Chapter 9
The Amphibian Clock System

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Abstract The variety of experimental approaches possible in amphibians, in particular, in the anuran species *Xenopus laevis*, have been crucial in discovering key regulators of circadian rhythms, such as melatonin and melanopsin. Differently from mammals, amphibians are characterized by the peculiar presence of multiple anatomical structures and cell types that feature photosensitive and self-sustained circadian activities. In particular, in amphibians, both the retina and the pineal complex are photosensitive and display circadian melatonin secretion. Furthermore, skin melanophores are light responsive and represent an exclusive model to study a peripheral circadian clock. In this chapter, we will review (1) the cellular and molecular mechanisms regulating circadian rhythms in amphibian retina, (2) the molecular bases of pineal circadian rhythms and its link to cell differentiation and cell proliferation, and (3) the *Xenopus* melanophore system as an example of a well-described peripheral, light-sensitive, clock.

9.1 Introduction

The presence in *Xenopus laevis* of multiple photoreceptive tissues including opsin-positive neurons of the hypothalamic magnocellular preoptic nucleus [1] and skin melanophores, in addition to the retina and the pineal complex, raised the question of the existence of a master circadian pacemaker in amphibians. Physiological experiments aimed at identifying circadian oscillators in *Xenopus*, and based on the ablation of specific structures, have shown that the pineal gland, the frontal organ, and the eyes are all required to maintain the normal period of free-running activity rhythms. However, light-entrainable circadian oscillators were still active even after removal of all three structures [2]. On the contrary, an intact suprachiasmatic area was found to be absolutely required for free-running

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behavioral rhythmicity, suggesting that the hypothalamic oscillator is the main regulator of circadian rhythms, which are in turn modulated by the pineal and retinal activities [3]. More recently, the majority of work performed in amphibians has focused on the identification of the molecular modulators of the circadian clocks in the retina, pineal gland, and melanophores, while the suprachiasmatic nucleus has not been further characterized in molecular terms. As for many other aspects of amphibian biology, the model of choice for these studies has been *Xenopus laevis* because it offers a variety of experimental approaches ranging from functional screens aimed at isolating novel genes, to the generation of cell-type-specific transgenesis [4].

9.2 The Retinal Circadian System in *Xenopus*

Autonomous circadian rhythms have been demonstrated in the retina of all vertebrate classes, indicating the presence of a local circadian clock. The easy accessibility of *Xenopus* retina, together with the large size of its cells and its high survival rate in culture, has made it a well-suited system to study retinal development and physiology. The mature amphibian retina displays the typical vertebrate organization into three main cell layers: outer nuclear layer (ONL), composed of rods and cones photoreceptors; inner nuclear layer (INL), containing different types of interneurons (amacrine, bipolar, and horizontal cells); and ganglion cell layer (GCL), whose axons fasciculate to form the optic nerve and send nervous signals to the brain (Fig. 9.1). Many of the factors that concur to retina development have been initially isolated and functionally described in *Xenopus* and subsequently

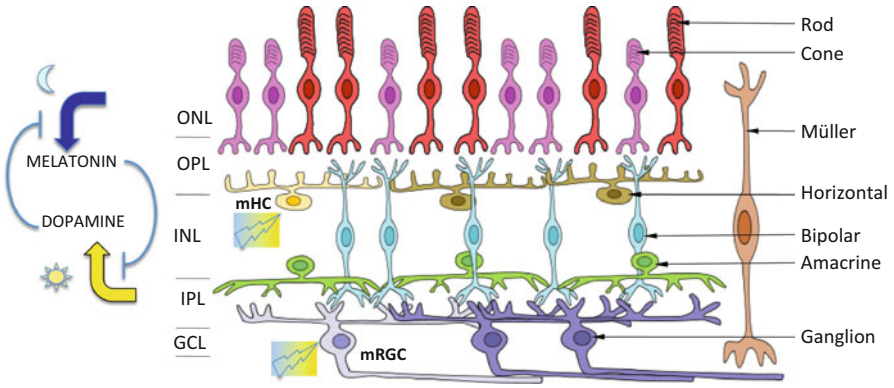

Fig. 9.1 Organization and neurochemical outputs of the retinal circadian system in *Xenopus laevis*. The mutual antagonism between melatonin secreted by photoreceptors (*blue arrow*) and dopamine released by amacrine cells (*yellow arrow*) is shown on the *left*. mHC and mRGC indicate photosensitive melanopsin-expressing horizontal cells and retinal ganglion cells, respectively. *GCL* ganglion cell layer, *INL* inner nuclear layer, *IPL* inner plexiform layer, *ONL* outer nuclear layer, *OPL* outer plexiform layer

demonstrated to be extremely conserved among vertebrates [5]. In particular, in this animal model, it was shown that the circadian clock controls several parameters of retinal physiology, including melatonin and dopamine synthesis, rod disk shedding, cone elongation, and light sensitivity [6, 7].

Taking advantage of the features of *Xenopus* retina, Besharse and Iuvone [8] were able to isolate eye cups and maintain them in culture. This allowed the authors to investigate, in isolated conditions, the circadian activity of retinal arylalkylamine N-acetyltransferase (AANAT), the penultimate enzyme in the melatonin biosynthetic pathway. As they showed, in cultured eye cups, AANAT activity is regulated rhythmically, increasing during the night and decreasing during the day. This rhythmicity is also observed in constant darkness and can be reset by light, indicating that the retina harbors an autonomous circadian oscillator.

In isolated eye cups, transcription of the tryptophan hydroxylase (TPH) gene, coding for the rate-limiting enzyme of melatonin biosynthesis, is also cyclic reaching a peak in the early night. Therefore, also TPH mRNA is regulated by the circadian retinal clock, although, differently from AANAT activity, it is not acutely affected by light [9].

Gene expression studies in *Xenopus* have shown that a number of known clock genes and clock-regulated genes, including AANAT, are expressed in the retina already during early neurogenesis and in some cases are even maternally inherited in the oocyte. However, their expression appears to become rhythmic only in advanced phases of retinogenesis [10–12].

Cultures of isolated photoreceptor layers from *Xenopus* retina showed that these cells produced melatonin rhythmically, with high levels at night and low levels during the day. The average period is 24.3 h, consistently with the cyclic phase of AANAT activity and TPH transcription. The same rhythms are maintained also in constant darkness and are resettable by light cycles or by cyclic treatment with quinpirole, an agonist of D2-like (D2, D3, and D4) dopamine receptors [13]. In the retina, light induces amacrine cells and interplexiform neurons to secrete the neurohormone dopamine, which, in turn, increases *per2* levels specifically in photoreceptors [14, 15]. Both quinpirole and light treatments reset the oscillator toward a daytime phase. However, while quinpirole was shown to activate a signaling pathway that leads to reduced levels of cyclic AMP, light appears to act independently of this molecule [16, 17]. Furthermore, melatonin and dopamine inhibit each other's release, thus, suggesting the existence of a dual-oscillator mechanism within the retina [18].

These data suggested for the first time that photoreceptors are circadian clock cells capable of generating melatonin rhythms, although for their light resetting, they appear to require a feedback from dopaminergic amacrine cells. However, to formally rule out the possibility that the clock properties of photoreceptors may depend from other cells in the retina, it was necessary to await for the generation of transgenic protocols in *Xenopus*. Using this approach, Green and collaborators inserted a dominant negative form of the key circadian transcription factor CLOCK (XCLΔQ) under the control of mouse interphotoreceptor retinoid-binding protein (IRBP) promoter, which drives expression specifically in the photoreceptors

[19]. The obtained transgenic tadpoles carried photoreceptors with inactivated circadian clocks, and culture of isolated eye cups displayed an arrhythmic melatonin production, thus indicating that the clock in these cells is necessary for this rhythmic output. It is worth noting that inactivation of the circadian oscillator in photoreceptors does not affect average total melatonin levels, suggesting that CLOCK acts in a more complex manner than simply turning on melatonin synthesis at night.

The *Xenopus* retina contains approximately equal numbers of rods and cones that interact with each other via gap junctions. More recently, to further determine the distinct contribution to the circadian rhythmicity of each of the two photoreceptor cell types, Green and collaborators generated transgenic tadpoles in which XCLΔQ expression was driven by either a rod (rod opsin)- or a cone (cone arrestin)-specific promoter [20]. By analyzing melatonin release in isolated eye cups, the researchers observed that while rod-specific expression of the XCLΔQ clearly alters melatonin rhythms in the retina, a significantly lower effect is observed when XCLΔQ is expressed in cones. Considering that rods are able to respond to dim light at night, when melatonin is secreted, it is reasonable to think that this cell type may play a predominant role in increasing melatonin release at night. However, as rods and cones are electrically coupled, they are likely to interact to coordinate circadian clocks in individual photoreceptors and orchestrate the circadian release of melatonin.

Three melatonin receptor subtypes (Mel1a, Mel1b, and Mel1c) have been identified in *Xenopus laevis* [21, 22]. The *Xenopus* Mel1a receptor is the ortholog of the mammalian MT1 receptor, and the *Xenopus* Mel1b receptor is the ortholog of the mammalian MT2 receptor. Mel1c is the homologue of mammalian melatonin-related receptor GPR50 and does not bind melatonin [23]. The retinal expression of Mel1a and Mel1b receptors was found to correlate with cone circuits, in particular, with Mel1b receptors being expressed by OFF-bipolar cells and Mel1a receptors by horizontal cell processes specifically at cone terminals. This selective localization of Mel1a and Mel1b receptors has suggested a role in reducing noise from signaling by cone terminals in darkness, when visual function is dominated by rods [24].

It should be noted that, although the retinal melatonin has a role in controlling a number of cellular and physiological function within the retina, it is rapidly metabolized within the *Xenopus* retina [25], and it is therefore likely to provide a paracrine and/or intracrine rather than an endocrine, circadian signal.

A first attempt to understand the molecular regulation of rhythmically expressed retinal genes was addressed by experiments that linked the advantage of the large, easily dissectable *Xenopus* retina, to the use of appropriate molecular biology strategies.

This was the case for *Nocturnin* (*Noc*), a gene isolated through a high-stringency differential display screen aimed at selecting mRNAs that exhibit rhythmic expression in the photoreceptors. *Noc* mRNA displays high-amplitude, rhythmic expression, reaching the highest level during the night in isolated *Xenopus* eye cups in cyclic light, as well as in conditions of constant dark or constant light. *Noc*, which in the *Xenopus* retina is specifically expressed in photoreceptors, shares sequence

similarity with the C-terminal deadenylase domain of the yeast carbon catabolite repressor 4 (yCCR4). The ability of Noc to remove 3′ polyA tails from mRNA was indeed confirmed in cell culture-based assays, indicating that Noc belongs to the family of magnesium-dependent family of nucleases. Although specific substrates for Noc in the photoreceptors have not been identified yet, the circadian expression of this deadenylase suggests that its targets could be mRNAs related to rhythmic processes within these cells. The extent of such regulation is not known; however, an estimate was determined in mouse liver, where it was shown that the poly (A) tails of 2.3 % of all expressed mRNAs cycle in length daily and that these rhythmic poly(A) tail lengths correlate strongly with the circadian protein expression profiles of this tissue [26].

Besides the canonical photopigments present in cone and rods, a screen originally performed in *Xenopus* photosensitive melanophores unraveled the presence of an atypical photopigment that was named melanopsin ([27], see below). Melanopsin is expressed in several tissues and displays a peak of light absorption at blue light wavelengths. Intriguingly, it is more closely related to invertebrate rhabdomeric opsins than to the vertebrate ciliary opsins typically expressed in cones and rods. Similarly to other nonmammalian vertebrates, *Xenopus* has two melanopsins: opn4x (also known as *Xenopus*-like melanopsin) and opn4m (also known as mammalian-like melanopsin). In mammals, only opn4m is present and its retinal expression is restricted to a small subset of photosensitive retinal ganglion cells (RGCs) that convey photic information to the "non-image-forming" (NIF) visual system, resulting in appropriate regulation of pupillary light reflex, circadian photo-entrainment, and sleep [28]. In *Xenopus* embryonic retina, opn4m and opn4x are mostly co-expressed in the outer half of the INL, in correspondence to the soma of specific horizontal cells (melanopsin-expressing horizontal cells, mHCs), and in a restricted number of retinal ganglion cells (melanopsin-expressing retinal ganglion cells, mRGCs. Fig. 9.1). Marker analysis identified mHCs as $Prox1^{+}/Otx2^{-}/Isl1^{-}/Pax6^{-}$ and mRGCs as $Pax6^{+}/Isl1^{-}$. About 80 % of these melanopsin-expressing cells are found in the peripheral retina, with mHCs located mainly in the dorsal retina and present in a ratio of 6:1 compared to mRGCs. To monitor the activation of embryonic NIF retinal circuit, Bertolesi and collaborators [29] assessed the expression of c-fos, an immediate early gene activated in second-order neurons following transduction of the light stimulus from photosensitive cells, after 30 min of light exposure. These experiments show that, as is the case for cones and rods in the classic image-forming (IF) circuit, light-sensitive mHCs do not express c-fos, which is instead activated in mRGCs, as well as in BCs, RGCs, and in a population of dopaminergic, tyrosine hydroxylase-positive amacrine cells (TH + ACs). A further analysis aimed at investigating c-fos activation by light with different wavelengths and possibly at discriminating between classic photoreceptor pathways and melanopsin-driven circuits. As a result, a situation more promiscuous than expected was revealed. In fact, photoreceptors of the IF circuit were shown to respond to blue light, while red and green light appeared as effective at turning on c-fos expression in mRGCs as blue light. Intriguingly, TH + ACs display preferential c-fos induction by blue light, suggesting that these cells participate to a

melanopsin-triggered circuit. A possible connection to this circuit might involve interaction of TH + ACs with (i) On-BCs via the rod/cone pathways; (ii) mRGCs, through a retrograde signal; and (iii) mHCs; in this case, TH + ACs would connect mHCs to mRGCs [29]. Differently from mammals, which develop an active retinal NIF system much earlier than vision, in *Xenopus* both the classic visual pathway and melanopsin NIF circuit become functional at the same developmental time, corresponding to the tadpole stage 37/38, concomitant with the establishment of retinal layers and synaptic connections and innervation of brain target by RGC axons. However, overall maturation of the NIF system in *Xenopus* still appears to precede that of the IF system because of the activity of the photosensitive pineal organ, which rhythmically secretes melatonin already at early tailbud stage (see below).

9.3 The *Xenopus* Pineal Circadian System

The amphibian pineal organ is an epithalamic vesicle connected to the roof of the diencephalon by a slender stalk. The pineal epithelium surrounds a central lumen, which is opened to the third ventricle and is thus filled with cerebrospinal fluid. The frog pineal organ is associated with the frontal organ, a more superficial extracranial photoreceptive structure located underneath an area of pigmentless skin, which shares similarities to the reptilian parietal eye [30]. The pineal organ of amphibians is primarily composed of photoreceptors, structurally similar to retinal cones, projection neurons, and interstitial glial cells. Pineal photoreceptors are highly polarized, with an outer segment in the apical part protruding in the pineal lumen and a basal extremity that establish synaptic contacts with the projection neurons whose axons enter the brain through the pineal stalk and innervate several brain areas. Pineal and retinal photoreceptors share a similar phototransduction machinery. Thus, the amphibian pineal organ resembles a simplified retina, mainly lacking interneurons, whose structure clearly indicates its role as a luminance detector [31]. Despite these remarkable similarities, the pineal organ cannot discriminate between rapid light changes, as the retina does, but rather acts as a mediator of gradual light intensity changes. This is consistent with the idea that the pineal is an indicator of day length and may provide input to the circadian clocks.

Integrated with inputs from the retina, the photosensitive activity of the pineal organ functions in resetting its circadian clock. A major output of this entrainment is the rhythmic activity of AANAT, which, as in the retina, results in the cyclic production of melatonin. This timekeeping hormone is secreted by pineal photoreceptors into the cerebrospinal fluid and blood at high level during the night and at low level during the day. Therefore, while the retinal melatonin appears to act and be catabolized locally [25], pineal melatonin is supposed to play a systemic role, synchronizing circadian rhythms in the entire organism.

Experiments based on in vitro cultured *Xenopus* pineal organs revealed that they are capable of synthesizing melatonin in a rhythmic pattern soon after they

evaginate from the diencephalon at early tailbud stage (stage 26). When kept in constant darkness, cultured pineal organs produce melatonin, although with low amplitude rhythms, suggesting the presence of a circadian clock in this tissue. However, after the third day of culture in constant darkness, melatonin release quickly damps to very low levels, indicating that cyclic light exerts a positive effect on melatonin synthesis in the pineal organ. In these in vitro organ culture assays, melatonin production by the pineal organ precedes of 1–2 days melatonin synthesis observed in dissected optic vesicles. However, when established, retinal melatonin secretion is maintained rhythmic both in cyclic light and in constant darkness [32]. Studies performed in the Japanese newt (*Cynops pyrrhogaster*), a urodele amphibian, indicate that, although the pineal organ plays a major role in generating the circadian locomotor activity, the pineal clock is not entrainable by extrapineal photoreception [33, 34]. These data suggest that, at least in this species, but possibly also in other amphibians, there is little or no mutual coupling between the pineal clock and the putative brain oscillator.

A relatively recent addition to the cellular events controlled by the circadian clock is the generation of daily rhythms of cell cycle progression [35], which is now documented virtually in every analyzed adult cell type [36]. Particularly, synchronized circadian oscillations of both the cell cycle [37] and cell cycle regulatory genes have been demonstrated to occur in zebrafish also during embryonic development [38].

In *Xenopus*, photoreceptor precursors of developing pineal organ expressing Crx-b (previously known as Otx5) undergo themselves a circadian-controlled entry in S-phase, reaching a peak in the second half of the light phase [39]. In this context, the homeodomain-containing transcription factor Bsx was found to display a rhythmic pineal expression with the highest levels during the night. Bsx rhythmicity is lost in embryos kept in constant darkness, suggesting that the transcription of this gene is controlled by the alternation of light and dark rather than by the circadian oscillator. Bsx expression inversely correlates with cell proliferation. In fact, besides displaying a cyclic expression profile that is complementary to the daily rhythms of S-phase entry, Bsx is expressed in non-proliferating pineal photoreceptor precursors, and its expression is repressed by cell proliferation induced by cyclin A2/cdk2 overexpression. Furthermore, its knockdown leads to increased S-phase entry during the night, whereas overexpression of a hormone-inducible Bsx protein causes a flattening of the cell proliferation profile to the lowest level [39]. In addition, Bsx plays an instructive role in pineal photoreceptor determination. Indeed, pineal organs of Bsx knocked-down tadpoles feature a decrease of differentiated photoreceptors, while Bsx overexpression leads to an increase of pineal photoreceptors. This effect of Bsx is specific to pineal photoreceptors, as projection neurons are not affected either by gain- or by loss-of-function experiments.

Although the transcriptomes of retina and pineal organ are extremely similar, Bsx is one of the few examples of transcription factor that are expressed in the pineal organ but not in the retina. Bsx expression in *Xenopus* pineal photoreceptor precursors follows the activation of Crx-b, Not2, and Rx1/Rax and is likely to act

downstream of these transcription factors, which specify the early pineal territory. Therefore, Bsx appears to play a later role, functioning as a link between daily circadian cell cycle and pineal photoreceptor fate determination. Bsx target genes remain to be identified. However, the enrichment of putative Bsx-binding sites in the flanking genomic regions of eight long noncoding RNAs (lncRNAs) displaying a circadian expression with nocturnal peak suggests that Bsx might specifically control the expression of nocturnal genes [40].

9.4 The *Xenopus* Melanophore Circadian System

Xenopus melanophore response to light has been an important paradigm to study how peripheral, photosensitive cells adjust to circadian light/dark cycles and to identify the molecules responsible for this phenomenon.

Skin cells respond to light variations by adjusting their color, as a consequence of specific pigment migrations. In fact, when the black pigment melanin disperses in the cytoplasm of melanophores, it causes darkening of the skin, while its aggregation around the melanophore cell nucleus results in a pale aspect of the skin. Two different mechanisms have been described: the "primary color response," which is due to a direct response to light by chromatophores [41], and the "secondary color response," which is mediated by the eyes and is controlled by the neural and/or endocrine system [42].

9.4.1 Primary Color Response

In the primary color response, chromatophores independently transduce light energy into the mechanical forces required for the processes of pigment and guanine crystals movements, which, in turn, induce color changes in the skin. A direct response to light is shown by melanophores of the posterior part of the tail fin of *Xenopus* late tadpoles (stage 51–56), which are fully aggregated under normal illumination, leading to the paleness of the tails. Conversely, in darkness pigment disperses, causing the tail to become dark. These effects are not mediated by the eyes or by the central nervous system [43–45]. Little is known about the molecular mechanisms mediating melanosome aggregation as a response to illumination, except that a rhodopsin-like molecule, distinct from melanopsin, expressed in cells of *Xenopus* tadpole tail fin is likely to be involved [46].

Differently from tail melanophores of late-stage tadpoles, in melanophores derived from *Xenopus* embryos, an opposite behavior is observed, with pigment dispersion under illumination and aggregation in the darkness. Working with cultured melanophores isolated from stage 30–35 tailbud embryos, Daniolos and collaborators [47] observed that melanin aggregation, induced by melatonin treatment in darkness, is reversed leading to dispersion when cells are irradiated with

white light. Furthermore, melanin photodispersion reached a peak when melanophores were stimulated with a 460 nm light, and this effect was found to be dependent on vitamin A, indicating the involvement of an opsin photopigment. This phenomenon was actually at the basis of melatonin discovery. Indeed, already in1917 McCord and Allen [48] reported that *Rana pipiens* tadpoles fed with a crude acetone extract of bovine pineal glands displayed lightened skin. It was only later, in 1958, that Lerner isolated the molecule responsible for melanin granule aggregation, a pineal indole, which he named melatonin [49].

Within the primary color response, the best characterized phenomenon is the one that occurs in cultured *Xenopus* melanophores, originally obtained from late tailbud embryos, which respond to light by pigment dispersion. The search for opsins expressed in dermal melanophores of *Xenopus laevis* led to the isolation of melanopsin, a photopigment that was shown to be synthesized not only in melanophores but also in other tissues displaying photosensitivity in amphibians, such as the hypothalamus, the iris and horizontal cells of the retina (see above; [27]). Photoactivated melanopsin initiates a phosphoinositide signaling pathway, similar to that found in invertebrate phototransduction, which causes the dispersion of melanosomes [50]. A first in vivo demonstration of melanopsin activity showed that melanophores of transgenic *Xenopus* constitutively overexpressing melanopsin are 100-fold more sensitive to light than control melanophores and induce a remarkable melanin dispersion [51]. This experimental evidence clearly indicates that melanopsin mediates the primary color response.

Xenopus embryonic dermal melanophores express the melanopsin gene *Opn4x* and *Opn4m* and display rhythmic expression of the circadian clock genes *Per1*, *Per2*, *Bmal1*, and *Clock*. The concomitant presence of the molecular core of the circadian clock and of photopigments suggested that these cells possess the main characteristics to be considered peripheral clocks. However, the neurohormonal signals potentially required to reset the melanophore clock by the central oscillator have not been clearly identified yet. In fact, although cycling light conditions are able to reset and synchronize *Xenopus* melanophores, neither melatonin nor α-melanocyte-stimulating hormone (α-MSH), which act as the main melanin-dispersing agent and master regulator of skin darkness, appear to significantly affect *Per1* cyclic expression. These data suggest that the presence of melanopsin might be sufficient to trigger melanin dispersion and modulate gene expression in these cells [52, 53], unless these hormones regulate the transcription of other clock genes such as *Per2* or *Bmal1* or control some later phases of gene expression, such as translational or posttranslational regulation.

9.4.2 Secondary Color Response

In the secondary color response, the link between skin pigmentation and circadian rhythms has remained elusive for a long time. In amphibians, α-MSH secretion is mediated by a simple neuronal circuit in which RGCs project to dopaminergic

suprachiasmatic melanotrope inhibitory neurons that innervate cholinergic α-MSH-releasing cells [42]. Recently, Bertolesi and collaborators [54] made an important step forward by demonstrating that melanopsin activation in mRGC is required to inhibit α-MSH secretion, resulting in skin lightening due to melanin aggregation in melanophores. This effect, which is relevant to correlate skin pigmentation both to background adaption and to circadian light/dark cycles, implicates retina-generated melanopsin as a trigger for the secondary color response.

9.5 Concluding Remarks

Studies performed in *Xenopus laevis* have been pivotal in identifying important molecules regulating amphibian circadian rhythms both in the central nervous system and in peripheral tissues. Future experiments in amphibians, exploiting also the genetically amenable *Xenopus tropicalis* system, as well as the recently developed genome editing techniques based on the CRISPR/Cas system, are expected to continue to break new ground in our understanding of vertebrate circadian rhythms.

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