# Chapter 5
# Neurocircuitry of Circadian Clocks


**Taishi Yoshii and Ayumi Fukuda**

**Abstract** Classical studies using several insect species have demonstrated that the principal circadian clock cells that generate circadian oscillations and control behavioral rhythms are located in a specific brain region. The discovery of a clock gene, *period* (*per*), in *Drosophila melanogaster* further facilitated the identification of specific cells by labeling gene expression. Since most of the *per*-expressing brain neurons display circadian molecular oscillations in the levels of *per* mRNA and its protein expression, they have conventionally been defined as "circadian clock neurons." In *Drosophila*, approximately 150 neurons (out of 200,000 brain neurons) have been identified as clock neurons. However, elucidating the role of clock neurons, even with the *Drosophila* model, has been a major challenge. In 1995, it was discovered that 16 clock neurons expressed a neuropeptide, pigment-dispersing factor (PDF), the most important neurotransmitter for the insect circadian clock. This was where *Drosophila* genetics and neuroscience met in chronobiology, leading to a significant development in the functional analysis of clock neurons in *Drosophila* and the identification of clock neurons in nonmodel insect species. This chapter will summarize the latest findings of the clock neuron network in *Drosophila* and other insect species.

**Keywords** Clock network · Clock neuron · Coupling · Neurotransmitter · PDF · PERIOD

## 5.1 Introduction

The most commonly observed circadian rhythms are shown in behavior, e.g., sleep-wake cycles or nocturnal/diurnal activity. Today, we know that cells containing circadian clocks are widespread throughout the body (Ito and Tomioka 2016; Chap. 6). Needless to say, however, the biggest question in the past was where in

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H. Numata, K. Tomioka (eds.), *Insect Chronobiology*, Entomology Monographs,
https://doi.org/10.1007/978-981-99-0726-7_5

the animal's body the clock was located. During the late 1960s and 1970s, several pioneering studies were conducted on insects to answer this question. The first study was performed by Nishiitsutuji-Uwo and Pittendrigh (1968), who surgically lesioned parts of the brain and measured locomotor activity rhythms in the cockroach *Rhyparobia* (*Leucophaea maderae*). The cockroaches in which a brain region called the optic lobe was bilaterally lesioned displayed arrhythmic locomotor activity. Later, the same conclusion was drawn from studies of other insects, such as beetles and crickets (Loher 1972; Fleissner 1982; Tomioka and Chiba 1984). Two significant findings have been made to reinforce the hypothesis that the optic lobe is the locus of the principal circadian pacemaker: (1) Transplantation of the optic lobes into the optic lobeless brain restored the activity rhythm of the donor cockroach in *R. maderae* (Page 1982). 2) The isolated optic lobes displayed circadian rhythms in neural activity in a self-sustained manner in the cricket *Gryllus bimaculatus* and *R. maderae* (Tomioka and Chiba 1986; Colwell and Page 1990; Tomioka and Chiba 1992). Thus, the optic lobes of these insects contain pacemakers that control the activity rhythm.

The optic lobes mainly consist of three neuropils, namely, the lamina, medulla, and lobula, which process visual information from the compound eyes and send the processed information to the midbrain. The anatomical relationship between the light input pathway and the circadian pacemaker is plausible, given the importance of light entrainment. In mammals, light information is conveyed directly from the eye via the retinohypothalamic tract to entrain the mammalian pacemaker located in the suprachiasmatic nucleus (SCN) (Panda et al. 2002; Ruby et al. 2002). This analogy between insects and mammals suggests that the origin of the pacemaker center should be tightly linked to photoreception.

## 5.2 Small Ventral Lateral Neurons in *Drosophila*

Classical lesion experiments have beautifully revealed the brain region important for rhythm generation. However, this method is not suitable for identifying the locus of the pacemaker at the cellular level. The discovery of the *period* (*per*) gene in the fruit fly *Drosophila melanogaster*, a model organism in genetics, overcame this difficulty by using modern cell labeling techniques, which enabled the identification of the cells expressing the *per* gene in the brain. In situ hybridization and immunohistochemistry, which label mRNA and protein expression, respectively, have revealed that *per* is expressed in cells distributed in a wide range of brain regions (Liu et al. 1988; Siwicki et al. 1988). Therefore, the difficulty in identifying pacemaker cells persisted.

Since then, developments in fluorescent immunohistochemistry, confocal laser microscopy, and transgenic fly lines, such as the GAL4-UAS system, have contributed significantly to determining the precise anatomical location of *per*-expressing brain neurons (Kaneko and Hall 2000; Helfrich-Förster 2003). Today, we know that *per* is expressed in approximately 150 brain neurons classified into nine groups

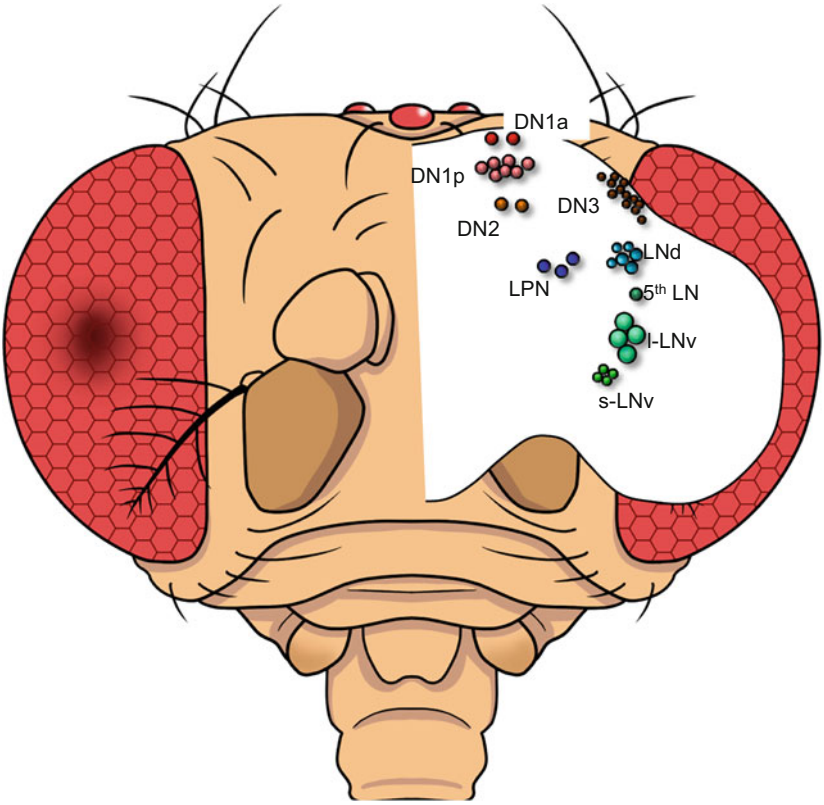


**Fig. 5.1** The brain of *Drosophila melanogaster* and the distribution of clock neurons. The *Drosophila* central clock consists of approximately 150 neurons in the brain. The clock neurons are divided mainly into nine groups based on their localization and size of cell bodies: small ventral lateral neuron (s-LNv), large ventral lateral neuron (l-LNv), fifth lateral neuron (fifth LN), dorsal lateral neuron (LNd), lateral posterior neuron (LPN), anterior dorsal neuron 1 (DN1a), posterior dorsal neuron 1 (DN1p), dorsal neuron 2 (DN2), and dorsal neuron 3 (DN3)

(Fig. 5.1). First, *per*-expressing neurons are divided mainly into the lateral and dorsal neuron groups. Lateral neurons are located between the optic lobe and midbrain and are further subdivided into small ventral lateral neuron (s-LNv), large ventral lateral neuron (l-LNv), fifth lateral neuron (fifth LN, also known as fifth s-LNv), dorsal lateral neuron (LNd), and lateral posterior neuron (LPN) groups. Dorsal neurons are located in the rim of dorsal brain regions and are further subdivided into anterior dorsal neuron 1 (DN1a), posterior dorsal neuron 1 (DN1p), dorsal neuron 2 (DN2), and dorsal neuron 3 (DN3) groups.

The *disconnected* (*disco*) gene, which encodes a $C_2H_2$-type zinc-finger transcription factor, plays a role in nervous system development. *disco* mutants lack many optic lobe neurons, including *per*-expressing lateral neurons, and are behaviorally arrhythmic (Dushay et al. 1989; Zerr et al. 1990; Hardin et al. 1992). Since the DN

groups are intact in *disco* mutants, researchers assume that lateral neurons are the central pacemaker neurons that control activity rhythms such as locomotion and eclosion. Within the lateral neuron groups, s-LNv and l-LNv neurons express a neuropeptide, pigment-dispersing factor (PDF) (Helfrich-Förster 1995). Only a few *disco* mutants retain some of the s-LNv neurons, and they are behaviorally rhythmic (Helfrich-Förster 1998), which further suggests that s-LNv neurons are the essential pacemakers.

The discovery of the *Pdf* mutant has also significantly advanced the functional analysis of s-LNv neurons. Wild-type *Drosophila* shows bimodal locomotor activity rhythms with two peaks in the morning and evening in light-dark cycles (LD) and the rhythms free run with a period of approximately 24 h in constant darkness (DD) (Konopka and Benzer 1971; Hamblen-Coyle et al. 1992). *Pdf* mutants display weak free-running activity rhythms with a period of approximately 22 h in DD for the first few days, and then the rhythms damp out (Renn et al. 1999). These phenotypes are attributed to the loss of PDF in s-LNv neurons, since *Pdf* knockdown only in s-LNv neurons (but not in l-LNv neurons) reproduces the weak activity rhythm of *Pdf* mutants (Shafer and Taghert 2009). This s-LNv master pacemaker hypothesis is further supported by the fact that *per* expression only in s-LNv neurons is sufficient for generating free-running activity rhythms in DD (Grima et al. 2004). Taken together, s-LNv neurons are the most influential clock neurons, and the PDF signaling output from s-LNv neurons conveys important circadian timing information to downstream neurons.

## 5.3 Outputs from the *Drosophila* Clock Neurons

Fourteen neurotransmitters, including PDF, have been identified in *Drosophila* cerebral clock neurons. Although studies on their functional roles are still in progress, those reported are listed in Table 5.1. Among them, nine neurotransmitters have been reported to be involved in intercellular communication between clock neurons (Fig. 5.2). Here, we summarize what we know about circadian outputs from clock neurons.

### *5.3.1 Pigment-Dispersing Factor*

PDF was found to be the first circadian neurotransmitter (Helfrich-Förster 1995). In 2005, three independent groups identified the PDF receptor (PDFR) gene (Hyun et al. 2005; Mertens et al. 2005; Lear et al. 2005). Interestingly, PDFR is expressed in many clock neurons, including PDF-positive s-LNv neurons, PDF-negative LNd, and other DN groups (Im and Taghert 2010). The function of PDF/PDFR signaling is to synchronize PDF-positive and PDFR-positive clock neurons to adjust the phase of molecular oscillations and $Ca^{2+}$ rhythms in the clocks (Peng et al. 2003; Lin et al.

**Table 5.1** Circadian neurotransmitters

| Neurotransmitter | Clock neuron group | Effect |
|---|---|---|
| PDF | s-LNv, l-LNv | Free-running rhythm<br>Morning activity<br>Evening activity |
| NPF | LNd, l-LNv | Evening activity<br>Sleep<br>Gene expression in the fat body |
| sNPF | s-LNv, LNd | Morning activity<br>Emergence rhythm |
| ITP | 5th LN, LNd | Free-running rhythm<br>Sleep |
| DH31 | DN1p, LPN | Sleep<br>Temperature preference rhythm<br>Free-running rhythm |
| CCHa1 | DN1a | Morning activity<br>Evening activity<br>Activity level<br>Sleep |
| AstA | LPN | Sleep<br>Feeding |
| AstC | LNd, DN1p, DN3, LPN | Evening activity<br>Oogenesis rhythm |
| CNMa | DN1p | Sleep |
| Trissin | LNd | Unknown |
| IPNa | DN1a | Unknown |
| Glutamate | DN1a, DN1p, DN3<br>Fifth LN?, LNd? | Free-running rhythm |
| Acetylcholine | 5th LN, LNd | Free-running rhythm |
| Glycine | s-LNv?, l-LNv? | Free-running rhythm |

2004; Shafer et al. 2008; Yoshii et al. 2009; Liang et al. 2016, 2017; Fig. 5.2a). The mammalian counterpart for PDF/PDFR signaling is vasoactive intestinal polypeptide (VIP)/VIP receptor signaling, which also functions to couple clock neurons in the SCN (Mieda 2020; Ono et al. 2021). Both PDFR and VIP receptors belong to a class II G-protein-coupled receptor family (Mertens et al. 2005), implying an evolutionarily conserved neural mechanism in a wide range of animal species.

PDF is expressed only in s-LNv and l-LNv neurons (Helfrich-Förster 1995). s-LNv and l-LNv neurons are morphologically different, although both groups are located very close to each other in the lateral brain (Helfrich-Förster 1997). s-LNv neurons have smaller cell bodies and send projections into the dorsal brain, where fifth LN, LNd, and DN1 neurons also send their projections, presumably to communicate with each other. l-LNv neurons have larger cell bodies and send the projections in two directions: one goes to the optic lobe with complex arborizations and the other to the contralateral hemisphere. Both s-LNv and l-LNv neurons also send fibers into the accessory medulla (Helfrich-Förster et al. 2007). The role of PDF

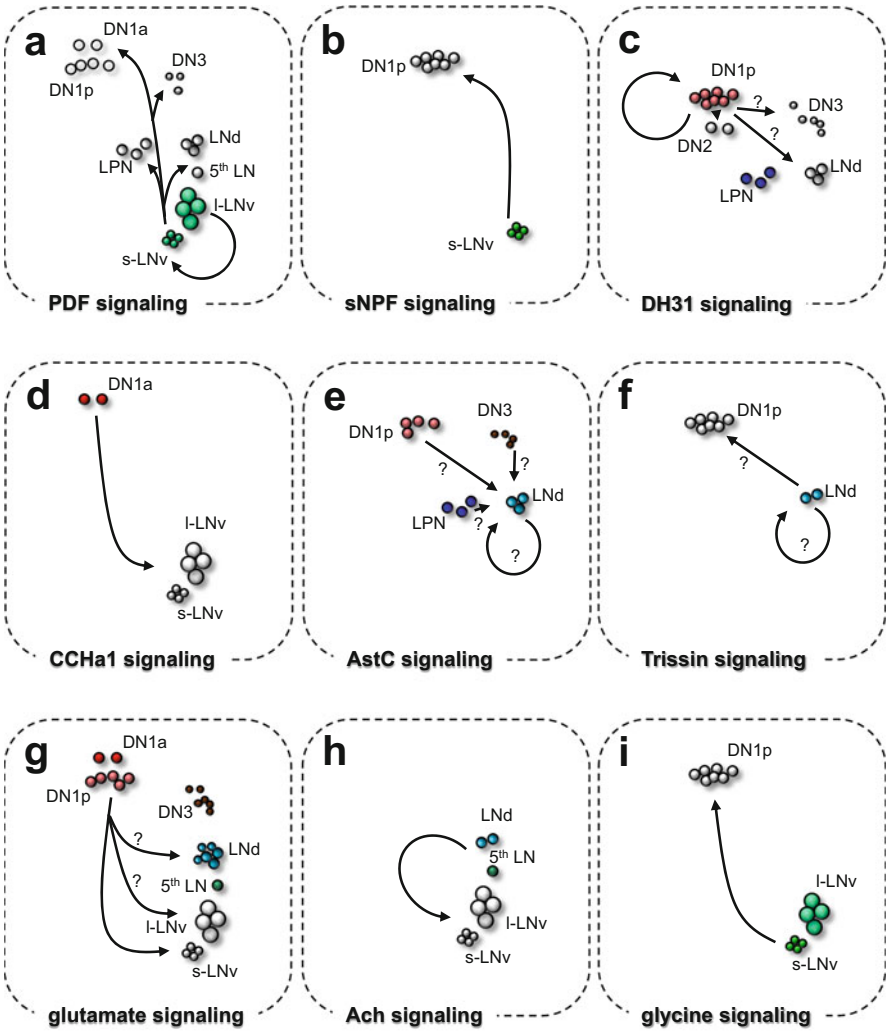


**Fig. 5.2** Intercellular communication pathways of the *Drosophila* circadian clock network. The colored clock neurons contain neurotransmitters indicated in each panel and transmit signals to other (or own) clock neurons (arrows). The colors correspond to those used in Fig. 5.1. Since the distributions of the receptors for some ligands are not identified, some correspondences between output and input neurons are uncertain

signaling from s-LNv neurons is to synchronize s-LNv neurons and other PDF receptor-positive clock neurons, such as fifth LN, LNd, and DN1 neurons, which is supposed to be important for the maintenance of free-running rhythms in DD (Peng et al. 2003; Shafer et al. 2008; Yoshii et al. 2009). The projection pattern of l-LNv neurons implies that l-LNv neurons send a signal into the visual processing neurons in the optic lobe and into the contralateral brain for bilateral clock

synchronization (Helfrich-Förster et al. 2007). The l-LNv neurons indeed receive a signal from contralateral l-LNv neurons to change membrane potential but via gap junctions, not via PDF (Cao and Nitabach 2008).

It has been inferred that l-LNv neurons are less important in free-running conditions. This is because clock protein oscillations are dampened in l-LNv neurons under DD (Yang and Sehgal 2001) and *Pdf* knockdown in l-LNv neurons does not affect free-running rhythms in DD (Shafer and Taghert 2009). $Pdf^{01}$ mutants display diminished morning activity and phase-advanced evening activity in LD. The rescue of PDF expression in l-LNv neurons restores the wild-type evening activity (Cusumano et al. 2009; Schlichting et al. 2016; Menegazzi et al. 2017; Schlichting et al. 2019b), suggesting that the role of PDF in l-LNv neurons is to set the phase of evening activity in LD. In contrast, PDF signaling from s-LNv neurons is essential for morning activity (Shafer and Taghert 2009). *pdfr* mutants also display phase-advanced evening activity (Hyun et al. 2005). The rescue of *pdfr* expression in DN1p or LNd and fifth LN neurons under the *pdfr* mutant background is sufficient for the wild-type morning and evening activity (Lear et al. 2009; Zhang et al. 2010a; Schlichting et al. 2016). According to the latest study by Schlichting et al. (2016), l-LNv neurons send PDF signaling to s-LNv neurons (PDF receptor-positive), and then s-LNv neurons pass it on to DN1p, LNd, and fifth LN neurons, which in turn generate wild-type evening activity. Since PDF is the first neuropeptide for the *Drosophila* circadian clock, it has been extensively studied. The functional analysis of PDF led directly to the analysis of s-LNv and l-LNv neurons, revealing the complexity of the circadian neural network.

### *5.3.2 Neuropeptides*

To date, 11 neuropeptides expressed in clock neurons have been identified in *Drosophila*. Johard et al. (2009) found that three neuropeptides, neuropeptide F (NPF), short-neuropeptide F (sNPF), and ion transport peptide (ITP), were expressed in subsets of clock neurons. *Drosophila* shows sleeplike behavior that is typically characterized as periods of quiescence lasting longer than 5 min (Hendricks et al. 2000; Shaw et al. 2000). NPF-positive clock neurons modulate evening activity and sleep behavior (Hermann et al. 2012; Chung et al. 2017). In addition, NPF signaling from LNd neurons entrains rhythmic gene expression in fat bodies, which are comparable organs to the mammalian liver (Chung et al. 2017).

sNPF and NPF are similar in name, but they are encoded by different genes. Immunohistochemistry using antibodies against the sNPF precursor revealed that it is expressed in s-LNv neurons and two of six LNd neurons (Johard et al. 2009). sNPF signaling from s-LNv neurons negatively affects the $Ca^{2+}$ level in DN1 neurons, which is correlated with the morning activity peak (Liang et al. 2017; Fig. 5.2b). sNPF also mediates circadian signaling from s-LNv neurons to prothoracicotropic hormone-expressing neurons, which control circadian emergence rhythms (Selcho et al. 2017).

One LNd and fifth LN neurons express the ITP neuropeptide (Johard et al. 2009). The ITP-positive LNd neuron is one of the three NPF-positive LNd neurons. Knockdown of *itp* expression in clock neurons reduces the evening activity peak in LD and lengthens the free-running period in DD (Hermann-Luibl et al. 2014). Furthermore, simultaneous knockdown of *itp* and *Pdf* increases the level of night activity in LD and makes arrhythmic. Taken together, ITP plays a role in the output of clock neurons, especially in relation to PDF signaling.

The neuropeptide diuretic hormone 31 (DH31) is expressed in DN1p and LPN neurons (Kunst et al. 2014; Reinhard et al. 2022). A loss-of-function allele of the *DH31* gene increases sleep late at night but shows normal circadian rhythms. However, DH31 does contribute to circadian activity rhythms. The double mutants of *Pdf* and *DH31* are nearly arrhythmic in DD (Goda et al. 2019), suggesting that DH31 plays a role in maintaining rhythms in the absence of PDF. DH31 receptor (DH31R) is expressed in DN1p neurons, and thus DH31 signaling from DN1p neurons may feedback on themselves (Goda et al. 2018; Fig. 5.2c). Flies change their preferred temperature over the course of a day, showing a so-called temperature preference rhythm with a peak in the evening (Kaneko et al. 2012). The DH31-PDFR signaling pathway in DN2 neurons plays a role in the temperature preference rhythm (Goda et al. 2016).

The neuropeptide CCHamide1 (CCHa1) is expressed in DN1a neurons (Fujiwara et al. 2018). The CCHa1 receptor (CCHa1R) is expressed in LNv neurons (Abruzzi et al. 2017; Fujiwara et al. 2018). Since PDFR is expressed in DN1a neurons and s-LNv neurons send projections to the dorsal brain in close proximity to DN1a neurons, DN1a and s-LNv neurons are reciprocally coupled via CCHa1 and PDF signaling (Fig. 5.2a, d). Mutant flies of *CCHa1* display diminished morning activity, reduced total activity, and enhanced sleep amount (Fujiwara et al. 2018). The mammalian homolog of CCHa1R is the receptor of gastrin-releasing peptide, which plays a role in the clock neuron network (Mieda 2020; Ono et al. 2021). Thus, similar to PDFR, the receptor (but not the ligand) is well conserved across animal species.

Allatostatin A (AstA) is a neuropeptide expressed in LPN neurons (Ni et al. 2019). Activation of LPN neurons promotes sleep and reduces feeding, and at least the sleep phenotype is partly mediated by AstA signaling (Chen et al. 2016; Ni et al. 2019; Reinhard et al. 2022). Since LPN neurons receive PDF signaling, LPN neurons are downstream of LNv neurons, and AstA signaling from LPN neurons may mediate coupling between LNv clock neurons and sleep-promoting neurons.

Allatostatin C (AstC) neuropeptide expression in clock neurons was first discovered by RNA-sequencing analysis (Abruzzi et al. 2017). Immunohistochemistry using an anti-AstC antibody further revealed that AstC is expressed in four to six DN1p, a subset of LNd, a subset of DN3, and LPN neurons (Díaz et al. 2019; Zhang et al. 2021a; Reinhard et al. 2022; Meiselman et al. 2022). Knockdown of *AstC* mRNA in clock neurons results in a phase-delayed evening activity, which is mediated by AstC-R2 (one of two AstC receptors) expressed in LNd neurons (Díaz et al. 2019). AstC is also involved in circadian rhythms in the progression of oogenesis in mated females (Allemand 1976). AstC signaling from DN1p neurons

outputs to the pars intercerebralis (PI) region, through which circadian oogenesis rhythms are generated (Zhang et al. 2021a, 2021b). Thus, AstC signaling is used in two directions. One is a signal to LNd neurons to communicate between clock neurons (Fig. 5.2e), and the other is an output of temporal information to downstream cells.

Transcriptome analyses in clock neurons reveal that two novel neuropeptides, CNMamide (CNMa) and Trissin, are expressed in DN1p and LNd neurons, respectively (Abruzzi et al. 2017; Ma et al. 2021). DN1p neurons input temperature information and modulate sleep in a temperature-dependent manner (Yadlapalli et al. 2018). CNMa signaling mediates the interaction between DN1p and PI neurons to control temperature-dependent sleep (Jin et al. 2021). In contrast, the function of Trissin has not yet been reported. Transcriptome analysis by Abruzzi et al. (2017) revealed that the receptor of Trissin is expressed in LNd and DN1 neurons, suggesting that Trissin mediates LNd-LNd and LNd-DN1 couplings (Fig. 5.2f).

IPNamide was discovered as the second circadian neuropeptide after PDF (Shafer et al. 2006). IPNamide is expressed in DN1a neurons, but its function has not been reported. IPNamide is encoded by the *neuropeptide-like precursor 1* gene (*Nplp1*), which encodes three other peptides, MTYamide, APK, and VQQ (Baggerman et al. 2002). Thus, it is difficult to analyze the function of IPNamide alone.

### 5.3.3 Glutamate, Acetylcholine, and Glycine

Vesicular glutamate transporter (VGlut) is expressed in DN1a, some DN1p, and DN3 neurons, suggesting that these clock neurons use glutamate as a neurotransmitter (Hamasaka et al. 2007). Glutamate signaling is mediated by two main receptors, a glutamate-gated chloride channel, GluCl, and a metabotropic G-protein-coupled receptor, mGluRA. The receptors are expressed in s-LNv, l-LNv, and LNd neurons (Hamasaka et al. 2007; Collins et al. 2012; Guo et al. 2016). Glutamate signaling from DN1p neurons synchronizes DN1p and LNv neurons (Collins et al. 2012; Guo et al. 2016; Fig. 5.2g). Since s-LNv neurons send PDF signaling to DN1p neurons (Shafer et al. 2008), there is reciprocal coupling between s-LNv and DN1p neurons. This coupling is essential for the robustness of molecular oscillations and normal activity rhythms (Hamasaka et al. 2007; Collins et al. 2014). *VGlut* may also be expressed in LNd and/or fifth LN neurons, as RNA interference for *VGlut* in LNd and fifth LN neurons influences activity rhythms (Duhart et al. 2020; Fig. 5.2g).

Johard et al. (2009) also reported that acetylcholine (Ach) is used as the circadian neurotransmitter, as choline acetyltransferase (ChAT) is expressed in two of six LNd and fifth LN neurons. ChAT is coexpressed with sNPF in the two LNd neurons, which is different from the three NPF-positive LNd neurons. Thus, six LNd neurons are divided into two sNPF- and ChAT-coexpressing neurons, two NPF-positive neurons, one ITP- and NPF-coexpressing neuron, and one with an unknown neurotransmitter. Cholinergic signaling, which is an excitatory input, from LNd neurons targets s-LNv and l-LNv neurons because its receptor is expressed (McCarthy et al.

2011; Lelito and Shafer 2012; Fig. 5.2h). Knockdowns of *ChAT* or *vesicular acetylcholine transporter* (*vAchT*) in LNd neurons do not change the speed of free-running activity rhythms in DD but reduce the robustness of the rhythms (Duhart et al. 2020).

The other fast neurotransmitter used in the *Drosophila* clock is glycine (Frenkel et al. 2017). Knockdown of the glycine transporter *dGlyT* in LNv (s-LNv and l-LNv) neurons results in lengthening of the free-running period in DD. Glycine applications on the cultured brain inhibit the neural activity of DN1p neurons. These results suggest that glycine is used in LNv neurons as a neurotransmitter and that its receptor is expressed in DN1p neurons. Since s-LNv neurons are responsible for the speed of the free-running period, one can assume that glycine mediates the neurotransmission from s-LNv neurons to DN1p neurons (Fig. 5.2i).

### *5.3.4 Gap Junctions*

Most studies of the neural network of the *Drosophila* circadian clock are concerned with chemical synapses. Perhaps this is because genetic screening targeting ligands or their receptors is an advantage of *Drosophila* research. However, insect clock neurons are known to couple at electrical synapses as well (Schneider and Stengl 2006; Li et al. 2018). In the case of *Drosophila*, the electrical synapse is composed of gap junctions by eight innexin proteins (innexins 1–8). Knockdown of *Innexin1* and *Innexin2* expression in clock neurons results in longer free-running periods than control strains in DD (Ramakrishnan and Sheeba 2021). Additionally, knockdown of *Innexin2* expression leads to a phase shift of PER oscillation and reduces PDF expression in the morning. These results suggest that gap junction-mediated signaling between clock neurons is important for maintaining circadian molecular oscillations.

### *5.3.5 Output Modes*

Clarifying when and how circadian neurotransmitters are released is still challenging, although some recent advances may have developed experimental methods to approach this long-lasting problem (Leopold et al. 2019; Ding et al. 2019). PDF immunostaining reveals that the PDF level in the terminals of the s-LNv projections cycles with a peak in the morning (Park et al. 2000). Similar observations have been made on ITP, CCHa1, DH31, AstA, and AstC neuropeptides, but the phases of their rhythms are different (Hermann-Luibl et al. 2014; Fujiwara et al. 2018; Díaz et al. 2019; Reinhard et al. 2022). The cycling of the neuropeptide contents may not reflect their synaptic release directly, but it implies that they are released in a circadian manner. A study using a fluorescent sensor for visualizing neuropeptide release has revealed that s-LNv neurons release neuropeptides in the morning with a slight delay

from the peak of PDF level at their axonal terminals (Klose et al. 2021). Thus, it is very likely that other circadian neuropeptides are also rhythmically released, by which timing information is transmitted to postsynaptic downstream neurons. However, the importance of rhythmic PDF release to activity rhythms is still controversial (Kula et al. 2006; Prakash et al. 2017).

The cyclic chemical transmissions can be complexly organized by the circadian structure remodeling of clock neurons. Fernández et al. (2008) found that the axonal terminals of s-LNv neurons change morphology, higher complexity during the day and lower complexity during the night, and the daily morphological changes are regulated by the molecular clock. Through rhythmic structure remodeling, s-LNv neurons change synaptic partners throughout the day (Gorostiza et al. 2014). A recent study proposed that s-LNv circadian remodeling is important for integrating light and temperature inputs (Fernandez et al. 2020). Similar morphological changes have been reported in fifth LN, LNd, and DN1a neurons (Duhart et al. 2020; Song et al. 2021). Thus, the circadian remodeling of axonal terminals may be a general property of clock neurons.

## 5.4 Functional Differentiation of Individual Clock Neuron Groups in *Drosophila*

### *5.4.1 Morning and Evening Oscillators in the* Drosophila *Circadian Clock*

*Drosophila* shows two distinct activity peaks in the morning and evening (Helfrich-Förster 2000), and the two activity peaks are controlled by two oscillators with different properties (Yoshii et al. 2012). Grima et al. (2004) and Stoleru et al. (2004) proposed that the two oscillators are separate groups of clock neurons: the morning oscillator (M oscillator) corresponds to s-LNv neurons and the evening oscillator (E oscillator) to LNd neurons. Later, the fifth LN neuron was identified (Rieger et al. 2006) and considered the evening oscillator. The M and E oscillators have different response modes to light. An exposure of dim light, which is equivalent to a light intensity of a quarter moon, in the night phase results in a phase advance of the M peak and a phase delay of the E peak (Bachleitner et al. 2007). This result fits well with the classical two-oscillator model proposed from studies performed in rodents (Pittendrigh and Daan 1976). In this model, the M oscillator accelerates, and the E oscillator decelerates the speed of oscillations upon light exposure. By changing the speed of oscillation depending on light exposure, both oscillators can flexibly adapt to different photoperiods, by which the circadian clock enables the measurement of day length to predict the coming season.

The *Drosophila* two-oscillator model has certainly inspired the functional analysis of clock neurons. Many studies support this model, but on the other hand, some studies note that it is oversimplified. For example, $per^{0}$ mutants with a *per* rescue

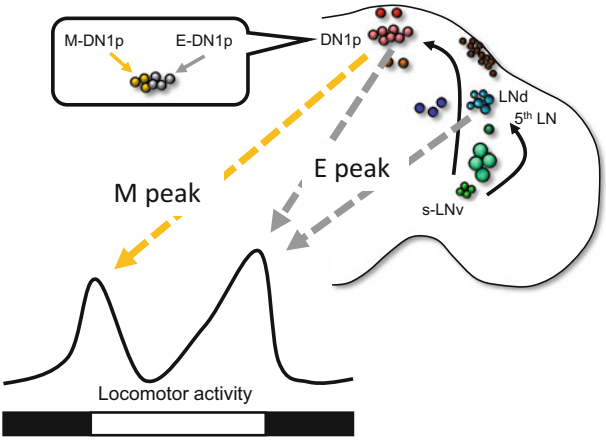


**Fig. 5.3** Current model for generating morning (M) and evening (E) activity peaks in *Drosophila*. *Drosophila* shows two activity peaks in the morning and evening under LD. In principle, s-LNv neurons and fifth LN and LNd neurons are designated as the M oscillator and E oscillator, respectively. DN1p neurons contain the M (M-DN1p) and E oscillators (E-DN1p) and mediate the output from s-LNv neurons

expression only in a subset of DN1p neurons display relatively normal morning and evening activity (Zhang et al. 2010b). Additionally, flies without s-LNv neurons still display a clear morning activity peak (Sheeba et al. 2010). $per^0$ mutants with a *per* rescue only in M or E oscillator do not completely restore typical morning and evening activity under various photoperiods (Menegazzi et al. 2020). On the other hand, CRISPR-mediated *per* or *tim* gene knockout only in M oscillator causes loss of the morning activity peak (Delventhal et al. 2019). These seemingly contradictory results are because it does not take into account the complex neural network between clock neurons (Jaumouillé et al. 2021). Since LNv and DN1 neurons interact intricately with each other through multiple neurotransmitters, it may be challenging to analyze functions only by manipulating specific clock neuron groups (Yao and Shafer 2014; Yao et al. 2016). Figure 5.3 shows a current M-E two-oscillator model that highlights the importance of the DN1p group for generating both M and E peaks (Chatterjee et al. 2018). In this model, two types of DN1p neurons (M-DN1p and E-DN1p neurons) control the M and E peaks. M-DN1p neurons receive a signal from the M oscillator (s-LNv neurons) and control M peak. E-DN1p neurons and E oscillators (fifth LN and LNd neurons) control the E peak, but they are concurrently influenced by the M oscillator.

### *5.4.2 s-LNv Neurons as the Master Clock?*

s-LNv neurons have been considered to be the master pacemaker clock. This hypothesis is based on the following facts: (1) the stability of the free-running

rhythm in DD is significantly weakened by the loss of PDF or PDF-positive LNv neurons, and (2) the free-running rhythm is restored by *per* rescue only in s-LNv neurons (Helfrich-Förster 1998; Renn et al. 1999; Grima et al. 2004; Cusumano et al. 2009). However, several lines of evidence point to different ideas. For example, $per^{0}$ mutant flies with a *per* rescue only in the E oscillator (fifth LN and LNd neurons) can display a free-running rhythm under constant dim light conditions (Rieger et al. 2009). Disruption of the molecular clock only in s-LNv neurons is insufficient to render flies arrhythmic in DD (Delventhal et al. 2019; Schlichting et al. 2019a, 2019b; Jaumouillé et al. 2021). Furthermore, silencing of neural activity in M or E oscillators, even with normal molecular oscillations, also renders fly arrhythmic (Bulthuis et al. 2019), which suggests that the disconnection of intercellular communication between clock neurons causes the loss of the free-running rhythm. Thus, s-LNv neurons remain essential for self-sustained DD rhythms, but the importance of other clock neurons has been overlooked.

### *5.4.3 DN1p Neurons as Circadian Output Centers*

DN1p neurons are composed of heterogeneous neurons. Six of 15 DN1p neurons express CRY and PDFR (Yoshii et al. 2008; Im and Taghert 2010), and they use AstC, DH31, glutamate, and CNMa as neurotransmitters (Kunst et al. 2014; Chatterjee et al. 2018; Ma et al. 2021). CRY-positive DN1p neurons are involved in sexual interactions (Fujii and Amrein 2010; Hanafusa et al. 2013), feeding (Barber et al. 2016), sleep regulation (Guo et al. 2016, 2017; Lamaze et al. 2018), memory extinction (Zhang et al. 2021b), activity rhythms (Nettnin et al. 2021), and reproductive rhythms (Zhang et al. 2021a). These reports suggest that CRY-positive DN1p neurons may be the output center of the circadian clock, which transmits timing information to downstream neurons to generate various behavioral rhythms. This may be why DN1p neurons have many different neurotransmitters.

## 5.5 Downstream Neurons of the *Drosophila* Circadian Clock

PI neurons have been considered the circadian output region for many years (Nishiitsutsuji-Uwo et al. 1967; Cymborowski 1973; Sokolove and Loher 1975; Takekata et al. 2018). In *Drosophila*, there are three distinct populations of PI neurons that express three different peptides: diuretic hormone 44 (DH44), SIFamide (SIFa), and *Drosophila* insulin-like peptide (dilp2). DN1p and LNd neurons directly or indirectly contact PI neurons (Cavanaugh et al. 2014; Barber et al. 2016, 2021). DH44-positive and SIFa-positive PI neurons mediate the output pathways to control circadian activity rhythms, whereas SIFa-positive and

dilp2-positive PI neurons control feeding rhythms and metabolism (Cavanaugh et al. 2014; Barber et al. 2016, 2021; Dreyer et al. 2019). Some DN1p neurons also contact tubercular-bulbar neurons that, in turn, connect ellipsoid body ring neurons (Guo et al. 2018; Lamaze et al. 2018), which include those that promote sleep and those involved in the output of activity rhythms (Liang et al. 2019).

DN1p neurons are not the only ones coupled to output pathways. As mentioned above, s-LNv neurons communicate with PTTH neurons via sNPF signaling (Selcho et al. 2017). PDF (and sNFP) signaling from s-LNv neurons plays roles in reproductive dormancy mediated by dilp2-positive PI neurons (Nagy et al. 2019) and memory mediated by the mushroom body (Flyer-Adams et al. 2020; Inami et al. 2021). The other output pathway from s-LNv neurons is the neuropeptide leucokinin (LK)-positive neurons (Cavey et al. 2016). Both *Lk* and *Lk receptor* mutants reduce the power of activity rhythms in DD. All the output pathways mentioned above have been analyzed morphologically, physiologically, and behaviorally. Recent electron microscopic data further revealed entire postsynaptic neurons of all clock neurons (Scheffer et al. 2020), showing that the output of the circadian clock spreads across a wide range of brain neurons.

## 5.6 Clock Neuron Networks in Other Insect Species

In insect species other than *Drosophila melanogaster*, immunostaining against PDF has provided the most reliable results for identifying putative clock neurons. This is because PDF antibodies are specific to many species due to the high conservation of PDF peptide sequences (Meelkop et al. 2011). Similar to all insect species studied, PDF cells reside in the lateral protocerebrum, and they extend neuronal processes toward the central brain and optic lobes (Helfrich-Förster 2005). In the cockroach *R. maderae*, ectopic transplantation of the accessory medulla, including PDF neurons, can restore activity rhythms in optic lobeless arrhythmic cockroaches, strongly suggesting the importance of PDF neurons (Reischig and Stengl 2003). Injections of synthetic PDF peptides into the brain phase shift activity rhythms in the cockroach (Petri and Stengl 1997) and cricket (Singaravel et al. 2003). The knockdown of *Pdf* mRNA expression by RNA interference or the knockout of the *Pdf* gene by CRISPR/Cas9 results in arrhythmicity or a short free-running period in the German cockroach (Lee et al. 2009), cricket (Hassaneen et al. 2011), and bug (Kotwica-Rolinska et al. 2022). Furthermore, circadian rhythms at the PDF level and the structural changes of the projections from PDF neurons have also been detected (Abdelsalam et al. 2008; Wei and Stengl 2011). Putting all these results together, it is likely that, as in *Drosophila*, PDF and PDF neurons are essential for circadian activity rhythms in insects. However, things are not so simple.

While the identification of PER-expressing cells in the brain has been attempted in several insect species, the locations of the PER-expressing cells are often different from those of *Drosophila* (Table 5.2; Helfrich-Förster 2005; Beer and Helfrich-Förster 2020). PER is not colocalized in PDF neurons in some insect species (Frisch

**Table 5.2** List of studies investigating insect clock neurons

| Reference | Species | Labeling method |
|---|---|---|
| Frisch et al. (1996) | Coleoptera (*Pachymorpha sexguttata*) | PER, PDF immunostaining |
| Sauman and Reppert (1996) | Lepidoptera (*Antheraea pernyi*) | PER immunostaining<br>*per* in situ hybridization |
| Wise et al. (2002) | Lepidoptera (*Manduca sexta*) | PER immunostaining<br>*per* in situ hybridization |
| Lupien et al. (2003) | Orthoptera (*Teleogryllus commodus*)<br>Orthoptera (*Teleogryllus oceanicus*) | PER, PDF immunostaining |
| Bloch et al. (2003) | Hymenoptera (*Apis mellifera*) | PER, PDF immunostaining |
| Závodská et al. (2003a) | Thysanura (*Thermobia domestica*) | PER immunostaining |
| Závodská et al. (2003b) | Archaeognatha (*Lepismachilis y-signata*)<br>Odonata (*Ischnura elegans*)<br>Ephemeroptera (*Siphlonurus armatus*)<br>Plecoptera (*Perla burmeisteriana*)<br>Orthoptera (*Locusta migratoria*)<br>Orthoptera (*Schistocerca gregaria*)<br>Hemiptera (*Aquarius paludum*)<br>Hemiptera (*Notonecta glauca*)<br>Hymenoptera (*Apis mellifera*)<br>Coleoptera (*Pachnoda marginata*)<br>Diptera (*Neobellaria bullata*)<br>Diptera (*Phormia regina*)<br>Trichoptera (*Hydropsyche contubernalis*) | PER immunostaining |
| Sehadová et al. (2004) | Lepidoptera (*Bombyx mori*) | PER, CRY, CYC, DBT immunostaining |
| Sauman et al. (2005) | Lepidoptera (*Danaus plexippus*) | PER, CRY, TIM immunostaining<br>*per*, *cry* in situ hybridization |
| Závodská et al. (2005) | Blattodea (*Periplaneta americana*) | PER immunostaining |
| Shao et al. (2006) | Orthoptera (*Dianemobius nigrofasciatus*)<br>Orthoptera (*Allonemobius allardi*) | PER, CRY, DBT immunostaining |
| Shao et al. (2008a) | Orthoptera (*Dianemobius nigrofasciatus*) | CLK, CYC immunostaining |
| Shao et al. (2008b) | Orthoptera (*Allonemobius allardi*) | CLK, CYC immunostaining |
| Zhu et al. (2008) | Lepidoptera (*Danaus plexippus*) | TIM, CRY1, CRY2 immunostaining<br>*cry2* in situ hybridization |
| Shiga and Numata (2009) | Diptera (*Protophormia terraenovae*) | PER, PDF immunostaining |
| Wen and Lee (2008) | | PER, PDF immunostaining |

(continued)

**Table 5.2** (continued)

| Reference | Species | Labeling method |
|---|---|---|
| | Blattodea (*Blattella germanica*)<br>Blattodea (*Blattella bisignata*) | |
| Vafopoulou et al. (2010) | Hemiptera (*Rhodnius prolixus*) | PER, TIM, PDF immunostaining |
| Mohamed et al. (2014) | Lepidoptera (*Antheraea pernyi*) | PER, CLK, CYC immunostaining |
| Kobelková et al. (2015) | Lepidoptera (*Ephestia kuehniella*) | PER immunostaining<br>*per*, *tim* in situ hybridization |
| Barberà et al. (2017) | Hemiptera (*Acyrthosiphon pisum*) | *per*, *tim* in situ hybridization |
| Fuchikawa et al. (2017) | Hymenoptera (*Apis mellifera*) | PER, PDF immunostaining |
| Beer et al. (2018) | Hymenoptera (*Apis mellifera*) | PER, PDF immunostaining |
| Kay et al. (2018) | Hymenoptera (*Camponotus floridanus*) | PER, PDF immunostaining |
| Kutaragi et al. (2018) | Orthoptera (*Gryllus bimaculatus*) | *per*, *cry2*<br>in situ hybridization |
| Colizzi et al. (2021) | Hemiptera (*Acyrthosiphon pisum*) | PER, CRY1, PDF immunostaining |
| Koide et al. (2021) | Hemiptera (*Riptortus pedestris*) | PER, PDF immunostaining |
| Barberà et al. (2022) | Hemiptera (*Acyrthosiphon pisum*) | *cry1*, *cry2*<br>in situ hybridization |

et al. 1996; Sauman and Reppert 1996; Závodská et al. 2003b; Koide et al. 2021). Furthermore, in the cricket, the surgical lesion of the outer medulla and lamina neuropils results in arrhythmicity even though the accessory medulla, including PDF neurons, is still intact (Okamoto et al. 2001). Therefore, we have to be cautious in simply concluding PDF neurons as clock neurons. It is quite possible that there is a diversity of clock neuron networks, along with the diversity of insect species. Even within the genus *Drosophila*, there are some variations in PDF/clock protein expression patterns (Hermann et al. 2013; Menegazzi et al. 2017). In contrast, several studies have shown clock neuron networks similar to those of *Drosophila* in the blowfly *Protophormia terraenovae* (Shiga and Numata 2009), the honeybee *Apis mellifera* (Fuchikawa et al. 2017; Beer et al. 2018), the ant *Camponotus floridanus* (Kay et al. 2018; Fig. 5.4a), and the aphid *Acyrthosiphon pisum* (Barberà et al. 2017; Colizzi et al. 2021; Fig. 5.4b). In addition, subsets of PER-positive neurons in the blowfly, ant, and honeybee also exhibit PDF, although this is not true in the aphid because it seems that the *Pdf* gene is lost in the aphid genome (Huybrechts et al. 2010).

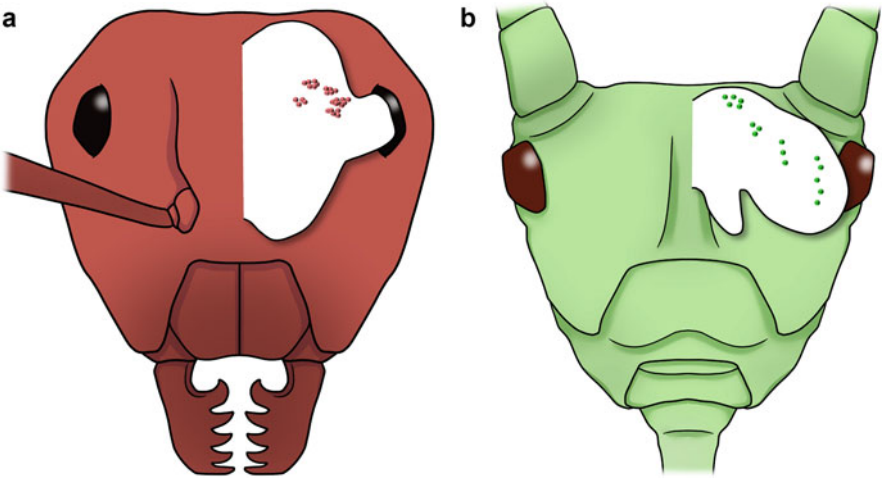


**Fig. 5.4** The brains of the ant *Camponotus floridanus* (**a**) and aphid *Acyrthosiphon pisum* (**b**) and their clock neurons. In both insects, clock neurons form clusters similar to *Drosophila* lateral and dorsal neurons (Fig. 5.1). The ant clock neurons consist of approximately 200 neurons, whereas the aphid clock neurons consist of approximately 40 neurons

## 5.7 Bilateral Coupling Between Two Optic Lobe Clocks

*Drosophila* is not always used as a model of insect chronobiology. The coupling between two clocks residing in the left and right brain is such a subject of study. Even in advanced *Drosophila* genetics, it is not possible to manipulate one side of the body asymmetrically. The tiny brain of *Drosophila* also makes it difficult to manipulate the brain surgically. In contrast, robust insect species with larger brains, such as crickets and cockroaches, are good models.

Page and his colleagues demonstrated that excision of one optic lobe (either right or left) in the cockroach *R. maderae* did not affect the ability to generate free-running activity rhythms, but their periods were longer than those of intact animals (Page et al. 1977; Page 1978). They proposed that two clock components that reside in the left and right optic lobes were mutually coupled and each clock worked to shorten the period of the other clock. The cricket clocks are more intriguing because the coupling between the two optic lobe clocks seems weaker than that of cockroaches. If the optic nerve is unilaterally disconnected from the optic lobe, this optic lobe should be blind and free-run as if it is in DD, while the contralateral optic lobe should be entrained by light cycles unless the two optic lobes exchange the light information. In this situation, crickets display two rhythms simultaneously, a phenomenon called "splitting" (Wiedenmann 1983; Tomioka et al. 1991; Tomioka 1993). The two rhythms do not run completely independently; the free-running period is modulated by the coupling of two optic lobe clocks (Tomioka et al. 1991; Tomioka 1993). Figure 5.5 shows a model of the bilateral optic lobe clocks that interact with

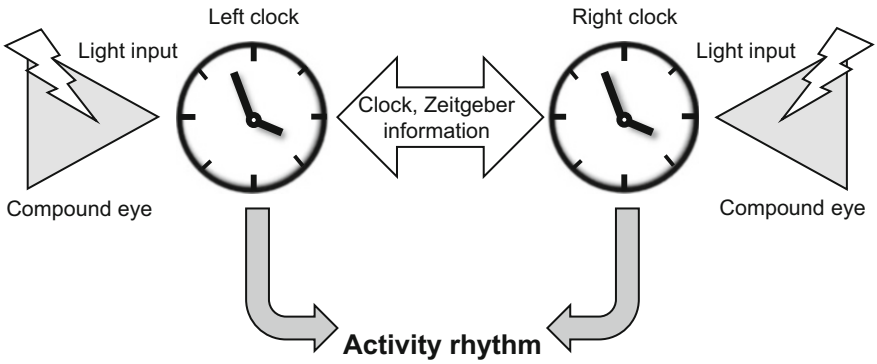


**Fig. 5.5** A model of two clocks located in bilateral optic lobes in the cockroach and cricket. The left and right clocks independently receive light information from the compound eyes on each side. Although the two clocks can separately drive activity rhythms, they mutually interact to exchange time and zeitgeber information, enabling the generation of a coherent activity rhythm

each other to exchange zeitgeber and time information. PDF and serotonin are used in this bilateral coupling pathway (Saifullah and Tomioka 2002, 2003). In particular, PDF neurons form commissures projecting in the contralateral optic lobe (Helfrich-Förster 1997; Reischig et al. 2004), which is suitable for the coupling pathway, and its morphology is conserved across many insect species. It should also be mentioned that there are many more interneurons that bridge two sides of the optic lobes and possibly mediate the coupling (Yukizane and Tomioka 1995; Reischig and Stengl 2002).

A series of studies on the bilateral coupling of two optic lobe clocks have left the detailed mechanisms unknown (Page et al. 1977; Page 1978; Wiedenmann 1983; Tomioka et al. 1991; Tomioka 1993). However, these studies provide key points into insect circadian networks: (1) coupling may be needed for exchanging zeitgeber and time information, and (2) the strength of coupling may vary among species.

## 5.8 Conclusion Remarks

There is still not enough data to summarize the whole picture of insect clock networks. It would be important to try immunostainings with specific antibodies in many more insect species. Even for species that have already been studied previously, it would be significant to perform the latest fluorescent immunostaining with a confocal microscope and newly generated antibodies. In addition, neurotransmitters other than PDF have not yet been focused on nonmodel insects. In *Drosophila*, the first immunostaining against PER was performed in the late 1980s (Siwicki et al. 1988), but the presently known classification of clock neurons is based on studies conducted approximately in the year 2000. Surprisingly, more detailed and precise classification is still an ongoing subject (Schubert et al. 2018; Reinhard et al. 2022).

Thus, the study of clock neuron networks will continue to be an active area of insect chronobiology.

**Acknowledgments** We thank Misako Yoshii for figure drawing and Charlotte Helfrich-Förster for comments on figures. This work was supported by JSPS KAKENHI (19H03265).

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