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Circadian rhythms of behavioral cone sensitivity and long wavelength opsin mRNA expression: a correlation study in zebrafish

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Summary

Using a behavioral assay based on visually mediated escape responses, we measured long-wavelength-sensitive red cone (LC) sensitivities in zebrafish. In a 24 h period, the zebrafish were least sensitive to red light in the early morning and most sensitive in the late afternoon. To investigate if the fluctuation of behavioral cone sensitivity correlates with opsin gene expression, we measured LC opsin mRNA expression at different times in the day and night under different lighting conditions. Under a normal light–dark cycle, the expression of LC opsin mRNA determined by real-time RT–PCR was low in the early morning and high in the late afternoon, similar to the fluctuation of behavioral cone sensitivity. This rhythm of LC opsin mRNA expression, however, dampened out gradually in constant conditions. After 24 h of constant light (LL), the expression of LC opsin mRNA dropped to levels similar to those determined in the early morning in control animals. By contrast, when the zebrafish were kept in constant dark (DD), the expression of LC opsin mRNA increased, to levels about 30-fold higher than the expression in the early morning in control animals. This day–night fluctuation in LC opsin mRNA expression was correlated to changes in opsin density in the outer segment of cone photoreceptor cells. Microspectrophotometry (MSP) measurements found significant differences in red cone outer segment optical density with a rhythm following the behavioral sensitivity. Furthermore, dopamine modulated the circadian rhythms in expression of LC opsin mRNA. Administration of dopamine increased LC opsin mRNA expression, but only in the early morning.

Key words: circadian rhythm, opsin mRNA expression, behavioral visual sensitivity, zebrafish.

Introduction

A variety of visual behaviors, such as photoreceptor cell disk shedding (Young, 1967; LaVail, 1976; Besharse et al., 1988; Bassi and Powers, 1990), rod and cone myoid movement (Pierce and Besharse, 1985; Dearry and Burnside, 1986), retinal pigment epithelium granule migration (Bruenner and Burnside, 1986), opsin expression (Korenbrot and Fernald, 1989; Pierce et al., 1993), and retinal sensitivity (Barlow, 1983; Bassi and Powers, 1986, 1987), display robust day–night rhythms. In frogs, for example, the expression of rod opsin mRNA fluctuates between the day and night; it is high in the day and low at night. This pattern of fluctuation persists in constant conditions and can be phase-shifted by light (Korenbrot and Fernald, 1989). In chicks, the expression of cone opsin varies diurnally; it is high in the afternoon and low in the early morning. This rhythm persists in cultured cells in the absence of external cues of time, suggesting that the circadian oscillators that regulate cone opsin gene expression are located in the photoreceptor cells (Pierce et al., 1993). In zebrafish, the absolute behavioral visual sensitivity is high in the late afternoon and low in the early morning. This pattern of behavioral sensitivity persists in DD or in LL, and can be phase-shifted by light (Li and Dowling, 1998).

Ample evidence suggests that in addition to the endogenous circadian control, external cues, such as light and dopamine, play important roles in circadian visual function. Light produces acute effects; shorter periods of light exposures may shift the circadian rhythms (Cahill and Besharse, 1991, 1993), whereas prolonged periods of light exposures may diminish them (Green and Besharse, 1996; Li and Dowling, 1998). Dopamine plays a modulatory role in circadian rhythms of photoreceptor sensitivity (Ko et al., 2003) or behavioral visual sensitivity (Li and Dowling, 2000). The underlying mechanisms of light and dopamine on circadian visual sensitivity may vary, e.g. by activating different second messenger pathways (Ribelayga and Mangel, 2003), or by

phase-shifting the expression of immediate-early circadian genes (Steenhard and Besharse, 2000).

While circadian oscillation in behavioral visual sensitivity and opsin expression has been well documented, the questions remain whether the behavioral visual sensitivity is correlated with opsin gene expression, and if a correlation exists, whether the cyclic expression of opsin is the sole factor limiting the circadian rhythm in behavioral visual sensitivity. We demonstrate that in zebrafish the circadian rhythm of behavioral cone (red cone) sensitivity was correlated with LC opsin mRNA expression, but only for about 24 h. The circadian cycle in LC opsin mRNA expression was also shown to be regulated by light and dopamine.

Materials and methods

Animals and maintenance

Zebrafish (*Danio rerio* Hamilton) were maintained as described in Westerfield (1995). Zebrafish used in this study were between 6 and 12 months of age. Normally, the fish were kept in a 14:10 light:dark (LD) cycle (light, 06:00–20:00 h; fluorescent room light). For LL (constant light) or DD (constant darkness) experiments, the fish were removed from the LD cycle at 20:00 h the day before the experiment and thereafter kept under designated conditions.

Behavioral analysis of zebrafish cone sensitivity

Methods for behavioral analysis of zebrafish visual sensitivity have been previously described (Li and Dowling, 1997; for a review, see Li, 2001). The test apparatus consisted of a circular plastic container, surrounded by a rotating drum. A black segment was marked on white paper that was attached to the inside of the drum. The drum was illuminated from above by a halogen lamp, filtered with a 620 nm band-pass interference filter (10 nm half max bandwidth; Oriel Instruments, CT, USA). The maximum light intensity at the water surface in the container was log 0=40 μW cm^{-2}. The light intensity was adjusted by changing neutral density filters at 0.5 log unit steps.

Zebrafish display robust escape responses to the black segment rotating outside the container, for example, they rapidly reverse their swimming direction when encountering the black segment (Li and Dowling, 1997). In this study, we measured red cone sensitivity by recording the lowest intensity of red light required to evoke escape responses. Red light (>600 nm) activates primarily the red cone photoreceptor cells (Robinson et al., 1993; Brockerhoff et al., 1997). Thus, the escape response evoked under the near-threshold red illumination is unlikely mediated by other types of photoreceptor cells such as rods, which have a λ_{max} at about 500 nm (Nawrocki et al., 1985; Cameron, 2002; Chinen et al., 2003). Prior to visual threshold measurements, the fish were dark adapted for 15 min. This timing is sufficient to dark adapt the cone system in zebrafish (Li and Dowling, 1997; Ren and Li, 2004). Normally, we observed the fish behaviors for 10–15 s, during which time the fish encountered the rotating segment 2–4 times. A minimum of two escape responses was required to score a threshold. The light illuminating the test apparatus was initially set at a dim level, log I=–3.0. If no escape response was observed, the light was increased by 0.5 log unit steps until an escape response was elicited. Infrared night vision goggles were used to handle the fish at night or when the experiments were performed in DD.

Drug administration

Drug treatments were performed using isolated eyecups. Eyecups (two eyecups for each sample collection) were prepared in the late afternoon the day before the experiments were performed. Zebrafish were anesthetized with 1% 3-aminobenzoic, then they were decapitated. Eyes were enucleated and cornea and vitreous body were removed. Eyecups were incubated in L15 media (Sigma, MO) overnight in the dark. Prior to drug treatment, dopamine or dopamine D_1 (SKF38393) or D_2 (quinpirole) receptor agonists (Sigma, MO) were dissolved in phosphate buffered saline, pH 7.0, and diluted in distilled H_2O to 100 μmol l^{-1} (Lin and Yazulla, 1994). Drugs were added to L15 culture media *via* micropipettes. The final concentrations of dopamine in the culture media were 0.1 μmol l^{-1}, 0.5 μmol l^{-1}, 1 μmol l^{-1} and 10 μmol l^{-1}, respectively, and for D_1 or D_2 agonist, 0.1 μmol l^{-1}, 1 μmol l^{-1}, and 10 μmol l^{-1}, respectively. After 30 min of drug treatment, the eyecups were removed from the media, and were transferred to RNA wiz (Ambion, TX, USA). Samples were stored at –80°C. All drug treatments were performed in the dark. Infrared night vision goggles were used to handle the samples in the dark.

Total RNA extraction and real-time RT–PCR

Total RNA was extracted from the eyes or cultured eyecups (in the case of drug treatment). Eyes or eyecups (two for each sample collection) were homogenized in 500 μl of RNA wiz (Ambion, TX, USA), followed by chloroform extraction. RNA was precipitated with isopropanol, washed with 75% ethanol, and re-suspended in 20 μl distilled H_2O (RNAse free). The concentration of total RNA was determined using a BioMate 3 series spectrophotometer (Thermo Spectromic, NY). Red cone opsin specific primers and probes (GenBank sequence accession number, AF109371; 5′-TGG AGC AGA TAC TGG CCT CAT-3′ and 5′-GGG TCC TCG CTT CCA CTG A-3′; TaqMan probe, 5′-TCT GAA GAC CTC CTG TGG CCC TGA TG-3′) were designed using the Primer Express Primers system (ABI, CA, USA).

Real time RT–PCR was performed using the TaqMan One-Step RT–PCR Master Mix Reagents Kit (ABI, CA, USA). The reaction (25 μl) contained 2 ng total RNA, 300 nmol l^{-1} primers, and 250 nmol l^{-1} probe. Reactants were mixed and transferred into a 96-well PCR plate, with 2 μl (1 ng ul^{-1}) of total RNA in each well. Each sample was run in duplicate along with control reactions, which did not include the addition of reverse transcriptase or template. TaqMan ribosomal RNA was used as an internal control. The thermo 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. Relative LC opsin mRNA expression was determined using the standard curve method provided by ABI. Standard dilution curves of cDNA were generated for both LC mRNA and rRNA control. The cDNA was synthesized using Superscript First-Strand Synthesis System (Invitrogen, CA, USA) using 5 µg of total RNA from each sample in 40 µl total volume. The reaction was performed by the same method described above except that there was no reverse transcriptase added. 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 LC opsin mRNA and rRNA were determined from the standard curve for each sample. The amount of LC opsin mRNA was divided by the amount of rRNA. Relative LC opsin mRNA expressions (during a 24 h period in LD or LL or DD) were determined by dividing the concentration of LC opsin mRNA obtained at each time in the day and night by the concentration of LC opsin mRNA obtained at 07:00 h.

Microspectrophotometry (MSP)

MSP was performed to determine the absorbance maximum (A_{max}) of the red cones. The microspectrophotometer used in this study has been previously described (Hawryshyn et al., 2001). In brief, short duration flashes (0.05 s) of full spectrum (300–800 nm) unpolarized (beam size, 2×3 µm) light from a 150 W xenon light source were delivered to the photoreceptor outer segments. The transmitted beam passed through a spectrometer (300 nm blazed grating; Acton Res Co, MA, USA) and onto a 1340×400 pixel Peltier cooled (–55°C) back-illuminated CCD detector (Princeton Instruments, NJ, USA). Photoreceptor absorbance [$\log_{10}$ ($1/T$)] was calculated by comparing the transmitted intensity through the photoreceptor (I_m) to the transmitted intensity through an area clear of debris adjacent to the photoreceptor [reference (I_r) thus, $T=I_m/I_r$]. A_{max} was recorded in mOD, which is a measure of optical density (1 mOD=10^{-3} OD).

Zebrafish were dark adapted for 1 h prior to dissection under infrared illumination. Once on the CCD-MSP microscope, samples were examined under infrared illumination and monitored by an infrared camera. The infrared image was transmitted to a computer that also served as the central control unit for the CCD-MSP device. While searching the retinal sample for long-wavelength sensitive cones the path of the motorized stage was plotted on screen to eliminate the possibility of recording from the same photoreceptor more than once, which would result in an underestimate of the A_{max}. Once recorded the absorbance spectra were stored for later analysis. Using an analysis program the A_{max} was automatically calculated from the peak of the λ_{max} to the baseline. The main estimate of λ_{max} was determined by a minimum variance fit to the upper 20% of the absorption spectrum (Govardovskii et al., 2000). An independent sample *t*-test was used to compare the mean A_{max} values determined in the day and night.

Results

Behavioral cone sensitivity was low in the morning and high in the afternoon

In a 24 h period, the zebrafish were least sensitive to red light in the early morning (a few hours before and after light onset) and most sensitive in the late afternoon (a few hours before light offset) (Fig. 1). At 07:00 h, the threshold light intensity required to evoke an escape response was log I=–0.9±0.2. During the day, cone sensitivity increased. Within 3 h after light onset, the behavioral cone sensitivity increased 0.7 log units. The increase of cone sensitivity continued in the mid-day and early afternoon. By 19:00 h, cone sensitivity peaked, during which time the light threshold to evoke an escape response was log I=–2.1±0.3. Shortly after light offset, the behavioral cone sensitivity began to decrease. By 04:00 h of the second day, cone sensitivity had returned to levels similar to those determined in the early morning of the previous day.

The fluctuation in behavioral cone sensitivity persisted in the zebrafish that were kept in constant conditions. In DD, cone sensitivity was low in the subjective early morning and high in the subjective late afternoon, similar to the fluctuation of cone sensitivity determined in LD. The threshold difference between the subjective early morning and late afternoon, however, was slightly reduced in DD as compared to the threshold difference determined in LD. In the first day of DD, for example, between subjective 07:00 and 19:00 h the difference in behavioral cone sensitivity was 0.8±0.2 log units. On the second day of DD, it was further reduced, to 0.6±0.2 log units (Fig. 1). It has been previously shown that the fluctuation of behavioral visual sensitivity persisted in DD for

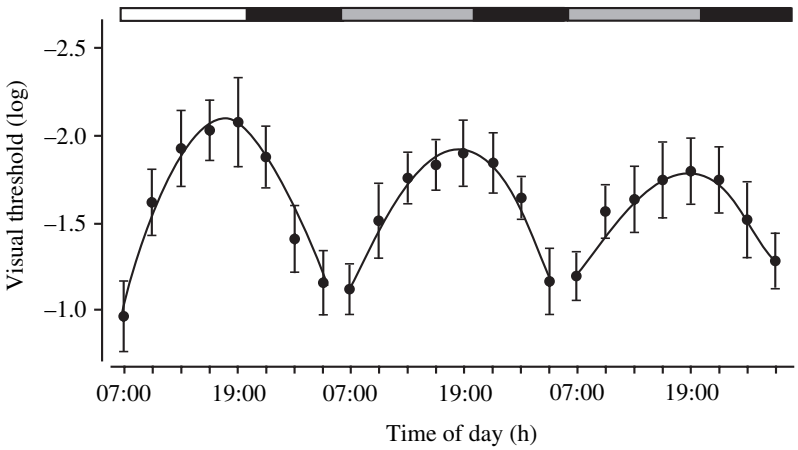

Fig. 1. Behavioral red cone sensitivity at different times in the day and night in zebrafish that were kept in LD and 2 days in DD. In both LD and DD, red cone sensitivity increased during the day and decreased at night. Horizontal bars at the top of the figure indicate lighting conditions; white bar, day; black bars, night; gray bars, subjective days without light. Data represent the mean ± S.E.M. (N=8).

5–7 days before it completely dampened out (see Li and Dowling, 1998).

LC opsin mRNA expression fluctuated in correlation with behavioral cone sensitivity

We measured LC opsin mRNA expression at different times in the day and night using real time RT–PCR. In LD (Fig. 2A), the expression of LC opsin mRNA was low in the early morning and high in the late afternoon. The lowest expression was observed at 07:00 h. The expression increased through the mid-morning and early afternoon, and reached the highest level in the late afternoon at 19:00 h. From 07:00–19:00 h, the expression of LC opsin mRNA increased approximately 50-fold. Shortly after light offset, the expression of LC opsin mRNA began to decrease. By 22:00 h, the expression had decreased to levels about one half of the peak level determined at 19:00 h. By 04:00 h in the second day, the expression of LC opsin mRNA decreased to levels about one half of the level seen at 22:00 h.

Under constant darkness, the circadian oscillations in the expression of LC opsin mRNA persisted for approximately 24 h. In the first day of DD (Fig. 2A), the expression of LC opsin mRNA was low in the subjective early morning and high in the subjective late afternoon and early evening, similar to the fluctuation of LC opsin mRNA expression in LD. During the subjective day, the expression of LC opsin mRNA increased. Between subjective 07:00 and 19:00 h, the expression of LC opsin mRNA increased about 40-fold. During the subjective night, however, the expression of LC opsin mRNA decreased only slightly. At subjective 04:00 h, the expression was significantly higher than the expression determined at 04:00 h in LD. In the second day of DD, no obvious fluctuations of LC opsin mRNA expression were seen, i.e. at all times during the subjective day and night, the expression was similar to that determined at the end of the first day of DD.

We also measured LC opsin mRNA expressions in LL. In the first 24 h of LL, the expression of LC opsin mRNA was low in the subjective early morning and high in the subjective late afternoon and early evening (Fig. 2B). Between subjective 07:00 and 19:00 h, the expression of LC opsin mRNA increased about 40-fold. At subjective night, the expression decreased, in a similar fashion as seen in LD. By 04:00 h, the expression of LC opsin mRNA decreased to levels similar to those determined in the early morning in control animals. In the second day of LL, no obvious fluctuations of LC opsin mRNA expression were seen, i.e. at all times between the subjective day and night, the expression was similar to that measured at 07:00 h in LD or at subjective 07:00 h in the first day of LL.

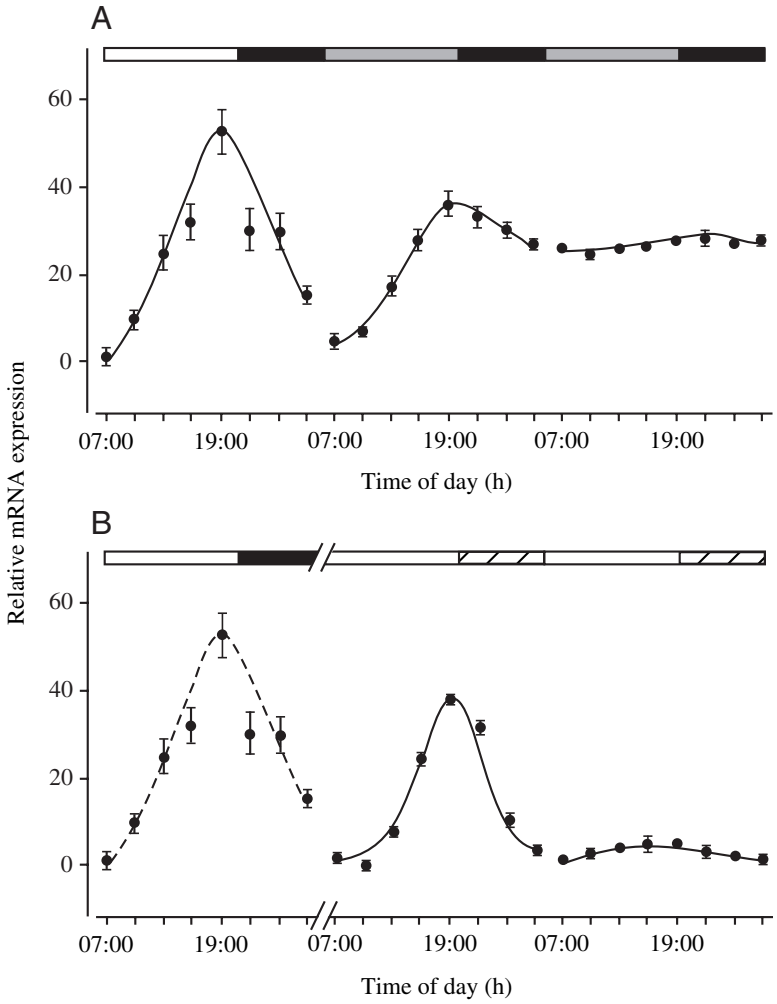

Fig. 2. Circadian rhythms of LC opsin mRNA expression under different lighting conditions. In LD, the expression of LC opsin mRNA was low in the morning and high in the afternoon. The fluctuation of LC opsin mRNA persisted in DD (A) or LL (B) for only one day. In the second day of DD or LL, the expression became flat. Horizontal bars at the top of the figure indicate lighting conditions in the day and night; white bar, day; black bars, night; gray bars, subjective days without light; hatched bars, subjective night with light. Data represent the mean ± S.E.M. (N=16).

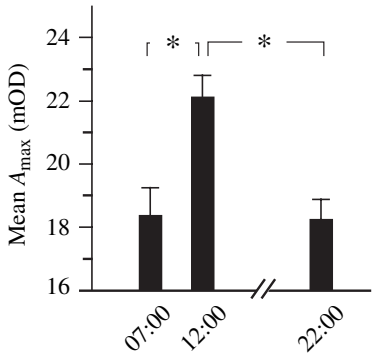

Fig. 3. Mean absorbance maxima (A_{max}) of zebrafish red cones measured at 07:00, 12:00 and 22:00 h. Each value is the mean of at least 13 photoreceptor cells recorded from each of three fish per time period. Data represent the mean ± S.E.M. *P<0.001.

LC opsin density was high in the day and low at night

We measured opsin expression using MSP to determine if the fluctuation of behavioral cone sensitivity and LC opsin mRNA expression was correlated with long-wavelength-sensitive cone opsin expression. Optical density of the long-wavelength sensitive cone was measured using MSP at 07:00, 12:00 and 22:00 h, respectively. The mean λ_{max} was 558 nm. Our estimate of λ_{max} is a close match to previous values reported for adult zebrafish (Nawrocki et al., 1985; Cameron, 2002; Chinen et al., 2003). All absorbance spectra conformed to the Govardovski et al. (2000) template for vitamin A_1-based visual pigments. The mean A_{max} at 07:00 and 12:00 noon was 18.38±0.99 mOD (N=44) and 22.17±0.76 mOD (N=75), respectively, and at 22:00 h, 18.34±0.70 mOD (N=59) (Fig. 3). An independent sample t-test showed that at 12 noon the long-wavelength sensitive cone opsin density (A_{max}) was significantly (P<0.001) increased relative to measurements made at 07:00 and 22:00 h.

Dopamine increased LC opsin mRNA expression in the early morning

We examined whether dopamine has an effect on the circadian rhythms of LC opsin mRNA expression. The experiments were performed at two different times, one in the early morning, when LC opsin mRNA expression is low, and the other in the evening, when LC opsin mRNA expression is high. Dopamine increased LC opsin mRNA expression in a dose-dependent manner, but only in the early morning (Fig. 4A). In the morning, when treated with 0.1 μmol l^{-1} dopamine, the expression of LC opsin mRNA increased to levels about 1.6±0.2-fold higher than the expression seen in control samples (P<0.01). When the dopamine concentration was increased to 10 μmol l^{-1}, the expression of LC opsin mRNA increased further to levels 2.0±0.5 fold of the control expression (P<0.01). No obvious changes occurred in LC opsin mRNA expression following dopamine administration when the experiments were repeated in the evening (P>0.01) (Fig. 4B).

The effect of dopamine on LC opsin mRNA expression is likely mediated by dopamine D_1 receptors. In the morning, LC opsin mRNA expression increased when the eyecups were incubated for 30 min with dopamine D_1 receptor agonist, SKF38393 (Fig. 5A). The increase of LC opsin mRNA expression by SKF38393 is dose-dependent. When treated with 0.1 μmol l^{-1} SKF38393, LC opsin mRNA expression

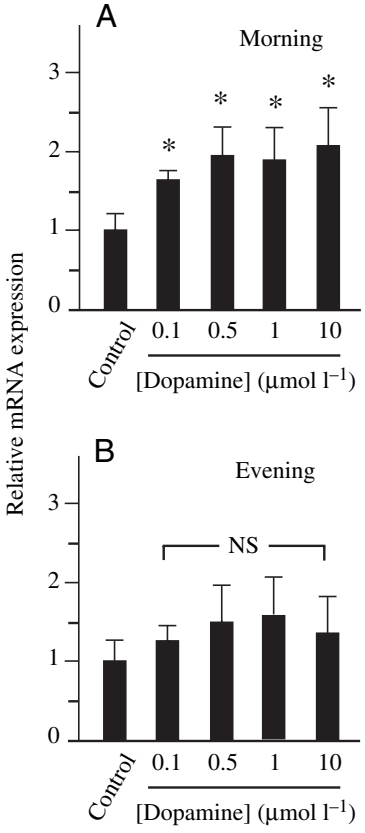

Fig. 4. Effect of dopamine (0.1, 0.5, 1.0, 10 μmol l^{-1}) on LC opsin mRNA expression in the morning (A) and in the evening (B). In the morning, dopamine increased LC opsin mRNA expression. In the evening, there was a tendency of increase, but it was not statistically significant. Data represent the mean ± S.E.M. (N=16). *P<0.01; NS, not significant.

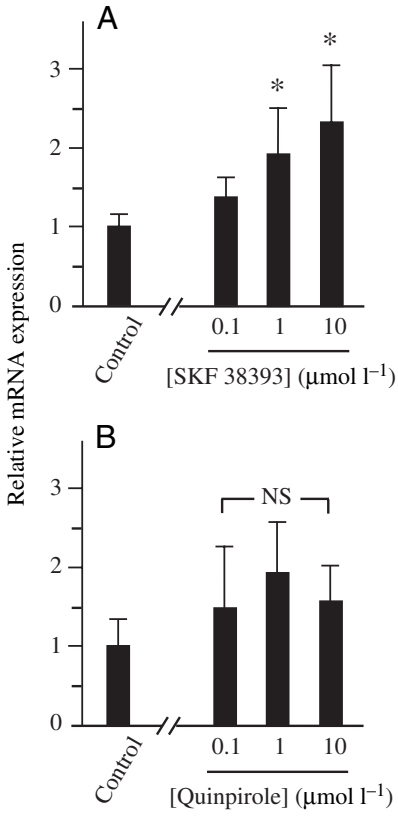

Fig. 5. Effect of dopamine D_1 or D_2 receptor agonist (0.1, 1.0, 10 μmol l^{-1}) on LC opsin mRNA expression determined in the early morning. Administration of dopamine D_1 receptor agonist (A) increased LC opsin mRNA expression at all tested concentrations. Administration of D_2 receptor agonist (B) produced little effect. Data represent the mean ± S.E.M. (N=16). *P<0.01; NS, not significant.

increased to levels about 1.3±0.2 fold higher than the control expression (P=0.08); when treated at 1.0 μmol l^{-1}, 1.8±0.7-fold (P<0.01); at 10 μmol l^{-1}, 2.3±0.8-fold (P<0.01).

Activation of dopamine D_2 receptors (by quinpirole) produced little effect on LC opsin mRNA expression (Fig. 5B). Although there was a tendency of increased LC opsin mRNA expression, in most cases the increase was not statistically significant (P>0.01). This increase may be due to a cross reaction between quinpirole and dopamine D_1 receptors. It has been shown that D_2 receptors are 2–3 orders of magnitude more sensitive to dopamine than D_1 receptors, and that D_2 receptors are expected to be activated at much lower concentrations, i.e. nanomolar (Witkovsky and Dearry, 1992; Missale et al., 1998).

Discussion

In zebrafish, red cone sensitivities determined by a behavioral assay and by LC opsin mRNA expression display robust day–night rhythms. In both cases, they were low in the early morning and high in the late afternoon. The circadian rhythms of behavioral cone sensitivity persisted for several days under constant conditions, but the rhythms of LC opsin mRNA expression dampened out after 24 h in the same conditions. The conclusions drawn from these data are twofold. First, the fluctuation of behavioral red cone sensitivity (1.2 log units) correlates with circadian expression of LC opsin mRNA (50-fold, which is about 1.7 log units) during the first 24 h of constant conditions. Second, circadian expression of LC opsin mRNA is not the sole factor limiting the circadian rhythms of behavioral cone sensitivity. In a previous study, Li and Dowling (1998) reported that in zebrafish, under constant conditions the absolute rod sensitivity fluctuated by approximately 2 log units between the day and night as measured behaviorally. However, when measured by electroretinograms (which measure outer retinal sensitivity), the difference in sensitivity was only one log unit. They proposed that the circadian oscillators expressed elsewhere in the central nervous system might play a role in circadian visual sensitivity. Recent studies have suggested that the expression of some early circadian genes, such as *period* or *clock*, and the expression of melanopsin in retinal ganglion cells may play a role in the regulation of circadian vision (Whitmore et al., 1998, 2000; Provencio et al., 1998; Cermakian et al., 2002; Morin et al., 2003). In mice, retinal ganglion cells that express melanopsin display distinct light responses (Hattar et al., 2002, 2003; Berson et al., 2002; Gooley et al., 2003; Jenkins et al., 2003). Activation of melanopsin-positive ganglion cells is sufficient to phase-shift the circadian rhythms of locomotory behaviors of the animal in the absence of both rod and cone photoreceptor cells (Ruby et al., 2002; Pando et al., 2001). It is possible that circadian signals produced by melanopsin-positive ganglion cells are passed to the outer retina *via* dopaminergic interplexiform cells. Dopaminergic interplexiform cells make numerous synapses in both inner and outer plexiform layers (Dowling, 1987).

Photoreceptor cells found in the pineal organ in the dorsal diencephalon (Kelly and Smith, 1964; Wilson and Easter, 1991; Forsell et al., 2001; Gothilf et al., 1999, 2002) may also play a role in circadian vision. The zebrafish pineal organ functions rhythmically in the absence of external time cues. For example, when cultured, the pineal organ continues to release melatonin in a circadian manner under constant lighting conditions (Cahill, 1996). Cone-like photoreceptor cells have been identified in the pineal (Hendrickson and Kelly, 1971; Pu and Dowling, 1981; Allward et al., 2001). These pineal cones express opsins as well as other genes that may or may not be expressed in retinal photoreceptor cells. In zebrafish *nrc* mutants, for example, the photoreceptor cells in the retina are degenerated, however, pineal photoreceptor cells are spared. In *nie* mutants, by contrast, degenerations are seen in both the retina and the pineal photoreceptor cells (Allward and Dowling, 2001). Circadian gene expression in the pineal photoreceptor cells may provide cues to the retina through centrifugal pathways. Centrifugal modulation of visual sensitivity has been previously reported in *Limulus* (Barlow et al., 1980; Battelle, 1991) and in zebrafish (Maaswinkel and Li, 2003).

The damping of LC opsin mRNA expression after 24 h of DD or LL may not be a result of a run-down of the circadian oscillators that control opsin expression. Rather, it may be due to un-couplings between the oscillator and the biochemical machinery responsible for expression of LC mRNA. Dalal et al. (2003) recently reported that in *Limulus* eyes, the expression of opsin mRNA displayed robust day–night rhythms when the animals were kept in normal LD conditions; opsin mRNA concentrations was low at night and high in late afternoon and early evening. This pattern of opsin mRNA expressions was regulated by light, regardless whether the eye received circadian efferent input.

Dopamine plays modulatory roles in the visual system (Baldridge et al., 1987; Witkovsky and Dearry, 1992; Ko et al., 2003, 2004). Alfinito and Townes-Anderson (2001) reported that in the retinas of tiger salamander, dopamine increased rod opsin mRNA expression. They further demonstrated that the effect of dopamine on opsin mRNA expression is mediated, *via* dopamine D_4 receptors, by cAMP-regulated protein kinase activities. In this study, we found that dopamine exerts its role on red cone opsin mRNA expression *via* D_1 receptors in zebrafish. Interestingly, such an effect of dopamine on LC opsin mRNA expression was seen only in the early morning. This could be explained by several possibilities. First, dopamine activates different types of receptors at different times in the day and night, which in turn, triggers different intracellular signaling pathways. Previous studies have shown that the effects of dopamine on photoreceptor cells or on inner retinal neurons are mediated by different receptors (Dearry and Burnside, 1986; Besharse et al., 1988; Fan and Yazulla, 1999, 2001). Ribelayga and Mangel (2003) recently revealed two separate but parallel dopamine mechanisms that regulate horizontal cell couplings in the goldfish retinas. Among those two pathways, one is mediated by an endogenous circadian

mechanism, and the other is controlled by light. In dark-adapted retinas, under circadian control the release of vitreal dopamine increased during the subjective day. This increase of vitreal dopamine is sufficient to activate D_2 receptors but not D_1 receptors. Conversely, light produced larger effects on dopamine release than the circadian clock. Under daylight, the release of vitreal dopamine further increased, thereby, activating D_1 receptors. Thus, dopamine may function differently, *via* different receptor pathways, at different times in the day and night.

The second possibility is that dopamine shifts the expression of early circadian genes. Steenhard and Besharse (2000) reported that in frog retinas, *per2* expression is acutely regulated by dopamine. In the early morning, administration of dopamine increased *per* expression threefold. The effect of dopamine on *per* expression can be mimicked by light *via* a different intracellular signaling pathway. It has been shown that functional expression of *period* plays a role in protein kinase activity (Cermakian et al., 2002), which in turn, regulates opsin mRNA expression (Cohen et al., 1992; Alfinito and Townes-Anderson, 2001). The third possibility is that *via* cAMP pathways dopamine regulates the circadian sensitivity of retinal photoreceptor cells. Ko et al. (2003) reported that in chick retinas, brief dopamine treatment (15 min) decreased the affinity of cGMP-gated channels on cone photoreceptor cells. This effect, however, was seen only during the day but not during the night. The fourth possibility is that dopamine cannot increase LC opsin mRNA expression in the evening because at this circadian time the expression is near saturation levels.

Light produces effects on circadian rhythms of visual sensitivity. It was unexpected, however, that prolonged light (>24 h) would result in decreased LC opsin mRNA expression. We can preclude the possibility of light damage to the retina due to prolonged light exposure, as histological sections revealed no alternations in the structure of the outer or the inner retinas. In fact, after 5 days of LL the absolute behavioral visual sensitivity is increased (Li and Dowling, 1998).

In summary, we have demonstrated that in zebrafish the circadian rhythms of behavioral cone sensitivity correlate with daily fluctuations of cone opsin gene expression and photoreceptor outer segment optical density in the normal LD and the first 24 h of LL and DD. We have also demonstrated that circadian expression of opsin is influenced by dopamine. It is of particular interest that this effect is seen only in the early morning, when LC opsin mRNA expression is low.

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