Chapter 3
Neural and Molecular Mechanisms of Entrainment

Kenji Tomioka and Taishi Yoshii

Abstract Synchronization or entrainment to daily environmental cycles is one of the important properties of the circadian clock, which is required to set an appropriate timing of physiology or behavior. Insects use various entraining agents or zeitgebers for entrainment, including light, temperature, food, and social cues. The mechanisms underlying entrainment have been studied extensively at cellular and molecular levels. For light and temperature, molecular components for their perception and how they reset the clock molecular oscillatory mechanism have been the main topics of chronobiology. This chapter will focus on the mechanism of light and temperature entrainment. The entrainment to restricted feeding, which has been progressing in recent years, will also be discussed.

Keywords Circadian clock · Clock gene · Entrainment · Phase shift · Zeitgeber

3.1 Introduction

Adaptation to daily and seasonally changing environments is of utmost importance for insects to live on this planet. The daily cycle includes not only geophysical factors but also biological/ecological factors (Dunlap et al. 2004). To confront these daily cyclical factors, insects must set their behavioral and physiological events at an appropriate time of day. For example, crickets and cockroaches exhibit stable nocturnal activity with the onset of activity at lights off under light to dark cycles, and the time of day mosquitoes come to the stable to suck blood varies from species to species (Katô and Triumi 1950). The timing is determined by the circadian clock that synchronizes or entrains to environmental cycles. Insects use various geophysical and biological factors for their clock synchronization to daily cycles. These include light (Pittendrigh and Minis 1964), temperature (Zimmerman et al. 1968), food availability (Xu et al. 2011), and social cues such as individual interaction

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(Bloch et al. 2013). Among them, light is the most powerful synchronizing agent or zeitgeber.

The mechanism for light entrainment has been extensively studied in various insects at physiological and molecular levels. Temperature is also known as an important zeitgeber, and the mechanism for temperature entrainment has recently been studied at cellular and molecular levels (Zimmerman et al. 1968; Glaser and Stanewsky 2005; Tomioka and Yoshii 2006; Sehadova et al. 2009). This chapter describes the behavioral, physiological, cellular, and molecular mechanisms for entrainment to light and temperature cycles. In addition, entrainment to restricted feeding is discussed. Social cues are also important entraining agents for social insects such as bees and ants (Bloch et al. 2013). Social entrainment is discussed in detail in Chap. 8.

3.2 Photic Entrainment and Circadian Photoreceptors

Among the zeitgebers or synchronizing agents that entrain the clock to environmental cycles, light is the most powerful (Saunders et al. 2002). Generally, light shifts the phase of circadian clocks in a phase-dependent manner: it delays the clock when given at early subjective night, whereas it advances at late subjective night (see Fig. 2.3) (Pittendrigh 1981). The transient cycles necessary for entrainment are dependent on light intensity and wavelength. In the cockroach *Periplaneta americana*, the most effective wavelength for phase shifts and ultimate entrainment is reportedly near 495 nm (Mote and Black 1981), suggesting the involvement of green-sensitive photoreceptors. Similar results were obtained in entrainment experiments in another cockroach species, *Blattella germanica* (Leppla et al. 1989). These results are quite consistent with our more recent results of RNA interference (RNAi) experiments in the cricket *Gryllus bimaculatus*, in which the green-light receptor, opsin-long wavelength (OpLW), is the circadian photoreceptor (Komada et al. 2015).

Photoreceptors necessary for photic entrainment were studied by occlusion with painting over the compound eyes and ocelli but later by surgical lesions or mutant screenings, which yielded more definitive results. Occlusion sometimes yielded inconsistent results. For example, occlusion of the compound eyes and ocelli in the cockroach *P. americana* resulted in arrhythmicity (Cloudsley-Thompson 1953), while in *Rhyparobia* (*Leucophaea*) *maderae* and *P. americana*, the same treatment reportedly caused free-running locomotor rhythms (Roberts 1960). In contrast, surgical lesions yielded clear results. Cutting the optic nerves that connect the compound eye and optic lobe clearly prevented the entrainment of activity rhythms in cockroaches and crickets (Nishiitsutsuji-Uwo and Pittendrigh 1968; Loher 1972; Tomioka and Chiba 1984) (Fig. 3.1a). In these experiments, ocelli were left intact so that ocelli were thought to have no important role in photic entrainment. However, they were shown to have some role in circadian control in singing rhythms of the cricket *Teleogryllus commodus* (Rence et al. 1988). In addition, the contribution of extraocular photoreceptors was later found in New Zealand weta *Hemideina*

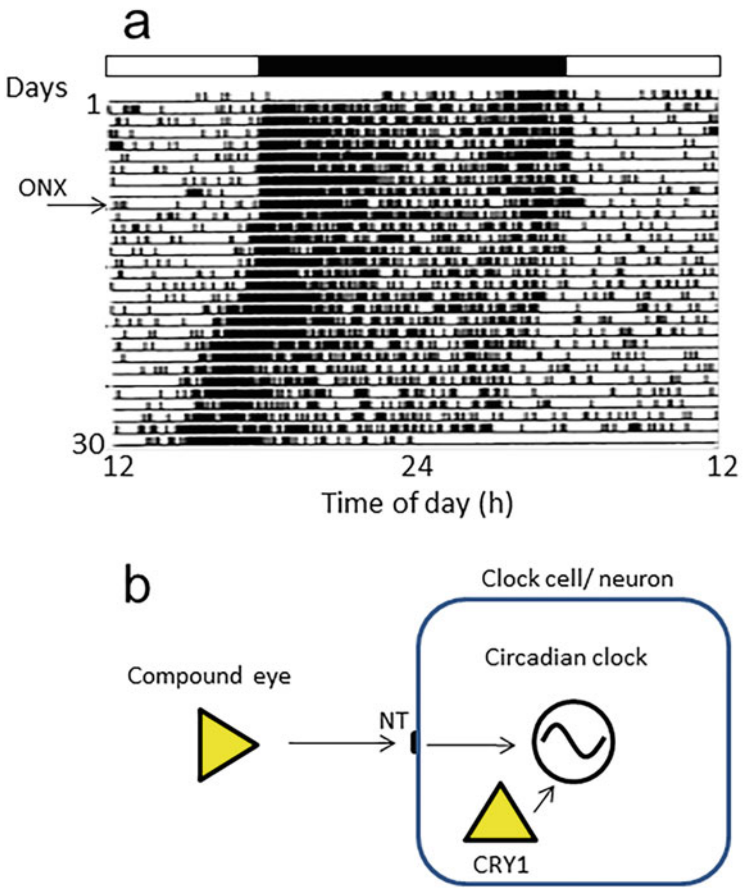

Fig. 3.1 Insect circadian photoreceptors. (**a**) Circadian locomotor activity rhythm of an adult male cricket, *Gryllus bimaculatus*, whose optic nerves were bilaterally cut on the day indicated by ONX (arrow). The cricket was kept under LD cycles, but after ONX, its rhythm free ran. (**b**) Two photic entrainment pathways in insects. CRY1 is an intracellular photoreceptor and directly acts on the clock machinery, whereas the light information perceived by the compound eye is conducted by the neural pathway and acts on the clock via neurotransmitters (NT)

thoracica and the cricket *Dianemobius nigrofasciatus* (Waddel et al. 1990; Shiga et al. 1999).

In *Drosophila melanogaster*, involvement of the extraocular photoreceptor in photic entrainment was clearly shown by mutant screening. Mutant flies carrying *sine oculis* with compound eyes genetically lost were shown to be entrained to light cycles (Helfrich and Englemann 1983). The photoreceptor molecule critical for photic entrainment was later found to be cryptochrome (CRY) by a gene screening experiment using *D. melanogaster* carrying a *per-luc* reporter (Emery et al. 1998; Stanewsky et al. 1998). The obtained *cry* mutant (*cryptochrome*^{baby}, *cry*^{b}) disrupted photic entrainment of the locomotor rhythm. Subsequent molecular studies revealed that CRY is a member of the photolyase family and plays an important role in resetting the oscillatory loop by perceiving blue/UV light (Lin and Todo 2005; Fogle et al. 2011) and is expressed in cerebral clock neurons, compound eyes, and peripheral tissues (Ito et al. 2008; Yoshii et al. 2008) (Fig. 3.1b). However, the compound eye and ocelli also have a significant contribution to photic entrainment in *D. melanogaster* (Rieger et al. 2003), so the mutant of *cry* can still be entrained by light cycles, albeit to a much slower extent than that of wild-type flies (Kistenpfennig et al. 2012). Hofbauer-Buchner (HB) eyelets that are the adult remnant of Bolwig's organ, the larval visual organ (Yasuyama and Meinertzhagen 1999), also play a role in photic entrainment. HB eyelets directly innervate the accessory medulla and affect the neuronal activity of clock neurons (Muraro and Ceriani 2015; Schlichting et al. 2016). When lacking all the external visual organs and *cry*, flies are not able to synchronize to light-dark cycles (LD) at all (Helfrich-Förster et al. 2001).

D. melanogaster has only one *cry* gene in the genome, but many other insects have two *cry* genes, the so-called *cry1* and *cry2*. The *cry1* gene is a homolog of the *Drosophila cry*, whereas the *cry2* gene is similar to the mammalian *cry* genes. An important question to be answered is why and when CRY1 has become to be used as a circadian photoreceptor in some insects. It seems premature to answer this question, but there is some important information available on this issue. Based on phylogenetic analysis, Yuan et al. (2007) suggested that both insect *cry1* and *cry2* homologs existed at the base of metazoan radiation and that gene duplication procedures occurred at least two times to acquire the *cry2* cluster. *cry1* was produced by the first duplication, and *cry2* and vertebrate *cry*, both of which lack photoreceptivity, occurred by the second duplication. CRY1 is suggested to be a circadian photoreceptor in higher-order insects, including flies and butterflies (Yuan et al. 2007). However, it is apparently not a major circadian photoreceptor in lower-order insects, including cockroaches and crickets. Moreover, many insect orders, including one of the most primitive insects, the firebrat *Thermobia domestica*, lacks *cry1* but possesses *cry2* (Kotwica-Rolinska et al. 2022). These circumstances suggest that loss of *cry1* may have occurred separately depending on different insect orders. Thus, the role of *cry*s in entrainment mechanisms should be thoroughly examined in various insect orders to understand its commonality and specificity.

3.3 Molecular Mechanism of Photic Entrainment

3.3.1 Cry-Dependent Pathway

In the photic entrainment mechanism, the *cry*-dependent pathway is best understood in insects (Fig. 3.2). As described in detail in Chap. 4, the *D. melanogaster* clock is based on rhythmic expression of so-called clock genes. The major loop consists of *Clock* (*Clk*), *cycle* (*cyc*), *period* (*per*), and *timeless* (*tim*). The product proteins of *Clk* and *cyc* form a heterodimer CLK/CYC, which promotes transcription of *per* and *tim* during late day to early night, and PER and TIM proteins accumulate in the

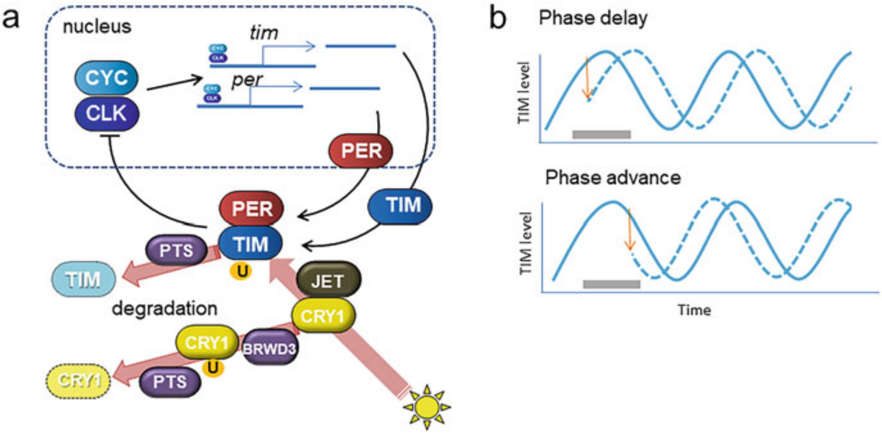

Fig. 3.2 Entrainment mechanism via the *cry* pathway in *Drosophila*. (**a**) Light-activated CRY1 works on TIM together with JET and ubiquitinates TIM. Ubiquitinated TIM leads to its degradation by proteasomes (PTS). CRY1 also leads to its degradation by PTS after ubiquitination by BRWD3. (**b**) Light-induced degradation of TIM results in phase delay in early subjective night (upper panel) but phase advance in late subjective night (lower panel). Gray bars indicate subjective night

cytoplasm during night. At late night, the PER/TIM heterodimer enters the nucleus to repress the transcriptional activity of CLK/CYC, reducing their own transcription and leading to a reduced level of their proteins, which corresponds to daytime. The reduction of PER/TIM releases CLK/CYC from the repression, and the clock oscillatory loop goes to the next round.

Molecular studies on CRY showed that CRY leads to degradation of TIM in a light-dependent manner (Fig. 3.2b). TIM degradation results in resetting of the molecular oscillation of the clock: at early night, the reduction in TIM delays the oscillation because the TIM level is necessary to increase to reach a level sufficient for repression of CLK/CYC. At late night, the reduction of TIM accelerates the oscillation to reach daytime earlier, advancing the clock's phase.

The CRY-dependent degradation of TIM requires another factor, JETLAG (JET) (Fig. 3.2a). JET is a member of the F-box and leucine-rich repeat protein (FBXL) family and a constituent of Cullin1-RING finger (CRL1) E3 ubiquitin ligases, recruiting substrates (Koh et al. 2006; Peschel et al. 2009). Light induces conformational changes in CRY, which enables CRY to bind to JET and TIM (Peschel et al. 2009; Ozturk et al. 2011; Zoltowski et al. 2011). The CRL1 E3 ligase ubiquitinates TIM, and ubiquitinated TIM is subsequently degraded by a proteasome-dependent mechanism (Koh et al. 2006; Peschel et al. 2009). In *D. melanogaster*, CRY is also degraded in a light-dependent manner (Fig. 3.2a). CRY degradation is also caused by another ubiquitin proteasome system, but the ubiquitination is caused by CRL4 E3 ligase, in which bromodomain and WD repeat domain containing 3, BRWD3, works as a component recruiting substrates (Ozturk et al. 2013).

3.3.2 Compound Eye-Dependent Pathway

Compound eyes are often used as a circadian photoreceptor necessary for photic entrainment of insect clocks. The neural pathway for compound eye-dependent entrainment has been studied in detail. As mentioned in Sect. 3.2, cutting the optic nerves prevents photic entrainment in crickets and cockroaches, which are solely dependent on the compound eye. In the cricket *G. bimaculatus*, partial destruction of the compound eye weakens entrainability, suggesting that the photic information received by photoreceptor cells in the compound eye is additively integrated in the entrainment pathway and supplied to the clock cells in the optic lobe (Tomioka et al. 1990).

Light information impacts the optic lobe clock cells through neurotransmitters and resets the clock's phase. The resetting mechanism has been unexplained for a long time but is now being rapidly elucidated in the cricket *G. bimaculatus* (Fig. 3.3). In this cricket, the clock molecular oscillatory mechanism consists of two major transcriptional/translational feedback loops, the *per*/*tim* loop and the *cry2* loop, which can oscillate independent of each other (Tokuoka et al. 2017). Both

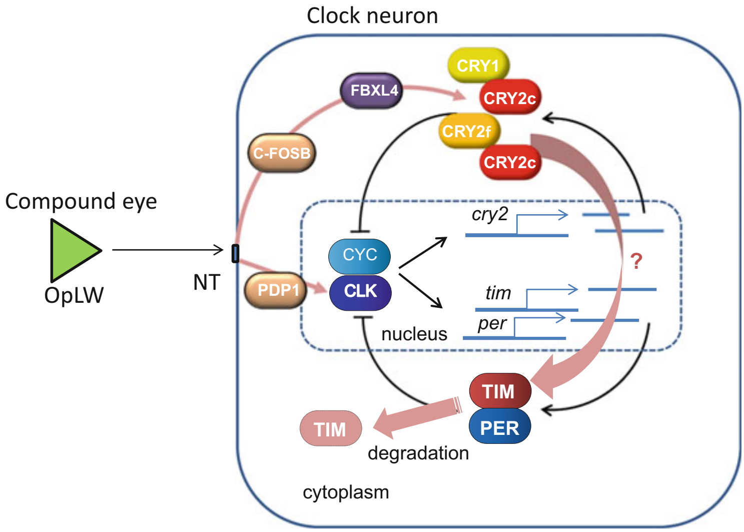

Fig. 3.3 Entrainment mechanism via the compound eye pathway in the cricket *Gryllus bimaculatus*. The cricket clock consists of the *per*/*tim*-loop and *cry2*-loop, which are coupled by sharing CLK/CYC as transcriptional activators (Tokuoka et al. 2017). In the *cry2*-loop, product proteins of *cry2* splicing variants and *cry1* form dimers and inhibit CLK/CYC. Light is perceived by green-sensitive opsin (opsin-long wavelength, OpLW). Light information acts on clock neurons in the optic lobe through neurotransmitters (NT), activating PDP1 or C-FOSB. *Pdp1* is upregulated only when the light off is delayed, leading to upregulation of *Clk*, which in turn upregulates *per* and *tim*, leading to prolonged subjective night, causing phase delay. C-FOSB induces FBXL4, which probably ubiquitinates CRYs and leads to degradation of TIM. Thereby, the *per*/*tim*-loop is reset, which subsequently resets the *cry*-loop

loops share the transcriptional activators CLK/CYC (Moriyama et al. 2012; Uryu et al. 2013; Tokuoka et al. 2017). The *per*/*tim* loop is similar to that of the *Drosophila* clock; *per* and *tim* are transactivated during late day to early night (Moriyama et al. 2008; Danbara et al. 2010). TIM also cycles in a similar time course to that in *D. melanogaster* (Moriyama et al. 2022). *cry2* oscillates in a similar time course to *per* and *tim*, but its product proteins form complexes between CRY2 variants and between CRY2 variants and CRY1, which negatively feedback to repress CLK/CYC transactivation (Tokuoka et al. 2017). In the photic entrainment pathway, light is perceived by a green light-sensitive photoreceptor, OpLW (Komada et al. 2015). The information resets the optic lobe circadian clock via two separate pathways: a *Pdp1* pathway and a *c-fos*/*cry* pathway. The former pathway is activated only when lights off is delayed (Kutaragi et al. 2016). Upregulation of *Pdp1* by delayed light off induces upregulation of *Clk*, which is followed by upregulation of *per* and *tim*, which prolongs the night phase to delay the clock (Kutaragi et al. 2016).

Once it switches to dark, the *Pdp1* pathway no longer functions, and the *c-fos*/*cry* pathway takes its place in resetting the clock (Kutaragi et al. 2018). In the *c-fos*/*cry* pathway, light induces *c-fosB*, which is a bZip transcription factor gene known to be upregulated by light exposure in mammalian circadian clocks (Kornhauser et al. 1990), in the optic lobe within 60 min of light exposure. Light-induced *c-fosB* mediates the induction of several *Fbxl* genes. RNAi-mediated gene silencing revealed that *Fbxl4* is involved in entrainment to light cycles by both advance and delay shifts (Takeuchi et al. 2023): RNAi of *Fbxl4* prevented or delayed entrainment to shifted light cycles. The photic entrainment is disrupted by double RNAi of *cry1* and *cry2* without any effect on light-dependent *c-fosB* induction, and RNAi of *c-fosB* strongly disrupted advance and delay shifts caused by light pulses given at late night and early subjective night, respectively (Kutaragi et al. 2018). These facts suggest that resetting of the clock by light in the dark starts with *c-fosB* induction and that *cry1* and *cry2* work downstream of *c-fosB* and are regulated by *Fbxl*.

We recently found that *tim* plays an essential role in photic entrainment via delay shifts (Moriyama et al. 2022). tim^{RNAi} disrupted reentrainment to delayed light cycles. Light pulse reduces TIM protein levels both at early and late night. TIM reduction at early night should be required for the delay shift of the clock. TIM reduction is probably coupled with the *cry2* oscillatory loop because *cry1*/*cry2* double knockdown by RNAi disrupted the normal entrainment to shifted light cycles in both advance and delay shifts (Kutaragi et al. 2018). This also suggests that TIM degradation is most likely downstream of CRYs. Interestingly, TIM degradation is shared by the compound eye-dependent pathway and the *cry*-dependent pathway, suggesting that TIM may have a common role in light entrainment of insect circadian clocks across species.

3.4 Temperature Entrainment

3.4.1 Role of Temperature in Phase Setting

Temperature is also an important entraining agent in insects. Temperature often cooperates with light cycles to set physiological or behavioral events to occur at an appropriate time of day. In the cricket *G. bimaculatus*, temperature is a weaker zeitgeber for circadian rhythm synchronization. Kannan et al. (2019) showed that *G. bimaculatus* entrains to temperature cycles of 25 °C and 30 °C under DD with a peak around the warm to cool transition and that when the temperature cycle was advanced by 6 h, the locomotor rhythm resynchronized to the shifted temperature cycle with long transient cycles. Temperature step-up and step-down by 5 °C shift the clock in the advance and delay directions, respectively, but no clear phase dependency was observed (Ikeda and Tomioka 1993). In addition to entrainment, temperature has substantial effects on activity rhythms in *G. bimaculatus*. Under 25 ° C or higher ambient temperature levels, adult crickets show nocturnal activity, while they switch to show diurnal activity at 20 °C (Ikeda and Tomioka 1993).

In the fruit fly *Drosophila pseudoobscura*, temperature regulates the timing of adult eclosion (Pittendrigh 1960). Eclosion usually occurs in the morning, when the humidity is high, to avoid death from desiccation. This timing is set by two clocks (oscillators). One is the light-entrainable A oscillator, and the other is the temperature-sensitive B oscillator; usually, the A oscillator entrains the B oscillator through an internal mechanism (Pittendrigh 1981; Tackenberg et al. 2017). This A-B two-oscillator model is also applicable to locomotor rhythms in *D. melanogaster*, which shows bimodal activity with a morning and an evening peak (Fig. 3.4). The rhythm is driven by several groups of clock neurons, called small ventrally located lateral neurons (sLNv), fifth sLNv, large-LNv (lLNv), dorsally located LNs (LNd), three groups of dorsal neurons (DN1, DN2, DN3), and lateral posterior neurons (LPN) (Yoshii et al. 2005). Under lower-temperature levels, the morning peak occurs later, and the evening peak occurs earlier. Experiments simultaneously entraining the clock by light and temperature cycles revealed that the light-entrainable clock neurons, including sLNv, fifth sLNv, lLNv, and LNd, set the framework of the activity to occur within the daytime, whereas the temperature-entrainable clocks, i.e., DNs and LPNs, tune the onset of the evening peak according to temperature cycles (Miyasako et al. 2007) (Fig. 3.4). Therefore, temperature plays an important adaptive role in the circadian system.

3.4.2 Molecular Mechanism of Temperature Entrainment

In *D. melanogaster*, the mechanism of temperature entrainment has been studied at cellular and molecular levels (George and Stanewsky 2021). The cerebral clock neurons are thought to have no direct entrainability to temperature cycles because

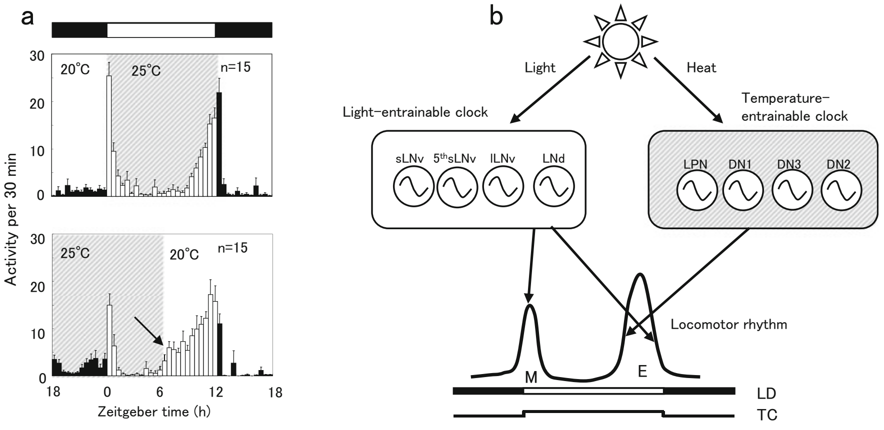

Fig. 3.4 *Drosophila* circadian locomotor rhythm is regulated by two groups of clocks, i.e., light-entrainable and temperature-entrainable clocks. (**a**) Average activity profiles of locomotor rhythm of adult male *Drosophila melanogaster* under a light-dark cycle (LD) (12 h light: 12 h darkness) and temperature cycle (TC) (12 h 20 °C: 12 h 25 °C). Gray shaded areas indicate 25 °C. White and black bars indicate light (while) and dark (black) phases. The white and black columns indicate activity in the light (white) and dark (black) phases, respectively. Error bars indicate SEM. When TC was advanced by 6 h, the onset of the evening peak advanced (arrow), but its offset stayed at lights off. (**b**) A cellular model of the *Drosophila* central clock system for light and temperature entrainment. Laterally located neurons (lLNv, sLNv, fifth sLNv, LNd) are all light-entrainable clocks, some of which determine the morning peak and the offset timing of the evening peak. Dorsally located neurons (DN1, DN2, and DN3) and lateral posterior neurons (LPNs) are temperature-entrainable clocks and regulate the onset timing of the evening peak. Redrawn from Miyasako et al. (2007)

those neurons in isolated brain kept in culture conditions cannot synchronize to temperature cycles. The temperature signal for entrainment is perceived by chordotonal organs, which are located at the joints between limb segments and antenna and are internally attached to the cuticle (Fig. 3.5). While the organs were originally thought to have functions as mechanoreceptors for stretching or vibration, they play an important role in temperature entrainment (Sehadova et al. 2009). The chordotonal organs express *no circadian temperature entrainment* (*nocte*) gene, encoding a large glutamine-rich protein, which is required for temperature entrainment (Glaser and Stanewsky 2005; Sehadova et al. 2009). NOCTE protein is thought to be required for the proper structural conformation and physiological function of the chordotonal organ, which is important for temperature entrainment. The temperature information perceived by the chordotonal organ is sent to the cerebral clock neurons. Among the clock neurons, the posterior DN1 (DN1ps) was recently demonstrated to monitor modest changes in environmental temperature that come not only from the chordotonal organs but also from the aristae of antennae (Yadlapalli et al. 2018).

The molecular mechanism for temperature entrainment is not yet fully understood, but some important information is available (Fig. 3.5b). Most likely, the temperature information is mediated by phospholipase C (PLC) because mutants

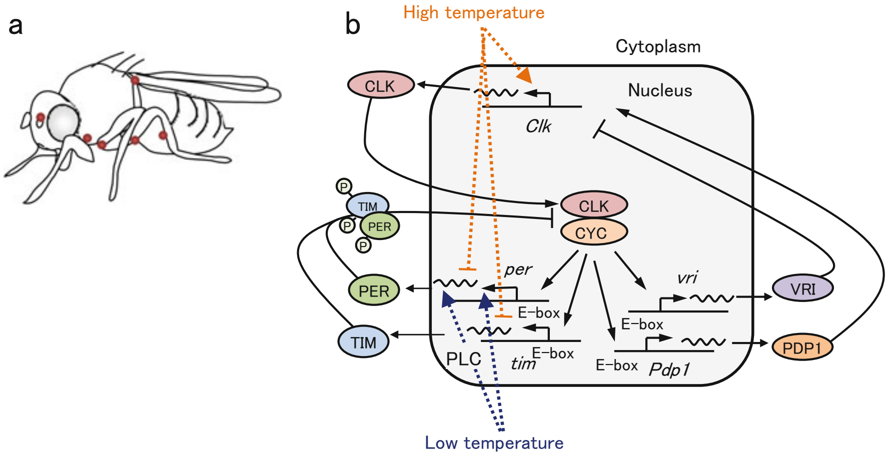

Fig. 3.5 Temperature entrainment mechanism in *Drosophila*. (**a**) Chordotonal organs that perceive ambient temperature. (**b**) A possible model for temperature entrainment. High temperature upregulates *Clock* (*Clk*) but downregulates *per* and *tim*, whereas low temperature upregulates *per* and stimulates its alternative splicing. The dotted arrow indicates upregulation, and the dotted lines with bar ends indicate suppression or downregulation. Redrawn with some modification from Tomioka and Yoshii (2006)

in *norpA* encoding PLC are not able to synchronize to temperature cycles (Glaser and Stanewsky 2005). Although molecular events downstream of PLC are less clear at present, changes in the expression of clock genes may be involved because PLC is suggested to be involved in 3′ splicing of *per* RNA (Collins et al. 2004). A similar temperature-dependent alternative splicing is also observed in the *tim* gene (Martin Anduaga et al. 2019). In fact, temperature has significant effects on the expression of clock genes in *Drosophila*.

Temperature entrainment of the molecular oscillation has been analyzed under constant light, in which the clock is stopped but the temperature cycle forces the clock to oscillate and entrain (Yoshii et al. 2005; Sehadova et al. 2009). Under continuous light conditions, light-activated CRY continuously leads to degradation of TIM, resulting in the arrest of the clock around circadian time (CT) 12. However, the temperature cycle induces molecular oscillation by stimulating clock gene expression. Temperature step-up and step-down have different effects on clock gene expression (Yoshii et al. 2007) (Fig. 3.5b). A temperature step-up from 20 °C to 30 °C stimulates *Clk* gene expression and reduction of *per* and *tim*. PER and TIM proteins are quickly degraded by exposure to a high temperature of 37 °C (Sidote et al. 1998), and TIM degradation is caused by the interaction between CRY and the PER/TIM complex (Fan et al. 2007). High-temperature-induced *Clk* upregulation is followed by upregulation of *per* and *tim* expression, whereas temperature step-down induces an increase in PER levels through upregulation of *per* mRNA (Yoshii et al. 2007). These facts may explain why temperature step-up induces phase advance and step-down phase delay in *D. pseudoobscura*

(Zimmerman et al. 1968). However, how temperature steps change the protein or mRNA levels of clock genes remains to be elucidated.

In other insects, knowledge of the molecular mechanisms of temperature entrainment is quite limited. Only fragmental information is available for molecular events underlying the temperature-induced phase shifts in the cricket *G. bimaculatus* (Kannan et al. 2019). QPCR analysis revealed that clock genes consisting of cricket's clock showed slightly earlier peaks under temperature cycles of 25 °C and 30 °C in DD compared with those under LD. When the temperature cycle was advanced by 6 h, they resynchronized to the shifted temperature cycle with a gradual phase advance. *per* and *tim* resynchronized more quickly than *cry2* and *cyc*. It is thus likely that clock genes play differential roles in resetting the clock in response to changes in environmental temperature cycles. However, further detailed analysis is necessary to clarify the temperature entrainment mechanism in *G. bimaculatus*.

3.5 Entrainment by Restricted Feeding

In mammals, daily scheduled time-restricted feeding induces food anticipatory behavioral rhythms: an increase in activity before feeding (Mistlberger 2011). Interestingly, similar food anticipatory rhythms can be observed even in mutants of canonical clock genes, suggesting the existence of distinct molecular mechanisms. A time-restricted feeding experiment has also been conducted in *D. melanogaster*, but activity rhythms were not clearly entrained by the feeding schedule (Oishi et al. 2004). From this result, one can conclude that the central brain clock is insensitive to feeding. However, it is well known that honey bees can memorize the time and place where food is available and forage at appropriate times, known as time-place learning (Renner 1960). This is a kind of anticipation and is associated with an increase in activity before food availability. The memory of timing can be transferred to another individual by transplanting a mushroom body (Martin et al. 1978). The same behavior has been observed in *D. melanogaster*, and clock genes are involved in time-place learning (Chouhan et al. 2015). At this point, however, it is an open question whether insect time-place learning is mediated by circadian entrainment.

Feeding is a potent zeitgeber for peripheral clocks in the fat body. Time-restricted feeding under DD conditions entrains rhythmic expression of clock and nonclock genes in the fat body in *Drosophila* (Xu et al. 2011) (Fig. 3.6). The fat body clock is not completely independent from the brain clock. Disruption of the brain clock attenuates free-running rhythms of gene expression in the fat body, which are mediated by a neuropeptide, neuropeptide F, produced in cerebral clock neurons (Erion et al. 2016). Additionally, misalignment of feeding and activity rhythms causes lower egg production (Xu et al. 2011). Therefore, it is suggested that the coordinated circadian rhythms between the fat body and central brain clocks are important for fitness.

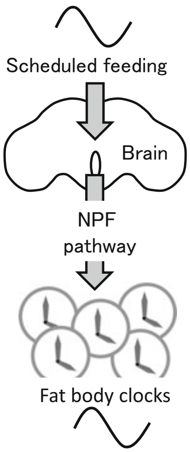

Fig. 3.6 Entrainment pathway for scheduled feeding in *Drosophila*. Zeitgeber information on time-restricted feeding is first transmitted to the brain and then transferred to fat bodies via the NPF signaling pathway. The entrained fat body clocks generate rhythmic gene expression

3.6 Future Perspectives

As we have reviewed in this chapter, the entrainment mechanisms of insect circadian clocks have been extensively studied at cellular and molecular levels, especially for light and temperature. In addition, the phase of the clock is also regulated by food availability and by social factors such as interactions among individuals. Insects live in diverse environments, and investigating which environmental factors they mainly use for entrainment of their clocks will help us understand the adaptive significance of the clocks and their zeitgebers. Zeitgebers may work together in complex ways to harmonize rhythms within the changing environmental cycle. The oscillatory mechanism of the clock is now being elucidated at the molecular level in various insects. Through investigating the entrainment mechanisms by these entraining agents, the commonalities and specificities of the mechanisms by which each agent acts on the oscillatory mechanism and controls the phase will be elucidated. Furthermore, in *Drosophila*, it has been shown that the zeitgebers used primarily vary from clock cell to clock cell. Thus, the mechanism of mutual phase control between these cells will be an important issue for the future.

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