Cellular/Molecular

Light-Driven Processes Control Both Rhodopsin Maturation and Recycling in Mosquito Photoreceptors

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Many invertebrates carry out a daily cycle of shedding and rebuilding of the photoreceptor's photosensitive rhabdomeric membranes. The mosquito *Aedes aegypti* shows a robust response, losing nearly all Aaop1 rhodopsin from the rhabdomeric membranes during the shedding process at dawn. Here, we made use of Aaop1 antibodies capable of distinguishing newly synthesized, glycosylated rhodopsin from mature nonglycosylated rhodopsin to characterize the fate of Aaop1 during the shedding and rebuilding processes. The rhabdomeric rhodopsin is moved into large cytoplasmic vesicles at dawn and is subsequently degraded during the standard 12 h daytime period. The endocytosed rhodopsin is trafficked back to the photosensitive membranes if animals are shifted back to dark conditions during the morning hours. During the daytime period, small vesicles containing newly synthesized and glycosylated Aaop1 rhodopsin accumulate within the cytoplasm. At dusk, these vesicles are lost as the newly synthesized Aaop1 is converted to the nonglycosylated form and deposited in the rhabdomeres. We demonstrate that light acts though a novel signaling pathway to block rhodopsin maturation, thus inhibiting the deglycosylation and rhabdomeric targeting of newly synthesized Aaop1 rhodopsin. Therefore, light controls two cellular processes responsible for the daily renewal of rhodopsin: rhodopsin endocytosis at dawn and inhibition of rhodopsin maturation until dusk.

Key words: GPCR cycling; photoreceptor sensitivity; rhabdomere shedding; rhodopsin; rhodopsin maturation

Significance Statement

Organisms use multiple strategies to maximize visual capabilities in different light conditions. Many invertebrates show a daily cycle of shedding the photoreceptor's rhabdomeric membranes at dawn and rebuilding these during the following night. We show here that the *Aedes aegypti* mosquito possesses two distinct light-driven cellular signaling processes for modulating rhodopsin content during this cycle. One of these, endocytosis of rhabdomeric rhodopsin, has been described previously. The second, a light-activated cellular pathway acting to inhibit the anterograde movement of newly synthesized rhodopsin, is revealed here for the first time. The discovery of this cellular signaling pathway controlling a G-protein-coupled receptor is of broad interest due to the prominent role of this receptor family across all areas of neuroscience.

Introduction

Many invertebrate photoreceptors remodel their light-sensitive rhabdomeric membranes extensively on a daily basis (Autrum, 1981; Barlow et al., 1989). A common feature is the shedding of rhabdomeric membranes at dawn, resulting in the accumulation of large multivesicular bodies within the cytoplasmic domain. This phenomenon has been documented in horseshoe crabs (Sacunas et al., 2002), mosquitoes (Brammer et al., 1978; Hu et al., 2012; Moon et al., 2014), and other invertebrate species (Blest et al., 1978; Autrum, 1981; Meyer-Rochow, 2001), but, notably, does not occur in the widely studied *Drosophila* model (Sapp et al., 1991). Rhabdomeric shedding initiates a daily renewal cycle of rhabdomeres and associated components. It also results in loss of rhodopsin from the light-sensitive rhabdomeric compartment, thereby providing a mechanism for changes in photoreceptor light sensitivity (Pieprzyk et al., 2003; Hu et al., 2012) on a daily basis.

Aedes aegypti mosquitoes provide an excellent experimental system with which to investigate the cellular mechanisms underlying these rhabdomeric renewal processes. The *A. aegypti* Aaop1 rhodopsin is expressed in all R1–R6 photoreceptors of the adult eye. During the shedding process at dawn, Aaop1 is endocytosed and brought into multivesicular bodies within the cytoplasmic region (Hu et al., 2012). In *Drosophila*, light-activated rhodopsin binds arrestin (Alloway et al., 2000) and the adapter protein AP-2

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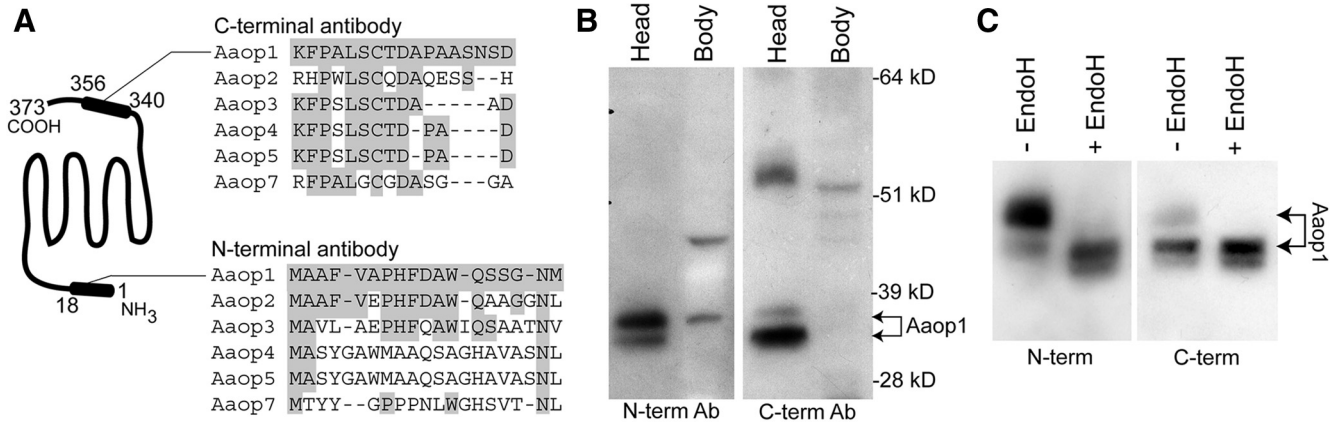

Figure 1. N-Aaop1 antiserum showing preferential staining of glycosylated Aaop1. ***A***, N-terminal and C-terminal domain sequence of Aaop1 used for production of antisera is compared with the corresponding sequences of other *A. aegypti* long-wavelength rhodopsins. For all sequences, the amino acids identical to the Aaop1 sequence are shaded in gray. ***B***, Protein blot of head and body protein extracts showing that both N-Aaop1 and C-Aaop1 antisera identify two head-specific proteins in the expected 35–37 kDa range of Aaop1 rhodopsin. ***C***, Protein blot comparing retinal samples with and without EndoH treatment (+EndoH and −EndoH, respectively) shows that the slower-migrating protein form prominently detected by the N-Aaop1 antiserum is N-linked glycosylated. After glycosidase treatment, Aaop1 proteins detected by N-Aaop1 and C-Aaop1 antisera possess a similar mobility. Protein extracts were prepared from mosquitoes in the afternoon (ZT = 9.5).

(Orem et al., 2006) to stimulate clathrin-mediated endocytosis. Only certain *Drosophila* mutants show high levels of rhodopsin internalization and, in these cases, the extensive rhodopsin internalization results in retinal degeneration (Orem and Dolph, 2002; Midorikawa et al., 2010). In contrast, mosquito photoreceptors must cope with the complete internalization of the Aaop1 rhodopsin content on a daily basis.

Cellular processes capable of returning G-protein-coupled receptors (GPCRs) to the plasma membrane (Drake et al., 2006) likely act to recycle internalized rhodopsin back to the rhabdomeric membranes. New rhodopsin synthesis must also make a contribution to the restoration of high rhabdomeric levels at dusk. In *Drosophila*, newly synthesized rhodopsin is subjected to *N*-linked glycosylation (Katanosaka et al., 1998; Webel et al., 2000), which is then lost during the maturation process (Rosenbaum et al., 2014) before movement into the rhabdomere. We show here that *A. aegypti* rhodopsin matures in a similar manner. We took advantage of the ability to distinguish newly synthesized rhodopsin from endocytosed rhodopsin to understand the dynamics of rhodopsin trafficking during the daily cycle. Our study reveals a novel signaling role for light as a negative regulator of rhodopsin maturation. The cytoplasmic accumulation of newly synthesized rhodopsin during the daytime provides a means to restore rhabdomeric rhodopsin levels rapidly after nightfall.

Materials and Methods

Mosquito rearing. Higgs White Eye (Wendell et al., 2000) *A. aeygypti* were reared in a 12 h light/12 h dark cycle at 27°C and 85% humidity. A 1 h transition period was used to increase the light gradually from 0 to 100 lux between zeitgeber time 0 (ZT0) and ZT1 and from 100 to 0 lux between ZT12 and ZT13 to mimic dawn and dusk light transitions, respectively. Mosquitoes were moved to 22°C and room humidity levels before protein extraction and retinal dissection. Females were used for all experiments because their larger size facilitated dissection and subsequent manipulation of retinal samples.

Antibody production. Two peptide antisera against the *A. aegypti* Aaop1 gene (GPRop1, AAEL006498) were used in this study. The peptide MAAFVAPHFDAWQSSGNM is an N-terminal domain sequence (aa 1–18) and the peptide KFPALSCTDAPAASNSD is a C-terminal domain sequence (aa 340–356). Peptide synthesis, antisera production, and affinity purification was performed by Biomatik (RRID:SCR_008944). The C-terminal Aaop1 antiserum (hereafter C-Aaop1 antiserum) was described previously (Hu et al., 2012). The N-terminal antiserum (hereafter N-Aaop1 antiserum) is newly described herein.

Protein blot analysis. SDS-PAGE and protein blot analyses were performed as described previously (Hu et al., 2012). To facilitate comparison of the glycosylation status of rhodopsin detected by the N-Aaop1 and C-Aaop1 antisera, five mosquito heads were prepared in 50 μl of standard SDS-PAGE 1× lysis buffer. Then, 10 μl of this protein lysate was incubated overnight at 37°C with 20 μl of endoglycosidase H (EndoH) solution (6 μl of 0.5 M sodium citrate, pH 5.5, 20 μl of water, 2 μl of 1% PMSF/isopropanol solution, 1 μl of 0.5 U/ml EndoH; Roche). A control sample was prepared and incubated in reaction buffer lacking the EndoH enzyme. After overnight incubation, 4 μl of 10× lysis buffer was added and the reaction tubes were heated at 95°C for 5 min. Next, 20 μl from each tube was loaded twice on a single SDS-PAGE gel. After electrophoresis and protein transfer, the membrane was cut in half. The ECL Western Blotting Detection System (GE Healthcare Life Sciences) was used with the N-Aaop1 (1:2000) antiserum on one of the two resulting membranes and with the C-Aaop1 (1:1000) antiserum on the other. Data images were uniformly adjusted for contrast and brightness using Adobe Photoshop CS6 (RRID:SCR_014199) software.

*Immunohistology with Aaop1 N-*terminal *and C-*terminal *antisera.* N-terminal antiserum was coupled to the Alexa Fluor 594 nm fluorochrome and C-terminal antiserum was coupled to the Alexa Fluor 488 nm fluorochrome using the APEX Antibody Labeling Kits (Life Technologies). To achieve the antibody concentrations (10–20 μg/10 μl) required for use of the manufacturer's protocol, the N- and C-terminal antisera were concentrated from 40 to 10 μl in a rotary evaporator. *A. aegypti* retinal samples were labeled simultaneously by these two antibody reagents (1:25) and Alexa Fluor 647 phalloidin (1:40, catalog at # A22287, RRID:AB_2620155; Thermo Fisher Scientific). Tissue preparation, antibody staining, and confocal imaging were performed as described previously (Hu et al., 2012).

Results

Aaop1 is N-linked glycosylated during maturation

The *A. aegypti* gene Aaop1 (GPRop1, AAEL006498) encodes a long-wavelength rhodopsin expressed in all the R1–R6 class of photoreceptors and the majority of R8 photoreceptors (Hu et al., 2012). We generated two different antisera against the Aaop1 rhodopsin (Fig. 1*A*) using peptide sequences derived from the N-terminal and C-terminal regions, respectively. In both cases, the peptide sequences were chosen so that the

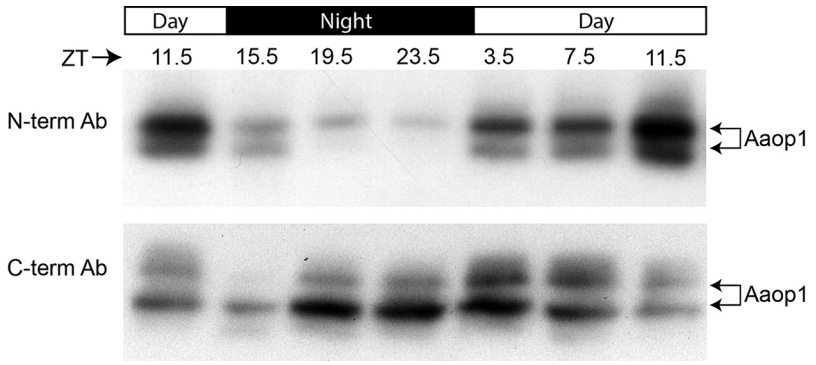

Figure 2. N-Aaop1 and C-Aaop1 antisera revealing daily fluctuations in the relative abundance of the glycosylated and nonglycosylated Aaop1 rhodopsin forms. Protein extracts of mosquito heads were obtained every 4 h of the 24 h light/dark cycle, separated by SDS-PAGE, blotted, and probed with N-Aaop1 antiserum (top) and C-Aaop1 antiserum (bottom). The first sample at ZT 11.5 was collected 0.5 h before the onset of dusk. The N-Aaop1 and C-Aaop1 antisera both recognize two mobility forms of Aaop1 marked by the dual arrows at the right side of the two panels. The slow-mobility (glycosylated) form is best detected by the N-Aaop1 antiserum and accumulates during the daylight hours. In contrast, the fast-mobility (nonglycosylated) form is best detected by the C-Aaop1 antiserum during the nighttime hours.

resulting antisera would be least likely to cross-react with the other long-wavelength rhodopsins. Aaop2, a rhodopsin expressed in a subset of adult R7 photoreceptors (Hu et al., 2009), and Aaop3, a rhodopsin expressed in a subset of photoreceptors of the larval stemmata (Rocha et al., 2015), possess the most sequence identity within these peptide epitopes (Fig. 1*A*). We verified the specificity of these antisera by showing they reacted with the Aaop1-expressing R1–R6 photoreceptors and not other photoreceptor types of the mosquito retina. Further, the N-terminal or the C-terminal Aaop1 antisera did not detect the Aaop2, Aaop3, or Aaop7 rhodopsins in the retinas of transgenic *Drosophila* expressing these rhodopsins.

Both N-terminal and C-terminal Aaop1 antisera recognize two *A. aegypti* head proteins sized in the 35–37 kDa range by SDS-PAGE analysis (Fig. 1*B*). The N-terminal antiserum consistently generated a stronger signal against the slower-migrating Aaop1 rhodopsin, whereas the C-terminal antiserum generated a stronger signal against the faster-migrating rhodopsin. *Drosophila* and other flies show multiple forms of rhodopsin due to the glycosylation and subsequent deglycosylation during maturation through the secretory pathway (Katanosaka et al., 1998; Webel et al., 2000; Rosenbaum et al., 2014). The faster-migrating rhodopsin is most abundant because it is the mature, nonglycosylated rhodopsin found in the rhabdomere. Therefore, results with the C-terminal antiserum, and not the N-terminal antiserum, reflected the expected relative abundance of the slower-migrating and faster-migrating forms.

To confirm that the N-terminal antiserum was labeling the glycosylated form of Aaop1 rhodopsin preferentially, we treated head protein samples with EndoH before SDS-PAGE and antibody detection. For both the N-Aaop1 and C-Aaop1 antisera, the EndoH treatment caused the loss of the slower-migrating Aaop1 form and the accumulation of Aaop1 in a faster-migrating form (Fig. 1*C*). It is noteworthy that the N-terminal antiserum shows stronger labeling of the faster-migrating Aaop1 form in EndoH-treated samples than in the untreated samples despite the expectation that the faster-

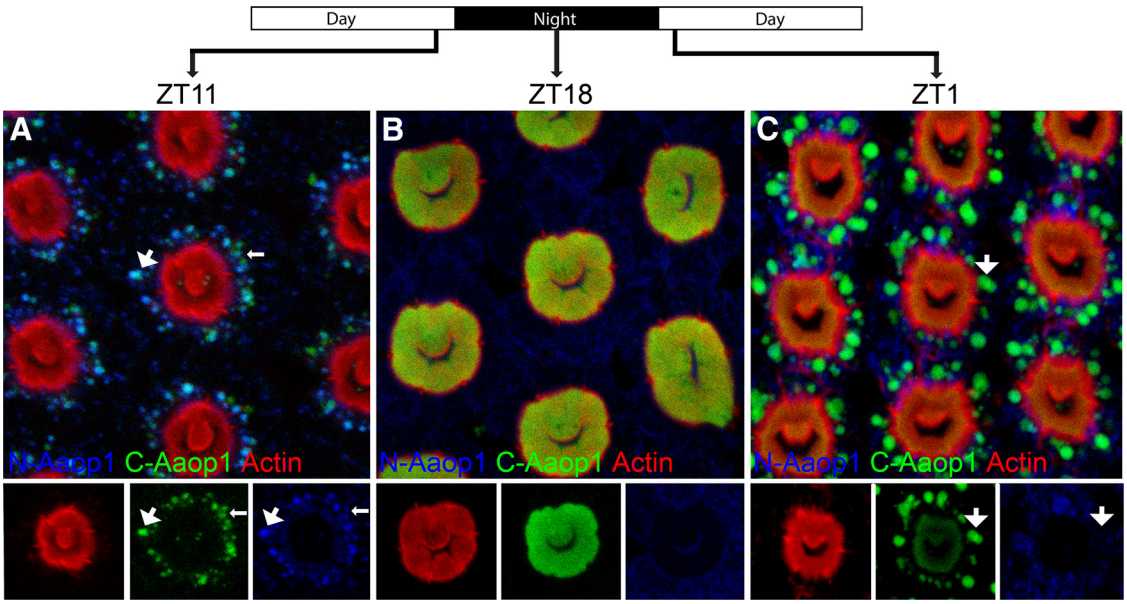

Figure 3. N-Aaop1 and C-Aaop1 antisera revealing daily changes in cellular distributions of glycosylated and nonglycosylated Aaop1. The displayed images are assembled from 3 sequential confocal images obtained from a 0.5 μm step through a whole-mounted *A. aegypti* retina stained simultaneously with 488 nm fluorochrome-conjugated C-terminal Aaop1 antiserum (displayed green), 594 nm fluorochrome-conjugated N-terminal Aaop1 antiserum (displayed blue), and 647 nm fluorochrome-conjugated phalloidin (displayed red). The bottom three panels are the three individual color channels of the central ommatidial unit. ***A***, At ZT11 (dusk), both N-Aaop1 (blue) and C-Aaop1 (green) antisera colabel small cytoplasmic vesicles (large arrow) sequestered around the actin-rich rhabdom (red). Neither antiserum detects rhodopsin within the rhabdom. A few cytoplasmic vesicles are labeled only by the C-Aaop1 antiserum (green, small arrow). ***B***, At ZT18, the C-Aaop1 antiserum (green) detects Aaop1 localized to the rhabdomeres. The N-Aaop1 antiserum (blue) shows only weak Aaop1 labeling exclusively within the cytoplasmic region. ***C***, At ZT1 (dawn), most of the Aaop1 is found in large cytoplasmic vesicles (arrow) and labels only the C-Aaop1 antiserum (green). The N-Aaop1 antiserum (blue) identifies only a small number of vesicles that are much smaller in size and colabeled by the C-Aaop1 antiserum.

migrating form is the most abundant form in untreated samples. These results suggest that N-Aaop1 antiserum readily detects the faster-migrating form produced by *in vitro* EndoH treatment, but detects the faster-migrating mature form of Aaop1 produced by the *in vivo* deglycosylation process poorly.

Differential Aaop1 labeling by N-terminal and C-terminal antisera during the daily cycle

We further investigated the differences in Aaop1 labeling by the N-Aaop1 and C-Aaop1 antisera by examining Aaop1 labeling at 4 h intervals through a daily cycle (Fig. 2). Both N-Aaop1 and C-Aaop1 analyses were performed on a common set of protein extracts to eliminate the possibility that differences in sample preparation might account for the observed differences. The N-terminal antiserum (Fig. 2, top) shows a preferential labeling of the slower-migrating glycosylated Aaop1 form, whereas the C-terminal antiserum shows preferential labeling of the faster-migrating nonglycosylated form at all time points. The strongest labeling of the faster-migrating form is found at the 19.5 and 23.5 nighttime time points, when all rhodopsin is sequestered in the rhabdomeres (Hu et al., 2012). At these time points, the N-terminal antiserum shows minimal labeling of the faster-migrating rhodopsin. This result provided strong evidence that the N-terminal antiserum recognizes poorly the mature rhabdomeric form of the Aaop1 rhodopsin produced by the *in vivo* deglycosylation process.

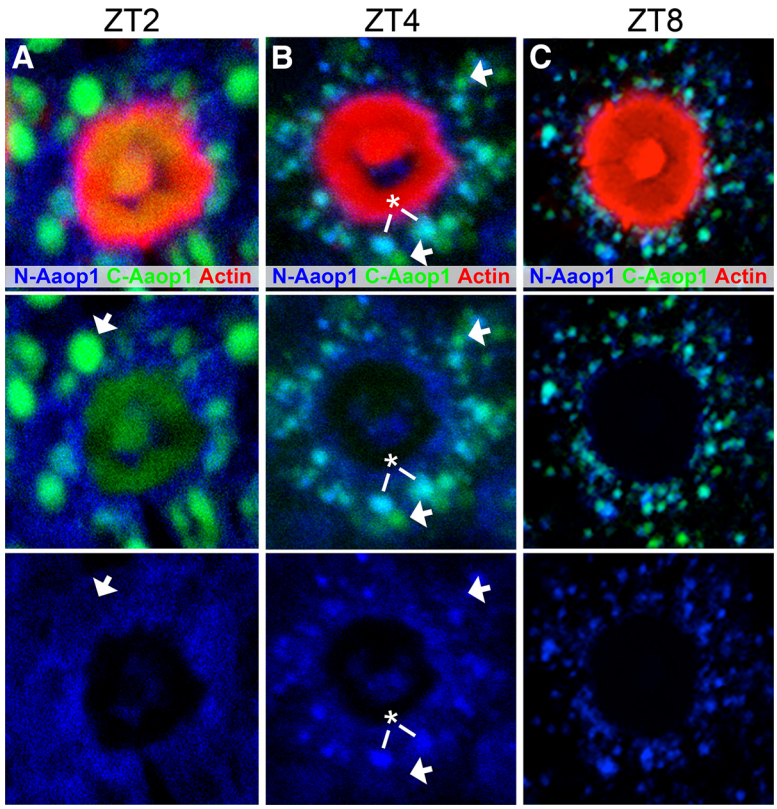

Figure 4. Photoreceptors showing a daily transition from large endocytic vesicles in the morning to small exocytic vesicles in the afternoon. ***A***, At ZT2, the C-Aaop1 antiserum detects Aaop1 rhodopsin within large cytoplasmic structures (green, middle, arrow). Little rhodopsin is found within the actin-rich rhabdom (red). In contrast, the N-Aaop1 antiserum generates weak cytoplasmic Aaop1 staining that is not localized to these large vesicles (bottom, arrow). ***B***, By ZT4, the actin-rich rhabdom shows a further loss of C-Aaop1 staining. The cytoplasm is now composed of two populations of vesicles: one population is light blue in appearance due to staining by both the N-Aaop1 and C-Aaop1 antisera (asterisk) and the other (arrow) is green in appearance because it is stained only by C-Aaop1 antiserum. ***C***, At ZT8, there is no Aaop1 staining within the rhabdom region. The cytoplasmic vesicles are smaller in size than those found earlier in the day. The majority of these vesicles are stained by both the C-Aaop1 or N-Aaop1 antisera.

To test directly the reactivity of the N-terminal and C-terminal antisera with the mature rhabdomeric form of Aaop1, we stained whole-mounted retinas at different times of day. At 1 h before dusk (ZT11), both the N-terminal and C-terminal antisera detected Aaop1 rhodopsin predominantly within small cytoplasmic vesicles (Fig. 3*A*, large arrow). In contrast, at night (ZT18; Fig. 3*B*), the majority of the Aaop1 rhodopsin is found within the actin-rich rhabdomeres (red) and is labeled only by the C-terminal antiserum (green). During the night, only the cytoplasmic regions are weakly labeled by the N-Aaop1 (blue). These results confirm that the N-terminal antiserum does not detect the mature rhabdomeric form of the Aaop1 rhodopsin.

The ZT1 time point imaged in Figure 3*C* shows the location of Aaop1 rhodopsin at 1 h after dawn. The majority of the Aaop1 rhodopsin is outside of the rhabdomere within large cytoplasmic vesicles labeled by the C-terminal antiserum, but not the N-terminal antiserum (Fig. 3*C*, arrow). This result confirms that the cytoplasmic vesicles present soon after dawn are generated by the endocytosis of Aaop1 rhodopsin from the rhabdomeric membranes.

Endocytosed rhodopsin is degraded during the daytime period

To investigate the fate of the Aaop1 rhodopsin endocytosed at dawn, retinas were prepared at the ZT2, ZT4, and ZT8 time points and labeled by N-terminal and C-terminal antisera. Representative confocal images of these retinas are shown in Figure 4. Retinas at the ZT2 time point are similar to those at ZT1 (Fig. 3*C*), showing the presence of large cytoplasmic vesicles labeled only by the C-terminal antiserum (Fig. 4*A*, arrow). Two hours later, at the ZT4 time point, the larger vesicles have been lost, replaced by two classes of smaller vesicles (Fig. 4*B*). One class is labeled only by C-Aaop1 (arrow), whereas the second class is labeled by both N-terminal and C-terminal antisera (asterisk). At the ZT8 time point, the majority of vesicles are smaller in size and colabeled by the N-terminal and C-terminal antisera (Fig. 4*C*). Very few vesicles are only labeled by the C-terminal antiserum at this time point.

We sought to determine the fate of the rhabdomeric rhodopsin endocytosed into the large vesicles during the morning. Protein blots showing a gradual loss of the lower Aaop1 band during the daytime (Fig. 2, C-terminal antiserum) suggested that the majority of the mature rhodopsin is normally degraded during the daylight hours. To determine whether the rhodopsin within these cytoplasmic vesicles has the potential to recycle back to the rhabdomere, we analyzed rhodopsin location in mosquitoes shifted back to dark conditions during the morning hours. Figure 5, *A* and *B*, in confirmation of our earlier analyses, shows the movement of rhabdomeric rhodopsin into cytoplasmic MVBs during the first 2 h after dawn. Four hours after dawn (Fig. 5*C*), the cytoplasm contains two populations of vesicles, one labeled

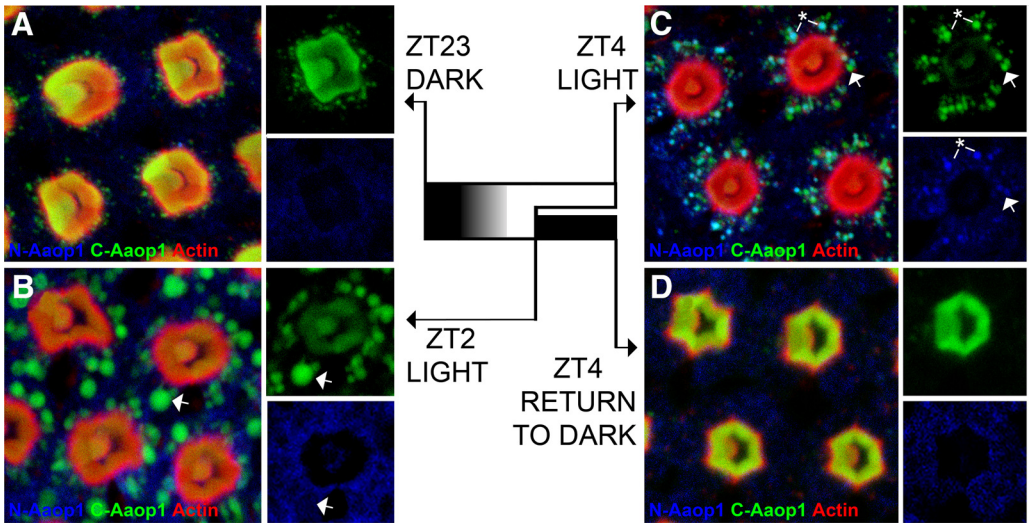

Figure 5. Endocytosed rhodopsin is retargeted to the rhabdomeric membranes when photoreceptors are returned to dark conditions. ***A***, At ZT23, 1 h before dawn, C-Aaop1 antiserum (green) detects rhodopsin largely localized within the actin-rich (red) rhabdom regions. The N-Aaop1 (blue) antiserum fails to detect rhodopsin within the rhabdom region or cytoplasmic vesicles. ***B***, At ZT2, 2 h after dawn, C-Aaop1 detects rhodopsin located within large cytoplasmic structures (green, arrow). These structures are not stained by the N-Aaop1 antiserum (arrow). ***C***, At ZT4, 4 h after dawn, the rhabdom lacks C-Aaop1 staining. Two populations of cytoplasmic vesicles are present: one is light blue in appearance due to staining by both the N-Aaop1 and C-Aaop1 antisera (asterisk) and the other (arrow) is green because it is stained only by C-Aaop1 antiserum. ***D***, For animals that are returned to the dark 2 h after dawn (at ZT2) and left in the dark for 2 additional hours (ZT4), the cytoplasm is devoid of vesicles stained by C-Aaop1 or N-Aaop1 vesicles. The C-Aaop1 antiserum heavily labels the rhabdom, whereas the N-Aaop1 antiserum gives only a low level of staining within the cytoplasmic regions.

only by the C-terminal antiserum (arrow) and another labeled by both the N-terminal and C-terminal antisera (asterisk). However, in animals returned to dark conditions at ZT2, neither population of vesicles is found at the ZT4 time point (Fig. 5*D*). These results show that, under dark conditions, trafficking pathways are able to move vesicular rhodopsin back to the rhabdomere, including the rhodopsin contained in vesicles stained only by C-terminal antiserum. Therefore, we conclude that trafficking pathways can return the endocytosed rhodopsin to the rhabdomeres efficiently even though this process does not play a large role in restoring rhabdomeric rhodopsin after the 12 h light period.

Rhodopsin maturation and rhabdomeric targeting is inhibited by light

The experiment presented in Figure 5 shows a second population of vesicles stained by both N-terminal and C-terminal antisera. We reasoned that these vesicles contain newly synthesized rhodopsin because the N-terminal antiserum does not recognize rhodopsin that has been modified by the cellular deglycosylation process and localized to the rhabdomere. Comparison of photoreceptors stained at different times of day (Figs. 3, 4) is consistent with the view that these vesicles increase in number during daylight hours and are largely absent during the dark periods. Further, returning animals to dark conditions during the morning hours caused rapid loss of these vesicles (Fig. 5*C*,*D*). These results suggest that Aaop1 is quickly matured and translocated to the rhabdomere only in the absence of light, suggesting that light is an effective inhibitor of the maturation process.

To determine whether light is capable of suppressing rhodopsin maturation, we continued light treatment beyond the ZT12 dusk period and examined the N-terminal and C-terminal population of rhodopsin vesicles. This sustained light exposure results in the presence of N-terminal-staining cytoplasmic vesicles at ZT14 (Fig. 6*A*) and ZT20 (Fig. 6*B*). There are no N-terminal-staining vesicles at the same time points if animals are dark-treated after dusk at ZT12 (Fig. 6*C*,*D*). Rhabdomeric accumulation of the mature rhodopsin labeled by the C-terminal, but not the N-terminal, antiserum is most evident in the dark-reared animals at these two time points.

We showed earlier that immature rhodopsin is also detected as the glycosylated and slower-migrating form on protein blots after SDS-PAGE. To estimate the amount of the immature rhodopsin held in the cytoplasm by sustained light treatment, we evaluated the SDS-PAGE rhodopsin profile at ZT16 for animals subjected to a dusk period and those maintained in sustained light. Figure 6*E*, left, shows that animals subjected to sustained light retain similar high levels of rhodopsin detected by the N-terminal antiserum late in the day (ZT11). In contrast, this rhodopsin is absent in control animals subjected to the standard light to dark shift at ZT12. The results in Figure 6*E*, right, show that EndoH treatment alters the mobility of the ZT16 rhodopsin detected by the N-terminal antiserum. Therefore, the rhodopsin retained in the cytoplasm by sustained light treatment is glycosylated rhodopsin.

Discussion

Photoreceptors of the mosquito *A. aegypti* exhibit a robust daily cycle of rhodopsin movement, being located in the light-sensitive rhabdomeric membranes at night and in cytoplasmic locations during the day. Here, we report the use of the *A. aegypti* model to characterize two light-regulated processes controlling the movement of the Aaop1 rhodopsin. A diagram summarizing these findings is presented in Figure 7. Whereas light-driven rhodopsin movement from the rhabdomeric membranes (Fig. 7, circle 1) has been described previously, our work documents for the first time a second process in which light acts to inhibit rhodopsin maturation (Fig. 7, circle 2). These two processes act together to keep rhodopsin levels low in the rhabdomeres during the day and restore rhodopsin to high levels in the rhabdomeres at night.

The shedding of rhabdomeric membranes at dawn occurs in the photoreceptors of many invertebrate species (Autrum, 1981). In *Limulus* and mosquitoes, it has been possible to document the oc-

currence of extensive rhodopsin endocytosis during the shedding process (Sacunas et al., 2002; Hu et al., 2012; Moon et al., 2014). In these species, the endocytosed rhodopsin accumulates in large multivesicular bodies (MVBs) within the cytoplasmic region (White, 1968; Sacunas et al., 2002; Hu et al., 2012). Other invertebrates accumulate MVBs during the shedding process (Eguchi and Waterman, 1967; Blest et al., 1978), thus making it likely that rhodopsin internalization always accompanies rhabdomeric shedding. *Drosophila* studies show that light-activated rhodopsin binds arrestin and other adapter components to initiate clathrin-dependent endocytosis and formation of rhodopsin-containing MVBs (Satoh and Ready, 2005; Orem et al., 2006). These observations led to the model that membrane shedding is a symptom of the robust burst of clathrin-dependent endocytosis initiated by light stimulation of rhodopsin at dawn. This is an attractive hypothesis because the underlying cellular mechanisms, from light activation of rhodopsin to the movement of rhodopsin through endocytic pathways, are well documented processes.

A second light-triggered mechanism was revealed by our study. An N-terminal antiserum reagent having the ability to distinguish newly synthesized and immature Aaop1 rhodopsin from rhabdomeric rhodopsin was critical to this analysis. This reagent recognizes an immunogenic site within the N-terminal domain of rhodopsin immediately preceding the N-linked glycosylation site of the protein. Studies in *Drosophila* have shown that N-linked glycosylation within this domain can be an essential step in the initial maturation of an invertebrate rhodopsin (O'Tousa, 1992; Webel et al., 2000), with subsequent maturation steps removing the attached polysaccharide. (Cao et al., 2011; Rosenbaum et al., 2014). Similarly, we show here that *A. aegypti* Aaop1 rhodopsin temporarily exists in a glycosylated form before the maturation and rhabdomeric localization. The N-terminal antiserum readily recognizes the glycosylated form of Aaop1 both before and after treatment with endoglycosidase H. Therefore, neither the presence of a larger carbohydrate structure nor the single GlcNAc residue remaining after enzymatic removal of this carbohydrate structure interferes with antibody recognition. For this reason, it is remarkable that mature Aaop1 rhodopsin, for which *Drosophila* studies suggest will have no sugar residues attached at this site (Rosenbaum et al., 2014), is not recognized by the N-terminal antiserum. We conclude that an additional modification not anticipated from analysis of

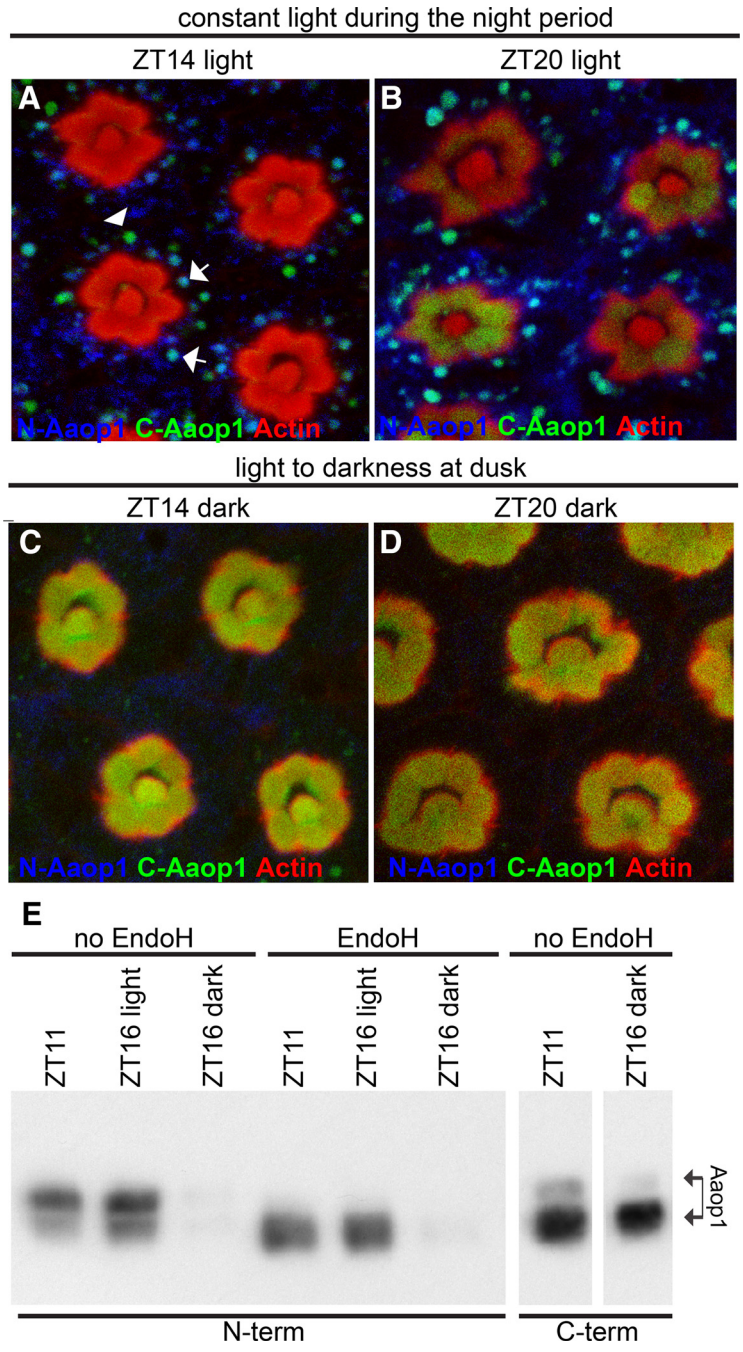

Figure 6. Light causes cytoplasmic retention of newly synthesized rhodopsin during the night hours. ***A***, ***B***, Photoreceptors at the ZT14 and ZT20 night time points but retained under light conditions show poor staining of rhabdomeric membranes by the C-terminal antiserum. The cytoplasmic regions contain punctate structures stained by C-Aaop1 antiserum (green, arrowhead) and by both N-Aaop1 and C-Aaop1 antisera (light blue, arrows). The few vesicles labeled only by the C-Aaop1 antisera (green) are likely generated by sustained light-triggered endocytosis of rhabdomeric rhodopsin. ***C***, ***D***, Photoreceptors at the ZT14 (***A***) and ZT20 (***B***) time points that transitioned from the normal light to dark transition at ZT12 showing rhabdomeric localization of the Aaop1 rhodopsin identified by C-Aaop1 antiserum. The N-Aaop1 antiserum detects weak staining only within the photoreceptor cytoplasmic area. ***E***, Protein blot analysis (left) showing the mobility and glycosylation profile of the Aaop1 rhodopsin recognized by the N-terminal antiserum before dusk (ZT11), after sustained light up to equivalent ZT16 time point (ZT16 light), and 4 h after dusk (ZT16 dark). The slower-migrating rhodopsin form present before dusk at ZT11 is retained by sustained light exposure 4 h into the night hours. EndoH treatment confirms that the slower-migrating rhodopsin at both of these time points is glycosylated. The N-terminal antiserum fails to detect Aaop1 rhodopsin for the normal dark conditions of the ZT16 time point. The C-terminal antiserum blot analysis (right) of the same protein samples for the ZT11 and ZT16 time points confirms the presence and mobility of other rhodopsin forms in these samples that are not recognized by the N-terminal antiserum.

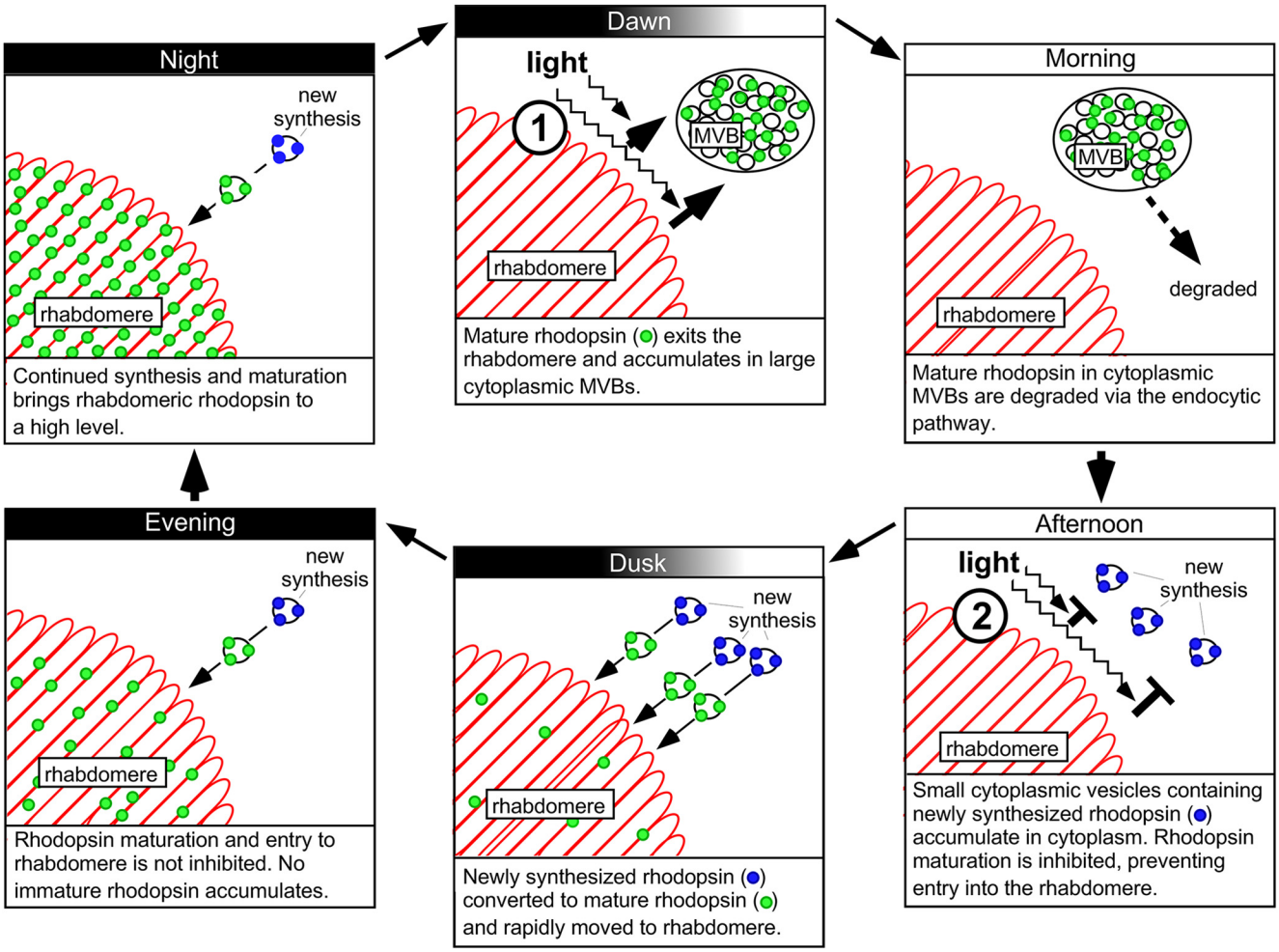

Figure 7. Two light-driven controls in the daily cycle of rhodopsin renewal in mosquito photoreceptors. Top left, At night, the mature deglycosylated form of rhodopsin (green circles) builds to a high level in the rhabdomere. Starting at the middle top and moving in a clockwise direction, at dawn, light triggers the extensive endocytosis of this rhabdomeric rhodopsin (circle 1). The rhodopsin initially accumulates in large cytoplasmic vesicles (MVBs) that are then degraded during the day. During the day, new rhodopsin synthesis results in the formation of smaller cytoplasmic vesicles containing glycosylated rhodopsin (blue circles). Light inhibits a maturation step before or at deglycosylation, thus preventing the rhabdomeric movement of the rhodopsin located in these vesicles (circle 2). These vesicles accumulate within the cytoplasm during the daytime hours. At dusk, inhibition is relieved, thus promoting the movement of newly synthesized rhodopsin to the rhabdomere. During the night, continued rhodopsin synthesis and maturation leads to high levels of rhabdomeric rhodopsin.

Drosophila rhodopsin occurs within the N-terminal domain during maturation of the Aaop1 rhodopsin.

During the day, rhodopsin is primarily localized within cytoplasmic vesicles and not within the rhabdomere. The presence of these vesicles cannot be explained by a cycle of rhodopsin maturation, rhabdomeric localization, and then light-triggered rapid internalization. Such a view is not consistent with the accumulation of vesicles containing the immature form of rhodopsin. Upon entry into the rhabdomere, all immature rhodopsin is converted to the mature form. This rhodopsin would no longer be recognized by the N-terminal antiserum nor run on SDS-PAGE with slower mobility due to glycosylation. Therefore, the accumulation of newly synthesized, glycosylated rhodopsin establishes that light mediates a cellular signal blocking maturation before rhodopsin deglycosylation and rhabdomeric localization.

A second class of cytoplasmic vesicles does not label with the N-terminal antiserum and therefore must represent vesicles containing the endocytosed rhodopsin. Recycling pathways for other GPCRs have been studied extensively (Gainetdinov et al., 2004). Our data show that similar pathways are capable of bringing the endocytosed rhodopsin back to the rhabdomeric membranes. The endocytosed *A. aegypti* rhodopsin is unique in that sustained replenishment of the rhabdomeric membranes does not occur until 12 h later at dusk. On protein blots, the faster-migrating Aaop1 band represents rhodopsin that has been endocytosed during the day period. In the protein blot shown in Figure 2, the amount of this protein at the end of the day (ZT11.5) is greatly reduced relative to the night and early morning hours. Therefore, it appears that a large amount of the endocytosed rhodopsin is eventually degraded during the 12 h of daylight.

We wondered whether degradation was the only possible fate of endocytosed rhodopsin. Cytoplasmic vesicles containing recycled rhodopsin are common in the morning at the ZT2 and ZT4 time points (Fig. 5), suggesting that rhodopsin degradation is a slow process requiring >4 h. This conclusion is consistent with protein blots (Fig. 2) showing no substantial decline in mature rhodopsin levels until the afternoon period. In contrast, no cytoplasmic rhodopsin can be detected, including vesicles containing recycled rhodopsin, after photoreceptors are returned to the dark for 2 h. These results provide evidence that endocytosed rhodopsin can be recycled to the rhabdomere. Our experimental approach was not capable of distinguishing be-

tween two possibilities: (1) that this pool of rhodopsin is subjected to continuous cycles of exocytosis/endocytosis during the typical day or (2) that exocytosis of the recycled rhodopsin is also suppressed by light.

The major insight from our analysis is the control of anterograde rhodopsin trafficking by ambient light conditions. This insight benefitted from the fortuitous development of antibody reagents that recognize newly synthesized rhodopsin preferentially. Determination of how common this mechanism is will require the development of similar capabilities in other invertebrate species. The cellular signaling pathways and effectors responsible for light-driven control of the maturation process should also be investigated. Prior investigations have characterized maturation steps controlled by specific Rab and ARF GTPases during the anterograde movement of rhodopsins and other GPCRs through the secretory pathway (Wang and Wu, 2012; Young et al., 2015). Light control of this process could be accomplished by regulating one of these steps with a second messenger generated during the phototransduction response. As an example, the rise in intracellular Ca^{2+} levels mediates a large number of light-driven responses in *Drosophila.* Among the known targets are the rhodopsin phosphatase (Lee and Montell, 2001), arrestin (Kahn and Matsumoto, 1997), the TRP light-gated channel (Gu et al., 2005), and phospholipase C (Hardie et al., 2001). Ca^{2+}, acting as a negative regulator of anterograde rhodopsin transport, would account for our observation that rhodopsin maturation is enhanced by dark conditions. Characterization of these control mechanisms will provide new approaches for modifying the activity of rhodopsins and other GPCRs.

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