light conditions. In the light episodes of coordinated motor activity occur regularly every few minutes, while in the dark (grey box) the preparation falls silent. (Biii) Graph of the time spent active in light and darkness, expressed as a percentage of the total recording period, for 23 larval preparations (light grey lines). The population mean is shown in black. (Biv) Other parameters of the ficitve motor activity remain unaltered by the lighting conditions – BD (N = 18), CP (N = 16) and ED (N = 23) are expressed as the mean percentage in the light relative to the value in the dark. (Bv) Graph of the mean latency to motor activity from 9 different preparations where at least 3 transitions between dark and light were recorded. In each example the latency to activity was measured following 10 minutes in the dark. See upper panel for an example response from a stage 54 larvae following 10 minutes in the dark (grey box). All error bars represent ± SEM. ***, $p = < 0.01$.

Fig. 2. – **Photosensitivity is tuned to short wavelengths.**

(Ai) Schematic depicting the brainstem and caudal diencephalon. Approximate area illuminated is shown by black dotted line. (Aii) A single ventral root trace from a stage 55 larva shows 200s before and 200s after a sequence of transitions from darkness (grey box) to light. In each case the preparation was illuminated following 10 minutes in the dark and the wavelength of light and its intensity is shown. (Aiii) Graph displays the average data of the time spent active 200s before illumination; 200s during illumination; and 200s after

illumination for each wavelength of light (UV, red, green and blue; N = 7). (Bi) A sequence of responses to different intensities of UV light following 10 minutes in the dark. (Bii) Graph shows the average data for time spent active during responses to UV light at maximum (Max; 39lux), medium high (MH; 21lux), medium low (ML; 10lux) and minimum (Min; 5lux) intensity (N = 4). (Biii) Graph shows the average data for the latency until the first activity following illumination with UV light of different intensity. All error bars represent ± SEM; ***, p = < 0.01; *, p = < 0.05.

Fig. 3. – **Photosensitive tissue resides within the caudal diencephalon.**

(Ai) Schematic of the normal dissection performed in these experiments. The forebrain is removed apart from a small portion of diencephalon caudal to the dorsal opening of the 3rd ventricle. Both dorsal and sagittal aspects are depicted. Scale bar represents 200μm. (Aii) A ventral root recording from a stage 54 larva show three consecutive responses to illumination with UV light (400nm; 39lux). (Aiii) Graph of the average data comparing the time spent active 200s before, during and after illumination (N = 7). (Bi) Schematic illustrates a dissection made flush with the optic tectum such that the diencephalon is completely removed. (Bii & iii) Equivalent data shown in A is displayed for preparations following removal of the diencephalon (N = 4). (Ci) Schematic illustrating the approximate location of focal illumination of 3 areas of the isolated nervous system (Cii) A sequence of responses to illumination of these different areas with UV light following 10 minutes in the dark (grey box). (Ciii & iv) Graphs show the average data for time spent active (Ciii) and mean episode number (Civ) for illumination of each area – comparison of the 200s before, during and after illumination are plotted (N = 4). (Di) Schematic illustrating the isolated nervous system before (upper panel) and after (lower panel) removal of the ventral portion of the diencephalon containing the hypothalamus and pituitary. (Dii) A ventral root recording from a stage 56 larvae before (upper trace) and after (lower trace) the dissection was performed. (Diii) Graph illustrating data from 3 different preparations. Swim % are shown both before (solid black lines) and after (dashed grey lines) removal of the ventral diencephalon. All error bars represent ± SEM; *, p = < 0.05.

Fig. 4. – **UV-sensitive proteins are located within the tadpole caudal diencephalon.**

(A) Schematic of a *Xenopus* tadpole brain showing the approximate position of sections taken for imaging. (B) OPN5-positive neurons within the caudal diencephalon of a stage 55 tadpole. (Bii) A cluster of neurons is located in the ventral half of the diencephalon in proximity to the hypothalamic ventricle (hv); also see and expanded view of the same area in (Biii) and a second more ventral image from a different preparation (C). (D) Negative controls lacking primary antibody; (Di) Texas Red secondary, (Dii) FITC secondary. (E) OPN5 (Ei) and CRY1 (Eii) plus merged image (Eiii) of immunoreactivity from the region surrounding the ventral portion of the hv. Scale bars represent 100μm.

Materials and Methods

Animals and husbandry

Experiments were performed on a range of pre-metamorphic and pro-metamorphic stages of the South African clawed frog, *Xenopus laevis*. Animals were obtained by human chorionic gonadotropin (hCG) hormone assisted injections (1,000 U/mL; Sigma) matings of adults selected from an in-house breeding colony. Fertilized ova were collected and reared in enamel trays until the first free-feeding stages, before being transferred to standard glass aquarium tanks. The tadpoles were fed at least once every 72 hours with powdered whole egg (AA Baits). Tanks were cleared of detritus approximately every 48 hours and the water was completely changed regularly – about every 14 days. The tanks were oxygenated with standard aquarium aerators and environmental enrichment was provided in the form of plastic aquarium plants. All procedures conformed to the UK Animals (Scientific Procedures) Act 1986 and the European Community Council directive of 24 November 1986 (86/609/EEC) and have been approved by the University of St Andrews Animal Welfare Ethics Committee (AWEC).

Extracellular electrophysiology apparatus

Prior to electrophysiological experiments, the animals were humanely killed via standard Schedule 1 methods: the tadpoles were first overdosed in approximately 230μg ml^{-1} Ethyl 3-aminobenzoate methanesulfonate (MS222); they were then transferred to a Sylgard-lined Petri dish containing ice-chilled 'RANA' saline (composition, mM: NaCl, 112; KCl, 2.0; $CaCl_2$, 5.6; $MgCl_2$, 1; $NaHCO_3$, 20; $C_6H_{12}O_6$, 17). Death was quickly confirmed, first via destruction of the heart and then by removal and destruction of the forebrain except for the most caudal portion of diencephalon.

Next, the remaining nervous system was dissected free of the carcass, apart from the caudal most portion of the tail, which was left attached in order to verify the preparation was capable of normal motor output. Ventral root data obtained from preparations in which the tail was completely removed (23); was indistinguishable from the preparations used here. The isolated brainstem and spinal cord was then transferred to a second Petri dish, containing fresh circulating saline that was bubbled with carbogen (95% O_2; 5% CO_2), for recording purposes. The carbogenated saline remained between pH 7.2-7.4. The recording dish was housed inside a Peltier cooling system in order to maintain the preparations at approximately 17°C, which has proved to be optimal for reliable extracellular recordings (see (42), for example). Using

sharpened tungsten wire, preparations were pinned down through the remaining tail muscle, and either the cranial nerves or a portion of tissue sometimes left intact around the brainstem. Glass suction electrodes, cut to approximately the diameter of the ventral root were used to record motor discharge.

Light sources

For experiments where the lighting conditions were manipulated, the recording apparatus was housed in a modified Faraday cage covered with aluminium foil and black-out cloth. The light level in the cage during lights-off was negligible (0 lux). Experiments with white light were performed with a standard halogen cold-light source (Olympus Highlight, 2000) which emitted broad spectrum light at approximately 13,000 lux (low voltage halogen projection lamp, 14.5V, 90W, Phillips, Germany).

When investigating the spectral sensitivity of the preparations, a series of LEDs were used (R-S components, UK – all catalogue numbers provided). The specifications were as follows: Blue LED (# 466-3532), peak λ was 468nm, brightness was 15,000 milli candela (mcd) or 461 lux; Green (# 671-6852), 523nm, 21,000mcd (136 lux); Red (# 496-6178), 635nm, 16,000mcd (36lux); UV (#713-5043), 400nm (39 lux).

Immunohistochemistry

Embedding and sectioning

For immunohistochemistry, tadpole brains were harvested from animals at stage 55. The nervous system was isolated from the rest of the animal as during electrophysiological experiments although the forebrain was left intact and the spinal cord was cut at approximately the 5th post-otic muscle block. Dissections were performed in a Petri dish containing 'HEPES' saline (composition in mM: 115 NaCl, 3 KCl, 2 $CaCl_2$, 2.4 $NaHCO_3$, 1 $MgCl_2$, 10 HEPES, adjusted with 4M NaOH to pH 7.4). The tissue was fixed overnight at 4°C in FAA fixative (50% v/v ethanol; 10% v/v 37-40% formaldehyde; 5% v/v acetic acid in dH_2O - the FAA was kept on ice prior to addition of tissue). Next, the fixed tissue was dehydrated through a graded alcohol series and cleared in chloroform. The tissue was then left overnight in a fresh change of chloroform. Tissue was exposed to 4 changes of molten paraffin wax (2 x 30 minutes; 2 x 1 hour) then embedded rostral end down and left overnight at 4°C. Sections were cut at 8μm on a rotary microtome and then mounted on electrically-charged slides.

Immunohistochemical staining

Sections were deparaffinised in xylene, rehydrated through a graded alcohol series and washed in PBS-T. Antigen retrieval was performed in 0.1M citrate buffer (pH 6.0) in a steamer (25 minutes). After being allowed to cool to room temperature the tissue was washed in PBS-T (3 x 3 minutes) then transferred to sequenza racks. 10% horse serum in PBS-T was used to block non-specific antibody binding (10 minutes) then the primary antibody (200µl 1:1000 rabbit anti-OPN5; 1:1000 rabbit anti-CRY1 or 1: 500 anti-CRY2, all Aviva Systems Biology Corporation) was introduced and left overnight at 4^{o}C. Previous to these experiments verification of the species cross-reactivity of this antibody with *Xenopus* OPN5 was carried out by BLAST searching (Aviva Biosystems Corporation) followed by verification that the antibody detected a protein of appropriate molecular weight in *Xenopus* samples (see S1) and that immunoreactivity could be abolished by pre-absorbing the antibody with a blocking peptide (S2). The cryptochrome antibodies had been commercially verified as able to cross-react with *Xenopus* proteins. The slides were again washed with PBS-T (2 x 5 minutes) before introducing the secondary antibody (200µl 1:200 FITC-anti-rabbit; Vector Labs, UK) and leaving overnight covered in tin foil. For double labelling the previous two steps were repeated with the second set of antibodies (200µl 1:1000 mouse anti-TH, Sigma Aldrich, UK and 200µl 1:200 TRITC-anti-mouse, Vector Labs, UK; or 1:1000 rabbit anti-OPN5 and 200µl 1:200 Texas Red-anti-rabbit, Vector Labs, UK). Following a final wash in PBS-T (5 x 5 minutes) the sections were mounted in citifluor and the coverslip was sealed with ethyl acetate (nail polish).

Imaging

Following immunohistochemistry, images were obtained on a Zeiss Axio Imager Ax10 at x40 magnification and neuronal measurements were made using Zen Imaging Pro (v10; Zeiss) software.

Data acquisition and statistical analysis

Extracellular signals were amplified using differential AC amplifiers (A-M Systems model 1700; low cut off, 300Hz; high cut off, 500Hz), digitized using a 1401 analogue-to-digitsal acquisition system (CED; Cambridge Electronic Design, Cambridge, UK) and stored and processed on a PC computer using Spike 2 (CED) software (sampling rate 8-10kHz).

Electrophysiological data were analyzed using Dataview software (v 8.62, courtesy of W. J. Heitler, School of Biology, University of St Andrews, St Andrews, UK), and then all raw data were imported into Excel (Microsoft).

Statistical analysis was performed in SPSS (v21). For comparison of average data either a paired t-test or a repeated-measures ANOVA with Bonferroni post-hoc corrections were used. Error bars represent standard error of the mean. Due to large inter preparation variation, data was sometimes normalised to the value in control (100%) for a more thorough comparison.