

Chapter 9

Circadian Rhythms and Vision in Zebrafish

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Abstract Zebrafish (*Danio rerio*) offer many advantages for studying vertebrate vision and circadian rhythmicity. They are amenable to high-throughput genetic and behavioral experiments, and the zebrafish retina is similar in structure and function to other vertebrates including mammals. Our goals here are to review some of the advances in our understanding of retinal circadian rhythm phenomena in zebrafish. We emphasize morphological and physiological rhythms, as well as circadian-regulated gene expression of the retina.

Keywords Zebrafish • Circadian rhythms • Vision • Retina • CLOCK genes • Photoreceptors • Horizontal cells • Electrical coupling • Dopamine • Synaptic ribbons

Abbreviations

6-OHDA	6-hydroxydopamine
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
<i>clk^{dg3}</i>	<i>clock1</i>
Cry	Cryptochrome
Cx35	Connexin 35

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dpf	Days postfertilization
ERG	Electroretinogram
hpf	Hours postfertilization
IRBP	Interphotoreceptor retinoid binding protein
<i>lag^{dg2}</i>	<i>lager and lime</i>
LC opsin	Long-wavelength opsin
MAPK	Mitogen-activated kinases
mPer	Mouse Period
mCry	Mouse Cryptochrome
<i>nbb</i>	<i>night blindness b</i>
OKR	Optokinetic reflex
Per	Period
PKA	Protein kinase A
RPE	Retinal pigment epithelium
VMR	Visual-motor response

9.1 Introduction

Zebrafish (*Danio rerio*) offer many advantages for studying vertebrate vision and circadian rhythmicity. They are amenable to high-throughput genetic and behavioral experiments, and the zebrafish retina is similar in structure and function to other vertebrates including mammals. Zebrafish have excellent color vision with red-, green-, blue-, and ultraviolet-sensitive cones as well as abundant rods. Embryonic development is rapid and in many respects synchronous within populations of embryos [1]: eggs are laid and fertilized at dawn, and by 24-h postfertilization (hpf), all the major organ systems have formed. Hatching takes place on the third day, and larvae begin coordinated swimming and feeding by 4–5 days of age. Key events in retinal differentiation occur at 48–50 hpf; photoreceptors first begin to express photoreceptor-specific markers including the expression of visual pigments. By 60 hpf outer segments and the adult cone mosaic have formed [2]. At this stage, zebrafish also exhibit light responses, and by day 4, the retina is functional [3].

One of the pioneers in promoting zebrafish as a useful model system for the genetic analysis of vertebrate circadian clock mechanisms was Gregory Cahill. He initially investigated the development of melatonin rhythmicity, one of the most studied manifestations of the circadian clock, in the embryonic zebrafish [4]. These studies revealed that a circadian oscillator that regulates melatonin synthesis becomes functional and responsive to light between 20 and 26 hpf [5]. Although both the retina and pineal contain circadian oscillators that drive rhythms in melatonin synthesis, as well as phototransduction mechanisms for entrainment of the oscillator, only the pineal photoreceptors have begun to differentiate at 20–26 hpf, suggesting that the first circadian melatonin rhythms are of pineal origin [4, 6]. Studies on cultured zebrafish pineals and retinas have shown that the pineal photoreceptors are capable of maintaining a robust circadian rhythm of melatonin

release with precise timing in constant darkness for 7 days. However, the retinal melatonin rhythm dampens out after a few cycles in constant darkness, indicating that retinal melatonin synthesis is regulated by a damped circadian oscillator [4]. This suggests that cellular signals from the pineal gland, which has been shown to drive rhythms of melatonin independent of any neuronal input or other master clock structure [7], may be essential for maintaining circadian rhythmicity in the retina [8].

9.2 Morphological Rhythms

9.2.1 *Photoreceptor Disc Shedding*

A fundamental characteristic of photoreceptor cells in all vertebrates is the continuous renewal of their outer segments. The basic mechanism of this process consists of formation of new membrane discs at the base of the outer segments and the shedding of old discs from their tips [9, 10]. The adjacent retinal pigment epithelial cells, which envelop the ends of the outer segments, ingest and degrade the detached membranes [9]. The shedding component of this renewal process follows a daily rhythm [11–13]. Groups of older rod membranes, assayed by an increase in the number of phagosomes in the retinal pigment epithelium (RPE), are shed early in the morning, shortly after light onset. Cone shedding also occurs in the morning in some species, but in others, cone shedding occurs after light offset. Disc shedding has not been studied in zebrafish, but studies in goldfish suggest that rods shed their membranes early in the light period, whereas cones shed membranes early in the dark period [14].

9.2.2 *Retinomotor Movements*

Since most fish lack strong pupillary reflexes to control the amount of light entering the retina, they rely on other mechanisms to adapt to changes in lighting conditions. Retinomotor movement is one such a mechanism, and it evolved early in the evolution of vertebrates. In fish and some lower vertebrates, photoreceptor inner segments elongate and contract according to changes in ambient lighting and the circadian cycle. Pigment granules in the RPE, found in fingerlike processes that surround photoreceptor outer segments, also disperse and aggregate in response to light and dark. These morphological movements in the photoreceptor layer are collectively known as retinomotor movements (see Fig. 9.1). Cone and rods display a reverse pattern of movements [15–18]. During the day or light adaptation, cones contract, rods elongate, and the pigment granules migrate to the apical part of the RPE and into the processes that surround the cone outer segments. This process

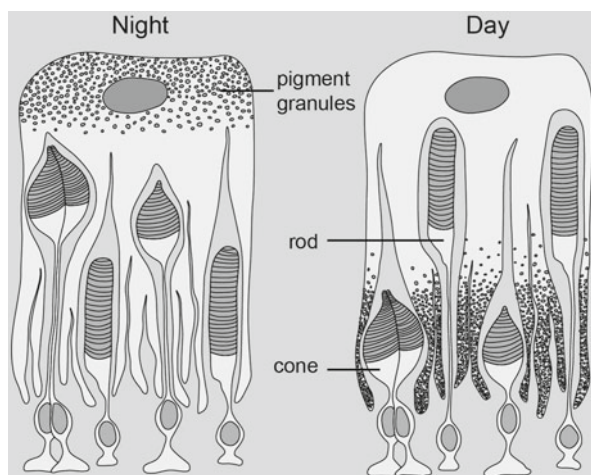


Fig. 9.1 Schematic drawing of retinomotor movements of cone and rod photoreceptors and pigment granules in the retinal pigment epithelium (RPE). Cone and rods display a reverse pattern of movements. During the day, cones contract, rods elongate, and the pigment granules migrate to the apical part of the RPE. This process allows the cone outer segments to be first in line for light absorption and shades the rod outer segments from excessive light exposure. In contrast, during the night or dark adaptation, cones elongate, rods contract, and pigment granules migrate to condense in the basal part of the RPE. This arrangement, in turn, leads to optimal light exposure for rod outer segments

allows the cone outer segments to be first in line for light absorption and shades the rod outer segments from excessive light exposure. In contrast, during the night or dark adaptation, cones elongate, rods contract, and pigment granules migrate to condense in the basal part of the RPE. This arrangement, in turn, leads to optimal light exposure for rod outer segments [19]. Elongation and contraction are mediated in the myoid region of the inner segments between the nucleus and the inner–outer segment boundary of the photoreceptor and are mediated by contractile mechanisms of microtubules and actin.

How these different types of movements (rods, cones, and pigment granules) are regulated by endogenous circadian oscillators and by light-dependent mechanisms vary among teleost species [16–18, 20]. In zebrafish, retinomotor movements follow a clear diurnal rhythm under normal light–dark cycles [21]. Rod inner segments elongate fully within 1 h after light onset, and all cones elongate maximally within the first hour after light offset. Both long single and double cones (blue- and red-, green-sensitive cones, respectively) remain elongated throughout the night until light onset, whereas short single (UV-sensitive) cones begin to contract during the last half of the night. Under continuous light conditions, retinomotor movements generally do not differ from those observed during the objective day, suggesting that light exposure overrides the endogenous circadian cycle. Under constant darkness, retinomotor movements of RPE pigment granules, long single and double cones

persist in the absence of light, suggesting that these types of movements are regulated by an internal circadian oscillator [21]. Also, double cones migrate quickly with the migration fully completed in 10–20 min in continuous darkness, whereas RPE pigment granules migrate relatively slowly (1 h) [22].

The circadian morphological changes in the retina are induced by the neuro-modulators melatonin and dopamine. Both neuromodulators are synthesized and released rhythmically, and the interplay between them results in two rhythms in counterphase: melatonin release is high during the night, and dopamine release is high during the day [23–26]. Melatonin acts to suppress dopamine release, whereas dopamine acts to inhibit melatonin production in photoreceptor cells [27]. In the fish retina, dopamine is released by interplexiform cells, which receive synaptic input from amacrine and bipolar cells in the inner plexiform layer and make synaptic contacts onto horizontal and bipolar cells in the outer plexiform layer [28, 29]. Dopamine receptors are G-protein-coupled receptors and are grouped into two families, D₁- and D₂-like receptors. The main functional difference between the two groups is based on their effects on cyclic AMP (cAMP) production [27]. Upon activation, the D₁ receptor family stimulates adenylate cyclase leading to an increase in cAMP, whereas the D₂ family inhibits adenylate cyclase leading to a decrease in cAMP production.

Myoid motility of the inner segment of photoreceptors is triggered by cAMP or calcium [30]. Earlier studies on teleost fish, such as the green sunfish, suggested that an increase of cAMP in exposed retinas or isolated cones induces cones to elongate [31, 32], whereas an increase in dopamine induces cones to contract [33, 34]. Thus, dopamine was proposed to mimic the effect of light. Studies on the blue acara, however, indicate that dopamine-depleted retinas (treated with 6-hydroxydopamine to selectively destroy dopaminergic cells) show no significant changes in rod retinomotor movements [35]. Similarly, studies on another fish, *Aequidens pulcher*, indicate that intraocular injections of dopamine have no significant effect on triggering retinomotor movements of the cones or RPE [17]. In *Xenopus laevis*, melatonin has been shown to mimic darkness by causing cone elongation under photic conditions, but this effect is blocked by an increase in exogenous dopamine concentration [36]. Thus, dopaminergic mechanisms appear to be involved in controlling the light-induced retinomotor migration in many animals; however, it is likely that additional control mechanisms are also involved.

9.2.3 Photoreceptor Synaptic Ribbons

After the discovery of retinomotor responses in fish, it was speculated that other structural changes seen in rods and cones might correlate with changes in their activity. Ultrastructural studies of cone photoreceptors in the cichlid fish revealed that the length of synaptic ribbons correlated with different degrees of light and dark adaptation [37]. Synaptic ribbons are electron-dense bars that are anchored at the

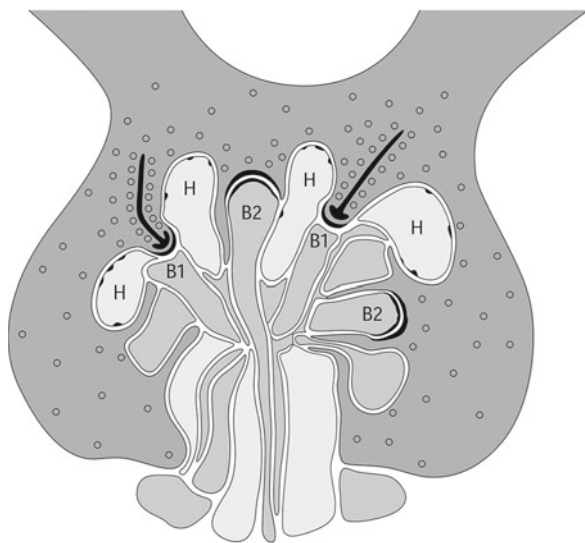


Fig. 9.2 Diagram of photoreceptor terminals in zebrafish larvae at 5 dpf. Bipolar and horizontal cell processes invaginate into the pedicle in a tight bundle to make two types of junctions: ribbon synapses (B1) and flat contacts (B2). Ribbon synapses are made onto presumed ON bipolar (B1) and horizontal (H) cell dendrites, whereas flat contacts are made onto OFF bipolar cells (B2). Synaptic vesicles surround the synaptic ribbon. Flat contacts are found between the ribbon synapses and have dense cytoplasmic material on both sides of the junction. This drawing was modified from [123] Emran et al. 2007. *PNAS*, 104(48):19126–31

synaptic terminal base by a structure called the arciform density to form a special type of synapse called the ribbon synapse [38, 39]. In the fish retina, bipolar and horizontal cell processes extend into a single invagination in rod and cone terminals in a tight bundle to make two types of junctions: ribbon synapses and flat contacts, also known as basal junctions (see Fig. 9.2). Ribbon synapses are made onto ON bipolar cell dendrites, whereas flat contacts are made onto OFF bipolar cell dendrites and are found between the ribbon synapses in fish. Cone pedicles typically contain several synaptic ribbons, whereas rod terminals usually contain just one or two. In many fish, synaptic ribbons completely disassemble at night and reform in the morning [37, 40]. Indeed, electron microscopic studies on larval zebrafish retinas demonstrated that the plasticity of synaptic ribbons in cone pedicles is a circadian phenomenon [41]. That is, whereas synaptic ribbons assemble and are more numerous during the day and disaggregate at night, this dynamic process of assembly and disassembly persists in constant darkness [41, 42]. How the ribbons are degraded is not well understood, but small round densities observed in the terminals at night suggest that ribbons are formed from an aggregation of subunits [41].

9.2.4 Spinule Formation in Horizontal Cells

In fish, such as the *Aequidens pulcher*, structural changes of synaptic ribbons on the presynaptic side in cone pedicles are correlated with changes on the postsynaptic side in horizontal cells. As mentioned above, horizontal and bipolar cells send their dendritic processes into the photoreceptor terminals to form synapses. In the fish retina, two distinct types of horizontal cells are observed, either cone or rod specific [43–46]. Dendrites of cone horizontal cells display numerous fingerlike projections, called spinules in the day. These spinules undergo rhythmic morphological changes: they extend during the day, but retract at dusk. Formation and disappearance of spinules takes about an hour and is circadian related [40]. Studies in goldfish revealed that both light-evoked and circadian-regulated cone spinule formation is dependent on dopamine [47]. When fish were treated with 6-hydroxydopamine (6-OHDA) to selectively destroy the dopaminergic neurons in the retina, formation of spinules was severely suppressed in the light. Thus, spinule formation in cone horizontal cells appears to be regulated by rhythmic exogenous dopamine levels in the retina [17]. As yet, spinule formation and disassembly have not been studied in zebrafish.

9.3 Physiological Rhythms

9.3.1 Behavioral Rhythms in Zebrafish

Another reason why zebrafish is a good model system to study not only vision but also circadian rhythms is their amenability for behavioral assays. For example, Cahill and colleagues examined circadian regulation of gross locomotor activity in adult and larval zebrafish [48, 49]. These authors automated the continuous recording of swimming movements in individual fish by using either infrared motion detectors for adult fish or video image analysis for larval fish. Locomotor activity rhythms in adult zebrafish are somewhat variable with about 70 % of fish showing strong activity patterns, with a characteristic diurnal activity profile; adult fish are more active during the light phase of the cycle than the dark cycle. Under constant conditions, such as continuous dark or light, circadian rhythmicity in locomotor activity continues for up to 10 days. Together these data suggest that zebrafish locomotor activity is regulated by a circadian clock [48].

The locomotor activity of larval zebrafish (5–18 days) is even more consistent, with over 95 % of larvae exhibiting a clear circadian rhythm. Similar to adult fish, larval zebrafish are more active during the subjective day and display a periodicity of about 25 h under dark-adapted conditions [49]. Cahill and colleagues used these behavioral rhythms as an assay to screen for mutants that display altered gross locomotor activity patterns. One such mutant is the *lager and lime* (*lag^{dg2}*) mutant, and its circadian periodicity is shortened by 0.7 h in heterozygotes and by 1.3 h in

homozygotes [50]. The *clock1* (*clk^{dg3}*) mutant, which has a mutation in the PAS domain of the Clock1 protein, also displays a shortened biological clock [51].

Behavioral assays designed to screen for deficits in the visual system of larval zebrafish include the optokinetic reflex (OKR) and the visual-motor response (VMR) assays [42, 52]. The OKR is a stereotyped eye movement, exhibited by all vertebrates in response to objects in motion when the head remains stationary. When zebrafish larvae are placed inside a rotating drum with alternating black and white bars moving in either direction, the animals move their eyes with a smooth pursuit until the bar moves out of the field of vision at which point their eyes rapidly move back to the starting position. The latter eye movement is called a saccade. In humans this reflex can be tested in newborns but in larval zebrafish as early as 4-day postfertilization (dpf). The use of this behavioral assay in large-scale genetic screens has led to the isolation of many mutant strains with specific defects in the visual pathway [52]. An especially interesting example is the belladonna mutation, where an axonal misrouting effect at the optic chiasm leads to a reversed OKR [53].

The VMR is a behavioral assay that is based on the ON and OFF responses of larval zebrafish in response to changes in illumination. Similar to the locomotor behavioral assay used to study circadian rhythms, zebrafish larvae are placed individually in 96 well plates and the motion of each fish recorded. However, instead of measuring gross locomotor activity, this assay measures the rate at which larval fish moves in each well with the use of a high-speed camera. The ON responses, i.e., the change in motor activity in response to lights on, consist of a sharp and robust increase of motor activity for the first 20–30 s after the start of the light stimulus. After the initial ON response, the larvae reduce their activity levels dramatically and show a partial freeze response, slowly returning to baseline activity levels within a few minutes. The OFF response, which is elicited by switching the lights off, is also characterized by a sharp and robust increase of motor activity that follows immediately after the illumination change. After the initial sharp OFF response, the animals reduce their activity to an intermediate level, and it typically takes 10–15 min to return to baseline levels [42]. Thus, the VMR ON and OFF responses consist of sharp increases in motor activity immediately after light increments or decrements. There are also small, slow changes in activity that occur, especially after light extinction, that do not appear retinal related. As expected baseline activity levels are higher during the subjective day than during the subjective night [54].

9.3.2 Visual Function and ERG Light Sensitivity in Adult Zebrafish

Do circadian rhythms influence visual function in adult zebrafish? To address this question, a behavioral assay based on the escape response of fish to avoid predators was developed [55]. Normally adult zebrafish swim around slowly when confined in a container that contains a post in its middle. However, when a threatening object

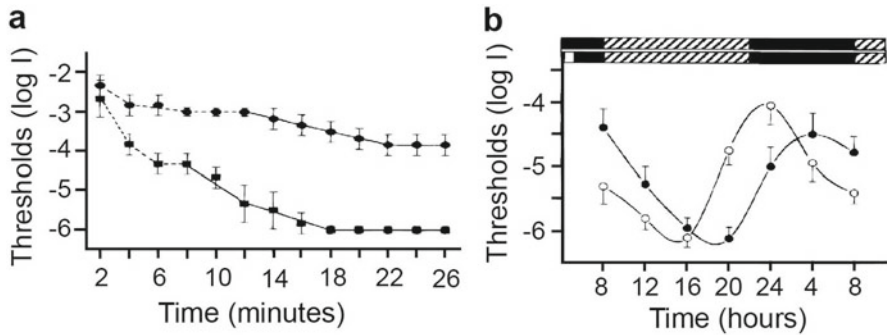


Fig. 9.3 Visual sensitivity of adult zebrafish. **(a)** Dark adaptation curves determined at 4 a.m. (circles) and 6 p.m. (squares) of cone and rod systems in adult zebrafish maintained in normal LD cycle. Dashed lines represent cone function; continuous lines represent rod function. Note that both cone and rod thresholds were significantly higher at 4 a.m. as compared to 6 p.m. **(b)** The variation of visual sensitivity during a 24-h period of constant darkness (DD) in control (closed circles) and test animals (open circles). In the control animals, thresholds were highest in the early morning hours (~4 a.m.) and lowest in the late afternoon hours (~6 p.m.). In the test animals, the rhythm was shifted forward by about 4 h by an early morning 1-h pulse; thresholds were highest at midnight and lowest at about 2 p.m. The black bars at the top of the panel represent the subjective night, and the hatched bars represent the subjective day. The small white bar indicates the 1-h light pulse given between 4 and 5 a.m. to the test animals. Data represents the means $\pm$ S.D. Modified from [55] Li and Dowling, 1998. *Visual Neuroscience*, 15(5):851–7. Copyright © 1998 Cambridge University Press

comes into view (i.e., a black segment on a rotating drum), the fish immediately turn and rapidly swim away to hide behind the post. Using this vision-mediated reflex of zebrafish, cone and rod light sensitivity was determined under both cyclic and constant lighting conditions [55]. Under a normal light–dark cycle, the visual system is strongly influenced by the circadian clock such that it is most sensitive in the late afternoon and least sensitive in the early morning hours. Over the course of 24 h, the threshold of light intensity shone on the drum required to elicit a positive response to the visual stimulus fluctuated by about 1.4 log units for cones and about 2.2 log units for rods (see Fig. 9.3a). This rhythm persisted in animals maintained in constant darkness for several days and could be phase shifted by light pulses (see Fig. 9.3b). Also, when fish are kept under constant darkness, the electroretinogram (ERG), a field potential generated primarily in the outer retina by photoreceptor and bipolar cells, shows similar changes in visual sensitivity as a function of time. However, the ERG visual sensitivity changes are about one log unit smaller than the visual changes measured behaviorally. Hence, not all aspects of visual sensitivity rhythmicity are reflected by changes in the outer retina, suggesting circadian regulation of the inner retina and/or higher visual centers is involved as well [55]. To study how dopamine affects visual sensitivity, Li and Dowling depleted the zebrafish retina of dopaminergic neurons (i.e., the interplexiform cells) by 6-OHDA treatment. Under dark-adapted conditions, only cone system function was observed, indicating that dopamine depletion leads to a loss of rod function as measured behaviorally.

However, ERG recordings were not affected, suggesting that rod photoreceptors are fully functional and transmit electrical signals to ON bipolar cells (as judged by the b-wave). Recordings of the ganglion cell discharges in dopamine-depleted retinas point to some inner retinal circuit dysfunction, such that rod signals are blocked in the inner plexiform layer [56]. Together, the results described above indicate that behavioral visual sensitivity is regulated by a circadian clock and that dopamine plays a crucial role in regulating the rhythmicity in both the inner and outer retina.

One of the major benefits of working on the zebrafish system is the relative ease of isolating mutants. For example, Li and Dowling identified a dominant mutation called *night blindness b* (*nbb*) that causes a disruption of the olfactoretinal centrifugal pathway and reduces the number of dopaminergic interplexiform cells in the retina [57]. After prolonged dark adaptation, this mutant displays fluctuations in its visual sensitivity by 2–3 log units from day to day. In contrast, wild-type fish maintain a constant level of visual sensitivity when measured at the same time on different days. ERG recordings indicate that outer retinal function is normal, whereas ganglion cell discharges suggest that the visual deficit relates to abnormalities in the inner retina. Also, ablation of the olfactory epithelium and olfactory bulb in wild-type fish phenocopy the visual abnormalities seen in the *nbb* mutant [57]. Thus, it is fair to conclude that dopaminergic interplexiform cells and the olfactoretinal efferents that innervate them are crucial in modulating circadian rhythmicity of the visual system.

9.3.3 Visual Responses and Light Sensitivity in Larval Zebrafish

To determine whether vision in larval zebrafish is regulated by a circadian clock, Emran et al. measured the OKR and VMR responses of 5–6-day-old zebrafish larvae at several time points during the day and at night [42]. During the day, fish respond normally in these behavioral tests, whereas during the night, fish display neither OKR nor VMR responses (see Fig. 9.4a). ERG responses recorded during the day and at night substantiated these results; responses were normal in amplitude throughout the day but were almost absent at night, suggesting that vision is severely reduced at night (see Fig. 9.4b). This phenomenon is largely circadian driven as fish show similar dramatic changes in visual responsiveness when raised in constant darkness from 4 to 7 dpf. However, light exposure at night partially restores the responses. Two mechanisms by which visual responsiveness might be decreased were examined: (1) photoreceptor outer segment activity was reduced at night as judged by a decrease in the ERG a-wave, and (2) synaptic ribbons in cone pedicles disassembled at night [42]. It should be pointed out that in this study mainly cone-mediated vision was examined during the day and at night, as rod responses are minimal in 5–6-day-old larvae. It is possible that rod vision is still present at night

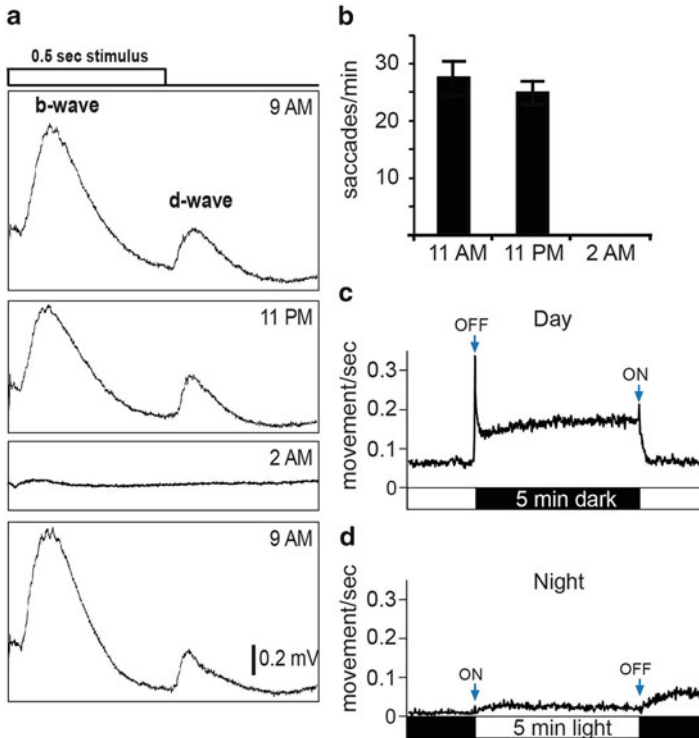


Fig. 9.4 Larval zebrafish exhibit a day/night cycle of visual responsiveness sensitivity. (a) Representative ERG recordings to a 0.5-s white light stimulus are shown at four different time points: at 9:00 a.m., when the ambient lights are turned ON; 11:00 p.m., shortly before the lights are turned OFF; 2:00 a.m., after the lights have been OFF for 3 h; and at 9:00 a.m. the next morning, after the lights are turned ON again. Note the large reduction of ERG amplitudes at 2:00 a.m. (b) Larval zebrafish exhibit a robust OKR at 11:00 a.m. and 11:00 p.m. (shortly before the lights are turned OFF at night) but not at 2 a.m. (3 h after the lights were turned OFF). (c) Larval zebrafish display ON and OFF visual-motor responses (VMR) during the day but fail to exhibit transient startle responses at night (each trace represents an average of 240 responses from 80 individual fish). During the daytime, motor output increased in response to a 5-min light off stimulus with prominent OFF and ON responses (first stimulus was introduced at 2:00 p.m.). (d) At night, the VMR responses to a 5-min light stimulus were abolished (first stimulus was introduced at 2:00 a.m.). Modified from [42] Emran et al., 2010. *PNAS*, 107(13):6034–9

and is not under the influence of the circadian clock, but rod vision does not become prominent until zebrafish are 2–3 weeks old.

Why do larval zebrafish turn off their retinas at night? It may be to save energy. The retina is extraordinarily active metabolically at night, because the photoreceptors are maximally active at night. A single dark-adapted mouse rod consumes 10^8 ATP molecules per second, and retinas contain thousands to millions of photoreceptors [58]. At 5–6 days of age, zebrafish have used up much of their yolk and are not

yet very robust feeders. Thus saving energy, especially at night when they are not using vision, may be essential for them to survive.

9.3.4 *Circadian Rhythms in Horizontal Cells*

Horizontal cells are electrically coupled to one another through gap junctions and form an extensive electrical network that dramatically increases the receptive field size of the cells [59]. Neuromodulators, such as dopamine, decrease horizontal cell receptive field size by triggering the uncoupling of gap junction channels [60, 61]. Work on zebrafish horizontal cells suggests that dopamine's main effect on gap junction channels is to modulate their spontaneous gating kinetics by reducing both the frequency and the duration of single channel openings. Thus on average, channels spend less time in the open state and, therefore, conduct less current as a population [62]. Electrophysiological recordings from single cells in intact fish retinas reveal that uncoupling of horizontal cells leads to an increase in response amplitude to small spots of light (because of reduced current shunting between the recorded and surrounding cells) as well as a decrease in the responses to annular stimulation, indicating that the size of the receptive field is reduced [61, 63]. Light adaptation of the retina, both flicker and sustained light stimulation, decreases horizontal cell receptive field size and gap junction coupling. However, only flicker-induced but not sustained light-induced uncoupling leads to an increase in dopamine levels in the retina [64, 65]. Recent work indicates that other neuromodulators, nitric oxide, retinoic acid, and zinc are also involved in the regulation of horizontal cell receptive field size [66–68].

The receptive field size of fish horizontal cells is altered by activation of dopaminergic receptors. As mentioned earlier, D_1 and D_2 are G-protein-coupled receptors that are classified based on their effects on cAMP production. Recordings from pairs of perch and catfish horizontal cells in culture showed that the electrical conductance across these cells is decreased by exogenous application of dopamine or by a D_1 agonist. This decrease in cell to cell communication is also observed when cAMP or agents such as forskolin, which increase the activity of adenylyl cyclase, are applied. Application of protein kinase A (PKA) blockers reverses the action of forskolin suggesting that dopamine uncouples horizontal cells by raising cyclic AMP levels and thereby activating PKA [61, 69]. Thus, dopamine activates D_1 receptors on fish horizontal cells and increases cAMP production and PKA activity, which subsequently leads to a decrease in gap junction coupling and receptive field size. Further studies in goldfish showed that specific activation of D_2 receptors by an agonist, quinpirole, has the opposite effect and increases the electrical coupling between horizontal cells, as indicated by a decrease in responses to centered spots or slits of light [70]. However, D_2 receptors are not found on horizontal cells but on dopaminergic interplexiform cells. Activation of these D_2 receptors decreases dopamine release from the interplexiform cells; hence, the effect of D_2 receptor

activation on horizontal cell coupling is indirect, mediated by a decrease of dopamine levels in the retina [70].

The effects of light and dark on horizontal cell coupling are murky and even disputed. Several reports have claimed that when a retina has been in the dark for a prolonged period, the horizontal cells are physiologically uncoupled [63, 71–73], and impressive histological evidence has been provided in support of this result. These data showed that Lucifer yellow, a gap junctional permeable dye, is confined to the cell in which the dye is injected and a few surrounding cells when retinas are thoroughly dark-adapted. As dim light illuminates the retina, however, the cells become coupled, shown both physiologically and histologically, but then with bright illumination of the retina, the cells uncouple again [73]. This has been termed triphasic adaptation and has been observed also in horizontal and amacrine cells of the rabbit retina [74, 75]. Others, on the other hand, claim that in dark-adapted retinas, the horizontal cells are extensively coupled and when light is presented to the retina, the cells uncouple [76, 77]. In other words, these later authors do not observe an uncoupling of the horizontal cells when the retinas are dark-adapted for prolonged periods of time.

There is general agreement that dopamine uncouples horizontal cells, and so one might suppose dopamine levels are high in retinas dark-adapted for a prolonged period, but that their levels decrease in dim to moderate light, but then increase again in bright light. Most (but not all) dopamine release studies [65] have claimed that dopamine release is low in the dark (although there is some ongoing dark release) and that dopamine levels increase substantially in the light. It is important to remember however that uncoupling of horizontal cells in fish retinas can be caused by other neuroactive substances including nitric oxide and retinoic acid, and so which agent is responsible for the uncoupling in prolonged darkness (assuming it does happen) is not known. Clearly more work is needed to sort out the effects of prolonged darkness, dim and bright light on horizontal cell coupling, and determining the role of modulators other than dopamine in regulating the coupling.

How does dopamine affect horizontal cell coupling? Studies in goldfish revealed that retinal dopamine release is under the control of the circadian clock, leading to increased dopamine production during the subjective day [78]. However, no effect on horizontal cell coupling was observed during subjective day when animals were kept continuously in the dark [76, 77]. Mangel and colleagues propose that in the goldfish retina two different dopamine systems are present. One system is controlled by an endogenous clock that activates low-threshold D_2 -like receptors during the subjective day—i.e., circadian-dependent release. The second is controlled by light and involves the activation of higher-threshold D_1 -like receptors. As mentioned earlier, light increases dopamine release to a greater extent than does the circadian clock. Hence, sufficient dopamine is released to activate D_2 -like receptors during the subjective day, but not D_1 -like receptors that are involved in horizontal cell uncoupling in the retina [76, 77]. Thus, horizontal cell coupling is not circadian regulated, at least by dopamine.

9.3.5 *Circadian Control of Photoreceptor Electrical Coupling*

Cone horizontal cells display a substantial increase in light sensitivity, as well as a spectral sensitivity shift from red sensitivity (i.e., cones) to green sensitivity (i.e., rods) under dark-adapted conditions [78–80]. These results suggest that cone horizontal cells receive input from rods when sufficiently dark-adapted since cone horizontal cells receive direct synaptic input only from cones. Consequently, rod input to cone horizontal cells must arrive via rod–cone synapses. Further, the light responses of dark-adapted cone horizontal cells vary in waveform, light sensitivity, and spectral sensitivity depending on the time of day [79, 81], suggesting circadian regulation of these junctions. Cone input to cone horizontal cells predominates in the day and rod input predominates at night, as indicated by the increase in light sensitivity as well as by the spectral sensitivity shift from red to green. Application of exogenous dopamine also induces a shift from rod to cone domination under constant darkness [78, 80, 82]. During the subjective day in animals maintained in the dark, endogenous dopamine levels increase and presumably activate D₂ receptors on both rods and cones. Activation of the D₂ mechanism decreases intracellular cAMP and PKA activity in photoreceptor cells, resulting in the decoupling of rods and cones, thus favoring cone input to cone horizontal cells [78].

Similar to horizontal cells, rods and cones are coupled to each other via gap junctions [83]. Using tracer coupling and recording in the goldfish retina, Ribelayga et al. measured the extent and strength of rod–cone electrical coupling during the day and night [82]. They report that the conductance of rod–cone gap junctions is regulated by a circadian clock and is increased at night. During the subjective day, activation of D₂ receptors decreases rod–cone coupling, whereas at night, rod–cone coupling is extensive and allows cones to receive light signals from rods [82]. Thus, in the goldfish retina, activation of D₂ receptors in the light decreases rod–cone coupling [78, 80, 82] and the same holds true in the rabbit [84]. It is worth pointing out, however, that in other animals, such as in the salamander retina, D₂ receptor activation or light adaptation increases rod–cone coupling [85, 86]. Similarly, rod–cone coupling is greater in the turtle retina when rods are slightly light-adapted as compared to fully dark-adapted rods [87, 88].

How are cells in the retina electrically coupled to each other? The building blocks for gap junctions are specialized proteins called connexins. Six connexins assemble into a connexon, which forms a cytoplasmic bridge by joining with another connexon on an adjacent cell. A gap junction typically consists of many connexons gathered in close proximity. There are many subtypes of connexins in the retina [89], all of which have phosphorylation sites. It is believed that the phosphorylated state of connexins modulates the electrical conductance across a junction, such that phosphorylation decreases conductance between horizontal cells, but increases it between photoreceptor cells [27, 90]. In fish, connexin 35 (homologous to connexin 36 of mammals) is found at both cone–cone and rod–cone gap junctions [91, 92]. A study in zebrafish found that phosphorylation of connexin 35 (Cx35) at serine residues 110 and 276 directly correlate with changes in photoreceptor coupling. Tracer

coupling of Neurobiotin is reduced during the day, when phosphorylation is low, and is extensive (especially in the cone network) at night when phosphorylation is high. Phosphorylation of Cx35 is regulated by PKA, as pharmacological manipulation of PKA activity mimics both the daytime and nighttime phosphorylation state and coupling of photoreceptors. Somewhat surprising is the observation that not only is rod–cone coupling enhanced at night, but the cone network is extensively coupled as well [92]. What function enhanced cone–cone coupling serves at night, when the cones are outside their operating range, is not clear.

9.4 Clock Genetics in Zebrafish

9.4.1 *Light-Sensitive Peripheral Oscillators*

Following the isolation of the first clock gene in mouse, *CLOCK* [93], the zebrafish homolog of this gene was discovered [6]. As expected, Clock is expressed in the brain, the eye, and the pineal gland in zebrafish. However, unlike its mammalian counterpart, zebrafish Clock displays robust circadian oscillation in gene expression in these defined pacemaker structures as well as in other tissues such as heart and kidney [6]. Moreover, cultured tissues from the heart and kidney maintain rhythmic expression of the *clock* gene under both continuous dark and light–dark conditions. Perhaps the most striking observation is that these cultured organs are themselves light sensitive and can be entrained by light/dark cycles. When the light–dark cycle is reversed, the cultured organs show a reverse cycle of Clock rhythm from day 2 onwards [94]. Cell lines derived from zebrafish embryos, PAC-2 and Z3, also contain an independent, light-entrainable circadian oscillator, suggesting that many cell types in zebrafish contain their own endogenous peripheral clocks [95]. The discovery of light-sensitive peripheral tissue circadian oscillators was first demonstrated in insects [96, 97]. For example, in *Drosophila* explanted tissues, including head, thorax, and abdominal tissues, show rhythmic expression of another clock gene *period* (*per*), are directly responsive to light [97], and can be entrained independently of the central pacemakers in the brain [98, 99]. This phenomenon has also been validated in mammals. Using a PER2::LUCIFERASE fusion protein reporter in mice, Takahashi and colleagues demonstrated that peripheral tissues express self-sustained circadian oscillations for more than 20 cycles in culture [100]. In mice, however, light cannot reset peripheral oscillators directly as is the case for *Drosophila* and zebrafish.

9.4.2 *Clock Genes*

The elucidation of the core circadian clock mechanism occurred first in the fruit fly, *Drosophila*, whereas Takahashi and colleagues, building upon the invertebrate work, showed that a similar mechanism works in vertebrates [101–103].

The prevailing model for the core molecular mechanism is a transcription–translation feedback loop that cycles with a period of about 24 h. In this feedback loop, a transcription factor, formed by heterodimerization of CLOCK and BMAL1, drives the expression of three Period genes (in mice, *mPer1*, *mPer2*, and *mPer3*) and two Cryptochrome genes (*mCry1* and *mCry2*). The mPER and mCRY proteins heterodimerize to inhibit their own transcription by translocating back to the nucleus, where they bind to the Clock:BMAL1 complex [104]. Due to a late whole-genome duplication event in the teleost lineage [105], extra copies of clock genes have been discovered in the zebrafish as compared to the mouse. For example, zebrafish have six rhythmically expressed cryptochrome genes (*cry1a*, *1b*, *2a*, *2b*, *3*, and *4*) [106]. Phylogenetic analysis revealed that *Cry1a*, *1b*, and *2b* share most sequence homology to mCRY1 and can repress transcriptional activation by Clock:BMAL1 dimers. However, *Cry3* and *Cry4* do not inhibit Clock:BMAL1-mediated transcription. *Cry3* shares some sequence similarities with *mCrys*, whereas *Cry4* is most divergent from all other vertebrate CRYs and resembles *Drosophila* CRY. As mentioned earlier (Sect. 4.1), many tissues throughout the body of both zebrafish and *Drosophila* are photosensitive. But what molecules are responsible for detection of light in these peripheral tissues? Two independent studies have identified the blue-sensitive pigment Cryptochrome (CRY) to be the major contributor for entrainment of peripheral oscillators in *Drosophila* [107, 108]. Similar results have been reported in zebrafish. Using the light-inducible embryonic zebrafish cell line Z3, Cermakian et al. reported that blue-sensitive pigments, probably Crys, are coupled to the MAPK (mitogen-activated protein kinase) pathway to elicit light-induced transcriptional activation of clock genes [109]. This was later confirmed by Tamai et al., demonstrating that overexpression of *Cry1a* protein in a *Per1:Luciferase* zebrafish cell line acts as a strong repressor of clock function and mimics the effects of sustained light [110]. Indeed, *cry1a* shows a light-driven pattern of expression and is not rhythmic in constant conditions, whereas the other *cry* genes are predominantly clock regulated [106].

Zebrafish homologs of the three mammalian *per* genes have also been identified: two *per1* homologs (*per1a* and *1b*) as well as a single copy of *per2* and *per3* genes. *Per1* and *per3* genes display a similar rhythm in expression pattern. However, *per2* is stimulated by light and is not rhythmic in constant conditions [95, 111]. Similar to *cry1a*, *per2* has also been suggested to act as a photoreceptor and be involved in entrainment pathways [110, 112]. Finally, three *clock* genes (*clock1a*, *1b*, and *2*) and three *bmal* genes (*bmal1a*, *1b*, and *2*) have been isolated and shown to interact as heterodimers [113–115]. Generally, the expression pattern for *bmal* and *clock* genes parallels each other with a peak just after the LD transition [116].

9.4.3 Clock-Regulated Genes in the Retina

In zebrafish retinas, the early circadian genes (*clock*, *bmal*, *per*, and *cry*) are expressed in several cell types, including photoreceptor cells. Opsin expression of

both rhodopsin and cone pigment has been shown to fluctuate between day and night in several vertebrates [117, 118]. Circadian rhythmicity in *long-wavelength cone (LC) opsin* mRNA levels has also been reported in zebrafish [119]. Expression levels reach a peak in the afternoon and a low in the early morning, and these fluctuations persist in constant darkness. To demonstrate that Clock is involved in regulating the circadian rhythms in photoreceptor cells, translation of clock mRNA was inhibited in zebrafish embryos [119]. This resulted in diminished circadian rhythms of *opsin* mRNA expression and also abolished the fluctuations in cAMP concentrations observed in photoreceptor cells. The authors proposed that the regulatory effect of Clock on *LC opsin* mRNA expression is mediated by cAMP-dependent cascades. That is, Clock regulates rhythmic synthesis of cAMP, which in turn regulates circadian expression of *LC opsin* mRNA. To examine whether rhodopsin gene expression also exhibits circadian fluctuation, Li and his team used a transgenic zebrafish line that expresses a short-life GFP under the transcriptional control of the rhodopsin promoter [120]. Similar to *LC opsin* expression, *rhodopsin* promoter expression in rod photoreceptor cells fluctuates rhythmically in constant darkness with an expression peak in the subjective late afternoon. However, not all rod photoreceptor cells are synchronized with each other, and in some cells *rhodopsin* promoter expression peaks in the subjective early morning, whereas in other cells it peaks in the afternoon or at night. Light synchronizes rod photoreceptor oscillations by transiently decreasing *rhodopsin* promoter expression. Application of dopamine or a D₂ receptor agonist also triggers synchronization of *rhodopsin* promoter expression in rod photoreceptor cells. In addition to light and dopamine, the circadian rhythms of *rhodopsin* gene expression may also be synchronized by central mechanisms, including the rhythmic production of melatonin by the pineal gland [120].

Two other retinal rhythms that have been studied in zebrafish include melatonin synthesis and mRNA for interphotoreceptor retinoid binding protein (Irbp). As discussed in more detail above (see Introduction), melatonin synthesis is circadian regulated and persists in retinal organ culture for a few cycles in constant darkness [4]. Irbp is synthesized and secreted by photoreceptors and mediates retinoid trafficking between photoreceptors and pigment epithelium. Expression of *irbp* mRNA is circadian regulated and is higher during the day than during the night. This rhythm persists for 2 days under constant conditions [121]. In addition, mRNA expression of this protein is regulated differentially in various photoreceptor subtypes. For example, *irbp* mRNA is expressed by all cone types and to a lesser extent by rods during the day. At night, *irbp* mRNA expression is restricted to the ultraviolet short single cones [121]. Despite the fluctuation in expression pattern of this protein at the level of photoreceptors, the total protein content in the retina remains constant throughout the day–night cycle [122]. It appears that the turnover rate of Irbp is faster during the day than during the night. Thus, the increased expression of mRNA and protein synthesis of this protein is to offset the increased turnover rate during the day to maintain constant amounts of the protein.

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