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1 SHORT COMMUNICATION

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4 **Light sensitivity in a vertebrate mechanoreceptor?**

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8 Gary E. Baker^{1†}, Willem J. de Grip², Michael Turton³, Hans-Joachim Wagner⁴,

9 Russell G. Foster³, Ron H. Douglas^{1*}

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13 ¹ Department of Optometry & Visual Science, School of Health Sciences, City University
14 London, Northampton Sq, London EC1V 0HB, UK

15 ² Department of Biochemistry, Radboud Institute for Molecular Life Sciences, Radboud
16 University Medical Center, Nijmegen, The Netherlands

17 ³ Nuffield Laboratory of Ophthalmology, University of Oxford, Levels 5-6 West Wing, John
18 Radcliffe Hospital, Headley Way, Oxford, OX3 9DU, UK

19 ⁴ Anatomisches Institut, Universität Tübingen, Österbergstrasse 3, 72074 Tübingen,
20 Germany

21

22 † Deceased

23

24 * Author for correspondence (r.h.douglas@city.ac.uk)

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28 **ABSTRACT**

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

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37 **KEY WORDS:** Melanopsin, lateral line, mechanoreceptor, photosensitivity,
38 multimodality, phototaxis

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42 **SUMMARY STATEMENT**

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

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47 INTRODUCTION

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

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

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

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

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80 RESULTS AND DISCUSSION

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

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

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

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

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

120 Many animals respond to dermal illumination with locomotor activity (Steven, 1963).
121 Some previous evidence suggests the lateral line of larval lamprey may mediate such dermal
122 photosensitivity. Their lateral line nerves generate electrophysiological responses following
123 illumination of the tail and the lesioning of these nerves disrupts the behavioural response to
124 such illumination (Deliagina et al., 1995; Ronan and Bodznick, 1991; Young, 1935). Our
125 results suggest that the photosensitivity of the lateral line might be conferred by melanopsin.
126 Interestingly, the light-driven electrophysiological response of the lamprey lateral line nerves
127 have a long latency, high threshold and do not adapt (Ronan and Bodznick, 1991), which are
128 also characteristics of melanopsin-based retinal photoreceptors in mammals (Bailes & Lucas,
129 2010; Hughes et al., 2012).

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

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

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

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

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

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

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181 **MATERIALS AND METHODS**

182 ***Immunocytochemistry & microscopy***

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

188 For immunostaining, tissue was rinsed in PBS, immersed in 0.3% H₂O₂-methanol for
189 30 minutes and rinsed again in PBS. Following immersion for 30 minutes in normal goat
190 serum diluted in a solution of 1% triton X-100 in PBS, tissue was incubated at 4°C for 24-48
191 hours in the primary antibody diluted 1:2000 or 1:4000 in PBS (both dilutions produced
192 identical staining patterns). This polyclonal antibody (CERN972) was raised against a 15-mer
193 peptide covering residues 216-230 of *Xenopus laevis* OPN4x (FLAIRSTGRNVQKLG)
194 (Provencio et al., 1998). The peptide was linked to rabbit serum albumin using SATA-MHS
195 chemistry (Schielen et al., 1989). The resulting construct was injected in albino female New
196 Zealand rabbits and processed as previously described (deGrip, 1985).

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

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

210

211 ***Molecular analysis***

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

214 pestle and mortar prior to homogenisation in 2% (w/v) SDS, 50mM DTT with mini complete
215 protease inhibitors (Roche). Samples were incubated at room temperature on a shaking
216 platform for 2h to improve solubilisation. The lysate was centrifuged at 23000xg for 30min at
217 20°C and the supernatant fraction used for SDS PAGE and Western blotting as described
218 previously (Pires et al., 2009).

219 Every effort was made to avoid contamination of lateral line stich samples with
220 dermal melanopores during dissection. If there was any minor contamination, this is unlikely
221 to have been sufficient to produce the strong immunoreactive bands observed. We also found
222 two clear bands in the stich lanes on SDS-PAGE, while previous studies (Provencio et al.,
223 1998; Bellingham et al., 2006) only detected one band in *Xenopus* melanophores. Hence even
224 if there was some contamination by melanophores, at least one of the observed bands is
225 derived from lateral line stitches.

226

227 ***Phototaxis***

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

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

247

248

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253 production.

254

255 **Competing interests**

256 The authors declare no competing financial interests

257

258 **Author contributions**

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

264

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335
336

337 **FIGURE LEGENDS**

338

339 **Figure 1 Melanopsin immunostaining of *Xenopus laevis* neuromasts**

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

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

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

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

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

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

356

357

358 **Figure 2 Western blot analysis of melanopsin expression in various tissues of *Xenopus***

359 *laevis*. The blots were screened with antibody CERN972. The upper immunoreactive bands

360 near 55 kDa represent full size OPN4m and/or OPN4x. The lower immunoreactive bands

361 may either represent full size OPN4m (complete sequence is not known currently), or smaller

362 splice variants of OPN4m and/or OPN4x. See body of text for further details.

363



