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# Synchronizing multiphasic circadian rhythms of rhodopsin promoter expression in rod photoreceptor cells

Chuan-Jiang Yu, Yan Gao, Ping Li and Lei Li*

*Department of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556, USA*

*Author for correspondence (e-mail: Li.78@nd.edu)

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## Summary

**Endogenous circadian clocks regulate day–night rhythms of animal behavior and physiology. In zebrafish, the circadian clocks are located in the pineal gland and the retina. In the retina, each photoreceptor is considered a circadian oscillator. A critical question is whether the individual circadian oscillators are synchronized. If so, the mechanism that underlies the synchronization needs to be elucidated. We generated a transgenic zebrafish line that expresses short half-life GFP under the transcriptional control of the rhodopsin promoter. Time-lapse imaging of rhodopsin promoter-driven GFP expression revealed that during 24 h in constant darkness, rhodopsin promoter expression in rod photoreceptor cells fluctuated rhythmically. However, the pattern of fluctuation differed between individual cells. In some cells, peak expression was seen in the subjective early morning, whereas in other cells, peak expression was seen in the afternoon or at night. Light transiently decreased rhodopsin expression, thereby synchronizing the multiphasic circadian oscillation. The application of dopamine or dopamine $D_2$ receptor agonist also synchronized the circadian rhythms of rhodopsin promoter expression. When the $D_2$ receptors were pharmacologically blocked, light exposure produced no effect. This suggests that the synchronization of the circadian rhythms of rhodopsin promoter expression by light is mediated by dopamine $D_2$ receptors. The mechanism that underlies the synchronization probably involves dopamine-mediated $Ca^{2+}$ signaling pathways. Light, as well as dopamine, lowered $Ca^{2+}$ influx into the rod cells, thereby resetting rhodopsin promoter expression to the initial phase.**

Key words: circadian clock, rod photoreceptor cell, rhodopsin promoter, retina, zebrafish.

## Introduction

Animals display robust day–night rhythms in behavior and physiology. The mechanisms that are responsible for generating the daily rhythms are similar across species. In most species, the daily rhythms are regulated by a central pacemaker, which expresses early circadian genes (*Clock*, *Bmal*, *Per1* and *Cry1*). The pacemaker functions autonomously and produces rhythmic gene expression in interlocked transcription–translation feedback loops. The circadian pacemaker is located in the suprachiasmatic nuclei (SCN) in mammals and in the pineal gland and neural retina in non-mammal vertebrates (Takahashi, 1995; Young and Kay, 2001; Schibler and Sassone-Corsi, 2002; Reppert and Weaver, 2002).

Recent studies have suggested that the timing of individual oscillators may fall in discrete phase groups (Welsh et al., 1995; Liu and Reppert, 2000; Quintero et al., 2003; Yamaguchi et al., 2003). In mice, for example, the rhythmicity of *Per1* expression varies in individual SCN cells. The expression cycles every 24 h, but each cell has a different peak time. In some cells, peak *Per1* expression is seen in the day, whereas in other cells, peak expression is seen at night (Kuhlman et al., 2003). In mice and rats, the spontaneous firing of SCN cells cycles every 24 h, but the firing of individual cells is not synchronized (Welsh et al., 1995; Yamaguchi et al., 2003). The multiphasic circadian oscillation of SCN firing can be synchronized by the application of neurotransmitter GABA (Liu and Reppert, 2000) or protein synthesis inhibitor cycloheximide (Yamaguchi et al., 2003). The mechanisms that underlie the synchronization of multiphasic circadian oscillation networks remain to be further studied.

Zebrafish (*Danio rerio*) have recently emerged as a model vertebrate for genetic studies of the circadian clocks (Whitmore et al., 1998; Whitmore et al., 2000; Cermakian et al., 2001; Pando et al., 2001; Cahill, 2002). In zebrafish retinas, the early circadian genes are expressed in several cell types, including photoreceptor cells. The photoreceptor cells are considered as independent circadian clocks (McMahon and Barlow, 1992; Cahill and Besharse, 1993; Cahill, 1996), but it remains unknown whether the individual clocks are synchronized. If so, the mechanisms need to be elucidated. In order to address these questions, we generated a transgenic zebrafish line [Tg(rhod::shGFP)] that expresses short half-life GFP under the

transcriptional control of the zebrafish rhodopsin promoter. By time-lapse imaging of rhodopsin promoter-driven GFP expression, we measured the circadian rhythms of rhodopsin promoter expression in individual rod photoreceptor cells. In a 24 h period, rhodopsin promoter expression fluctuated rhythmically. However, the pattern of fluctuation differed in individual cells. In some cells, peak expression was seen in the subjective early morning, whereas in other cells, peak expression was seen in the afternoon or at night. The multiphasic oscillation of rhodopsin promoter expression was synchronized by light, probably *via* dopamine $D_2$ receptor-coupled $Ca^{2+}$ signaling pathways.

## Materials and methods

### *Animals and maintenance*

Zebrafish *Danio rerio* Hamilton were maintained in our animal facility as described previously (Westerfield, 1995). Unless otherwise specified, the fish were kept in a 14 h:10 h light:dark cycle (light, 07:00–21:00 h; fluorescent room light). The fish were fed with freshly hatched brine shrimp twice a day. All the experimental procedures adhered to the NIH Guidelines for Animals in Research.

### *The transgenic fish*

A DNA fragment that contained 1.2 kb of zebrafish rhodopsin promoter (Kennedy et al., 2001) was cloned into the pd2EGFP-1 vector (Clontech, Mountain View, CA, USA). The expression cassette (restriction sites, *Eco*RI and *Sal*I) was recovered with the Qiagen gel extraction kit (Qiagen, Valencia, CA, USA). The DNA was dissolved in 1× Danieau's buffer (58 mmol $l^{-1}$ NaCl, 0.7 mmol $l^{-1}$ KCl, 0.4 mol $l^{-1}$ $MgSO_4$, 0.6 mmol $l^{-1}$ $Ca(NO_3)_2$, 5 mmol $l^{-1}$ Hepes, pH 7.6) and was injected (4.6 nl, 50 ng $\mu l^{-1}$) into 1-cell stage embryos. Germline transmission was confirmed by polymerase chain reaction (PCR) with genomic DNA from the next generation.

### *PCR*

Genomic DNA was extracted by lysing the embryos or adult tail clippings in 100 mmol $l^{-1}$ Tris pH 8.3, 200 mmol $l^{-1}$ NaCl, 0.4% SDS, 5 mmol $l^{-1}$ EDTA, and 200 μg $ml^{-1}$ proteinase K. Primers were designed for *Gfp* (forward 5′-GGGCGAG-GAGCTGTTCACCGG, reverse 5′-CGGCGGCGGTCAC-GAACTCC-3′, which amplify a 674-bp band) and *Wnt* (forward 5′-CAGTTCTCACGTCTGCTACTTGCA, reverse 5′-ACTTCCGGCGTGTTGGAGAATTC-3′, which amplify a 387-bp band). *Wnt* was used as an internal control. The PCR was run in 1× PCR buffer with 0.25 i.u. of Taq polymerase (Invitrogen, Carlsbad, CA, USA), 1.5 mmol $l^{-1}$ of $MgCl_2$, 0.2 mmol $l^{-1}$ of dNTP, and 0.1 μmol $l^{-1}$ of each primer. The reaction was performed with an initial 2 min denaturation step at 94°C followed by 30 cycles of 45 s at 94°C, 30 s at 65°C, and 1 min at 72°C, and a final extension of 10 min at 72°C.

### *Real time RT–PCR*

Total RNA was extracted from the zebrafish retinas as described previously (Li et al., 2005). RNA was precipitated with isopropanol, washed with 75% ethanol, and re-suspended in 20 μl distilled water (RNAse free). Rhodopsin-specific primers and probes (GenBank accession number, AF109368; 5′-CCTCACGCTGTACGTCACCAT-3′ and 5′-CAGGTTC-AGCAGGATGTAGTTGA-3′; TaqMan probe, 5′-AGCAC-AAGAAGCTGCGCACACCC-3′) were designed using the Primer Express system (ABI, Foster City, CA, USA).

Real-time RT–PCR was performed using the TaqMan One-Step RT–PCR Master Mix Reagents Kit (ABI). The reaction (25 μl) contained 2 ng total RNA, 300 nmol $l^{-1}$ primers and 250 nmol $l^{-1}$ probe. Each sample was run in duplicate along with control reactions, which did not include reverse transcriptase and template. TaqMan ribosomal RNA was used as an internal control. The thermal cycling conditions were 30 min at 48°C, 10 min at 95°C, 45 cycles of 15 s at 95°C, and 1 min at 60°C. Standard dilution curves of cDNA were generated for both opsin mRNA and rRNA. The cDNA was synthesized using the Superscript First-Strand Synthesis System (Invitrogen) with 5 μg of total RNA from each sample in a total volume of 40 μl. The reaction was performed by the same method described above, without the addition of reverse transcriptase. The dilution values of 1, 0.25, 0.0625, 0.0156, 0.0039, 0.0010 and 0.00025 were used to generate the standard curve. To normalize the data to the endogenous control rRNA, the amount of rhodopsin mRNA and rRNA were determined from the standard curve for each sample. Relative rhodopsin mRNA expressions at different times in the day and night were determined by dividing rhodopsin mRNA concentration obtained at each time point by the lowest mRNA concentration (obtained at 07:00 h).

### *Time-lapse imaging*

Isolated retinas (from adult transgenic zebrafish, between 6 and 8 months of age) were embedded in low-melting point agarose and were cut using a vibroslicer (WPI, Sarasota, FL, USA). Retinal slices of 250 μm were cultured in a medium containing 140 mmol $l^{-1}$ NaCl, 5 mmol $l^{-1}$ KCl, 2.5 mmol $l^{-1}$ $CaCl_2$, 0.5 mmol $l^{-1}$ $MgCl_2$, 0.3 mmol $l^{-1}$ $NaH_2PO_4$, 0.3 mmol $l^{-1}$ $Na_2HPO_4$, 0.5 mmol $l^{-1}$ $MgSO_4$, 10 mmol $l^{-1}$ glucose and 10% fetal calf serum (Sigma, St Louis, MO, USA). Rhodopsin promoter-driven GFP expression was detected using a Zeiss Axiovert S100TV microscope with a 40× plan-NeoFluar oil objective lens. We measured GFP expression in cell soma using the MetaMorph software (average pixels, unsigned 16 bits grayscale; Universal Imaging, Downingtown, PA, USA). The same areas were used for calculating GFP fluorescence intensities at different time points. For each rod cell, we compared the average pixel values with the normalized value obtained before the treatment (designated as 1.0).

Time-lapse images were taken at 15-min intervals and controlled by a Lambda 10-2 shutter (Sutter Instrument Co., Novato, CA, USA). At each time point, 20 z-series images were taken at steps of 1 μm. The stocks were projected to one image. A minimum exposure time of 25 ms was used to avoid bleaching the GFP. Under our experimental conditions (e.g.

20°C room temperature in the dark); in the presence of RNA synthesis inhibitor DRB, the half-life of the GFP we observed in live zebrafish rod photoreceptor cells was approximately 45 min (fit by the exponential decay equation; rate constant, 0.99±0.22).

Cytoplasmic free $Ca^{2+}$ was labeled by X-Rhod-1 AM (Invitrogen). Retinal slices were incubated with the dye for 30 min, and then were washed in an indicator-free medium to remove the dye that was nonspecifically bound to cell membrane.

### *Light and drug treatments*

Retinal slices were transferred to the recording chamber on the microscope stage, and were allowed to settle for 30 min before light (room fluorescent light) or drug (e.g. dopamine, dopamine receptor agonist or antagonist, cGMP analog) treatments. Drug solutions were freshly prepared each day before the experiment. Drugs were dissolved in distilled water and were added to the culture medium by slow perfusion through the input tubing at a flow rate of 5 ml $min^{-1}$. Drug treatments were performed in the dark. Infrared night vision goggles were used to handle the samples in the dark.

### *Immunolabeling*

Protocols for immunolabeling were similar to those described previously (Schmitt and Dowling, 1996). In brief, the fish eyes were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and embedded in OCT compound (Polysciences, Warrington, PA, USA). Cryostat sections of 16 μm were mounted on gelatin-treated glass slides. Specimens were incubated briefly with blocking solutions that contained 5% normal goat serum and 0.1% Tween 20 in PBS, and then were incubated with anti-rhodopsin antibody (1:500) (Vihtelic et al., 1999) and rhodamine-conjugated secondary antibody (1:200; Chemicon, Temecula, CA, USA). Specimens were viewed under a microscope connected to a fluorescent light source.

### *Data analysis*

We used one-way ANOVA followed by a *post-hoc* Tukey test to compare the time-lapse data at different time points. A paired *t*-test was used to compare the changes in GFP expression in individual cells before and after light or drug treatments. An unpaired *t*-test was used to compare the changes in GFP expression between groups that received different treatments (e.g. different concentrations of drug treatment).

## Results

### *Circadian rhythms of rhodopsin expression in the retina*

Zebrafish display robust circadian rhythms in behavioral visual sensitivity. In a 24-h period, for example, zebrafish are most sensitive to light in the late afternoon and early evening, and are least sensitive in the early morning (Li and Dowling, 1998). To determine whether the day–night fluctuation in behavioral visual sensitivity correlates with rhodopsin gene expression, we measured rhodopsin mRNA expression using real-time RT–PCR. Total RNA was isolated from adult zebrafish retinas at different times in the day and night while the fish were kept in constant darkness (DD). The fish were placed in the dark at 21:00 h the day before they were killed for RNA isolation. In a 24-h period in DD, the level of rhodopsin mRNA expression fluctuated rhythmically. The expression was low in the early morning, increased in the mid-day, peaked in the evening and decreased at night (Fig. 1).

### *Transgenic zebrafish that express short half-life GFP in rod photoreceptor cells*

To further characterize the circadian rhythms of rhodopsin expression, we generated transgenic zebrafish that expressed short half-life GFP under the transcriptional control of the zebrafish rhodopsin promoter. We cloned a 1.2 kb fragment of zebrafish rhodopsin promoter into the pd2EGFP vector (Fig. 2A) and injected the DNA into 1-cell stage zebrafish embryos. After 72 h, the embryos were examined for transgene (rhodopsin::shGFP) expression. Approximately 70% of the injected embryos (*N*=40) showed transient transgene expression (Fig. 2B). By performing PCR with genomic DNA we identified, among the injected fish that survived to adulthood, three founders that showed GFP expression. We crossed each founder with wild-type zebrafish, and screened their progeny for germline transmission. We identified one founder fish that showed stable germline transmission of the transgene; approximately 50% of its progeny showed GFP expression (Fig. 2C). The progeny of this founder were raised to adulthood and were used for breeding colonies.

To determine whether the transgene is expressed in rod photoreceptor cells, we labeled the retinas of transgenic fish with antibodies against zebrafish rhodopsin (Vihtelic et al., 1999). Fig. 3 shows images of a cryostat section across the outer retina of an adult transgenic fish that was labeled with rhodopsin antibody. The expression of the transgene (rhod::GFP) was seen in the cell bodies and inner segments

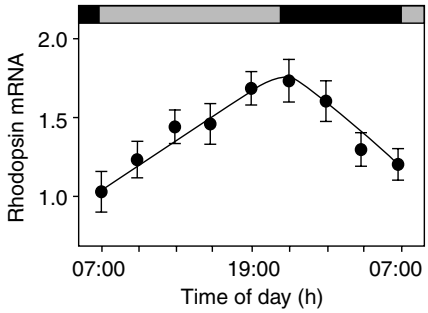


Fig. 1. Circadian rhythms of rhodopsin mRNA expression in isolated zebrafish retinas in constant darkness (DD). Between the subjective day and night, the expression of rhodopsin mRNA fluctuated. The expression increased steadily during the day, peaked in the late afternoon and then decreased at night. Horizontal bar: black indicates night; gray, subjective day without light. Values are means ± s.e.m. (*N*=8 at each time point).

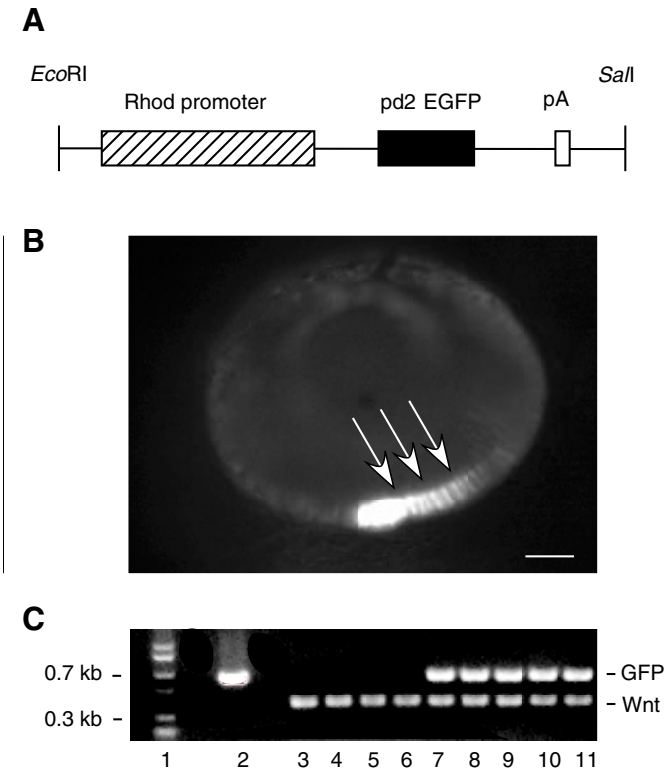


Fig. 2. Construction and expression of rhodopsin::GFP. (A) A diagram of the DNA fragment that contained 1.2 kb of zebrafish rhodopsin promoter and 0.7 kb of pd2EGFP cDNA with a short poly(A) (pA) tail. (B) Rhodopsin promoter-driven GFP expression in rod photoreceptor cells in the ventral patch of the retina (arrows) at 4 days post-fertilization. Scale bar, 40 μm. (C) Germline transmission of the transgene determined by PCR. Lane 1, molecular markers. Lane 2, positive control (plasmid DNA that contained 0.7 kb of pd2EGFP cDNA). Lanes 3–11, PCR of genomic DNA from nine embryos that were selected from a cross between a transgenic and a wild-type fish. Lanes 3–6, non-transgenic siblings. Lanes 7–11, transgenic siblings. Each lane represents PCR from an individual embryo. *Wnt* (internal control) was detected in every embryo.

(left panel). The antibody labeled rhodopsin in both the inner and outer segments (middle panel). The merged image shows the co-localization of the transgene and rhodopsin (right panel).

### *The expression of rhodopsin promoter is not synchronized*

To measure the circadian rhythms of rhodopsin promoter expression, we took time-lapse images of rhodopsin promoter-driven GFP expression in individual rod cells from retinal slice preparations. The experiments were performed in DD. In 24 h of DD, GFP intensity fluctuated rhythmically in each rod photoreceptor cell. However, the pattern of fluctuation differed among individual cells. Fig. 4A shows time-lapse imaging data of rhodopsin promoter-driven GFP expression in several rod cells in the first and second DD cycles, respectively. Each cell had a different fluctuation pattern of GFP intensity. In some cells, for example, peak expression was seen in the subjective early morning (e.g. cell 2, 5, 7, 8), whereas in other cells, peak expression was seen in the afternoon (cell 1) or at night (cell 3). The expression in some cells remained high at night and in the early morning but decreased in the afternoon (cell 4, 6).

Fig. 4B shows time-lapse images of rhodopsin promoter-driven GFP expression in two rod cells from the same slice preparation during 24 h in DD. In cell 1, the GFP intensity was low at night and in the early morning. It gradually increased in the middle of the day, peaked in the early afternoon (13:00 h), and decreased thereafter. In cell 2, the highest GFP intensity was seen in the early morning (07:00 h). During the day, GFP intensity gradually decreased.

### *Light transiently decreases rhodopsin promoter expression via dopamine $D_2$ receptor-coupled mechanisms*

The multiphasic circadian oscillation of rhodopsin promoter expression among individual rod cells can be synchronized by light. This was observed in all the rod cells, regardless of

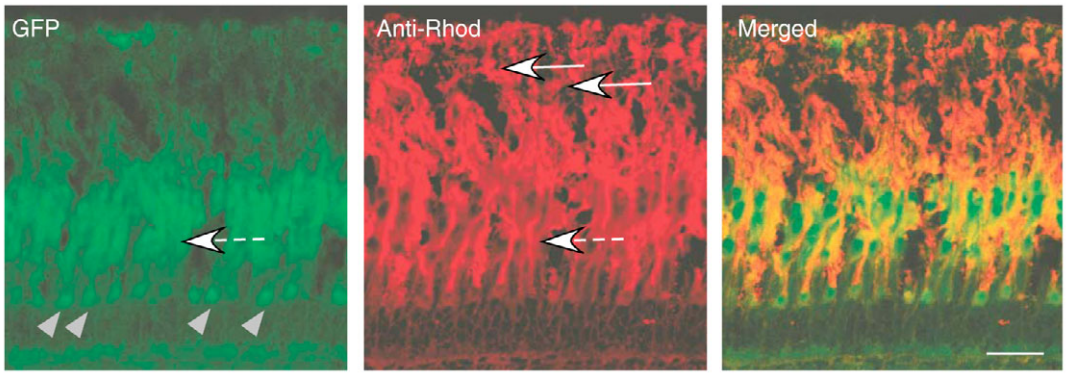


Fig. 3. A cryostat section across the outer retina of a transgenic fish showing GFP (left), anti-rhodopsin immunoreactivity (middle), and the co-localization of GFP and rhodopsin (right). GFP was found in the soma (arrowheads) and inner segment (arrow with broken line) of rod photoreceptor cells. The antibody labeled rhodopsin in the inner (arrow with broken line) and outer (arrow with solid line) segments. The merged image shows the co-localization of GFP and rhodopsin. Scale bar, 40 μm.

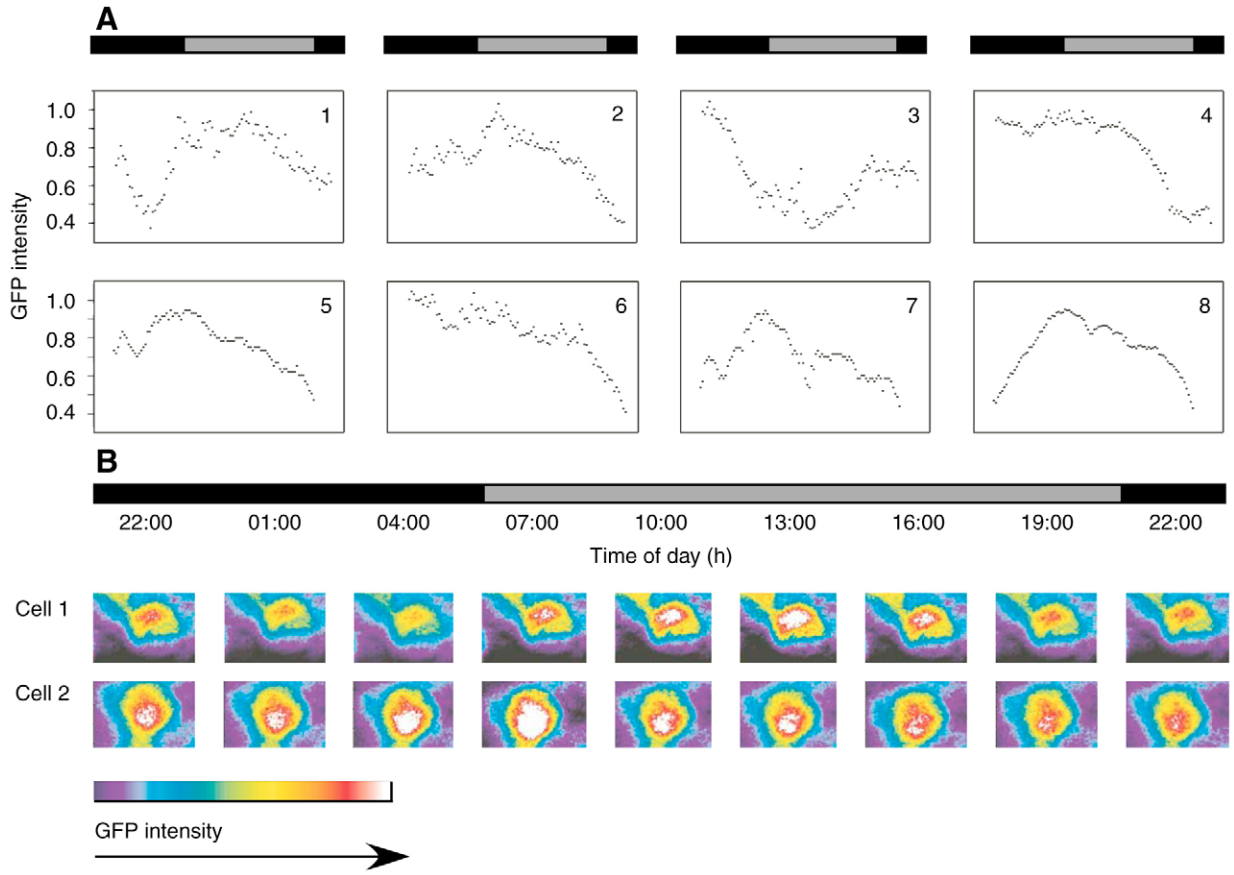


Fig. 4. Circadian rhythms of rhodopsin promoter-driven GFP expression in individual rod photoreceptor cells. (A) GFP intensity in individual rod cells during the first (top row) and second cycles (bottom row) in constant darkness (DD). Note that the times of peak expression (normalized to 1.0) varied in different cells. (B) Time-lapse images of rhodopsin promoter-driven GFP expression in two rod cells from the same slice preparation in 24 h of DD. The highest expression was detected at 13:00 h and 07:00 h, respectively, in cell 1 and cell 2. Black bars, night; gray bars, subjective day without light.

whether rhodopsin promoter expression was in the rising or descending phase at the onset time of light exposure. Fig. 5A shows time-lapse imaging data of rhodopsin promoter-driven GFP expression in 24 h of DD, except at 22:00 h, at which time a 30-min light pulse (room fluorescent light, 92 Lux) was applied. Before light exposure, rhodopsin promoter-driven GFP expression in individual cells was not synchronized. Between 17:00 h and 22:00 h, for example, the expression increased in some cells, but decreased in other cells. After light treatment, the expression in all the rod cells increased. By 05:00 h on the second day, the expression peaked. Afterwards, the expression gradually decreased and became desynchronized.

Light synchronized the circadian rhythms of rhodopsin promoter expression by decreasing the expression. The effect, however, was only transient. The effect was maximal at 30 min, at which time the expression had decreased by 22.1±0.8% ($P$<0.001). After 30 min of light exposure, the expression began to increase (Fig. 5B).

Dopamine, which is often considered an intra-retinal light signal, produced a similar but long-lasting effect. After 60 min of dopamine treatment (100 μmol $l^{-1}$), rhodopsin promoter-driven GFP expression decreased by 19.3±1.3% ($P$<0.001; Fig. 5B). Activation of dopamine $D_2$ receptors with quinpirole (10 μmol $l^{-1}$) also decreased rhodopsin promoter expression, for example, by 16.5±2.7% ($P$<0.001; Fig. 5B). Selective activation of dopamine $D_1$ receptors (with 10 μmol $l^{-1}$ SKF 38393) produced no effect on rhodopsin promoter expression (not shown).

To determine whether the effects of light and dopamine on rhodopsin promoter expression is mediated by the same or different signaling pathways, we measured rhodopsin promoter-driven GFP expression in response to light while the slice was treated with dopamine $D_2$ receptor antagonist (sulpiride; 10 μmol $l^{-1}$). In the presence of sulpiride, light produced no effect on rhodopsin promoter expression (Fig. 5B). This suggests that the effect of light on rhodopsin expression is mediated by dopamine through dopamine $D_2$ receptor-couple signaling pathways. Inactivation of dopamine $D_1$ receptors (with 10 μmol $l^{-1}$ SCH23390) did not affect light-induced synchronization of rhodopsin promoter-driven GFP expression (not shown).

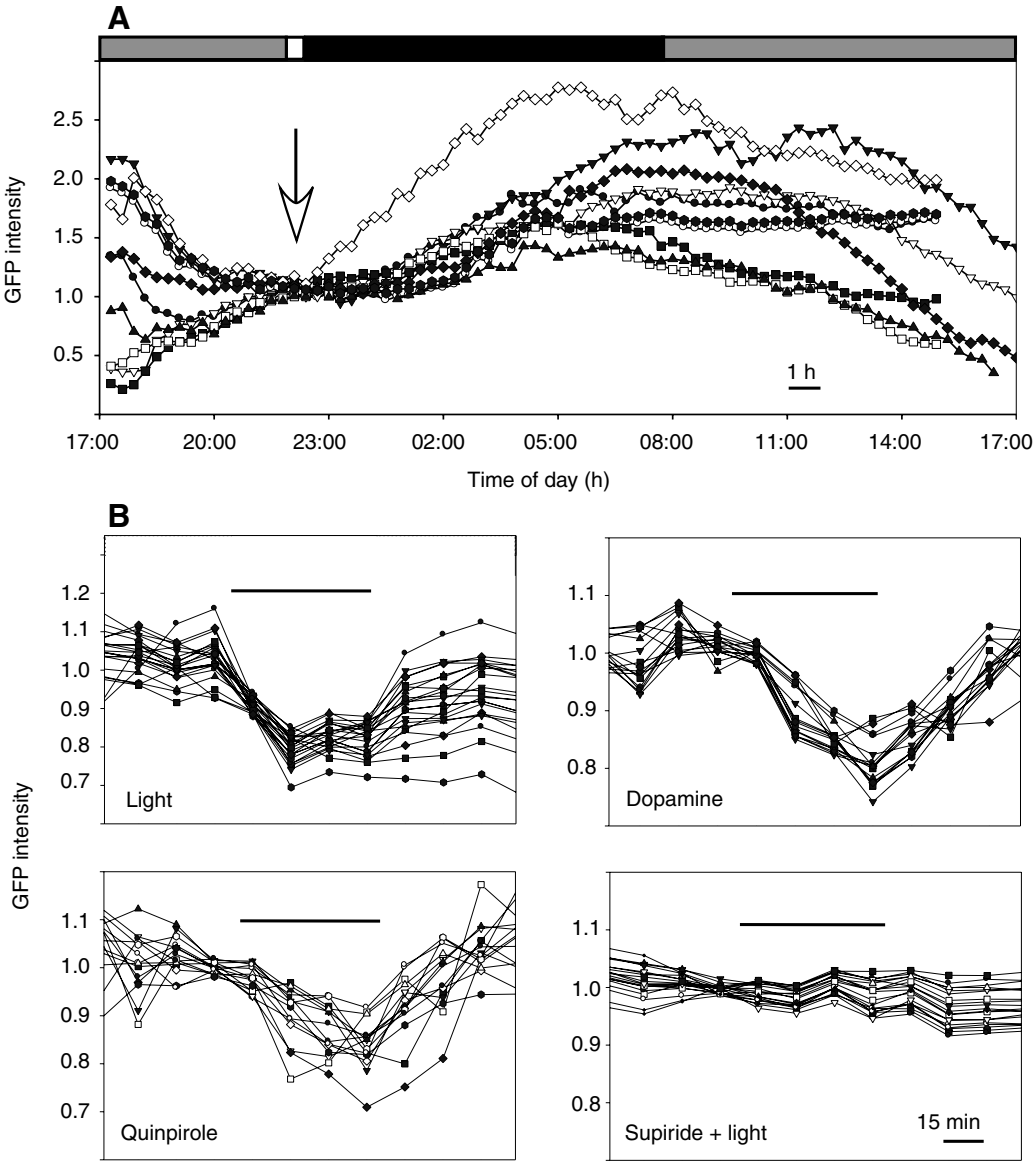


Fig. 5. Synchronization of rhodopsin promoter expression in individual rod photoreceptor cells. (A) Rhodopsin promoter-driven GFP expression in 24 h in constant darkness (DD), except at 22:00 h, when a 30-min light pulse was applied. Exposure to light (indicated by the arrow) synchronized the expression in all rod cells ($N$=10). After light treatment, the expression began to increase. (B) Light exposure ($N$=21) or the application of dopamine ($N$=16) or dopamine $D_2$ receptor agonist quinpirole ($N$=14) decreased rhodopsin promoter expression. In the presence of the dopamine $D_2$ receptor antagonist sulpiride, light produced no effect on rhodopsin promoter expression ($N$=19). Each line represents time-lapse imaging data from one rod cell. Horizontal lines represent the duration of treatment.

### *Correlations between $Ca^{2+}$ influx and rhodopsin promoter expression*

The mechanisms behind light-induced synchronization of rhodopsin gene expression probably involve dopamine $D_2$ receptor-coupled $Ca^{2+}$ signaling pathways. In the dark, $Ca^{2+}$ crosses the cell membrane through cGMP-gated cation channels. Light closes cGMP-gated channels, thereby decreasing $Ca^{2+}$ currents (Stryer, 1986). We recorded decreased cytoplasmic $Ca^{2+}$ concentrations in zebrafish rod photoreceptor cells after light treatment. After 30 min of light treatment, cytoplasmic $Ca^{2+}$ (labeled by X-Rhod-1 AM) concentrations decreased by 16.2±2.8% ($P$<0.001; Fig. 6A). Activation of dopamine $D_2$ receptors (with 10 μmol $l^{-1}$ quinpirole) produced a similar result, for example, a decrease in cytoplasmic $Ca^{2+}$ concentration by 9.8±1.6% ($P$<0.001; Fig. 6B).

To determine whether the decrease of cytoplasmic $Ca^{2+}$ concentration after dopamine treatment is due to an effect of dopamine on cGMP-gated cation channels, we measured the $Ca^{2+}$ concentration in rod cells in response to 8-pCPT-cGMP (a membrane permeable cGMP analog) and dopamine in the

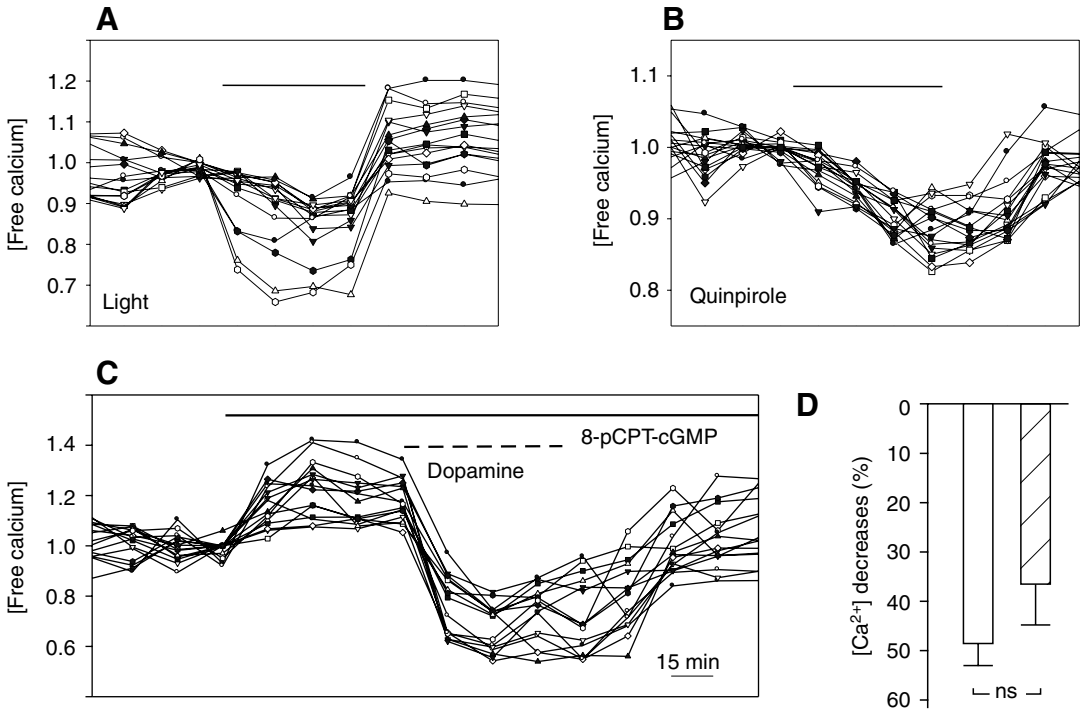


Fig. 6. Cytoplasmic free $Ca^{2+}$ concentrations in individual rod cells measured before, during and after exposure to light (A; *N*=16), quinpirole (B; *N*=17) or 8-pCPT-cGMP and dopamine treatment (C; *N*=17). (A,B) Light or quinpirole treatment decreased cytoplasmic $Ca^{2+}$ concentrations. Horizontal lines represent the duration of light or drug treatment. (C) The application of 8-pCPT-cGMP (a membrane-permeable cGMP analog) increased $Ca^{2+}$ influx. However, when dopamine was added to the medium, cytoplasmic $Ca^{2+}$ concentrations decreased. Horizontal solid and broken lines represent 8-pCPT-cGMP and dopamine treatments, respectively. Each line represents time-lapse imaging data from one rod cell. (D) Decreases in cytoplasmic $Ca^{2+}$ concentrations after 8-pCPT-cGMP and dopamine treatments in the absence (*N*=17; open bar) or presence of $Co^{2+}$ (*N*=8; hatched bar). Values are the means ± s.e.m.; ns, no significant difference.

absence or presence of $Co^{2+}$, which is known to block voltage-gated $Ca^{2+}$ channels (Pan, 2000). The application of 8-pCPT-cGMP (500 μmol $l^{-1}$) increased cytoplasmic $Ca^{2+}$ concentration by 21.3±2.8% ($P$<0.001; Fig. 6C). After the application of dopamine (100 μmol $l^{-1}$), cytoplasmic $Ca^{2+}$ concentration decreased. After 30 min of dopamine treatment, for example, cytoplasmic $Ca^{2+}$ concentration decreased by 49.0±3.5% as compared with the concentration measured at 30 min after the application of 8-pCPT-cGMP (Fig. 6C,D). The application of $Co^{2+}$ ($CoCl_2$; 0.5 mmol $l^{-1}$) did not change the increase/decrease patterns of cytoplasmic $Ca^{2+}$ concentrations in response to 8-pCPT-cGMP and dopamine (Fig. 6D), suggesting that the decrease of cytoplasmic $Ca^{2+}$ concentration is due to the effect of dopamine on cGMP-gated channels.

## Discussion

Here, we report a study on the circadian rhythms of rhodopsin promoter expression in zebrafish rod photoreceptor cells. In each rod cell, the expression of rhodopsin promoter is regulated by an independent circadian oscillator. Interestingly, each oscillator functions by its own timing. In some cells, peak rhodopsin promoter expression is seen in the early morning hours, whereas in other cells, peak expression is seen in the afternoon or at night. The multiphasic circadian oscillation of rhodopsin promoter expression can be synchronized by light and dopamine. In the presence of dopamine $D_2$ receptor blockers, however, the effect of light is blocked. This suggests that the synchronization of the circadian rhythms of rhodopsin promoter expression by light is mediated by dopamine $D_2$ receptor-coupled signaling pathways.

A transient increase of vitreal dopamine concentration in the early morning, promoted by light or endogenous circadian pacemakers (Whitkovsky and Dearry, 1992; Ribelayga et al., 2003; Puppala et al., 2004), seems to be essential for synchronizing the circadian rhythms of rhodopsin promoter expression. Dopamine down regulates rhodopsin promoter expression by decreasing cGMP-gated $Ca^{2+}$ currents. Previous studies have shown that dopamine has a role in the regulation of cGMP-gated channels. In chicks, for example, dopamine modulates the affinity of cGMP-gated channels in cone photoreceptor cells (Ko et al., 2003; Ko et al., 2004). Depending upon the duration of dopamine treatment and the time of day, the effect of dopamine on cGMP-gated channels may vary. During the day, for example, brief activation of dopamine $D_2$ receptors decreases the affinity of cGMP-gated cation channels. At night, however, exposing the cone cells

to dopamine for 2 h increases the affinity of cGMP-gated channels (Ko et al., 2003). Other mechanisms, such as the rhythmic production of melatonin by the photoreceptor cells (Cahill, 1996; Tosini and Menaker, 1998; Doyle et al., 2002; Ribelayga et al., 2003) or the expression of early circadian genes (Steenhard and Besharse, 2000), may also have a role in synchronizing the circadian rhythms of rhodopsin promoter expression. It is possible, for example, that the increase of retinal dopamine concentration is partially due to the decrease in melatonin production (Behrens et al., 2000).

In fish retinas, the only cell types that release dopamine are dopaminergic interplexiform cells (DA-IPCs) (Yazulla and Zucker, 1988; Dowling and Ehinger, 1978; Li and Dowling, 2000). DA-IPCs are located in the distal inner nuclear layer, and their processes (dendrites and axons) are found in both the outer and inner plexiform layers. Dopamine plays important roles in the regulation of photoreceptor cell functions. For example, activation of dopamine D2 receptors regulates daily photomechanical movement of both rod and cone myoids (Douglas et al., 1992; McCormack and Burnside, 1992; Hillman et al., 1995). Rod and cone photoreceptor cells may synapse with each other *via* gap junctions. However, we may rule out the possibility that gap junctions play a role in synchronizing this multiphasic circadian oscillation, because light or dopamine un-couples gap junctions (Lasater and Dowling, 1985).

In addition to the light and dopamine signals described here, the circadian rhythms of rhodopsin gene expression may also be synchronized by the well defined central mechanisms, including the rhythmic production of melatonin by the pineal gland. In zebrafish that were kept in DD, for example, the circadian rhythms of rhodopsin mRNA expression in the whole-retina fluctuated in a synchronized pattern (Fig. 1).

Of particular interest, we demonstrated in this and other studies that light may regulate opsin expression in different ways, depending on the duration and intensity of light treatment. When applied for a short period of time (e.g. up to 30 min), light transiently decreases rhodopsin promoter expression. After the transient decrease, light produces no further effect in rhodopsin promoter expression. During subsequent light or dark adaptation, rhodopsin promoter expression increases (Yu et al., 2007). By contrast, when applied for a long period of time, light decreases the expression and diminishes the circadian rhythms of opsin expression. In zebrafish, for example, after 24 h of light exposure, the expression of long wavelength-sensitive (red cone) opsin mRNA at all times in the subjective day and night decreased to the lowest level normally seen in the early morning in control fish (Li et al., 2005).

In summary, this study provides insight into the mechanisms for synchronizing multiphasic circadian oscillation in photoreceptor cells. In zebrafish, the circadian oscillators that regulate rhodopsin promoter expression appear to act independently in individual rod photoreceptor cells. Light synchronizes the multiphasic circadian expression of rhodopsin *via* dopamine $D_2$ receptor-coupled $Ca^{2+}$ signaling pathways. The synchronized circadian rhythms of rhodopsin mRNA expression may play a role in the regulation of the circadian rhythms of behavioral visual sensitivity.

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