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

Stephen P. Currie, Gayle H. Doherty & Keith T. Sillar

University of St Andrews, School of Psychology and Neuroscience, St Andrews, United Kingdom

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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 400 nm 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 light-sensitive proteins OPN5 and/or cryptochrome 1, since populations of OPN5-positive and cryptochrome-positive cells reside 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.

photoreception | locomotion | CPG | opsin5 | cryptochrome

**INTRODUCTION**

Animals utilise spatiotemporally patterned light information to form images using their eyes, whilst slower changes in illumination 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 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 colour in response to light (3). In addition it was demonstrated that lesions to the diencephalon removed this response implying 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),

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 (10). In vertebrates the first evidence for extra-retinal, extra-pineal ‘photomotor’ behaviour came from experiments on blinded and pinealectomised lampreys (11, 12). A similar study of blinded, pinealectomised eels (*Anguilla anguilla*) showed they too responded to illumination of the head with a change in motor behaviour (13). In zebrafish, both positive and negative phototaxis occurs (14, 15). The fish will swim away from a bright light and generally prefer dark conditions, but in a dark environment 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 (16). 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) (17). 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 occurs transiently during development and is mediated by cells within the caudal hindbrain, which are both necessary and sufficient for the behaviour (18).

We have studied the effects of ambient light on spontaneously generated fictive locomotion produced by the isolated nervous system of pro-metamorphic *Xenopus laevis* larvae (19). This preparation, devoid of visual and pineal afferent inputs, retains photosensitivity; episodes of locomotor activity occur spontaneously in the light, but preparatory/and relative quiescent or completely silent in the dark. The response is found to be tuned to short-wavelength (390-410 nm) UV illumination and 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), and cryptochrome 1, a blue-light sensor found in *Drosophila* (20, 21), reveals cells in this region of the tadpole diencephalon that express proteins with an appropriate spectral sensitivity. Together these results suggest that *Xenopus* larvae are equipped with short-wavelength sensitive neurons deep within the brain that directly 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 laevis* tadpoles (Fig. 1Aii) generates periodic episodes...
of rhythmic locomotor-like activity (Fig. 1Bi; 23). As previously shown at embryonic and early larval stages of development (22), motor bursts recorded from spinal ventral roots display left-right alternation opposite 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 occur spontaneously (19).

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), 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. 1Bi; 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 2.60 ± 2.29%; and the episode duration (ED) was 112.75 ± 100.72; while in the dark (grey box) the preparation falls silent. (Bii) Graph showing 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 showing average data for latency to first activity after illumination with UV light of different intensities. Error bars are ± SEM; ***, p = <0.01; *, p = < 0.05.
of the fibre optic light pipe which was positioned approximately 10 cm from the recording bath.

Since classical light sensitivity in the nervous system depends on opsins proteins which have ‘stereotypical spectral fingerprints’ (1), a first step in exploring the phototransduction mechanism was to test representiveness to different wavelengths of light. The halogen light source used in the initial experiments 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 (390–410 nm) 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. 2Ai & iii - purple). Illumination of the same area with blue (468 nm) light, green (523 nm) or red (635 nm) light did not increase activity above the value recorded in the dark (Fig. 2Aii & iii - colour corresponds to wavelength).

The intensity of light applied 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 (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 < 0.05). 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.

As well as the total time spent active, the intensity of UV light also dictated the latency to the first swimming episode when the illumination is turned on (Fig. 2Bi & iii). The mean latency to activity was significantly shorter at 39 lux (32.63 ± 11.27s) than at 5 lux (121.50 ± 48.5s; 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 above the value recorded in the dark (Fig. 2Aii & iii - colour corresponds to wavelength).

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on the spinal cord alone, no response could be elicited at any intensity or wavelength, including broad spectrum white light. 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 were rendered light insensitive. In preparations that were spontaneously active (see the episode of activity in the dark period, 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. 3Bii; N = 4).

In a parallel set of experiments, a smaller diameter light guide was used to locally illuminate three different areas of the light-sensitive, diencephalon-attached preparation. Illumination of area 1 (see Fig. 3C), the rostral extent of the preparation including the caudal diencephalon, produced a significant increase in both the time spent active (Fig. 3Cii) and the number of swim episodes (Fig. 3Civ; also Fig. 3Ci). The time spent active increased to 18.37 ± 2.18% compared with 8.55 ± 0.80% before illumination (Fig. 3Ci; N = 5, 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, 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. 3Ciii–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. 3Ciii–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 resides.

Since most 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. 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. Immunofluorescent labelling of OPN5 positive neurons was therefore performed. Both longitudinal (Fig. 4Bi) and coronal (Fig. 4Bi–iv) 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 ± 0.73um (measuring only clearly defined somata, n = 30 neurons; N = 3 animals). The cluster was located at the level of the 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 matches the spectral sensitivity of the light-triggered locomotor behaviour.

In addition to OPN5, cryptochrome proteins have been reported as blue light sensors (20, 21) with a spectral sensitivity that also 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 isolated larval CNSs and report widespread, protein-specific expression. CRY2 expression is abundant only in non-neuronal cells (microvascular; 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 low level of labelling that was widely distributed, including the OPN5 positive region of the diencephalon (Fig. S4A, B N = 8). In addition, we found intense CRY1 labelling in ventrally and caudally located diencephalic structures including the hypothalamus and pituitary, located ventral to the brainstem proper (Fig. 3Di, S4 A, Cii), suggesting that CRY1 could be responsible for or contribute to the light sensitivity we describe.

To test this idea further we first recorded photic activation of swimming in control isolated CNSs (Fig. 3Dii, upper panels). Next we surgically removed the ventral diencephalon to excise the structures with the strongest 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 still recorded from spinal ventral roots (Fig 3Diii lower panels, Diii; n=3). Therefore, whilst this intensely-labelled CRY1 population does not appear to be necessary for the photoreceptive response, we cannot rule out the possibility that CRY1 is sufficient to play a role via the lower intensity expression observed in the dorsal portion of the diencephalon. In addition, 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 (16). We found no evidence that OPN5 was located within dopaminergic neurons (S7). However, we did identify a cluster of dopaminergic neurons in the same region of the hypothalamus, located just dorsal to the OPN5-positive somata. These TH-positive neurons are the rostral-most members of a population of dopaminergic neurons contiguous with the dopaminergic neurons of the posterior tuberculum (PT), found more caudally in the hypothalamus (S7).

**DISCUSSION**

We have demonstrated that the isolated nervous system of 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 phototransducers in the lights on response are OPN5 and cryptochrome. 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 region with the strongest CRY1 expression, leaving the periventricular OPN5 neurons intact, does not eliminate light sensitivity. Nevertheless, while we cannot differentiate unambiguously between the contributions...
of OPN5 and CRY1 to the locomotor response, strong CRY1 expression in the hypothalamus and pituitary suggests that it may play a role in slower, hormonal and/or diurnal changes in tadpole response to store 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 using the CRISPR/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 expressing the UV-specific opsin, OPN5, strongly implies this is an important 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 (23). An important facet of these experiments is that 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.

This deep brain light sensitivity could function as 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 (24). In addition it may help to avoid the brightest lit areas of the environment where detection by predators is likely to occur. This form of light avoidance strategy is found in many fish species where it is thought to confer a specific advantage in the face of diurnal predation (25). 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 (26, 27). 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 confer some 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.

An important next step will be to determine which neuronal pathway links the photoreceptive neurons to the activation of the motor system. The expression of the UV/blue light sensitive proteins, OPN5 and CRY1, was located in relatively close proximity to a set of dopaminergic neurons potentially related to the A-11-type population, known to project to the spinal cord and express A11-type population, known to project to the spinal cord and express melatonin (16), which has been shown to be present in the PT (16), 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 projection in a motor control (28). However, they are unlikely to mediate the phototransduction we document here. Firstly, the original work that identified melatonin as a phototransduction was carried out in Xenopus and while it was found in both the pre-optic and suprachiasmatic nuclei, there is no evidence for it being present in the caudal hypothalamus (31). Secondly, since the photomotor behaviour in Xenopus is tuned to short-wavelength UV light, it does not correspond to the profile of a melatonin-mediated response, which peaks around 480nm (1, 32, 33).

Alternatively, OPN5 is a UV-specific opsin that has recently been shown to be a component of the photoperiodic response in quail (8, 9). OPN5 was located within the quail PVO, a caudal hypothalamic structure also present in the photosensitive tadpole preparation. Moreover, the PVO cells of other species have been shown to contain dopamine, noradrenaline and/or serotonin (34), all known modulators of locomotion in Xenopus (35–37). An 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 (38). This more caudal group are thought to be homologous to the dopaminergic neurons of the mammalian zona incerta, which makes up the subthalamic diencephalic locomotor region, an area important in the supraspinal control of locomotion (39, 40).

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 around 13,000 lux, within the range of intensities experienced during the day while not in direct sunlight (10,000-25,000 lux; (41)); the brightest LED (blue: 468nm) was approximately 460 lux and so similar to the light intensity experienced at sunrise or sunset; 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.

Deep brain photoreception may promote light avoidance behaviour by increasing locomotor activity relative to light intensity, and so increasing the likelihood of navigating to and settling in dimly lit areas. A role for deep brain photoreception in negative phototaxis has been shown in eels (13), however the generalised increase in locomotor activity seen in the isolated Xenopus nervous system is more similar to the darkphotokinesis behaviour displayed by larval zebrafish (16). In the eel, deep brain photoreception was also shown to mediate phototropism to a circadian cycle of increased nocturnal activity (13). While there is no evidence for circadian variation in activity during larval life, adult Xenopus are nocturnal (42). Tadpoles of the American toad (Bufo americanus) display increased activity and feeding during the day and are generally inactive overnight (43). In tadpoles of Xenopus laevis we propose that deep brain photoreception serves the dual purpose of reducing exposure to the damaging influences of both predation and UV on the one hand and automatically adjusting energetically expensive bouts locomotor activity to diurnal changes in light intensity on the other hand.

**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 as described previously (19) from an in-house breeding colony. 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 (AAWC) Extracellu
electrophysiology apparatus. After removal of the forebrain apart from the most caudal portion of the diencephalon, the remaining nervous system was dissected free of the carcass, apart from the caudal most portion of the tail, which was left attached to verify the preparation was capable of normal motor output. Recording conditions were as described previously (19). 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 periods of 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). (Note with small light level for photoreceptors in the sparrow. II. Photophase of testis growth. Proc Natl Acad Sci USA 60:146–151.


