Chapter 6
Molecular Approach to the Circadian Clock Mechanism in the Cricket

**Kenji Tomioka, Outa Uryu, Yuichi Kamae, Yoshiyuki Moriyama,
ASM Saifullah, and Taishi Yoshii**

Abstract Circadian rhythms are physiological and behavioral changes that follow a roughly 24-h period, responding primarily to daily cycles in an organism's environment. Crickets have provided a good model to study the neural mechanisms controlling the circadian rhythm, because they have a large central nervous system. Neurobiological studies revealed that the circadian clock is located in the optic lobe and the photoreceptors necessary for light entrainment are in the compound eye. Recent progress in molecular technology enabled us to use crickets for dissection of the circadian system at a molecular level. The oscillatory mechanism of the circadian clock has been studied in *Drosophila* and a few higher order insect species, but the results from those insects are often inconsistent. We employed a reverse genetic approach to the cricket clock. We first obtained clock genes, *period* (*per*), *timeless* (*tim*), and *Clock* (*Clk*) with molecular cloning and then analyzed their functions with RNAi technology. The obtained results could be only partially explained by the *Drosophila* model. The central oscillatory mechanism of the cricket clock will be discussed together with the peripheral oscillators and the involvement of pigment-dispersing factor as a neurotransmitter in regulating the locomotor rhythm.

K. Tomioka (✉) • O. Uryu • Y. Kamae • Y. Moriyama • T. Yoshii
Graduate School of Natural Science and Technology, Okayama University,
Okayama 700-8530, Japan
e-mail: tomioka@cc.okayama-u.ac.jp

A. Saifullah
Bangladesh Atomic Energy Commission, Dhaka 1212, Bangladesh

6.1   Circadian Rhythms in the Cricket

Most animals show daily rhythms in their lives. The rhythm is thought to be acquired as an adaptive mechanism to daily environmental cycles associated with the Earth's rotation (Dunlap et al. 2004). The rhythm is generated by an endogenous mechanism called the circadian clock. Crickets have been a good model for studying the mechanisms underlying the circadian rhythm (Loher 1972, 1974; Tomioka et al. 2001; Tomioka and Abdelsalam 2004). They show clear circadian rhythms in a variety of physiological functions such as locomotor and stridulatory activity, spermatophore formation, retinal photosensitivity, and responsiveness of visual interneurons (Loher 1972, 1974; Tomioka and Chiba 1982a, b, 1992; Tomioka et al. 1994; Abe et al. 1997). It would be advantageous to search for the mechanisms underlying these rhythms in the cricket.

6.2   The Optic Lobe Is the Circadian Clock Locus

The relatively large size of crickets prompted the search for the tissue that generates the circadian behavioral rhythms. Experimental lesions revealed that the optic lobe is the candidate tissue that generates the activity rhythm: removal of the optic lobe or transection of the optic tract abolishes the locomotor or stridulatory rhythm in *Teleogryllus commodus*, *Gryllus bimaculatus*, *Gryllodes sigillatus*, and *Dianemobius nigrofasciatus* (Loher 1972; Sokolove and Loher 1975; Tomioka and Chiba 1984; Shiga et al. 1999; Abe et al. 1997). The optic lobe was later unequivocally shown to generate the circadian rhythm by measuring its efferent electrical activity in isolated and culture conditions (Tomioka and Chiba 1992). The isolated optic lobe maintains an electrical activity rhythm with a peak during the subjective night.

   The photoreceptive area necessary for synchronization to environmental light dark cycle is the compound eye. Disruption of the photic input through the compound eye via bilateral optic nerve transection abolishes the photic entrainment (Tomioka and Chiba 1984). The photoreception in the compound eye is also necessary for mutual synchronization between the bilaterally paired circadian clocks; the dorso-caudal region of the eye plays the most important role since surgical lesion of this area disrupts the synchronization (Tomioka and Yukizane 1997). The photic information is most likely mediated by a group of neurons called medulla bilateral neurons. They show a circadian rhythm in their responsiveness to light stimuli presented to the compound eye, and sectioning of their axonal tract prevents the mutual entrainment of the circadian pacemaker in the optic lobe (Yukizane and Tomioka 1995; Yukizane et al. 2002).

6.3   Molecular Oscillatory Mechanism of the Circadian Clock

The circadian clock machinery has been studied in several insects such as *Drosophila*, monarch butterflies, honeybees, and mosquitoes. The basic clock mechanism is believed to be composed of transcriptional/translational feedback loops. In *Drosophila*, the major players in the loop are *period* (*per*), *timeless* (*tim*), *Clock* (*Clk*), and *cycle* (*cyc*; Fig. 6.1). In brief, transcriptional activators CLOCK (CLK) and CYCLE (CYC), which are protein products of the *Clk* and *cyc* genes, heterodimerize and bind to the promoter regions of *per* and *tim* to activate their transcription (Allada et al. 1998; Darlington et al. 1998; Rutila et al. 1998). The resultant PER and TIM proteins accumulate during the night, form PER/TIM heterodimers, and translocate to the nucleus at late night to repress their own transcription through inactivation of CLK/CYC (Curtin et al. 1995; Saez and Young 1996; Darlington et al. 1998; Lee et al. 1998; Rutila et al. 1998). PER and TIM are subsequently subjected to a degradation process. These processes eventually reduce the PER and TIM levels and release the CLK/CYC from the PER/TIM-dependent inactivation. Thus, the loop goes to the next cycle.
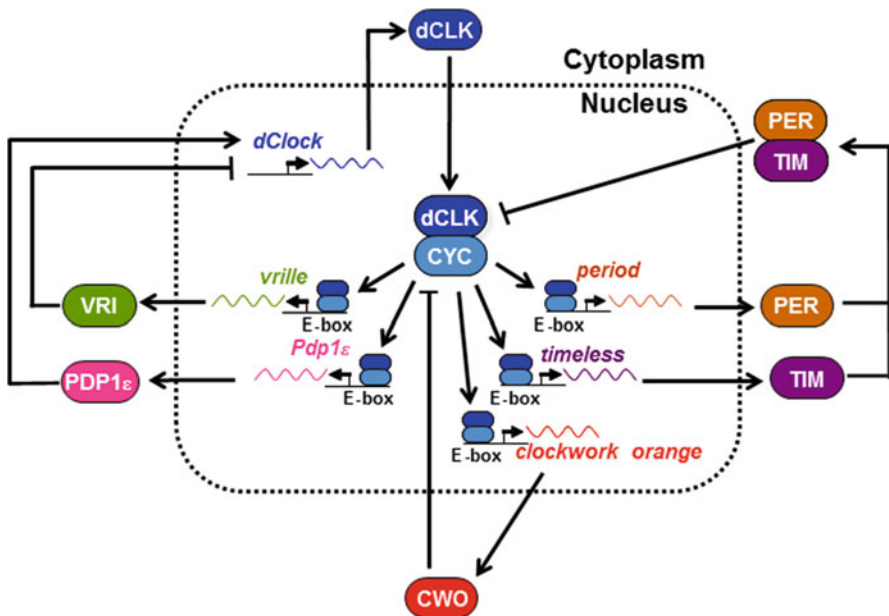

Fig. 6.1 The molecular oscillatory mechanism of the *Drosophila* circadian clock. It includes three transcriptional/translational loops consisting of *period* (*per*), *timeless* (*tim*), *Clock* (*Clk*), *cycle* (*cyc*), *Par domain protein 1ε* (*Pdp1ε*), *vrille* (*vri*), and *clockwork orange* (*cwo*). For details, see text

Transcription of the *Clk* gene is also under circadian regulation. There are two other factors, *vrille* (*vri*) and *Par domain protein 1ε* (*Pdp1ε*), involved in this mechanism (Cyran et al. 2003; Glossop et al. 2003). Their transcription is activated by CLK/CYC during the early night, but VRI accumulates earlier than PDP1ε and represses the *Clk* transcription through binding to a V/P-box, which lies on the regulatory region of the *Clk* gene. Later, accumulating PDP1ε then activates *Clk* transcription by competitively binding to the V/P-box. These processes result in the oscillation of *Clk* mRNA levels, which increase during the late night to early day.

The third loop involves *clockwork orange* (*cwo*), a transcription factor belonging to bHLH *orange* family (Lim et al. 2007; Matsumoto et al. 2007). *cwo* mRNA levels oscillate through a negative feedback of its product protein CWO and regulates the oscillation amplitude of other clock genes such as *per* and *tim*.

However, the *Drosophila* clock model is not completely supported in most of the other insects that have been examined. For example, there is no evidence that PER enters the nucleus in moths, cockroaches, or bloodsucking bugs (Sauman and Reppert 1996; Vafopoulou et al. 2007; Wen and Lee 2008). In moths, it is hypothesized that antisense and sense RNAs are transcribed simultaneously from the *per* gene and that antisense RNA is involved in the rhythmic expression of the PER protein (Sauman and Reppert 1996). *tim* does not exist in the hymenopteran genome, which includes honeybees (Rubin et al. 2006; Zhan et al. 2011). Instead of *tim*, mammalian-type *cryptochrome* (*cry2*) is thought to act as a negative component together with *per*. However, most of the studies in these non-model insects did not make rigorous functional analyses of the clock genes because of the limitation of available genetic techniques.

6.4 Molecular Approach to the Circadian System

The greatest advantage of using crickets is that RNAi is quite effective in analyzing the gene function. We thus use the cricket *Gryllus bimaculatus* to dissect the molecular oscillatory mechanism of the circadian clock as well as its output system regulating the overt activity rhythm.

6.4.1 Dissection of the Molecular Oscillatory Mechanism of the Circadian Clock

We first completed molecular cloning of the clock genes by a degenerate PCR strategy. The primers were designed based on known insects' clock genes. We have succeeded in obtaining cDNA fragments of the clock genes, *per*, *tim*, and *Clk*. 5′ and 3′ RACEs were performed for each of these genes and their full length cDNAs
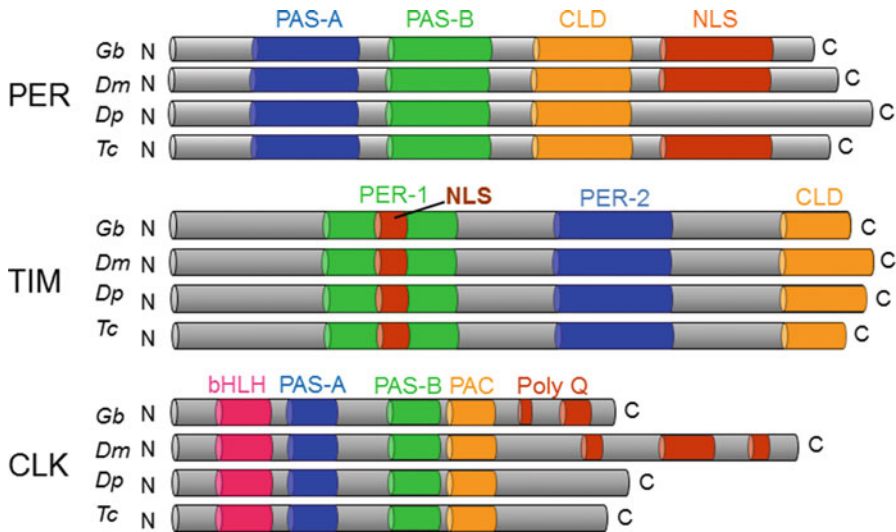
Fig. 6.2 Molecular structure of the protein products of *per*, *tim*, and *Clk* in the cricket *Gryllus bimaculatus* (*Gb*) aligned with those of *Drosophila melanogaster* (*Dm*), *Danaus plexippus* (*Dp*), and *Tribolium castaneum* (*Tc*). PER includes PAS-A, PAS-B, nuclear localization signal (NLS), and cytoplasm localization domains (CLD). TIM includes PER-1, PER-2, NLS, and CLD, and CLK is characterized by bHLH, PAS-A, PAS-B, PAC, and poly-Q. For details, see text

were obtained. The structural analyses revealed their resemblance to those of *Drosophila* and other insects (Fig. 6.2) (Moriyama et al. 2008, 2012; Danbara et al. 2010). *per* is characterized by four functional domains: PAS-A and PAS-B domains that are involved in the protein-protein interaction (Allada et al. 1998; Darlington et al. 1998), a cytoplasmic localization domain (CLD), and a nuclear localization signal (NLS). *tim* has two regions for dimerization with PERIOD (PER-1, PER-2) (Gekakis et al. 1995; Myers et al. 1995; Saez and Young 1996), NLS, and CLD. *Clk* includes a basic helix-loop-helix (bHLH) domain for binding to DNA (Allada et al. 1998; Darlington et al. 1998), PAS-A, PAS-B, and a polyglutamine repeat (poly-Q) in the C-terminal region that is implicated in transcriptional activity in *Drosophila* (Allada et al. 1998; Darlington et al. 1998). These structural similarities suggest that the clock genes *per*, *tim*, and *Clk* may have similar roles to those that they play in the *Drosophila* clock.

We measured the mRNA levels of those clock genes. Figure 6.3 summarizes the expression profiles of the clock gene mRNAs in the optic lobe. The mRNA levels of *per* and *tim* oscillate with a peak at early night. The profiles are similar to those reported for *Drosophila* and other insects. Interestingly, unlike in *Drosophila*, *Clk* is not rhythmically expressed. This is more similar to the mammalian clock than that of the *Drosophila* clock.

To investigate their importance in rhythm generation, we performed RNA interference. We synthesized dsRNA from the cDNA of the clock genes each of

Fig. 6.3 mRNA expression patterns of *per* (**A**), *tim* (**B**), and *Clk* (**C**) and the effect of specific dsRNA on the mRNA levels of respective genes in the optic lobe of the cricket *Gryllus bimaculatus*. In control crickets (*filled symbols*), *per* and *tim* show a rhythmic expression with a peak during the late day to early night, while *Clk* shows rather constitutive expression. Systemic treatment with dsRNA (*open symbols*) effectively knocked down mRNA levels of the respective clock gene and abolished the rhythmic expression in *per* and *tim*. *White* and *black bars* indicate light and dark phases, respectively. **C**: after Moriyama et al. (2012)
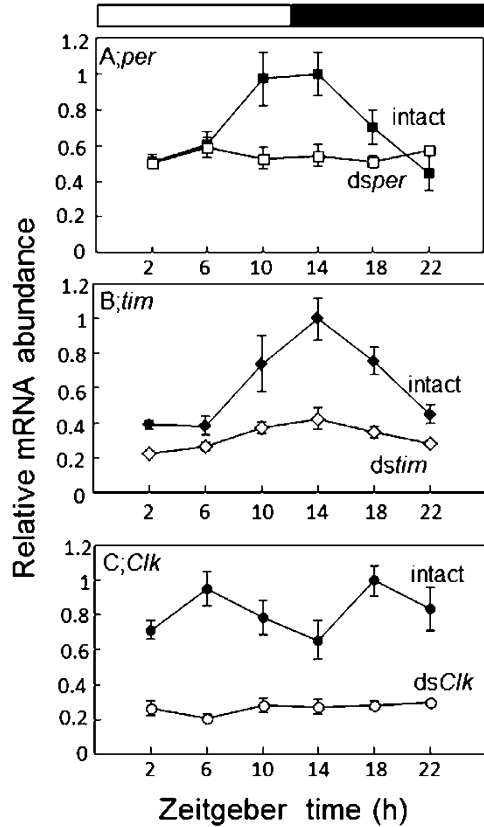
which was injected into the abdomen of nymphal or adult crickets. The mRNA levels were gradually reduced over several days after dsRNA injection and a substantial, significant reduction was observed 7 days after the treatment (Uryu et al. 2013). The dsRNA injection successfully reduced the levels of respective clock gene mRNAs and abolished the rhythm in *per* and *tim* as shown in Fig. 6.3. Thus, RNAi against the clock genes has the desired effects in the cricket *G. bimaculatus*.

We then examined the role of clock genes in rhythm generation by measuring locomotor activity of the RNAi-treated crickets. The RNAi against *per* and *Clk* resulted in a loss of rhythmicity in constant darkness (Fig. 6.4). The arrhythmicity persisted for more than 30 days until the end of the experiment. Only a small fraction of the treated crickets maintained the rhythm but the power of the rhythm was significantly reduced. *tim* RNAi crickets maintained the rhythm but with shortened free-running periods (Fig. 6.4) (Danbara et al. 2010), when compared with those of control crickets injected with dsRNA against *DsRed2*, which is derived from a coral species.
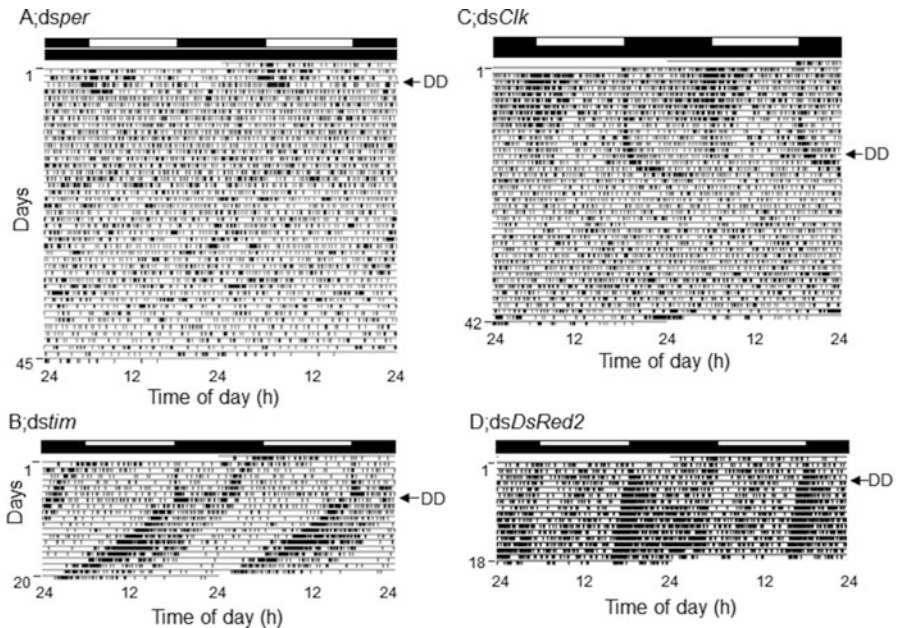
Fig. 6.4 Effects of *per* (**A**), *tim* (**B**), or *Clk* (**C**) RNAi on the locomotor rhythm of the cricket *Gryllus bimaculatus*. The locomotor rhythm disappeared in *per* and *Clk* RNAi crickets in DD, while it persisted with a shortened free-running period in the *tim* RNAi cricket. Control crickets treated with ds*DsRed2* showed a rhythm similar to that of intact crickets (**D**). *White* and *black bars* above actograms indicate light and dark phases, respectively. **A**, after Moriyama et al. (2008); **B** and **D**, after Danbara et al. (2010); **C**, after Moriyama et al. (2012)

6.4.2 The Role of Clock Genes in Molecular Oscillatory Mechanism

To know the role of clock genes in the molecular clock machinery, we examined the effects of knocking down one clock gene on the expression of other clock genes. In *Clk* RNAi crickets, both *per* and *tim* transcripts were significantly reduced, and the daily expression was lost, suggesting that it may act as a transcriptional activator as in *Drosophila*. In *per* RNAi, *tim* and *Clk* transcripts were both significantly reduced and rhythmic expression of *tim* was abolished. Similarly, in *tim* RNAi crickets, expression levels of both *per* and *Clk* were significantly reduced. These data suggest that the expression of clock genes is regulated by a complex mechanism, probably through some complex gene network. Based on the results, we assume that the cricket circadian clock functions as follows (Fig. 6.5). Since we have already confirmed the existence of the *cycle* (*cyc*) gene and its rhythmic expression under LD in the cricket optic lobe (Uryu et al. 2013), we assume that *Clk* and *cyc* work together as transcriptional activators as in *Drosophila*, even though their expression profiles are more similar to those of their counterparts in mammals.
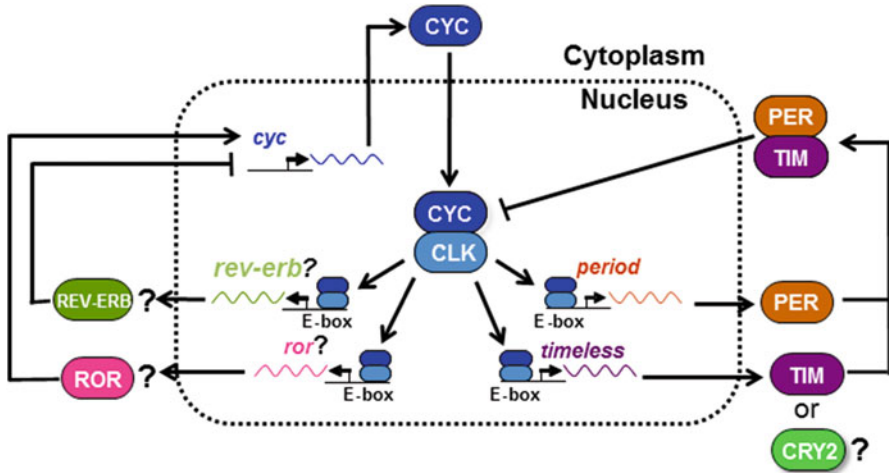
Fig. 6.5 A schematic model of the cricket circadian clock. It probably consists of at least two feedback loops for rhythmic expression of *per* and *tim* and for *cyc*. In analogy to the mammalian clock, *rev-erb* and *ror* may be involved in regulation of *cyc* expression. For further explanation, see text

Probably, *per* and *tim* act as negative factors to repress their own transcription through their negative effect on CLK/CYC, although there may be an additional complex pathway(s) that regulates the rhythmic expression of the *cyc* gene. *tim* RNAi knocked down *per* and *Clk* mRNA levels and stopped the oscillation in *per*; nevertheless, a locomotor rhythm persisted. We speculate that some other loop involving *cwo* or *cryptochrome* might retain its oscillation, independent of the oscillation of *per* and *tim*. The diversity and commonality of the circadian oscillatory mechanism in insects is a challenging issue to understand.

6.5 Peripheral Oscillators

In addition to the central clock tissues regulating activity rhythms, peripheral tissues also show circadian rhythms in crickets. The compound eyes have a circadian rhythm in their responsiveness to light (Tomioka and Chiba 1982a) and spermatophore formation occurs in a rhythmic, circadian manner (Loher 1974). Peripheral rhythms are also known in other insects. Cuticle secretion is known to occur in a rhythmic manner in cockroaches and flies (Wiedenmann et al. 1986; Ito et al. 2008), and antennal odor sensitivity is under a regulation of the circadian clock in cockroaches and flies (Krishnan et al. 2001; Page and Koelling 2003). The controlling mechanism for these peripheral rhythms has been investigated only in a few species. For example, in *Drosophila* the rhythms are controlled by circadian clocks located in those related tissues (Plautz et al. 1997), while in cockroaches at

least the rhythms in the compound eyes and antennae are under the control of the central clock that is located in the optic lobe (Wills et al. 1985; Page and Koelling 2003). However, the oscillatory mechanism of the peripheral oscillator and the relationship between the central and peripheral oscillators remain to be elucidated.

Crickets again provide a good model to address these issues because the central clock has been localized and some peripheral tissues show circadian rhythms (Tomioka and Abdelsalam 2004). We first analyzed the peripheral oscillations in various tissues by measuring circadian expression of the clock genes *per* and *tim* in the cricket *Gryllus bimaculatus*. mRNA levels of both *per* and *tim* genes showed a circadian rhythmic expression in the brain, terminal abdominal ganglion (TAG), anterior stomach, midgut, and testis in DD, suggesting that they include a circadian oscillator (Uryu and Tomioka 2010). However, the amplitude and the levels of the mRNA rhythms varied among those tissues. Removal of the optic lobe, the central clock tissue, differentially affected the rhythms. The rhythm of both*per*and *tim* was lost in the anterior stomach, while in the midgut and TAG, *per* maintained rhythmic expression but *tim* expression became arrhythmic. In the brain, both *per* and *tim* mRNA retained rhythmic expression but with a shifted phase (Uryu and Tomioka 2010). These data suggest that rhythms outside the optic lobe are controlled by the optic lobe to varying degrees and that the oscillatory mechanism may be different from that of the central clock in the optic lobe.

6.6 Molecules Involved in the Output Pathway

Molecules involved in the output pathway regulating insect activity rhythms are still largely unknown except for a few neurotransmitters. The pigment-dispersing factor (PDF), an octadeca-neuropeptide, is one such molecule and is believed to be a principal neurotransmitter regulating locomotor rhythms in various insects including *Drosophila* and cockroaches (Renn et al. 1999; Helfrich-Forster et al. 2000; Park et al. 2000; Lee et al. 2009). In the cricket, however, PDF seems unessential for locomotor rhythms, because partial removal of the optic lobe, which spares the PDF cells in the medulla, abolishes the locomotor rhythm (Okamoto et al. 2001). But PDF is apparently involved in the regulation of the responsiveness of the circadian rhythms of the visual system (Saifullah and Tomioka 2003).

We thus used RNA interference to study the role of the *pigment-dispersing factor* (*pdf*) gene in the regulation of circadian locomotor rhythms in the cricket, *Gryllus bimaculatus* (Hassaneen et al. 2011a). Injections of *pdf* dsRNA effectively knocked down the *pdf* mRNA and PDF peptide levels. The treated crickets maintained the rhythm both under LD and DD, confirming our previous assumption that PDF is not an essential neurotransmitter in the cricket circadian system (Okamoto et al. 2001). However, the RNAi-treated crickets showed reduced nocturnal activity and synchronized to the shifted LD more quickly than control crickets. The free-running periods of the *pdf* RNAi crickets were shorter than

those of control crickets in DD. Molecular oscillations of *per* and *tim* in the optic lobe showed a reduced amplitude and an advanced phase, corresponding to the weak and shorter free-running rhythms in the *pdf* RNAi crickets (Hassaneen et al. 2011b). These results suggest that PDF is not essential for rhythm generation but plays an important role in the control of nocturnality, photic entrainment, and fine-tuning of the free-running period of the circadian clock. To further understand the role of PDF at a molecular level, we need to investigate the role of PDF receptors, which have been studied in detail in *Drosophila* (Im and Taghert 2010), in our future studies.

6.7 Future Perspective

Crickets provide a unique model to study important aspects of the circadian rhythm. They have clocks in peripheral tissues (Uryu and Tomioka 2010) and are suitable for studies investigating the relationship between the central and peripheral clocks, in addition to the oscillatory mechanism of the peripheral clocks. They also show ontogenetic changes from nymphal diurnal to adult nocturnal rhythm (Tomioka and Chiba 1982b), providing a model to study phase regulation by the clock, which has been a question for years. With molecular techniques, we will be able to approach these questions. The key will be to clarify the molecular oscillatory mechanism of the central circadian clock. As described in this chapter, we have cloned some clock and clock-related genes in the cricket *Gryllus bimaculatus* and investigated their roles in the clock machinery (Moriyama et al. 2008, 2012; Danbara et al. 2010). However, for complete elucidation of the circadian clock machinery, we need to obtain many other clock genes known in other species, such as *Pdp1ε*, *vrille*, *rev-erb*, and *ror*, and to reveal their functional roles in the circadian clock. Elucidation of the cricket clock would lead to understanding how the insect circadian clock diversified evolutionarily, since the cricket is phylogenetically more primitive than flies, butterflies, and moths and has a clock that at least partially resembles the mammalian clock.

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