Expression of rhodopsin and arrestin during the light-dark cycle in Drosophila

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Purpose: To determine the protein and transcript levels for rhodopsin (Rh1), arrestin 1 (Arr1), and arrestin 2 (Arr2) over a 12 h light/12 h dark cycle in the retina of the fruit fly, Drosophila melanogaster. This information is important for understanding the process of photoreceptor membrane turnover.

Methods: Drosophila were entrained for several generations to a daily 12 h light/12 h dark cycle. They were sacrificed at 4 h intervals, beginning at the time of onset of the light phase. Proteins were resolved by polyacrylamide gel electrophoresis (PAGE) and subjected to immunoblot analysis using antibodies directed to rhodopsin, NinaA, Arr1, and Arr2. Northern blots were incubated with riboprobes corresponding to the rhodopsin gene (ninaE), arrestin1(arr1), and arrestin2 (arr2).

Results: In entrained Drosophila, protein and mRNA levels for rhodopsin, arrestin1, and arrestin2 were constant during a 12 h light/12 h dark cycle.

Conclusions: These results indicate that rhodopsin and arrestin protein synthesis in Drosophila photoreceptors do not fluctuate on a daily cycle. These findings are similar to those obtained in Xenopus laevis, but in contrast to a variety of other vertebrate and invertebrate species.

Photoreceptor membrane turnover involves a precise balance between the biosynthesis and assembly of new membrane constituents and the process of photoreceptor membrane shedding [1-3]. These events often display a daily rhythm in both vertebrates and invertebrates [4-6].

In many organisms, the biosynthesis of several components of the phototransduction cascade is regulated as a function of the 12 h light/12 h dark cycle (12L/12D cycle). Diurnal rhythms of opsin and arrestin mRNA levels have been observed in mice, rats, fish (Haplochromis burtoni), and toad (Bufo marinus) [7-11]. In other organisms, such as Xenopus laevis, levels of opsin and arrestin are continuous during the 12L/12D cycle [12,13]. In the chicken, cone opsin mRNA is rhythmic in the same preparations that lack a rhythm in rod opsin mRNA even though the shedding of rod and cone photoreceptor cell membrane is circadian [14]. In vertebrates as well as many invertebrates, biosynthesis can occur over a broad period of time or be continuous. It is often temporally separated from shedding, which often occurs within a small window of time [6,13]. Therefore, the mechanisms controlling biosynthesis and assembly of photoreceptor membrane may be regulated separately from the processes of membrane shedding. Even though these events may be regulated differently, a precise balance of protein synthesis and targeting coordinated with membrane turnover is essential for visual performance.

A number of invertebrates also undergo dramatic daily cycles of membrane turnover and visual sensitivity, while some do not [6,15-18]. For example, although many arthropods undergo dramatic daily fluctuations in photoreceptor membrane turnover, daily variations have not been detected in Drosophila [6,18-20]. Drosophila and human visual systems have many well-recognized similarities as well as differences [21,22]. For example, rhodopsin and arrestin play comparable roles in phototransduction and retinal degeneration diseases in both organisms [23-31]. Since the processes of photoreceptor membrane biosynthesis and shedding may be regulated by different mechanisms, even within the same organism, we investigated whether the levels of rhodopsin (Rh1), arrestin1 (arr1), and arrestin2 (arr2) varied over the 12L/12D cycle in Drosophila.

METHODS

Drosophila strains, genetics, transgenic animals, and sample collection: The wild-type strains of Drosophila melanogaster used in this study are Canton S and w1118. The white-eyed w1118 flies are wild type in all aspects except that they lack the red eye color pigment. The flies were raised on a standard medium of cornmeal and yeast at 21 °C and entrained to a 12 h light/12 h dark cycle (lights on at 0800, off at 2000) for several generations. The flies were reared in a Precision Scientific 818 incubator (Precision Scientific, Chicago, IL) equipped with 34 watt cool white fluorescent bulbs. To assess expression over the 12L/12D cycle, animals were sacrificed at 4 h intervals during the 24 h period (0400, 0800, 1200, 1600, 2000, and 2400). More precise sampling was also carried out at 1 h intervals beginning at the time of light onset (0800) or the time of lights off (1600). Three independent fly collections were carried out on separate days, and all experiments were conducted at least three times. Immunoblots and North-
ern blots shown are representative of the results obtained. Flies were frozen in liquid nitrogen and stored at -80 °C. Heads were separated from other body parts using screens in liquid nitrogen, as described [32].

**SDS-PAGE and immunoblotting:** All SDS-PAGE and immunoblotting was carried out according to published procedures [33,34]. Heads from flies were placed in cold sample buffer containing protease inhibitors. Samples were sonicated, separated by electrophoresis in 10% SDS-polyacrylamide gels [35] and electroblotted onto nitrocellulose filters [36]. The nitrocellulose was stained with 0.05% Ponceau S stain to visually evaluate the protein loading per lane. The nitrocellulose was incubated for 1 h with the 4C5 mouse monoclonal antibody directed to Rh1 [37], a rabbit polyclonal antibody directed to NinaA [33,34,38], or rabbit polyclonal antibodies directed to peptides that are specific to the COOH-terminus of either Arr1 or Arr2 protein (gifts of A. Becker and C. S. Zuker) [26]. The 4C5 antibody, Arr1 antibody, Arr2 antibody, and the NinaA antibody were all used at a 1:500 dilution. The immunoreactive proteins were visualized using horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG (Jackson ImmunoResearch, Westgrove, PA) used at 1:1,000 dilution followed by ECL detection (Amersham Pharmacia Biotech, Piscataway, NJ). X-ray film was scanned into the computer and peak areas were integrated using Sigmagel software (Jandel Corporation, San Rafel, CA). At least four to five exposure times were visually evaluated to ensure that the ECL was in the linear range of the film. Gels were stained with Coomassie Brilliant Blue R250.

**Northern analysis and preparation of riboprobes:** Northern blot analysis was carried out according to published procedures [39]. RNA was isolated using the Ultraspec RNA isolation system (Biotex, Houston, TX). Ten micrograms of RNA from each sample was run on a denaturing 1% agarose gel as described in Maniatis et al. [40]. Each gel was stained with ethidium bromide and photographed on an UV transilluminator. The mRNA was transferred overnight by capillary action from each sample was run on a denaturing 1% agarose gel as described in Maniatis et al. [40]. Each gel was stained with ethidium bromide and photographed on an UV transilluminator. The mRNA was transferred overnight by capillary action from each sample to a positively charged nylon membrane (Nytran Plus, Schleicher and Schuell, NH). The mRNA was transferred overnight by capillary action from each sample to a positively charged nylon membrane (Nytran Plus, Schleicher and Schuell, NH). The mRNA was transferred overnight by capillary action from each sample to a positively charged nylon membrane (Nytran Plus, Schleicher and Schuell, NH). The mRNA was transferred overnight by capillary action from each sample to a positively charged nylon membrane (Nytran Plus, Schleicher and Schuell, NH).

**Protein expression by time (mean ± standard deviations)**

<table>
<thead>
<tr>
<th>Protein</th>
<th>0800</th>
<th>1200</th>
<th>1600</th>
<th>2000</th>
<th>2400</th>
<th>0400</th>
</tr>
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<tbody>
<tr>
<td>Rh1</td>
<td>6646±77</td>
<td>6814±64</td>
<td>6527±31</td>
<td>6701±37</td>
<td>6448±20</td>
<td>6074±14</td>
</tr>
<tr>
<td>Arr1</td>
<td>4212±32</td>
<td>4477±26</td>
<td>4160±82</td>
<td>4150±17</td>
<td>4107±12</td>
<td>3852±62</td>
</tr>
<tr>
<td>Arr2</td>
<td>5042±94</td>
<td>5017±100</td>
<td>4790±343</td>
<td>4880±96</td>
<td>4215±34</td>
<td>3358±17</td>
</tr>
<tr>
<td>NinaA</td>
<td>6122±332</td>
<td>752±520</td>
<td>689±333</td>
<td>707±32</td>
<td>665±220</td>
<td>593±199</td>
</tr>
</tbody>
</table>

**Figure 1.** Expression of Rh1, arrestin, and NinaA over the 12L/12D cycle. Immunoblot analysis reveals protein expression over the 12L/12D cycle. Heads were collected at 4 h intervals: 0800, 1200, 1600, 2000, 2400, and 0400. Each band was scanned three times and the peak area was integrated with Sigmagel software. The lights were turned on at 0800 and off at 2000. White boxes represent the time of lights on and black boxes represent lights off.
of rRNA loaded and the peak area measured in the Northern blot.

RESULTS

To study the influence of the light-dark diurnal cycle on Rh1 and arrestin expression in the *Drosophila* eye, we entrained flies to a 12L/12D cycle for several generations. We carried out immunoblot analysis on samples collected at 4 h intervals during the 12L/12D cycle. The amount of Rh1 protein expressed does not fluctuate during the 12L/12D cycle (Figure 1A). Rh1 is also present in the mature, 34 kD form. The immature, high molecular weight (MW), glycosylated forms of Rh1 were not detected [33]. The lack of the high MW form demonstrates that Rh1 biosynthesis is not occurring in detectable amounts at any time point, consistent with previous suggestions of low continuous turnover and high stability of Rh1 in flies [43].

We studied the expression of arrestin during the 12L/12D cycle. Figure 1B,C show immunoblots probed with antibodies directed to Arr1 and Arr2 [26]. Arr1 and Arr2 are present continuously during both the light and dark phases of the 12L/12D cycle. These data are consistent with those obtained for *Xenopus*. In *Xenopus* neither principal rod opsin nor arrestin are significantly rhythmic in the 12L/12D cycle [13,44].

Since NinaA is critical as a chaperone for Rh1 biosynthesis [33,34], we investigated the levels of NinaA over the 12L/12D cycle. NinaA protein levels do not fluctuate (Figure 1D). The peak area for each band was integrated using Sigmagel software and values did not vary significantly over the 12L/12D cycle (Figure 1). These findings are consistent with continuous expression of Rh1 during the 12L/12D cycle in *Drosophila*. To evaluate the variation in loading per lane, the blots were stained for protein. Figure 1E shows uniform protein profiles in a Coomassie Blue stained gel. When samples are corrected for loading of protein by comparison with the Ponceau S staining of the nitrocellulose, any apparent difference diminishes.

To further refine our analysis to smaller intervals at the time of light onset and lights-off, we determined the expression of Rh1, Arr1, and Arr2 at 1 h intervals at light onset (0800-1100) and at lights-off (2000-2300). Immunoblot analysis reveals that expression of Rh1 is continuous at the time of light onset (Figure 2A) and lights off (Figure 2D). In addition, expression of Arr1 (Figure 2B,E) and Arr2 (Figure 2C,F) was also indistinguishable at all time points at light onset and lights off. These data confirm that fluctuations in protein levels do not occur at the time of light onset and lights-off further sup-

![Figure 2](image-url)  
**Figure 2.** Expression of Rh1 and arrestin at the time of light onset and lights-off. Immunoblot analysis reveals Rh1 and arrestin expression at the time of light onset (A-C) and lights off (D-F). A-D: Rh1 is detected with the 4C5 antibody (34 kD, 2 heads per lane); B,E: Arr1 (37 kD, 20 heads per lane); C,F: Arr2 (48 kD, 20 heads per lane). The flies were maintained on a 12L/12D cycle. Heads were collected at 1 h intervals: 0800, 0900, 1000, and 1100 at the time of light onset, and at 2000, 2100, 2200, and 2300 at the time of lights-off. When band density was normalized to Ponceau S staining of the nitrocellulose, no significant variation was observed between lanes. The lights were on at 0800 and off at 2000. White boxes represent the time of lights on and black boxes represent lights off.

![Figure 3](image-url)  
**Figure 3.** Northern blot analysis. Northern blot analysis reveals expected transcripts of 1.5 kb for *ninaE* (Rh1) [41,62], 1.45 kb for *arr1* and 1.65 kb for *arr2* [42,63,64]. The flies were maintained on a 12L/12D cycle. Heads were collected at 4 h intervals: 0800, 1200, 1600, 2000, 2400, and 0400. The lights were on at 0800 and off at 2000. White boxes represent the time of lights on and black boxes represent lights-off. 10 µg of total RNA was loaded per lane and 18S rRNA was used as a loading control. Each band was scanned three times and the peak area was integrated with Sigmagel software. The mean areas were normalized by calculating the ratio of the mean area and the amount of rRNA loaded.
porting that Rh1 and arrestin do not vary during the 12L/12D cycle.

Since protein levels reflect both biosynthesis and degradation, we carried out Northern blot analysis to determine transcript levels. We determined the expression of \textit{ninaE} (Rh1), \textit{arr1}, and \textit{arr2} at 4 h intervals during the 12L/12D cycle. Figure 3 reveals that mRNA expression for Rh1, Arr1, and Arr2 does not vary significantly during the light/dark cycle. In the Northern blot shown in Figure 3, it appears that mRNA levels for Rh1 at 1600 and 0400 may have increased, but when those samples are corrected for loading of RNA by comparison with the amount of 18S RNA in each sample, the apparent difference diminishes (see figure legend for values). These findings suggest that transcript levels for the \textit{ninaE} and the two arrestins are the same throughout the 12L/12D cycle. There is no detectable daily rhythm or light-evoked impact on Rh1 or arrestin expression in \textit{Drosophila}.

**DISCUSSION**

\textit{Drosophila} displays locomotor activity rhythms close to 24 h and their behavioral rhythms resemble mammalian sleep and wake cycles [45]. Flies also display rhythms in olfactory responses as well as other physiological and biochemical processes [46]. The two best characterized clock genes, \textit{period} (\textit{per}) and \textit{timeless} (\textit{tim}) were initially identified in genetic screens in \textit{Drosophila} and \textit{per} homologs have been identified in mammals [45]. Here, we have entrained flies to a 12L/12D cycle and investigated rhodopsin and arrestin biosynthesis in \textit{Drosophila} photoreceptor cells. We demonstrate that protein levels and mRNA levels for both rhodopsin (Rh1) and arrestin are constant throughout the 12 L/12D cycle.

In \textit{Drosophila}, each eye unit of the compound eye contains eight photoreceptor cells (R1-R8) and four cone cells, surrounded by a sheath of pigment cells, as well as a pseudocone and a corneal lens located apically [47,48]. \textit{Drosophila} photoreceptor cells contain specialized portions of the plasma membrane, called rhabdomeres, consisting of numerous tightly packed microvilli containing rhodopsin photopigments and other components of the phototransduction cascade. The microvillar processes of the rhabdomeres are functionally equivalent to the phototransducing disc membranes present in the vertebrate photoreceptor outer segments [21,49]. We previously found in \textit{Drosophila} that mutations in \textit{ninaE} (Rh1), \textit{ninaA}, and \textit{arrestin} lead to severe retinal defects often causing retinal degeneration [23,26,33]. The major rhodopsin, Rh1, is expressed in the R1-6 photoreceptor cells. During biosynthesis, Rh1 is synthesized and core glycosylated in the endoplasmic reticulum (ER) and transported via the Golgi to the rhabdomeres for its role in phototransduction [23,33,37,50]. Here we have shown that Rh1 is present at constant levels and in the mature form over the 24 h L/D cycle.

As part of the photoreceptor renewal process in vertebrates, rhodopsin is synthesized in the proximal inner segment and is transported to the outer segment via the connecting cilium [1,51,52]. The shedding process involves the phagocytosis and degradation of the tips of outer segments by the retinal pigment epithelium (RPE) [2,53,54]. However, \textit{Drosophila} do not have an equivalent to the RPE, and photoreceptor membrane is shed into the photoreceptor cell [6,19,55]. Thus, rhabdomere membranes are renewed by a process of addition of new membrane at the base of the rhabdomere, and the shedding of old microvillar membrane material that subsequently accumulates as multivesicular bodies in the photoreceptor cells [19]. Multivesicular bodies fuse with lysosomes, and later, residual bodies are evident in the photoreceptor cells [19,20]. The subretinal cisterna (SRC) is a membrane system located beneath the microvillar processes of the rhabdomeres [56,57]. The close proximity of the SRC to the photoreceptive microvilli has lead to the proposal that the SRC plays a role in rhodopsin transport to the rhabdomeres as well as shedding and turnover of photoreceptor membrane [6,56]. In certain arthropods, dynamic changes in the SRC parallel the diurnal changes in rhabdomere turnover [6]. However, in \textit{Drosophila}, the structure of the SRC in dark-adapted flies is indistinguishable from that in light-adapted flies [57]. Although visual sensitivity fluctuates in \textit{Drosophila}, membrane turnover is continuous over the 24 h light-dark cycle [18-20]. In another fly, the muscid fly, \textit{Lucilia}, membrane turnover is also continuous [58]. Consistent with this concept, we have shown here that Rh1 protein and mRNA levels are continuous during the 12L/12D cycle. In addition, Rh1 is detected solely in the mature, 34 kD form at all times during the 24 h L/D cycle supporting the concept that Rh1 undergoes low levels of turnover and is highly stable in \textit{Drosophila} [43].

In \textit{Xenopus}, neither principal rod opsin nor arrestin are significantly rhythmic in the 12L/12D cycle [13,44]. In addition, chicken rod opsin mRNA is not rhythmic in the same tissue preparations that are rhythmic for cone opsin mRNA [14]. Diurnal rhythms of opsin and arrestin mRNA levels have been observed in mice, with peak levels for opsin in the morning, about the time of light onset, just prior to the time of rod disc shedding [7,9,10]. Rats display similar fluctuations in opsin expression [8]. In fish (\textit{Haplochromis burtoni}) and toad (\textit{Bufo marinus}), the reverse pattern was found; opsin mRNA levels are high during the day and decrease at night [11]. In organisms that display circadian rhythms in biosynthesis and membrane shedding, the day and night states of photoreceptor cells must precisely follow the needs for performance of the eyes and fluctuations generally reflect the day and night demands for vision [6,13,59,60]. In other organisms, such as \textit{Drosophila}, that do not display daily rhythms in protein biosynthesis or membrane shedding [18-20], the day and night states of the photoreceptors do not differ significantly.

Two strains of mice that display inherited retinal degeneration, retinal degeneration (\textit{rd}) mice and peripherin/retinal degeneration slow (\textit{rds}) mice, both display diminished or absent cycling of opsin (\textit{rd} and \textit{rds} mice) and arrestin expression (\textit{rd} mouse) [7,10,61]. Disruptions in rhythmic expression of opsin and arrestin suggest that a complex set of factors influence protein expression and that pathology can dramatically influence this strategy. Even though Rh1 and arrestin expression do not fluctuate on a daily cycle in \textit{Drosophila}, a balance between the synthesis and targeting of new protein and membrane constituents with the breakdown of existing membrane
must exist [18-20]. Although there are differences as well as similarities between Drosophila and mammals, studies in Drosophila continue to provide insights and mechanistic explanations for visual function and clinically relevant retinal diseases.

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REFERENCES


