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RESEARCH ARTICLE

Rhodopsin coexpression in UV photoreceptors of *Aedes aegypti* and *Anopheles gambiae* mosquitoes

Xiaobang Hu, Matthew T. Leming, Michelle A. Whaley and Joseph E. O'Tousa*

ABSTRACT

Differential rhodopsin gene expression within specialized R7 photoreceptor cells divides the retinas of *Aedes aegypti* and *Anopheles gambiae* mosquitoes into distinct domains. The two species express the rhodopsin orthologs Aaop8 and Agop8, respectively, in a large subset of these R7 photoreceptors that function as ultraviolet receptors. We show here that a divergent subfamily of mosquito rhodopsins, Aaop10 and Agop10, is coexpressed in these R7 photoreceptors. The properties of the *A. aegypti* Aaop8 and Aaop10 rhodopsins were analyzed by creating transgenic *Drosophila* expressing these rhodopsins. Electroretinogram recordings, and spectral analysis of head extracts, obtained from the Aaop8 strain confirmed that Aaop8 is an ultraviolet-sensitive rhodopsin. Aaop10 was poorly expressed and capable of eliciting only small and slow light responses in *Drosophila* photoreceptors, and electroretinogram analysis suggested that it is a long-wavelength rhodopsin with a maximal sensitivity near 500 nm. Thus, coexpression of Aaop10 rhodopsin with Aaop8 rhodopsin has the potential to modify the spectral properties of mosquito ultraviolet receptors. Retention of Op10 rhodopsin family members in the genomes of *Drosophila* species suggests that this rhodopsin family may play a conserved role in insect vision.

KEY WORDS: Mosquito vision, Photoreceptor, UV sensitivity, Visual pigment, Rhodopsin coexpression

INTRODUCTION

Aedes aegypti (Linnaeus 1762) and *Anopheles gambiae* Giles 1902 are the major vectors of mosquito-borne diseases. Visual input underlying adult mosquito behaviors, including mating, host seeking, resting and ovipositioning are mediated by the compound eye (Allan, 1994; Day, 2005). The mosquito compound eye is composed of approximately 200 to 300 ommatidia, each containing a stereotypical arrangement of eight (R1–R8) photoreceptors. Each photoreceptor cell contains a rhabdomere, a highly compact microvillar structure that houses the components for visual transduction. The rhabdomeres of all R1–8 photoreceptors assemble to form a single rhabdom. The R1–6 cells are located on the perimeter and project rhabdomeres inward, while the R8 cell occupies the central region and projects its rhabdomere outward, into this rhabdom. The R7 photoreceptor cell occupies a unique position within the ommatidium because it projects its rhabdomere only at the distal surface of the fused rhabdom structure (Brammer, 1970; Hu et al., 2009).

Department of Biological Sciences and the ECK Institute for Global Health, Galvin Life Science Building, University of Notre Dame, Notre Dame, IN 46556, USA.

*Author for correspondence (jotousa@nd.edu)

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The rhodopsins expressed in the photoreceptors provide a second means to describe the organization of the mosquito retina. The *A. aegypti* and *A. gambiae* genome projects identified 10 rhodopsin genes in each of these mosquitoes (Hill et al., 2002; Nene et al., 2007). In *A. aegypti*, the long-wavelength rhodopsin Aaop1 is expressed in R1–6 photoreceptors throughout the retina (Hu et al., 2012), whereas expression of different rhodopsins in R7 and R8 cells divides the retina into the dorsal, central, ventral stripe and ventral regions (Hu et al., 2009; Hu et al., 2011). The ultraviolet (UV) rhodopsin Aaop8 is expressed in the R7 cells of the central region and the ventral region. The long-wavelength rhodopsin Aaop2 and the short-wavelength rhodopsin Aaop9 are coexpressed in the R7 cells of the dorsal region and the ventral stripe (Hu et al., 2011). The *A. gambiae* retina appears to be similar, with the only identified difference being that the UV rhodopsin Agop8 is only expressed in the R7 cells of the central region (Hu et al., 2009).

Both *A. aegypti* and *A. gambiae* genomes contain an orthologous rhodopsin: Aaop10 and Agop10, respectively. Sequence comparison (Nene et al., 2007) showed that this Op10 rhodopsin belongs to a divergent group with greatest similarity to *Drosophila* Rh7, a rhodopsin of unknown function (Posnien et al., 2012). To initiate the study of this rhodopsin group, we characterized the expression profile of these mosquito genes. Here we show that in both *A. aegypti* and *A. gambiae*, Op 10 rhodopsin is coexpressed in the Op8-expressing R7 cells. Analysis of transgenic *Drosophila* showed that Aaop8 is a UV rhodopsin while Aaop10 is a long-wavelength rhodopsin. Our results provide a second example in which rhodopsins with different spectral properties are coexpressed in a mosquito photoreceptor.

RESULTS

Op10 rhodopsin is coexpressed with Op8 rhodopsin in R7 photoreceptor cells of *A. aegypti* and *A. gambiae*

Orthologous genes identified in the mosquito genome projects, *A. aegypti* GPROP10 (AAEL005322) and *A. gambiae* GPROP10 (AGAP007548), will be referred to as Aaop10 and Agop10, respectively, and *A. aegypti* GPROP8 (AAEL009615) and *A. gambiae* GPROP8 (AGAP006126) are referred to as Aaop8 and Agop8, respectively. To characterize the expression of mosquito Op10 rhodopsins, we generated polyclonal antiserum against the C terminus of the *A. aegypti* Aaop10 protein (Fig. 1A). *Aedes aegypti* retina stained with this antiserum detected a discrete site of Aaop10 immunoreactivity (green) within the fused rhabdom structure (red) of individual ommatidial units (Fig. 1B). Pre-incubation of the antiserum with the Aaop10 fusion protein eliminated this staining, providing evidence that the antisera is specific to the Aaop10 C-terminus sequence. To identify the photoreceptor cell type expressing Aaop10, longitudinal retinal sections were prepared and simultaneously stained with Aaop10 and Aaop8 antisera. Fig. 1C shows that Aaop10 is detected in a discrete central area within the ommatidial units (arrow). The observation that Aaop8 colocalized

The Journal of Experimental Biology (2014) doi:10.1242/jeb.096347

List of abbreviations

ERG	electroretinogram
ORF	open reading frame
PBS	phosphate-buffered saline
PBT	phosphate-buffered saline with Tween-20
UV	ultraviolet

at this site (Fig. 1D,E) provided evidence that the Aaop10 is present in the same R7 rhabdomere as Aaop8.

The *A. aegypti* Aaop10 sequence used to generate the antisera retains significant sequence identity with the corresponding *A. gambiae* Agop10 sequence (Fig. 1A). To test the possibility that the antisera would also detect Agop10, we carried out similar experiments on sectioned *A. gambiae* retina. The results show that Aaop10 antisera recognizes Agop10, and Agop10 is also coexpressed with Agop8 in R7 cells of *A. gambiae* (Fig. 1F–H).

Op10 rhodopsin is co-expressed with Op8 rhodopsin in distinct retinal regions of *A. aegypti* and *A. gambiae*

Prior work established that the differential rhodopsin expression in the R7 photoreceptors divides the *A. aegypti* retina into four distinct regions (Hu et al., 2009). These are the dorsal region, the central region, the ventral stripe and the ventral region. Aaop8 is expressed in both the central and ventral regions. To reveal whether the Aaop10 expression in R7 cells conforms to this pattern, we imaged intact whole retinas labeled with the Aaop10 antisera. Fig. 2A shows that Aaop10 expression is localized to the central and ventral regions, and absent from the dorsal region (labeled DR) and the ventral stripe (labeled VS). To confirm that these central and ventral regions are exactly those regions previously defined by the pattern of Aaop8 expression, whole retinas were doubled labeled for Aaop8 and Aaop10. The results (Fig. 2B) showed that cells expressing Aaop10 are precisely those that express Aaop8, and these two rhodopsins are not expressed in the dorsal region or ventral stripe.

Differential rhodopsin expression in the R7 photoreceptors of *A. gambiae* also divides its retina into different regions. One difference between the two mosquito species is rhodopsin expression in the R7 cells of the ventral region. In this region, *A. gambiae* specifies Agop2 expression in the R7 photoreceptors whereas the *A. aegypti* pattern specifies Agop8 expression in the R7 photoreceptors (Hu et al., 2009). In an *A. gambiae* retina stained with the Aaop10 antiserum (Fig. 3A), staining is seen within the central region but not within the dorsal (arrowhead) and ventral regions (small arrow). Analysis of retina costained with Aaop8 antibody and actin (Fig. 3B–D) demonstrates that cells expressing Agop10 are those previously identified Agop8-expressing R7 cells. Thus, in both mosquito species, the Op8 and Op10 rhodopsins are coexpressed in the subset of R7 photoreceptors previously identified as UV receptors.

Although Op8 and Op10 rhodopsins are coexpressed in R7 photoreceptors, they exhibit different subcellular distributions. For *A. aegypti*, both sectioned retina (Fig. 1C–E) and whole-mount (Fig. 2C–E) analyses detected Op8 in both the rhabdomere and cell body of R7 cells. The same two analyses detected Op10 rhodopsin mainly within the rhabdomere.

Aedes aegypti Aaop8 and Aaop10 rhodopsins possess different spectral properties

To characterize the spectral properties of *A. aegypti* Aaop8 and Aaop10 rhodopsins, we created transgenic *Drosophila melanogaster* strains expressing the Aaop8 or Aaop10 genes under the control of *Drosophila* Rh1 rhodopsin promoter. This allows for expression of the mosquito rhodopsins in the major class of *Drosophila* photoreceptors (R1–6 cells). For Aaop10, a previously undocumented exon 1 sequence was identified 179 kb upstream of exon 2. The inclusion of this exon extends its sequence identity with Agop10 (Fig. 4A) and includes the coding information for the first transmembrane domain of the Aaop10 protein.

To confirm the expression of these rhodopsins in *Drosophila*, we stained retinas of the transgenic strains with Aaop8 and Aaop10 antisera. This analysis established that the Aaop8 protein was expressed in transgenic *Drosophila* and that the Aaop8 protein localized as expected within rhabdomeric membranes and not within the lamina synaptic region (Fig. 4B). In contrast to Aaop8, the Aaop10 rhodopsin was poorly expressed in transgenic *Drosophila* and located in diffused and punctate structures within the cell body

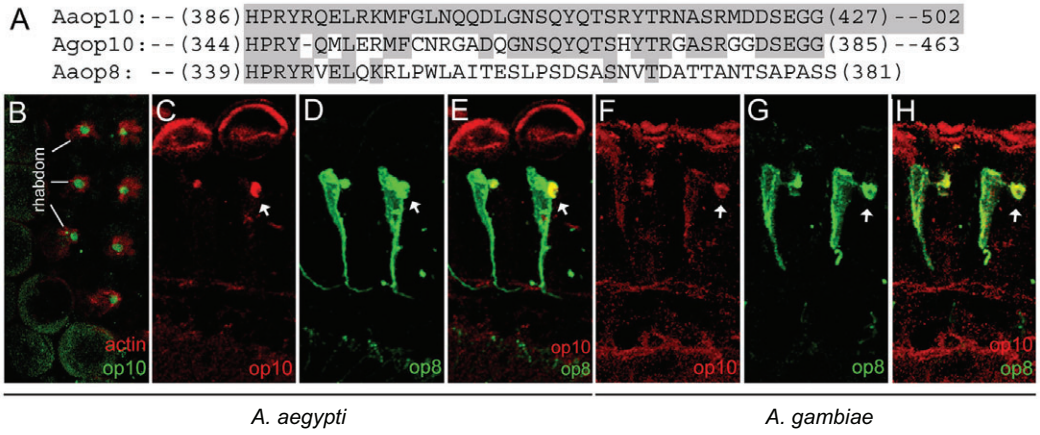

Fig. 1. Op10 rhodopsin is co-expressed with Op8 rhodopsin in *Aedes aegypti* and *Anopheles gambiae* R7 photoreceptor cells. (A) The sequence alignment of the C-terminal regions of *A. aegypti* Aaop10, *A. gambiae* Agop10 and *A. aegypti* Aaop8 rhodopsins. Amino acids identical to the Aaop10 sequence are shaded gray. Only the first 42 amino acids showing substantial sequence identity are displayed. (B) A cross-section of the *A. aegypti* retina labeled for Aaop10 (green) and actin (red) shows Aaop10 is detected at a central location within the actin-rich rhabdom of individual ommatidial units. (C–E) Longitudinal view of two ommatidial units in *A. aegypti* labeled for Aaop10 (C,E; red) and Aaop8 (D,E; green). Aaop10 and Aaop8 are colocalized in the R7 rhabdomere (arrows). Aaop10 is localized mainly within the R7 rhabdomere, while Aaop8 is found in both the R7 rhabdomere and cell body. (F–H) Longitudinal view of two ommatidial units in *A. gambiae* labeled with the Aaop10 antiserum (F,H; red) and Aaop8 antiserum (G,H; green). The antisera detect Agop10 and Agop8 colocalization within the R7 rhabdomere (arrows).

The Journal of Experimental Biology (2014) doi:10.1242/jeb.096347

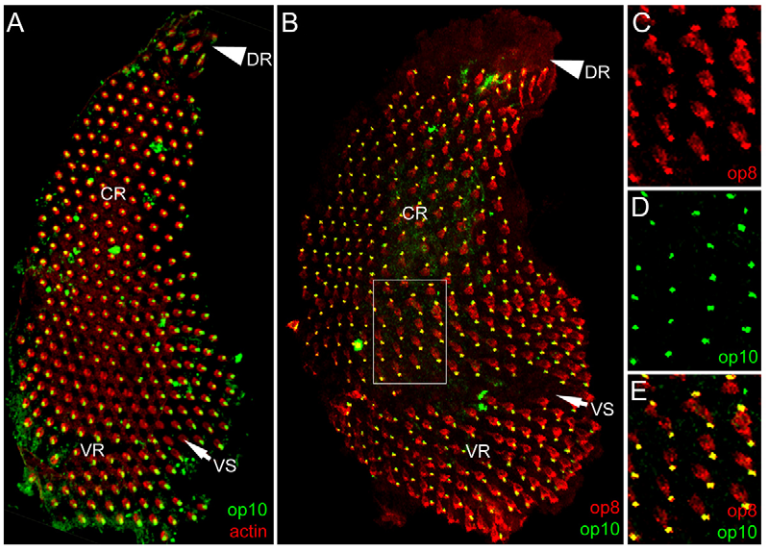

Fig. 2. Aaop10 rhodopsin is co-expressed with Aaop8 rhodopsin in distinct retinal regions of *A. aegypti*. (A) An *A. aegypti* retina labeled for Aaop10 (green) and actin (red). Aaop10 is expressed in the R7 photoreceptors of the central region (CR) and ventral region (VR), but not within the R7 photoreceptors of the dorsal region (DR) and ventral stripe (VS). Part of the dorsal region of this retina was lost during dissection so the dorsal region is larger in B. (B) *A. aegypti* retina labeled for Aaop10 (green) and Aaop8 (red). Aaop10 and Aaop8 are co-expressed in the R7 cells of ommatidial units within the central (CR) and ventral (VR) regions, but not within the cells of the ventral stripe (VS) and dorsal region (DR). (C–E) A magnified view of the boxed region of the image in B. Aaop10 is mainly localized within the R7 rhabdomere, while Aaop8 is located in both the R7 rhabdomere and cell body.

(arrowheads) and lamina compartments (Fig. 4C). Only a small amount was present within the expected location, the photoreceptors' rhabdomeric membranes (stained red).

To determine the spectral properties of Aaop8 and Aaop10 rhodopsin, we carried out electroretinogram (ERG) analysis of the transgenic flies. For this analysis, we created *Drosophila* strains in which a transgene was the only rhodopsin expressed in the R1–6 cells and there was no light response from R7 and R8 photoreceptors (Ahmad et al., 2006). In the absence of a rhodopsin transgene, no light response is evoked using 600–350 nm light stimuli, with intensities adjusted so that similar photon contents were delivered. Expression of the Rh1 rhodopsin (Fig. 5A) in this genetic background evoked dual peak responses consistent with published maximal sensitivities near 480 and 350 nm (Salcedo et al., 1999). In *Drosophila* expressing Aaop8, the ERG analysis shows a single peak response at 350 nm (Fig. 5B), indicating that Aaop8 is a UV-sensitive rhodopsin.

To further characterize Aaop8's spectral properties, we prepared membrane extracts from Aaop8 transgenic fly heads and subjected the extracts to spectrophotometric assays (Paulsen, 1984). Difference spectra obtained from blue- and UV-adapted membranes (Fig. 5C) showed that blue-adapted membranes (generating rhodopsin) possess a peak absorbance near 330 nm, whereas UV-adapted membranes (generating metarhodopsin) displayed a peak absorbance near 460 nm. These results indicate that Aaop8 is a

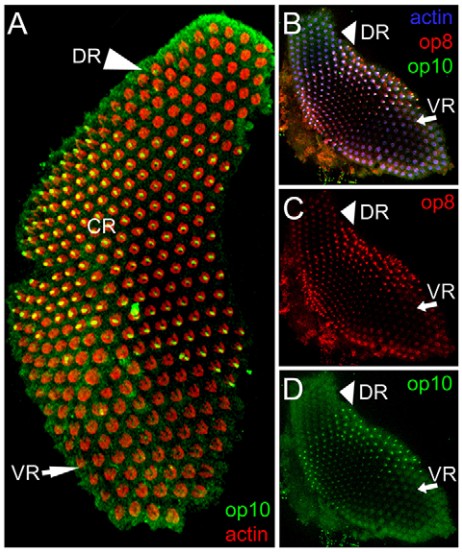

Fig. 3. *Anopheles gambiae* Agop10 and Agop8 co-expression in R7 photoreceptor cells. (A) *Anopheles gambiae* retina labeled for Agop10 (green) and actin (red). Agop10 is expressed in the ommatidial units of the central region (CR) but not in the dorsal region (DR) and the ventral region (VR). (B–D) *Anopheles gambiae* retina simultaneously labeled for actin (B; blue), Agop8 (B,C; red) and Agop10 (B,D; green). No Agop8 or Agop10 expression is seen within the dorsal or ventral regions.

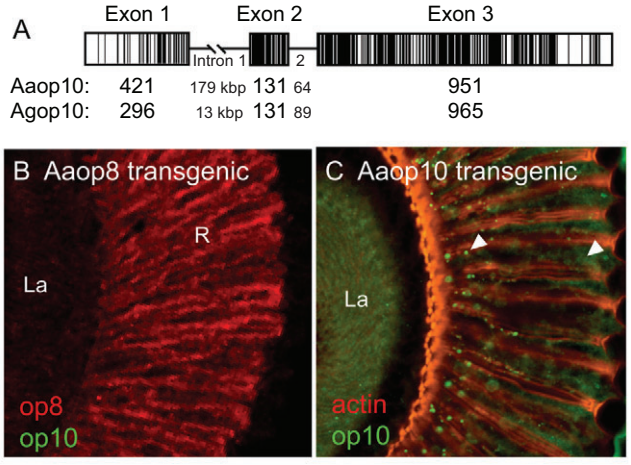

Fig. 4. Expression of *A. aegypti* Aaop8 and Aaop10 rhodopsins in transgenic *Drosophila*. (A) Proposed organization of the Aaop10 gene in *A. aegypti*. Within the three exons, black bars indicate amino acid identity and gray bars indicate amino acid conservation in the *A. aegypti* and *A. gambiae* Op10 proteins. Sizes of the introns and exons shown below the diagram are in base pairs except, as noted, the size of intron 1 is shown in kilobase pairs. (B) Micrograph showing a retinal section of an Aaop8 transgenic *Drosophila* stained for Aaop8 (red), and Aaop10 (green). Aaop8 is localized within the photoreceptor rhabdomeres of the retina (R) but not in photoreceptor axonal projections extending into the lamina (La). The Aaop10 antiserum fails to label the Aaop8 transgenic *Drosophila*. (C) Micrograph showing a retinal section of an Aaop10 transgenic *Drosophila* stained for Aaop10 (green) and actin (red). Aaop10 is detected at low levels within the rhabdomeric membranes. Aaop10 is also detected within the photoreceptor cell body (arrowheads) and the photoreceptor axonal projections extending into the lamina.

The Journal of Experimental Biology (2014) doi:10.1242/jeb.096347

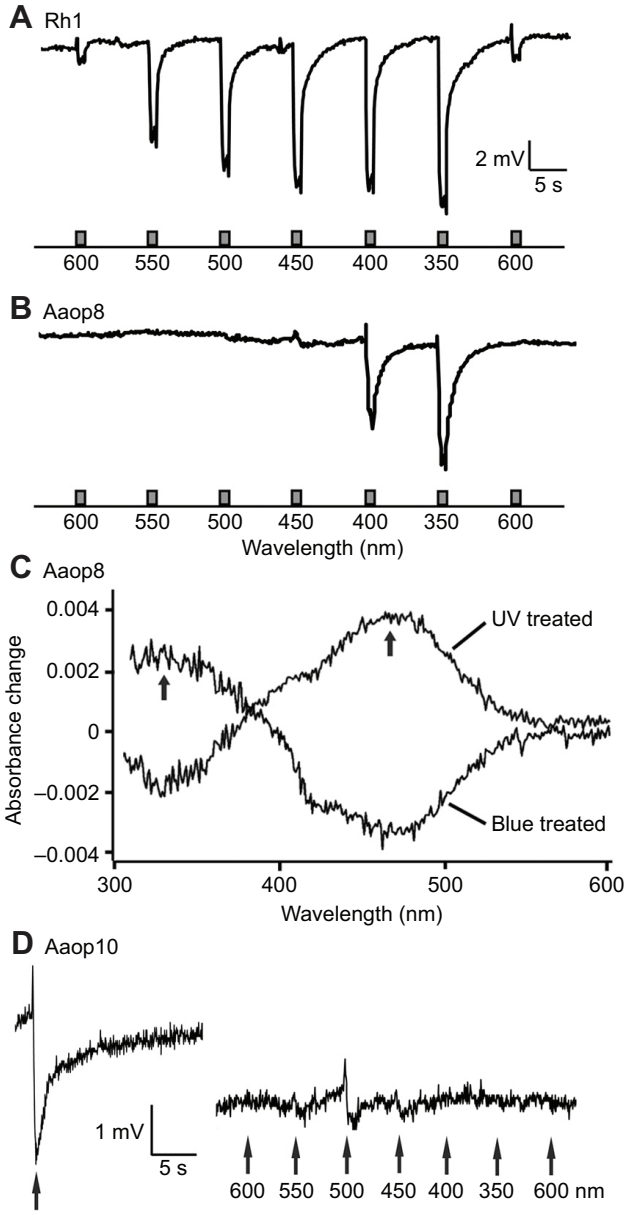

Fig. 5. Spectral analysis of *A. aegypti* Aaop8 and Aaop10 rhodopsins expressed in transgenic *Drosophila*. (A) An electroretinogram (ERG) of the positive control *Drosophila* genotype *norpA*^{P24}; *cn bw*; <pRh1:norpA >*ninaE*^{I17}/*ninaE*$^{+}$ (Rh1 expressed) shows a dual peak response in the blue (500–450 nm) and UV (350 nm) regions. Stimuli were adjusted to contain similar photon flux (20 µE m^{-2} s^{-1}) at the indicated wavelengths and were administered as depicted in the diagram under the trace. (B) An ERG of the *Drosophila* genotype *norpA*^{P24}; *cn bw* <pRh1:Aaop8 >/*cn bw*; <pRh1:norpA >*ninaE*^{I17}/*ninaE*^{I17} shows that flies expressing Aaop8 are maximally sensitive to UV light. The stimuli were identical to those used in A. (C) Difference spectra obtained from Aaop8-expressing *Drosophila* head membrane extracts. Blue light treatment generates the rhodopsin form of Aaop8 with a λ_{max} at ~330 nm (left arrow). UV light treatment generates the metarhodopsin form of Aaop8 with a λ_{max} at ~460 nm (right arrow). (D) ERG of the *Drosophila* genotype *norpA*^{P24}; *cn bw* <pRh1:Aaop10 >; <pRh1:norpA >*ninaE*^{I17}. At left, a recording of the response of a Aaop10 transgenic *Drosophila* following a bright 100 ms stimulus of 10^4 lx broadband white light (arrow) shows a small response of 2–3 mV with slow response and recovery times. At right, a recording of the responses following light stimuli at the indicated wavelengths shows the maximum response at 500 nm. The intensity of these light stimuli at all wavelengths was ~10-fold higher than those used in A and B, with the sole exception being that the intensity of the 350 nm stimulus remained the same.

bistable pigment that photoconverts between 330 nm rhodopsin and 460 nm metarhodopsin forms.

The transgenic Aaop10 flies failed to produce a light response at any of the stimulus intensities that were used in the analysis of Aaop8. However, delivery of a bright white light produced small ERG responses of 2–3 mV that featured slow response and recovery times (Fig. 5D, left). Because of these observations, the spectral response analysis was carried out using light intensities at 600 through 400 nm that were 10-fold higher than those applied in the Aaop8 study (Fig. 5D, right). This approach allowed small responses to be recorded, with a maximum sensitivity at 500 nm light (n=6, average response 0.6 mV). These results indicate that Aaop10 is a long-wavelength rhodopsin.

DISCUSSION

Photoreceptors possessing UV-sensitive rhodopsins are widespread and phylogenetically ancient in both invertebrates and vertebrates (Briscoe and Chittka, 2001; Shi et al., 2001). The detection of UV light is known to be involved in many animal behaviors such as foraging, navigation, intraspecific communication and the control of circadian rhythms (Goldsmith, 1994; Tovée, 1995; Hunt et al., 2001). Both *A. aegypti* Aaop8 and *A. gambiae* Agop8 rhodopsins retain primary protein sequence motifs that are characteristic of invertebrate UV pigments, including K110 lysine, the shorter third cytoplasmic loop CL3 and the DRY motif (Salcedo et al., 2003). Based on these properties, Aaop8 and Agop8 were assigned as UV-sensitive pigments by genome studies (Hill et al., 2002; Nene et al., 2007). This expectation was confirmed by results reported here. We show by ERG analysis that Aaop8 rhodopsin confers sensitivity to UV light in transgenic *Drosophila*. Further, absorbance spectra obtained from membrane extracts of the Aaop8-expressing *Drosophila* heads revealed that Aaop8 rhodopsin has a peak absorbance in the UV range near 330 nm, and it is photoconverted to a stable metarhodopsin with a peak absorbance near 460 nm.

Both *A. aegypti* and *A. gambiae* express a second rhodopsin, Aaop10 and Agop10, respectively, in these UV-sensitive photoreceptors. Phylogenetic analysis shows that these Op10 rhodopsins are orthologs of the *Drosophila* Rh7 rhodopsin (Nene et al., 2007). This rhodopsin family is known to be present in the three mosquito species and the 12 *Drosophila* species for which there are characterized genomes (Posnien et al., 2012). The conservation of this rhodopsin family suggests that they may have a unique role in mosquito visual system function.

We created transgenic *Drosophila* that express Aaop10 in the R1–6 class of photoreceptors, an approach used for spectral analysis of many invertebrate rhodopsins (Feiler et al., 1988; Feiler et al., 1992; Townson et al., 1998; Salcedo et al., 1999; Knox et al., 2003). Using this approach, we determined that the Aaop10 rhodopsin elicits only small and slow responses when expressed in *Drosophila*. The basis of this behavior requires further investigation. One possibility is that the Aaop10 rhodopsin we expressed in *Drosophila* possesses an altered N terminus, as this structure was deduced solely from sequence homology to other rhodopsins. A second possibility is that the Aaop10 rhodopsin is poorly compatible with *Drosophila* visual components, which might be required for maturation of the visual pigment or initiating the G-protein coupled cascade. A third possibility is that Aaop8 and Aaop10 rhodopsins may retain distinct signaling capabilities within the mosquito UV-sensitive photoreceptors. R7 photoreceptor cells show both rhabdomeric and cell body localization of Op8, but mainly rhabdomere localization of Op10. We previously reported that the long-wavelength rhodopsin Aaop1 shows extensive light-induced movement from the

The Journal of Experimental Biology (2014) doi:10.1242/jeb.096347

rhabdomere to the cell body (Hu et al., 2012). Our finding that Op10 rhodopsin does not behave in a similar manner suggests that it may play a specialized role in these R7 photoreceptors.

The analysis of the transgenic *Drosophila* suggests that Aaop10 is a long-wavelength rhodopsin. The Rh7 rhodopsins (Op10 rhodopsin homolog) of all 12 *Drosophila* species possess K110, the DRY motif and the shorter version of the CL3 loop characteristic of invertebrate UV pigments. For *Drosophila* Rh3 rhodopsin, a mutation of K110 to E110 shifted the spectral sensitivity from the UV to the visible range (Salcedo et al., 2003). Thus the presence of K110 in *Drosophila* Rh3 rhodopsins is consistent with expectation that these are UV rhodopsins. In contrast, the *A. aegypti*, *A. gambiae* and *Culex pipiens* Op10 rhodopsins, while retaining the DRY motif and the shorter CL3 loop, possess E110. This is consistent with our data indicating that Aaop10 is not a UV rhodopsin but a long-wavelength rhodopsin.

Retinas of both vertebrates and invertebrates consist of multiple classes of photoreceptors, each typically expressing a different rhodopsin with unique spectral properties that provides the basis for color vision. However, there are now many examples of rhodopsin co-expression. In vertebrates, the mouse, rabbit, guinea pig and Syrian hamster all express both green-sensitive (M) and short-wavelength-sensitive (S) rhodopsins in a transitional zone between the superior and the inferior regions of cone photoreceptors (Röhlich et al., 1994; Glösmann and Ahnelt, 2002). The UV-sensitive cone of the salamander retina co-expresses three functional rhodopsins (Makino and Dodd, 1996). Examples in invertebrates include co-expression of rhodopsins in butterflies (Kitamoto et al., 2000; Arikawa et al., 2003; Sison-Mangus et al., 2006), co-expression of Rh3 and Rh4 UV-sensitive rhodopsins in *Drosophila* (Mazzoni et al., 2008), and co-expression of multiple long-wavelength rhodopsins in the horseshoe crab *Limulus* (Katti et al., 2010).

We previously reported that the shortwavelength rhodopsin Aaop9 and the long-wavelength rhodopsin Aaop2 are co-expressed in one class of R7 photoreceptors and thereby may impart broadband specificity to these photoreceptors (Hu et al., 2011). In this report, we show that the other class of *A. aegypti* R7 photoreceptors, those capable of UV sensitivity, co-express the long wavelength Aaop10 rhodopsin along with the UV-sensitive Aaop8 rhodopsin. This co-expression is also found in *A. gambiae* UV-sensitive R7 cells, indicating that this rhodopsin co-expression is a conserved feature in these mosquito UV photoreceptors. Further studies are needed to characterize the possible physiological function that Op10 and Op8 rhodopsins co-expression may provide in these two mosquito species.

MATERIALS AND METHODS

Production of antisera specific to mosquito Aaop10 rhodopsin

A 354 bp C-terminal coding region of Aaop10 was amplified from *A. aegypti* genomic DNA using the primers 5′-G*GAATTC*CACCCTCGC-TACCGACAG-3′ and 5′-ATTT*GCGGCCGC*CTAGTGATTGCTGTTGGG-3′. The italicized sequences indicate *Eco*RI and NotI restriction sites used to ligate this open reading frame (ORF) into pET32a(+) expression vector (EMD Biosciences, San Diego, CA, USA). The construct was confirmed by DNA sequencing. Protein containing the C-terminal Aaop10 sequence was expressed in *BL21DE3-PlysE* cells using standard procedures and purified by affinity chromatography using His-Bind columns (EMD Biosciences, San Diego, CA, USA). Purified Aaop10 fusion protein was injected into mice using Titer Max Gold Adjuvant (Sigma-Aldrich, St Louis, MO, USA) and rabbits (Proteintech, Chicago, IL, USA) for the production of polyclonal antiserum. The rabbit polyclonal serum was affinity-purified by using AminoLink Plus immobilization kit (Pierce, Rockford, IL, USA). The Aaop8 mouse polyclonal antibody was made as described previously (Hu et al., 2009).

Immunostaining of mosquito and fly head sections and whole-mounted retinas

The *A. aegypti* white-eyed Kh^{w} strain and the *A. gambiae* Mali NIH (red-eyed) strain were used in this study. Mosquito heads and *Drosophila* heads were cut and fixed in 4% paraformaldehyde/5% sucrose/1× phosphate-buffered saline (PBS) overnight at 4°C, rinsed three times for 10 min in 5% sucrose/1× PBS, placed in 5% sucrose/1× PBS overnight at 4°C, then placed in 30% sucrose/1× PBS overnight at 4°C, and finally in 30% sucrose/1× PBS:Tissue Freezing Medium (1:1; Triangle Biomedical Sciences, Durham, NC, USA) for 4 h at room temperature or overnight at 4°C. Tissue was then embedded and frozen in 100% Tissue Freezing Medium and used to prepare 10–12 µm sections. Slides were dried at 50°C for 2 h. Sections were rehydrated with 1× PBS for 20 min, blocked with 1× PBS/2.5% normal goat serum/0.3% Triton X-100/1% DMSO for 1 h, and incubated overnight at 4°C with a 1:100 dilution of anti-Aaop10 and/or anti-Aaop8 polyclonal antisera. After three 10 min washes in phosphate-buffered Tween-20 (PBT: 1× PBS/0.1% Tween-20), samples were incubated in goat anti-mouse/rabbit Alexa Fluor 488 (1:500) and/or goat anti-rabbit/mouse Alexa Fluor 594 (1:500) and phalloidin-Alexa Fluor-594 (1:40) (Molecular Probes, Carlsbad, CA, USA) diluted in blocking buffer for 1 h at room temperature. Sections were washed three times for 10 min in PBT, and then mounted using Vectashield (Vector Laboratories, Burlingame, CA, USA).

For preparation of retinal whole mounts, adult mosquito heads were bisected, leaving one eye undamaged, and fixed overnight with 2% paraformaldehyde at 4°C. Retinas were dissected in PBS, washed three times in PBT, and incubated with the primary antisera (anti-Aaop10 and/or anti-Aaop8 polyclonal antisera, 1:100) diluted in BNT (1× PBS/0.1% BSA/0.1% Tween-20/250 mmol l^{-1} NaCl) for 48–72 h at 4°C. After three 10 min washes with PBT, retinal tissues were incubated with secondary antibodies (Alexa Fluor 488 goat anti-mouse/rabbit IgG and/or Alexa Fluor 594 goat anti-rabbit/mouse IgG: 1:500 in BNT) and Alexa Fluor 594/633 phalloidin (1:40) for 2 h at room temperature. After three 10 min washes and an overnight wash in PBT, retinal tissues were mounted in Vectashield and imaged by confocal microscopy.

Cloning the ORF of *A. aegypti* Aaop8 and Aaop10 rhodopsins

The cDNA clone NABN432 generated by the *Aedes* genome project contained the entire Aaop8 ORF. To create the Aaop10 ORF sequence, three PCR amplification reactions were performed to independently amplify each exon from genomic DNA. For these reactions, primers were engineered to allow a mixture of these three PCR products to anneal and create the complete Aaop10 ORF sequence in a second PCR reaction. These primers were: exon 1, 5′-CACCATGAAGCTTATCCTATTTTTC-3′ and 5′-GGAT-TTGAACCTGAAGAACATCAGAATCAC-3′; exon 2, 5′-TGTTCTTCAG-GTTCAAATCCCTCCGTACTC-3′ and 5′-ATCGTGCACCAAACATTC-CCGGTAG-3′; and exon 3, 5′-GGGAATGTTTGGTGCACGATCTACG-3′ and 5′-AATACGACTCACTATAGGG-3′. The reconstructed DNA was then placed in the pENTR/DTOPO (Invitrogen) vector, and the Aaop10 ORF sequence was confirmed by DNA sequence analysis.

Transgenic *D. melanogaster* expressing *A. aegypti* Aaop8 and Aaop10 in R1–6 photoreceptors

The Aaop8 ORF was directionally cloned into a modified pCaSpeR4 transformation vector, in which the polylinker region was replaced with the *Drosophila* Rh1 rhodopsin (*ninaE*) promoter, and a 0.7 kb 3′ untranslated region of the *ninaE* gene (Ahmad et al., 2006). For Aaop10, we modified the pCaSpeR4 vector by adding an attR1-ccdB-attR2 Gateway recombination site, then the Aaop10 ORF plasmid described above was inserted into this plasmid using Gateway technology (Life Technologies, Grand Island, NY, USA). This effort created P element transformation vectors containing the ORFs of Aaop8 and Aaop10 under control of the *Drosophila* Rh1 promoter. Transgenic *Drosophila* strains carrying the Aaop8 or Aaop10 transgenes were then generated using standard procedures.

ERG analysis of transgenic *Drosophila*

Genetic crosses similar to those described previously were used to create white-eyed flies in which the only rhodopsin expressed in R1–6 photoreceptors was *A. aegypti* Aaop8 or Aaop10, and other photoreceptor

cell classes did not respond to light (Hu et al., 2009). The ERG analysis was carried out using standard procedures (Washburn and O'Tousa, 1992), using narrow bandpass and neutral density filters (Oriel, Stratford, CT, USA) to deliver 600, 550, 500, 450, 400 and 350 nm light stimuli with comparable photon content (20 μE m^{-2} s^{-1}).

Spectral analysis of transgenic Aaop8 *Drosophila* head membranes

Transgenic Aaop8 flies were made white-eyed by placing a *cn bw* second chromosome in the genetic background. The complete genotype of the flies used for analysis was *w*; *cn bw*; $ninaE^{I17}$/ <pRh1:Aaop8 >$ninaE^{I17}$. The membrane isolation and spectral analysis were performed according to a published protocol (Kiselev and Subramaniam, 1994). Briefly, ~1000 adult transgenic flies were incubated in dark overnight, then rapidly frozen in liquid nitrogen, and subjected to vigorous shaking to remove fly heads. The heads were selectively recovered by filtering through a 710 μm mesh sieve. Heads were disrupted on ice in a Teflon-glass homogenizer in 10 ml of homogenization buffer. The resulting suspension was centrifuged twice at 1000 ***g*** for 5 min at 4°C to remove particulate material. Membranes were collected from the supernatant by centrifugation at 45,000 ***g*** for 1 h and resuspended in 1.5 ml of homogenization buffer. Difference spectra were recorded at room temperature using a Cary 300 spectrophotometer equipped with the CA-30 Internal Diffuse Reflectance Accessory. A 150 W fiber-optic illuminator (Cole-Parmer, Chicago, IL, USA) was used with a broadband UV filter (UG-11, Oriel, Stamford, CT, USA) to convert Aaop8 rhodopsin to metarhodopsin, and with a broadband blue light filter (BG-18) to convert Aaop8 metarhodopsin to rhodopsin. All manipulations were performed in the dark or under dim red light.

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Competing interests

The authors declare no competing financial interests.

Author contributions

All authors contributed to the conception, design and execution of this work, the interpretation of the findings, and drafting and revising this article.

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