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Review Article (Invited)

Regulation of long-term memory by a few clock neurons in *Drosophila*

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Identification of the neural circuits in the brain regulating animal behavior and physiology is critical for understanding brain functions and is one of the most challenging goals in neuroscience research. The fruitfly *Drosophila melanogaster* has often been used to identify the neural circuits involved in the regulation of specific behaviors because of the many neurogenetic tools available to express target genes in particular neurons. Neurons controlling sexual behavior, feeding behavior, and circadian rhythms have been identified, and the number of neurons responsible for controlling these phenomena is small. The search for a few neurons controlling a specific behavior is an important first step to clarify the overall picture of the neural circuits regulating that behavior. We previously found that the clock gene *period* (*per*), which is essential for circadian rhythms in *Drosophila*, is also essential for long-term memory (LTM). We have also found that a very limited number of *per*-expressing clock neurons in the adult brain are required for the consolidation and maintenance of LTM. In this review, we focus on LTM in *Drosophila*, introduce the concept of LTM regulation by a few clock neurons that we have recently discovered, and discuss how a few clock neurons regulate *Drosophila* LTM.

Key words: memory consolidation, memory maintenance, *period*, circadian clock, courtship conditioning

Significance

Identifying neurons involved in behavioral control is crucial to understanding the operating principles of neural circuits. There has been a rapid development in the use of neurogenetic tools in *Drosophila* to identify a small number of neurons regulating specific behaviors and then use these identified neurons as clues to clarifying the complex neural networks. This review outlines the importance of research strategies to identify complex neural circuits from the small number of neurons involved in the control of long-term memory, which have recently been identified.

Introduction

The animal brain comprises many neurons connected to each other to form complex neural circuits and control behavior and physiology (e.g., instinctive and learned behaviors, maintenance of physiological state, and adaptation to the external environment). Identifying neurons and neuronal circuits regulating specific behavior or physiology is extremely important for elucidating brain functions. Since 2000, the fruitfly *Drosophila melanogaster*, for which vast genetic tools are available, has been frequently used to clarify brain function, and neurons or circuits associated with specific behaviors and physiology have been identified [1-9]. In particular, circadian rhythms found in many animal species have been best

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investigated in *Drosophila*, and their molecular and cellular bases have been elucidated. The gene *period* (*per*), which is conserved in many animal species and essential for the generation of circadian rhythms, was identified in *Drosophila* as the first clock gene [10,11]. Subsequent studies have revealed additional clock genes, and the details of the molecular mechanisms of circadian rhythms have been elucidated [8,12,13]. The fly brain contains approximately 150 *per*-expressing neurons, which are referred to as clock neurons. The clock neurons can be divided into at least six neuronal clusters: small ventral–lateral neurons (s-LNvs), large ventral–lateral neurons (l-LNvs), dorsal–lateral neurons (LNds), and three types of dorsal neuron (DN_1, DN_2, and DN_3) [8,12]. Among them, s-LNvs are considered the pacemaker neurons that play a crucial role in the generation of circadian rhythms of locomotor activity in *Drosophila* [8,12,14,15]. Although about 150 clock neurons exist in the fly brain, only four pairs of s-LNv neurons function as the circadian pacemaker [8,12]. This is a surprising finding that a very small number of brain neurons control fly physiology and its related behaviors. Furthermore, in *Drosophila*, a small number of command neurons directly control behaviors, not only in circadian rhythms but also in male courtship or feeding behavior [1,16]. Thus, as a first step toward fully understanding the neural network of the brain that controls specific behaviors, it would be useful to identify a small number of neurons that have a significant impact on particular behaviors. In this article, we refer to the small number of neurons that trigger specific physiological and behavioral events as "singularity cells", and summarize our current findings that only a very limited number of clock neurons function as the singularity cells regulating *Drosophila* memory processes.

Courtship Conditioning to Measure *Drosophila* Memory

Several behavioral assays have been established to measure *Drosophila* memory [17-19]. One of the behavioral assays, courtship conditioning, first reported by Siegel and Hall in 1979 [18], is still frequently used in *Drosophila* memory research [7,20-22]. In this assay, virgin males and previously mated females are paired in an observation chamber. Mated females release an inhibitory sex pheromone called cis-vaccenyl acetate (cVA) which suppresses male courtship and simultaneously makes females vigorously rejects male courtship [22]. Males initiate courtship when exposed to the female sex pheromone, but when they approach a mated female, they receive the inhibitory sex pheromone, and their courtship activity is reduced. Even if they court a mated female, the female rejects them, and remating rarely occurs. Conditioned males who have undergone this experience do not court very much, even if paired with a virgin female. In *Drosophila*, there are many mutants that cannot form memories. Even when such memory mutants had been used for courtship conditioning, they do not show experience-dependent courtship suppression. Therefore, courtship suppression induced by courtship conditioning is considered to be based on memory of past experiences. The memory generated by courtship conditioning is called courtship memory. When courtship conditioning is applied to wild-type males for 1 h (1 h conditioning), courtship suppression persists for at least 8 h after 1 h conditioning [7,23]. However, after 24 hours, male courtship activity is restored, and they actively court females again [7,23]. On the other hand, when courtship conditioning is applied for 7 h (7 h conditioning), courtship suppression lasts for at least 5 days (Fig. 1) [7]. Thus, 1 h conditioning is used to measure short-term memory (STM), whereas 7 h conditioning is used to measure LTM.

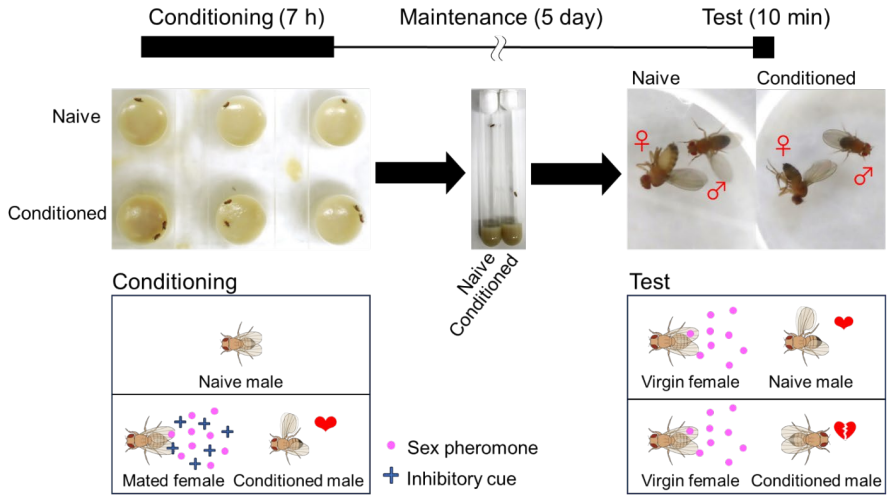

Figure 1 Courtship conditioning in *Drosophila*. A male and a mated female are introduced into an acrylic conditioning chamber (15 mm diameter, 5 mm depth). Males are conditioned for 7 h and subsequently kept for 5 d until the test. In the test, male courtship activity toward virgin females is measured for 10 min.

Drosophila Courtship Memory and the Clock Gene *per*

Throughout the day, animals acquire many memories, but newly acquired memories are unstable, many of which are lost the next day (STM). However, acquired memory is converted from STM to LTM in the brain under certain conditions, resulting in a stable LTM. Once acquired, LTM is maintained in the brain for a long time. Genetic studies of courtship memory in *Drosophila* have identified many genes essential for LTM (hereafter, courtship LTM genes) [7]. We found the clock gene *per* as the first courtship LTM gene [24]. Our finding suggests that *per*-expressing clock neurons are critically involved in *Drosophila* LTM. *per* null mutant males establish STM normally but cannot acquire LTM. The adverse effect of *per* mutations on courtship LTM was also confirmed by Donlea et al. [25]. When *per* is expressed in males with the *per* null mutant background during 7 h conditioning, LTM is established [24]. However, the induction of *per* expression after 7 h conditioning cannot restore LTM [24]. In addition, *per* overexpression during 7 h conditioning enhances courtship LTM [24]. Taken together, it is most likely that *per* is essential for memory consolidation to establish LTM.

In the generation of circadian rhythms, the Per protein forms a heterodimer with its partner protein Timeless (Tim); if the Per/Tim heterodimer does not form normally, the circadian rhythms are lost [11,13]. The Clock (Clk) protein forms a heterodimer with the Cycle (Cyc) protein [11,13]. The Clk/Cyc heterodimer acts as a transcription factor for *per* and *tim* , and circadian rhythms are lost in *Clk* and *cyc* mutants [11,13]. Thus, these four clock genes (*per*, *tim*, *Clk*, and *cyc*) are essential for the generation of circadian rhythms. Interestingly, *tim*, *Clk*, and *cyc* mutants show no specific defects in LTM [24]. Therefore, Per regulates LTM by molecular mechanisms distinct from circadian rhythms (Fig. 2). Since only *per* is involved in LTM among the four clock genes mentioned above, which has also been confirmed in LTM induced by associative learning of odors and electrical shocks [26], Per plays a critical role in LTM consolidation, regardless of the type of *Drosophila* memory.

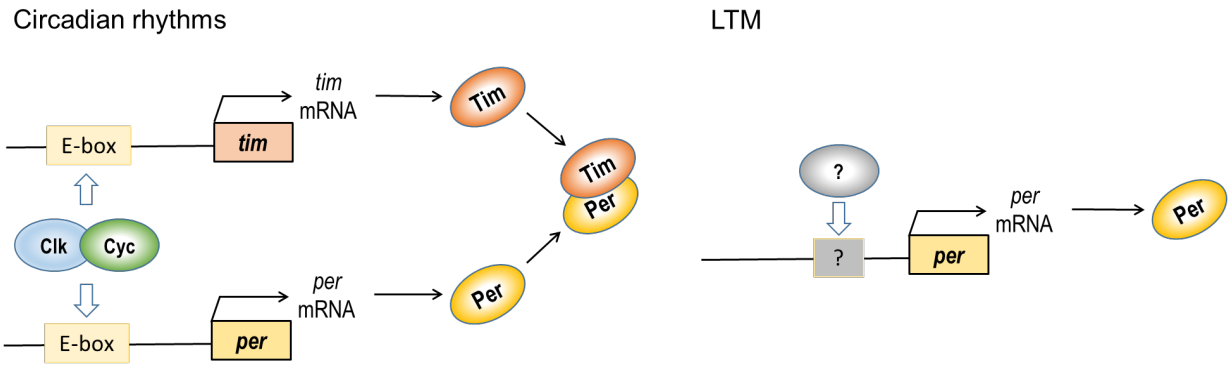

Figure 2 Differential molecular mechanisms of circadian rhythms and LTM in *Drosophila*. Clk/Cyc-meditated expression of Per and Tim is essential for the generation of circadian rhythms. Of the four clock proteins, only Per is essential for LTM.

Two Pairs of LNds Essential for the Regulation of Courtship LTM

Since 2004, many courtship LTM genes have been identified, and the majority of them are expressed in the mushroom body (MB), which is considered to be the memory center of *Drosophila* [7]. Thus, courtship LTM in *Drosophila* is considered to be consolidated and maintained in the MB neurons. Nevertheless, no evidence has been obtained indicating that Per is expressed in MB. Thus, the discovery of *per* as a courtship LTM gene suggests that neurons other than MB are also essential in regulating courtship LTM. Since *per* plays a crucial role in LTM consolidation, among the approximately 150 clock neurons, there should be neurons that play critical roles in memory consolidation. Which clock neurons regulate courtship LTM? One of the clock neuron clusters, LNds, is critically involved in courtship LTM [27]. LNds are composed of six clock neurons in one hemisphere, all of which express Per [12,27]. We have established an experimental system that can induce gene expression in only two pairs of neurons among LNds (Fig. 3) using the so-called split-GAL4 system, which is a *Drosophila* gene expression system [27,28]. Although the electrical silencing of two pairs of LNds does not affect circadian rhythms or courtship STM, it impairs courtship LTM, indicating that the electrical activity in the two pairs of LNds specifically modifies courtship LTM [27].

One of the neurogenetic tools in *Drosophila*, *shibire*^{ts1} (*shi*^{ts1}), can disrupt the neurotransmission of specific neurons in a temperature-dependent manner [29,30]. The *Drosophila* gene *shi* encodes a Dynamin protein, which regulates the

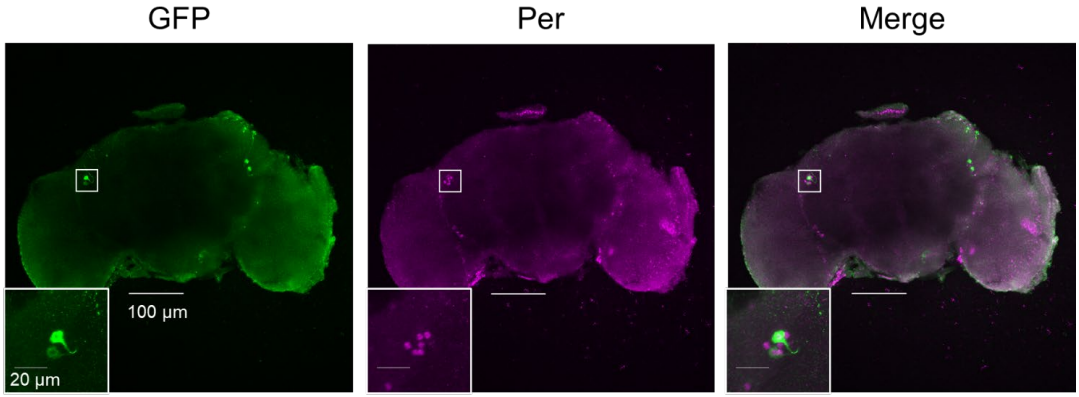

Figure 3 Dorsal–lateral clock neurons (LNds) in LNd-split-GAL4/UAS- *mCD8::GFP* flies. Scale bars, 100 μm (whole brain) or 20 μm (LNds); green, GFP; magenta, Per.

synaptic vesicle recycling, and the temperature-sensitive allele shi^{ts1} functions as a normal dynamin at permissive temperatures (19–25°C), but it is dysfunctional at restrictive temperatures above 30°C, resulting in the disruption of neurotransmission. Experiments using transgenic flies expressing shi^{ts1} in two pairs of LNds to disrupt neurotransmitter release from LNds during the consolidation, maintenance, or recall phases revealed that neurotransmission from LNds is essential for maintaining courtship LTM [27]. LTM is maintained for at least 5 days when neurotransmission from the LNds is disrupted for 24 h the day after courtship conditioning. However, if neurotransmission is inhibited on the second day after courtship conditioning, LTM is not maintained and is lost (Fig. 4). Interestingly, LTM is maintained even when neurotransmission is inhibited from day 3 to day 4 after courtship conditioning. In other words, the neurotransmitter released from LNds on day 2 after courtship conditioning maintains LTM for at least 5 days. In mutants or transgenic flies that we have found unable to maintain LTM [23], LTM is confirmed on the day after courtship conditioning but lost on the second day. Therefore, it is most likely that LTM is consolidated by the day after courtship conditioning and that the maintenance phase of LTM begins on the second day (Fig. 4). Considering these results, neurotransmission from LNds may trigger the start of the LTM maintenance phase.

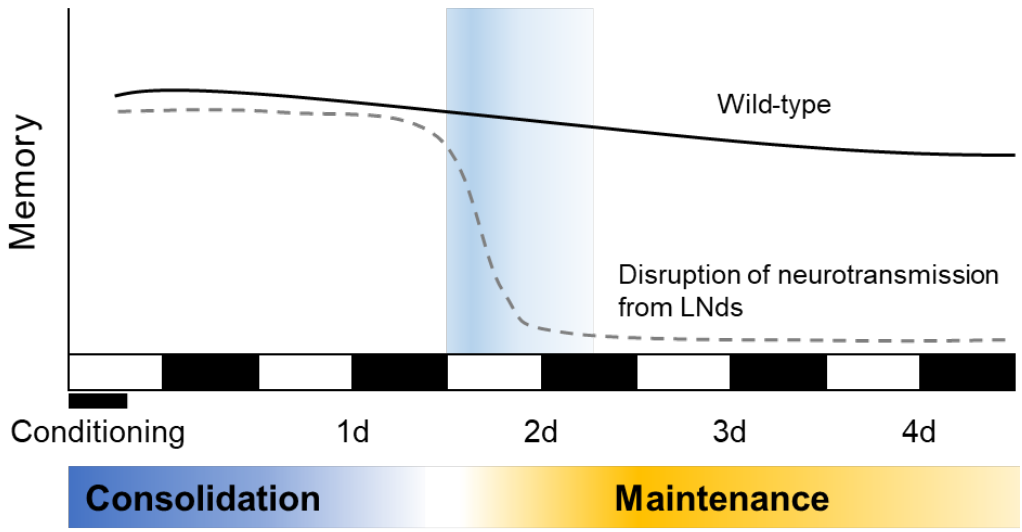

Figure 4 Schematic diagram of courtship memory processing. After courtship conditioning, wild-type flies maintain LTM (block line). When neurotransmission from LNds is disrupted on the second day after courtship conditioning, LTM is not maintained (dotted line).

Since MB neurons are crucial for *Drosophila* courtship memory, LNds likely modulate physiological properties in MB. Do LNds project to MB neurons directly? Previous circadian rhythm research does not support the idea that LNds project directly to MB neurons. According to the database of *Drosophila* connectomics (*neu*Print,

https://neuprint.janelia.org/?dataset=hemibrain%3Av1.0.1&qt=findneurons), it seems unlikely that LNds synaptically project and transmit information to MB neurons directly [27]. Therefore, further research is needed to clarify how information is transmitted from LNds to MB neurons. If the neurotransmitters released from LNds can be identified, it will be possible to identify the neurons involved in LTM maintenance from among the neurons that express their receptors. In addition, clarifying the connections of the identified neurons to MB may reveal the part of the neural circuitry that regulates LTM maintenance.

Molecular Functions of Per in LTM Consolidation

Does Per expression in LNds affect courtship LTM? Inhibition of *per* expression in two pairs of LNds inhibits LTM consolidation but not LTM maintenance or recall [27]. Furthermore, the induction of the dominant-negative transgene of *per* (*per*$^{\Delta PAS}$), which lacks the two PAS domains required for binding to Tim (Fig. 5), in two pairs of LNds also impairs LTM consolidation [27]. These findings show that Per in two pairs of LNds is involved in LTM consolidation. However, the molecular function of Per remains unclarified. Per in the nucleus binds to the transcription factor Clk/Cyc through the Clk–Cyc interactive domain (CCID) of Per, resulting in the inhibition of Clk/Cyc-mediated *per* transcription [31,32]. Since the Per$^{\Delta PAS}$ used in our study lacks the PAS domains but retains the CCID (Fig. 5), induction of Per$^{\Delta PAS}$ may trap endogenous Clk/Cyc and inhibit endogenous *per* expression in LNds. In *Drosophila*, the Doubletime (Dbt) protein (a *Drosophila* homolog of Casein kinase 1ε) phosphorylates Per, and subsequently, the phosphorylated Per is degraded in the proteasome [12]. Kim et al. have reported that Dbt binds to the *Drosophila* Per–Dbt binding domain (dPDBD), and Per lacking the dPDBD is present at constant high levels throughout a daily cycle and undergoes little phosphorylation [32]. Since Per$^{\Delta PAS}$ retains the dPDBD (Fig. 5), the targeted expression of Per$^{\Delta PAS}$ in LNds may trap endogenous Dbt and consequently inhibit endogenous Per–Dbt binding. Thus, it will be interesting to examine whether the disruption of Per/Dbt complex formation in LNds affects LTM consolidation. However, it remains unclarified how Per contributes to cellular functions in two pairs of LNds, and how LNds not directly connected to MB neurons physiologically regulate LTM consolidation in MB neurons will need to be clarified.

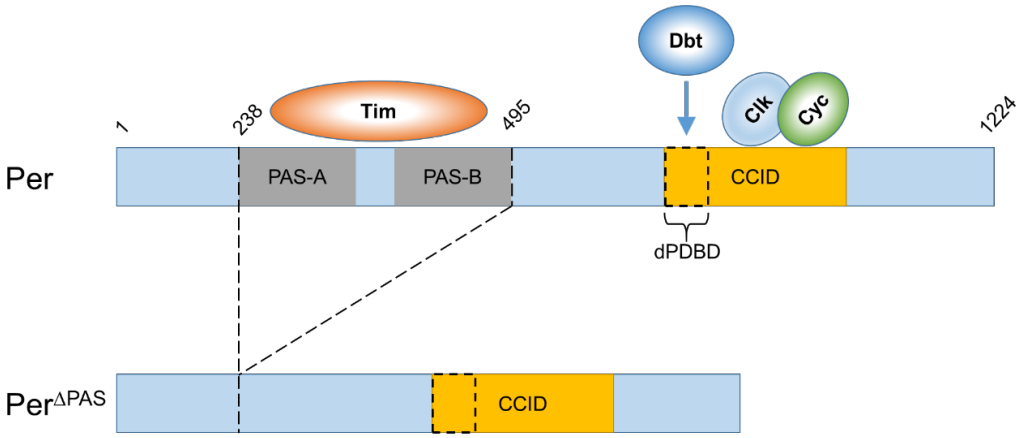

Figure 5 Schematic representation of the full-length Per and Per$^{\Delta PAS}$ lacking two PAS domains. CCID, Clk–Cyc interactive domain; dPDBD, Per–Dbt binding domain.

Possible Model of the Regulation of LTM Maintenance via LNds

We have recently found that environmental light is required for LTM maintenance in *Drosophila* [33]. The circadian photoreceptors Cryptochrome (Cry) and Rhodopsin 7 (Rh7) are involved in light-dependent LTM maintenance [21]. Cry and Rh7 are expressed in s-LNvs and l-LNvs [34,35]. s-LNvs and l-LNvs can directly receive light, and their activity is directly activated by light [33,35,36]. In particular, the light-dependent release of the neuropeptide Pigment-dispersing factor (Pdf) from l-LNvs is essential in light-dependent LTM maintenance [33].

Interestingly, some neurons in LNds express Cry [12]. Thus, LNds may be directly activated by light (Fig. 6A). We will be able to test this hypothesis by determining whether two pairs of LNds express Cry and show light-dependent activation. Alternatively, LNd activity may be triggered via l-LNvs in a light-dependent manner because Pdf is released from l-LNvs, and the Pdf receptor is expressed in a subset of LNds [37,38]. Thus, the intercellular communication from l-LNvs to LNds may also modulate LTM consolidation (Fig. 6B) [27].

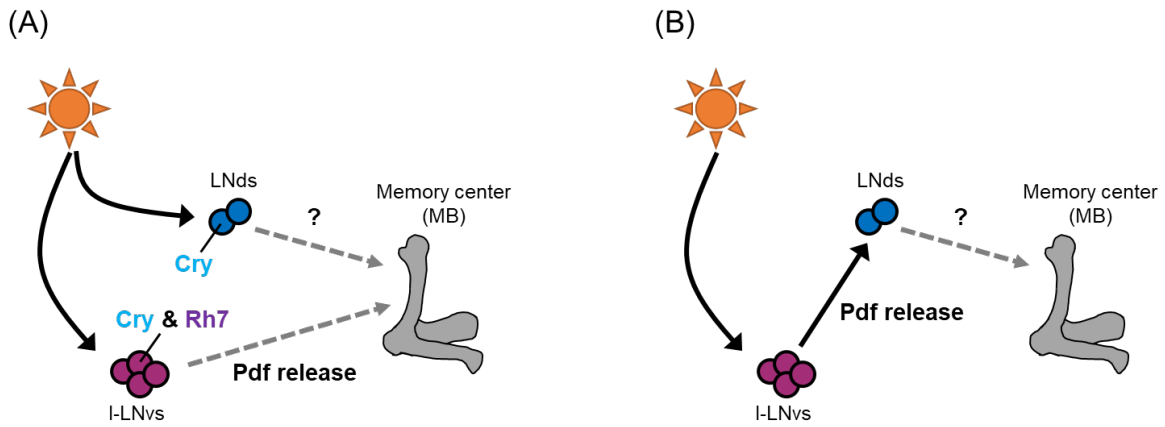

Figure 6 Possible model of regulation of courtship LTM via l-LNvs and LNds. MB, mushroom body; LNds, dorsal lateral neurons; l-LNvs, large ventral-lateral neurons; Cry, Cryptochrome; Rh7, Rhodopsin 7. (A) l-LNvs and LNds regulate MB activity in parallel. (B) l-LNvs and LNds regulate MB activity sequentially.

Concluding Remarks

The inhibition of *per* expression or the induction of the dominant-negative transgene of Per in two pairs of LNds impairs LTM consolidation. On the other hand, the disruption of neurotransmission in two pairs of LNds impaires LTM maintenance. Thus, at least two pairs of LNds play vital roles in the regulation of LTM consolidation and maintenance. As shown in Figure 6, it is unclear what pathways the LNds use to control the memory center, while it is likely that LNds ultimately control the function of the memory center. Thus, we can assume that two pairs of LNds are the singularity cells regulating *Drosophila* memory processes. However, it is still unclear whether the remaining four pairs of LNds are also involved in courtship memory processing, and further research will be needed.

The fact that *per* affects *Drosophila* LTM has been confirmed not only in courtship memory but also in memory induced by aversive olfactory learning using odors and electric shocks [26]. *per* expression is essential for establishing 1 d memory induced by aversive olfactory learning [26]. Furthermore, as was observed in courtship LTM, Yin et al. reported that light is required for the maintenance of LTM induced by aversive olfactory learning and that a clock gene other than *per*, *vrill* is also required for the consolidation of LTM induced by aversive olfactory learning [39]. These findings imply that clock genes and clock neurons play an important role in the regulation of *Drosophila* LTM, regardless of the learning method. Since clock genes and clock neurons are mainly involved in LTM rather than STM in *Drosophila*, LTM is likely consolidated and maintained by the interaction between the clock neuron network and the memory center. Future work is needed to clarify the neural connections between the clock neuron network and the memory center and how the clock neuron network regulates the activity of the memory center in *Drosophila*.

Our previous studies suggest that Per has different molecular functions from those revealed by chronobiology research and that these as yet unknown molecular functions of Per may be involved in the regulation of LTM. Per1, the mammalian Per homolog, is expressed in the hippocampus, the mammalian memory center [40]. Although the function of Per1 in mammalian LTM is poorly understood, the results of *Drosophila* memory research focusing on Per may provide new insights into the mechanisms of mammalian LTM.

Conflict of Interest

The author declares no conflicts of interest.

Author Contributions

R.S., and U.K. prepared the figures for this review. R.S., U.K., and T.S. wrote the manuscript.

Data Availability

The evidence data generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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