SHORT COMMUNICATION

Light sensitivity in a vertebrate mechanoreceptor?

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ABSTRACT

Using immunohistochemistry and Western blot analysis we demonstrate that melanospin is localised in cells around the central pore of lateral line neuromasts in the African clawed frog, *Xenopus laevis*. Since melanopsin is a known photoreceptor pigment with diverse functions in vertebrates, we suggest that the lateral line of *Xenopus laevis*, which is primarily a mechanorecptor, may also be light sensitive. Potential functions of such photosensitivity are discussed, including its role in mediating locomotor responses following dermal illumination.

KEY WORDS: Melanopsin, lateral line, mechanoreceptor, photosensitivity, multimodality, phototaxis

SUMMARY STATEMENT

Lateral lines are sense organs on the bodies of aquatic vertebrates sensitive to water displacement. In the African clawed frog they contain the photopigment melanopsin, suggesting they may also be light sensitive.

INTRODUCTION

Although photoreceptors within the outer retina of vertebrate eyes are used by animals for image forming (IF) light detection, extraretinal photoreceptors are widespread among non-mammalian vertebrates, occurring mainly in the brain, but also evident elsewhere in the body (Foster & Hankins, 2002). Such non-image forming (NIF) photoreceptors serve diverse functions including; the regulation of circadian rhythms, mediating locomotor responses to dermal illumination, influencing pigment migration in chromatophores and conferring direct light sensitivity to muscles within the iris.

Until relatively recently, it has been assumed that the only pigments capable of conferring photosensitivity to photoreceptors, even those located in structures outside the eye, use rod and cone opsins. However, in the last two decades a number of opsins have been identified that are different enough to those of traditional photoreceptors to constitute separate gene families (Shand and Foster, 1999). One such photopigment opsin is melanopsin (OPN4). Initially shown to contribute to light-evoked pigment migration within dermal melanophores of *Xenopus laevis* (Provencio et al., 1998), melanopsin has since been implicated in a number of roles including conferring light-sensitivity to a subset of photoresponsive retinal ganglion cells (pRGCs) in mammals which measure overall irradiance and underlie various non-imaging photoreceptive tasks (Hankins et al., 2008; Bailes and Lucas, 2010).

A chance observation during an investigation into iris photosensitivity suggested that the lateral line neuromasts of *Xenopus laevis* might contain melanopsin. Lateral line neuromasts are mechanoreceptors sensitive to water displacement, distributed across the body of many aquatic vertebrates (Dijkgraaf, 1962). In *Xenopus laevis* they are grouped into raised 'stitches' arranged in characteristic patterns on the skin's surface (Murray, 1955). The localisation of melanopsin within lateral line neuromasts suggests they may be sensitive to photic as well as mechanical stimuli.

Here we report on the presence and distribution of melanopsin within *Xenopus laevis* lateral lines and speculate on the functional significance of light sensitivity within this mechanoreceptor.

RESULTS AND DISCUSSION

Immunostaining using a polyclonal antibody (CERN972), raised against a *Xenopus laevis* melanopsin peptide, showed the majority of neuromasts on both dorsal and ventral surfaces of adult male and female pigmented and albino *Xenopus laevis* to be immunopositive (Fig 1A). No differences in distribution of melanopsin were observed between the different phenotypes.

Individual neuromasts showed dense immunopositive staining surrounding the central pore, with fine processes radiating outwards (Fig 1B). In light- (Fig 1C) and electron-microscopic (Fig 1E) sections, dense immunopositive staining was located intracellularly in epidermal cells at the margins of the neuromast pore. As evident from wholemounts (Fig 1B), immunostaining was not confined to the margin of the pore. In serial reconstructions of individual neuromasts we also identified melanopsin in peripheral cells lying slightly deeper in the neuromast (Fig 1D).

Immunoreactivity was also detected by the CERN972 antibody in a Western blot analysis of *Xenopus* brain and stitch samples at a mass consistent with melanopsin (Fig. 2). This is in agreement with previous identification of melanopsin expression in tadpole melanophores and adult *Xenopus laevis* brain and ocular structures (Provencio et al., 1998). Most samples present an upper immunoreactive band near 55 kDa and a lower band at 45-50 kDa. There are 2 isoforms of melanopsin in *Xenopus laevis* (OPN4x and OPN4m)(Bellingham et al., 2006), both of which would be detected by CERN972 and may be represented by the two bands in the stitch samples (Fig 2). This would indicate that the two melanopsin orthologs most commonly found in non-mammalian vertebrates (Bellingham et al., 2006; Davies et al., 2011) are present in *Xenopus laevis* lateral line stitches. The predicted mass for OPN4x is 60 kDa, but membrane proteins usually migrate with a somewhat lower apparent mass in SDS-PAGE. The full sequence of OPN4m is unknown but comparison of OPN4x and m isoforms in other species suggests that they migrate with a similar apparent mass in SDS-PAGE (Davies et al., 2011; Bailes and Lucas, 2013). Bellingham et al (2006) did not detect the OPN4x message in adult *Xenopus* skin tissue, which may either be due to the low quantity of OPN4x message, being only expressed in stitches, or it may indicate that the two strong bands we observe in stitch samples represent two splice variants of OPN4m. This phenomenon has already been observed in some mammalian species (Pires et al., 2009), in chicken (Torii et al., 2007) and in elephant shark (Davies et al., 2012). The immunoreactivity at higher molecular masses is consistent with

formation of oligomeric complexes (dimers, trimers, etc.) which is common under the conditions used for SDS PAGE analysis.

Since melanopsin is a known photopigment, the presence of melanospin immunoreactivity within *Xenopus laevis* lateral line neuromasts suggests that apart from being sensitive to mechanical stimuli, these sense organs may also be light sensitive. It is natural to speculate about the potential functional significance of such lateral line photosensitivity.

Many animals respond to dermal illumination with locomotor activity (Steven, 1963). Some previous evidence suggests the lateral line of larval lamprey may mediate such dermal photosensitivity. Their lateral line nerves generate electrophysiological responses following illumination of the tail and the lesioning of these nerves disrupts the behavioural response to such illumination (Deliagina et al., 1995; Ronan and Bodznick, 1991; Young, 1935). Our results suggest that the photosensitivity of the lateral line might be conferred by melanopsin. Interestingly, the light-driven electrophysiological response of the lamprey lateral line nerves have a long latency, high threshold and do not adapt (Ronan and Bodznick, 1991), which are also characteristics of melanopsin-based retinal photoreceptors in mammals (Bailes & Lucas, 2010; Hughes et al., 2012).

A previous report suggests adult *Xenopus laevis* are negatively phototactic (Denton and Pirenne, 1954). However, it is not known if they react to localised dermal illumination with locomotor activity. We confirmed the negative phototaxis of this species by observing their behaviour in an aquarium, only half of which was illuminated. In 89.8% trials (n=49) where the animals started in the lit half of the aquarium they moved to the dark half of the tank within three minutes (average latency 63 secs). When they started in the dark half of the aquarium (n=39), on the other hand, the frogs normally remained there for the duration of the experiment, spending on average 86.6% of their time in darkness and only rarely venturing into the light for brief periods of time.

We investigated whether focal illumination of the animal's ventral surface, which could not be detected by their eyes, would induce a locomotor avoidance response. While they did appear to react to such stimuli, this was no more frequent than in control animals simply maintained in darkness. Thus, using focal ventral illumination, there was no evidence of dermally-induced locomotor activity in adult *Xenopus laevis*. It could be argued that ventral illumination is not the ideal stimulus, as in the wild the underside of the animal will receive less illumination than other areas of the body. However, ventral neuromasts stained as heavily with melanopsin antibody as neuromasts elsewhere on the body. Furthermore, using

focal ventral illumination was the only way to be certain that the illumination was not detected by the dorsally directed eyes of intact animals. Less systematic focal illumination of other areas of the body also failed to induce consistent locomotor responses

Since focal illumination of the body surface did not induce a behavioural response, it seems likely that melanospin in lateral line neuromasts of *Xenopus laevis* serves a function other than dermally-driven locomotor activity.

The activity of lateral line neuromasts is known to be modulated by the central nervous system using efferent neurons (Russell, 1971). For example, the activity of toadfish lateral line nerves is rapidly suppressed by visual stimuli such as the sight of prey species (Tricas and Highstein, 1991). Outer retinal photoreceptors (rods and cones) are required for such IF processes as identifying prey and thus efferent innervation is essential if the lateral line is to be affected by such stimuli. However, the physiological properties of rods and cones make them less suited for monitoring overall light levels and this is thought to be the primary reason the mammalian retina contains a population melanopsin-containing pRGCs, whose sluggish but long lasting responses make them ideal for detecting overall irradiance (Bailes & Lucas, 2010; Hughes et al., 2012). It is therefore conceivable that melanopsin within the lateral line serves a similar role and modulates lateral line activity in response to longer term changes in ambient light levels. Lateral line sensitivity might, for example, be increased in darkness when photic stimuli are not available. Alternatively, the sensitivity of neuromasts might be adjusted by variations in light level associated with depth as the nature of the vibratory information changes.

Co-localisation of mechano and photosensory function is not unique to *Xenopus laevis* and lamprey lateral lines. It has also been reported in invertebrates. Larval *Drosophila* abdominal mechanosensory neurones also respond to light and contribute to light avoidance behaviour (Xiang et al., 2010). Based on the distribution of developmental *Pax* genes, it has been suggested that ears, mechanoreceptors closely related to lateral lines, and eyes share a common evolutionary lineage (Fritzsch and Piatigorsky, 2005). Multimodality of sense organs involving photoreception and mechanoreception might therefore not be that unusual or surprising.

MATERIALS AND METHODS

Immunocytochemistry & microscopy

Five *Xenopus laevis* (Daudin) were euthanized by overdose of tricaine methanesulfonate (Sigma) followed by decapitation and pithing. The skin was immersed in phosphate buffered (pH7.3) 4% paraformaldehyde at 4°C for 3-4 hours. Patches containing lateral line stitches were stored in Phosphate Buffered Saline (PBS) until further processing or in 30% sucrose for cryosectioning.

For immunostaining, tissue was rinsed in PBS, immersed in 0.3% H_2O_2-methanol for 30 minutes and rinsed again in PBS. Following immersion for 30 minutes in normal goat serum diluted in a solution of 1% triton X-100 in PBS, tissue was incubated at 4^oC for 24-48 hours in the primary antibody diluted 1:2000 or 1:4000 in PBS (both dilutions produced identical staining patterns). This polyclonal antibody (CERN972) was raised against a 15-mer peptide covering residues 216-230 of *Xenopus laevis* OPN4x (FLAIRSTGRNVQKLG) (Provencio et al., 1998). The peptide was linked to rabbit serum albumin using SATA-MHS chemistry (Schielen et al., 1989). The resulting construct was injected in albino female New Zealand rabbits and processed as previously described (deGrip, 1985).

After primary antibody incubation, labelling was visualized using an avidin-biotinylated horseradish peroxidase second antibody procedure (Vector Elite ABC kit; Vector Laboratories, Peterborough, UK) applying diaminobenzidine as the chromagen (Sigma Fast; Sigma-Aldrich, Gillingham, Dorset, UK).

Skin segments were viewed in wet mount to identify immunopositive regions. Some were prepared as wholemounts, while segments for fine structural observation were immersed in 2% aqueous osmium tetroxide for 1 hour, before processing for araldite embedding. Semithin (1μm) sections were cut (Ultracut E; Reichert-Jung, Depew, New York, USA) and counterstained with toluidine blue. Images were collected using an Olympus BH2 photomicroscope equipped with a Spot RT Color digital camera (Diagnostic Instruments inc., Sterling Heights, Michigan, USA). For electron microscopy no further enhancement to the contrast of the HRP-label was required and sections were viewed on a LEO-EM912 electron microscope (Zeiss, Oberkochen, Germany) and recorded with a digital camera.

Molecular analysis

Samples of lateral line stitches, eye, and various brain regions were removed from 2 animals euthanized as described above and frozen. All tissue was ground using a pre-chilled

pestle and mortar prior to homogenisation in 2% (w/v) SDS, 50mM DTT with mini complete protease inhibitors (Roche). Samples were incubated at room temperature on a shaking platform for 2h to improve solubilisation. The lysate was centrifuged at 23000xg for 30min at 20°C and the supernatant fraction used for SDS PAGE and Western blotting as described previously (Pires et al., 2009).

Every effort was made to avoid contamination of lateral line stich samples with dermal melanopores during dissection. If there was any minor contamination, this is unlikely to have been sufficient to produce the strong immunoreactive bands observed. We also found two clear bands in the stitch lanes on SDS-PAGE, while previous studies (Provencio et al., 1998; Bellingham et al., 2006) only detected one band in *Xenopus* melanophores. Hence even if there was some contamination by melanophores, at least one of the observed bands is derived from lateral line stitches.

Phototaxis

Individual animals were removed from their home tank, during the light phase of their light/dark cycle, and put in an experimental aquarium (20x30x20 cm). The sides of this aquarium were covered by black card and animals were observed from above. After 10 minutes acclimation in dim room light the animal was placed in total darkness for 2 mins, before one half of the aquarium was illuminated (3.41W/m^2) from below by a 'light box', consisting of two fluorescent tubes (Phillips 20W/47 Graphic A; Guildford, Surrey, UK) behind a white diffusing surface, for three minutes followed by 2 minutes of darkness before being exposed to light once more for another 3 minutes, for a maximum of 10 trials per animal. The half of the tank that was illuminated was varied randomly. 7 pigmented and 2 albino animals were tested.

We also investigated the ability of focal illumination to induce locomotor activity. The ventral surface of 4 animals was illuminated using the same protocol as above, but instead of illuminating half the aquarium the light source was covered except for a 1cm round aperture that was positioned near the centre of the animals ventral surface when it was resting on the bottom of the tank. The time of any movement after the spot was turned on was noted (n=18). A similar number of control observations were made with the stimulating spot in position but not switched on. Less systematically, we also tried directing light onto various parts of the body with both a narrow torch beam and low power lasers and observing any reaction.

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Competing interests

The authors declare no competing financial interests

Author contributions

G.E.B and R.H.D. conceived the study. G.E.B. performed the immunohistochemistry, for which W.J.dG. provided the antibody. H.-J.W. performed most of the microscopy. R.G.F. and M.T. carried out the Western blot analysis and R.H.D. performed the photactic experiments. All authors contributed to the interpretation of data. R.H.D. drafted the manuscript , which was edited by all authors, except G.E.B., prior to submission.

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FIGURE LEGENDS

Figure 1 Melanopsin immunostaining of *Xenopus laevis* neuromasts

A: Low power wet mount of the dorsal skin showing linear arrays of stained neuromasts (arrows) forming lateral line stitches.

B: Higher power wet mount of three neuromasts within one stitch emphasising dense staining at the centre of the neuromast, with fine processes emanating from it. Arrowheads indicate putative melanopsin positive melanophores.

C: Light microscopic section through the centre of a stained neuromast highlighting the presence of melanopsin in cells at the pore margin (arrows). The superficial immunostaining in the region of hair cell stereocilia and kinocilia (asterisk) is most likely artefactual.

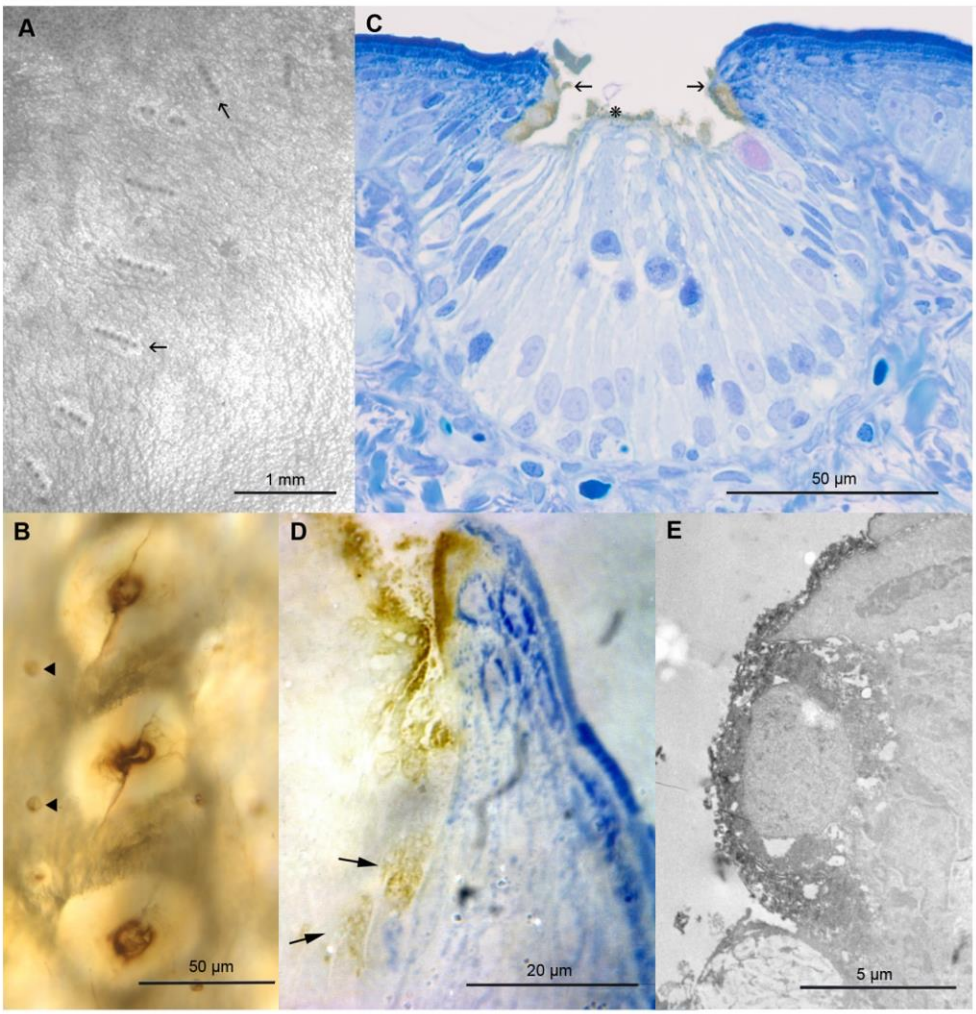
D: Section showing the lateral region of a stained neuromast highlighting melanopsin location deeper in peripheral cells of the organ (arrows).

E: Electron micrograph through a stained cell at the pore margin. The darker areas of the cytoplasm indicate the intracellular location of melanopsin. The nucleus is the spherical lighter (unstained) area

The regions depicted in D and E are from an area equivalent to the stained (brown) tissue at the upper right corner of the neuromast shown in C

Figure 2 Western blot analysis of melanopsin expression in various tissues of *Xenopus laevis*. The blots were screened with antibody CERN972. The upper immunoreactive bands near 55 kDa represent full size OPN4m and/or OPN4x. The lower immunoreactive bands may either represent full size OPN4m (complete sequence is not known currently), or smaller splice variants of OPN4m and/or OPN4x. See body of text for further details.

364 **Figure 1**



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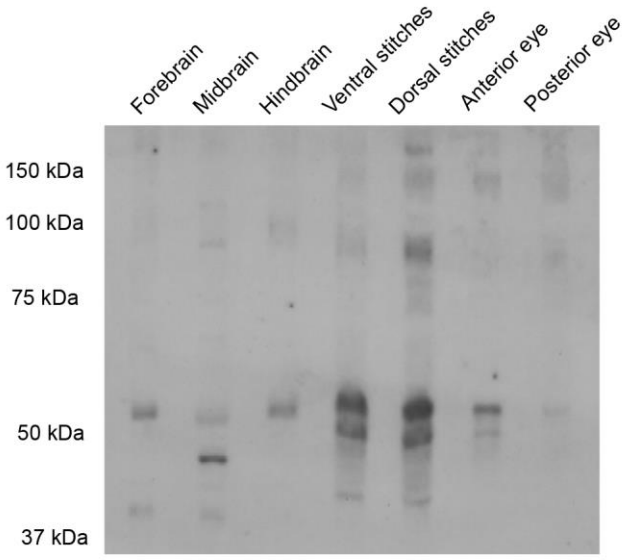
367 **Figure 2**



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