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The clock gene *period* differentially regulates sleep and memory in *Drosophila*

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Abstract

Circadian regulation is a conserved phenomenon across the animal kingdom, and its disruption can have severe behavioral and physiological consequences. Core circadian clock proteins are likewise well conserved from *Drosophila* to humans. While the molecular clock interactions that regulate circadian rhythms have been extensively described, additional roles for clock genes during complex behaviors are less understood. Here, we show that mutations in the clock gene *period* result in differential time-of-day effects on acquisition and long-term memory of aversive olfactory conditioning. Sleep is also altered in *period* mutants: while its overall levels don't correlate with memory, sleep plasticity in different genotypes correlates with immediate performance after training. We further describe distinct anatomical bases for Period function by manipulating Period activity in restricted brain cells and testing the effects on specific aspects of memory and sleep. In the null mutant background, different features of sleep and memory are affected when we reintroduce a form of the *period* gene in glia, lateral neurons, and the fan-shaped body. Our results indicate that the role of the clock gene *period* may be separable in specific aspects of sleep or memory; further studies into the molecular mechanisms of these processes suggest independent neural circuits and molecular cascades that mediate connections between the distinct phenomena.

Keywords

Drosophila; sleep; memory; Period

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Introduction

A major goal of neuroscience is to understand how the brain encodes and stores learned information for time frames that range from seconds to lifespans. Among the brain processes known to be involved in memory consolidation, sleep is thought to be especially important (reviewed in Rasch and Born, 2013, Dissel et al., 2015b). For example, the altered brain-wide activity during specific sleep stages permits reactivation of neural memory ensembles, an event thought to be crucial for consolidation (Yang et al., 2014). While the importance of sleep on a systems level is well established, less is known about the molecular mechanisms connecting sleep and memory. Complicating the mechanistic understanding of the connection between sleep and memory is the fact that both sleep and memory have dissociable aspects that are supported by distinct cellular and molecular interactions. In all animal models studied, mechanistic differences discriminate between acquisition, short-term memory (STM), and distinct phases of long-term memory (LTM) (reviewed in Kandel et al., 2014). Likewise, behavioral and physiological studies on sleep have identified separable regulators of homeostatic and circadian factors on sleep amount and intensity (Saper et al., 2005). Further, while time-of-day (TOD) effects have been described for learning, memory formation and sleep in flies, it is unclear whether similar mechanisms underlie the TOD effects on these phenomena (Lyons and Roman, 2008; Fropf et al., 2014; Chouhan et al., 2015).

The clock gene *period* has been studied in several *Drosophila* behavioral paradigms investigating either sleep or memory. *period* null mutants and RNAi directed against *period* have been shown to disrupt memory, while overexpression of wild-type *period* has been shown to rescue these memory impairments and enhance memory in wild-type flies (Sakai et al., 2004, Donlea et al., 2009, Chen et al., 2012, Sakai et al., 2012). Initial characterizations of sleep behavior in flies examined circadian mutants and found, as expected, that arrhythmic *period* mutants have relatively constant sleep levels under free-running conditions (Hendricks et al., 2000, Shaw et al., 2000). After a bout of deprivation, mutants have intact sleep homeostasis and show transient increases in sleep before returning back to pre-deprivation baseline levels (Shaw et al., 2000). However, *period* mutants have established deficits in sleep plasticity after memory formation; while wild-type flies increase sleep transiently after memory training, *period* null mutants do not show post-memory sleep increases (Donlea et al., 2009). Though the memory effects of Period manipulations are postulated to result from changes in sleep regulation (Sakai et al., 2012), this theory has not been explicitly tested.

Localizing specific subsets of cells important for either sleep or memory has greatly improved our ability to study the molecular mechanisms of each phenomenon, but a mechanistic understanding of the connection between sleep and memory remains poorly understood. Finding cellular substrates that underlie the connection between sleep and memory is a first step toward a fuller understanding on this interaction. Given the important role of *period* in both sleep and memory, it is plausible that Period is involved in the connection between these two phenomena. Therefore, identifying the cells in which Period regulates specific aspects of sleep and memory may help elucidate the connection between sleep and memory. A better understanding of the specific role of Period activity, in the

appropriate cell types, could serve as a starting point to study the molecular connections between different behavioral processes.

In both mammals and flies, glial cells play an important role regulating brain functions including neuronal excitability, memory formation, behavioral rhythmicity, and sleep (reviewed in Jackson 2015). Early characterizations of Period in the *Drosophila* brain document extensive rhythmic clock gene activity within glia (Siwicki et al., 1988, Liu et al., 1988, Zerr et al., 1990). Further, intact clock-related activity within glia has been shown to be important in many glial-regulated functions, including behavioral rhythmicity (Ewer et al., 1992, Ng et al., 2011). While glia play an established role in sleep homeostasis in both flies and mammals (reviewed in Jackson, 2015), less is known about a possible role for glia regulating the circadian aspect of sleep and whether clock genes within glia may play a role in sleep regulation. Similarly, glia have a documented role modulating memory formation in *Drosophila* (Yamazaki et al., 2014, Matsuno et al., 2015), but memory-related roles for clock genes within glia remain unexplored.

The fan shaped body (FB) is a major component of the central complex in *Drosophila*, a premotor complex with predominant visual input. As such, the FB plays a well-established role in visual processing, pattern recognition, and visual memory (Liu et al., 1996, Wang et al., 2008, Pan et al., 2009). Additional behaviors regulated by the FB include courtship activity, temperature preference, and responses to directionally-specific visual motion (Weir 2014, Sakai et al., 2004, Sakai et al., 2012). More recently, the FB has garnered much attention as a center controlling arousal and sleep in the *Drosophila* brain. Different groups have demonstrated tight control over the arousal state of animals by using various methods to manipulate the dorsal FB (Donlea et al., 2011, Liu et al., 2012, Ueno et al., 2012, Kottler et al., 2013, Donlea et al., 2014, Dissel et al., 2015a; Liu et al., 2016; Pimental et al. 2016). As a result of FB-based sleep alteration, these groups report memory enhancement after increasing sleep and memory deficits as a result of reducing sleep. A recent publication from Dissel et al. (2015a) finds that sleep increases, using whole-brain as well as FB manipulations, can rescue memory deficits in classic *Drosophila* memory mutants as well as Alzheimer's models without addressing the underlying genetic dysregulation. Collectively, these datasets provide evidence for the argument that more overall sleep benefits the brain and can enhance the process of memory formation. While detailed studies into the role of the FB indicate that it is involved in the homeostatic aspect of sleep regulation (Pimental et al., 2016), it is unclear if the memory effects of FB manipulation result from altered sleep plasticity or simply different overall amounts of sleep. Further, it is unknown whether Period plays any circadian role in memory formation via the FB (Lyons and Roman, 2008; Fropf et al., 2014).

The Lateral Neurons (LNs) are a group of ~150 diverse neurons, distributed throughout the brain, that form the circadian pacemaker cells in *Drosophila* (Taghert, 2009). A subset of these LNs express the neuropeptide pigment dispersing factor (pdf), and the rhythmic release of pdf, as well as plasticity within the pdf-expressing neurons, is important for appropriate behavioral cycling (Fernandez et al., 2008, Lear et al., 2009, Yoshii et al., 2009). These pdf-releasing circadian pacemaker cells have also been shown to be important for onset and maintenance of sleep (Parisky et al., 2008, Sheeba et al., 2008). Furthermore,

plasticity within pdf neurons is necessary for experience-dependent changes in sleep (Donlea et al., 2009) while sleep manipulations alter pdf neuron architecture (Donlea et al., 2011). Although Period function within pdf neurons is known to be important for control of rhythmic behavior, it is unknown what role Period might play in these neurons in sleep regulation. Likewise, any memory-related role for Period in pdf neurons is not yet described.

Period function has been shown to be important for memory formation within a pair of cells called the Dorsal Anterior Lateral (DAL) neurons. The DAL neurons, which extensively interact with the Mushroom Body in *Drosophila*, have recently been shown to be an important anatomical substrate of LTM for particular time windows after memory training (Chen et al., 2012). Period expression within the DAL is altered after memory formation, and Period interference within the DAL disrupts memory. However, the full behavioral effects of Period manipulation within the DAL remain relatively untested.

We undertook the present study to better understand the role of the clock gene *period* in both sleep and memory. Given the established role of Period in these behavioral phenomena, as well as its extensively documented role in circadian rhythms, we hypothesized that a fuller understanding of period's function in specific aspects of these behaviors may provide an avenue to study the molecular connection between sleep, memory, circadian rhythms. To this end, we examined specific behavioral effects of Period manipulation in *Drosophila* mutant and transgenic animals.

Materials and Methods

Drosophila stocks and genetics

Fly stocks were maintained at 22°C on standard food supplemented with yeast paste. Experimental animals were collected 1–3 days post eclosion, housed at 100 flies per vial, and entrained to a 12:12 light:dark schedule for 3–5 days before behavioral experiments.

Wild-type flies in all experiments were the *YW* strain. *period* mutant flies were previously described to have shortened, lengthened, or arrhythmic locomotor oscillations under dark-dark conditions (Konopka and Benzer, 1971). For transgenic experiments, stable lines of doubly-transgenic flies were created with UAS-*per^S* combined with Repo-, 104Y-, or pdf-Gal4. Doubly-transgenic males were crossed to *per⁰* mutant females and male progeny were used for experiments.

Olfactory Conditioning Assays

Flies were trained in the olfactory avoidance-training paradigm developed by Tully and Quinn and modified to allow for automated training sessions. A single-cycle of training consists of 90 sec exposure to ambient air; 60 sec of electric shock (the unconditioned stimulus); 70-V pulses lasting 1.5 sec and administered every 5 sec (12 total) accompanied by simultaneous exposure to 1 odor (the conditioned stimulus condition, CS+); 45 seconds of ambient air exposure to clear the first odor; 60 seconds of exposure to the second odor, with no shock (the CS– condition), 45 sec of ambient air to clear the second odor. Testing was done by placing flies in a choice point and allowing them to decide between the CS+ and CS– stimuli for 2 min. Spaced training consists of 10 single cycles separated by 15-min

rest intervals. We used 3-octanol and 4-methylcyclohexanol as odors. The performance index=[the number of flies making the correct choice] – [the number of flies making the incorrect choice]/total number of flies, multiplied by 100. To avoid odor-avoidance biases, we calculate the performance index of each single N by taking an average performance of 2 groups of flies, 1 trained with 3-octanol as CS+, the other with 4-methylcyclohexanol.

Sleep Assays

Flies were collected upon eclosion and entrained to a 12:12 light dark cycle for three days before assaying for sleep. At 3 days post eclosion (DPE), individual flies were loaded into tubes and placed in the *Drosophila* Activity Monitor (DAM) System under CO₂ anesthesia. For post-memory sleep experiments, flies were loaded using aspiration to avoid anesthesia that might interfere with memory formation. Raw activity counts at 1-minute intervals for each fly were collected over a 24-hour period. An Excel Macro program was used to calculate the various sleep measures presented.

Statistical analysis

Data are presented as mean $\pm$ SEM.

For all behavioral experiments, two-sample t-tests were performed to provide between-subjects comparisons of different genotypes. All statistical tests are performed using R (R Development Core Team, 2012) software. For all assessments, the alpha value was set to 0.05.

Results

For all experiments, wild-type (YW), *period* mutant, and transgenic flies were assessed for differences in memory and sleep. Memory assays were performed using a classical aversive olfactory conditioning paradigm with flies trained and tested as indicated. Sleep was measured in individual flies using the *Drosophila* Activity Monitoring (DAM) apparatus.

Period mutants have specific alterations in immediate and 24-hour memory

It has been shown that *Drosophila* display time-of-day (TOD) differences in memory performance but it is unclear the extent to which the circadian clock influences these effects (Fropf et al., 2014). To clarify the relationship between the circadian clock and TOD variation in memory, we trained and tested a set of isogenic YW flies and *period* mutants at different times of day while animals were entrained to a light/dark (LD) schedule. Based on our previous behavioral and molecular data, we decided to train flies at four different time points: early day (ZT2), late day (ZT8), early night (ZT14), and late night (ZT20). Flies were trained, returned to normal housing, tested 24 hours later, and performance index (PI) values were calculated (Figure 1A). Wild type flies showed a peak in memory performance during the early night ($p<0.001$ for all comparisons) and trough in late night ($p<0.05$ for all comparisons), which matched well with previously published data. Compared to WT, *per^S* flies showed a shifted peak and trough in memory performance, with best performance during the late day ($p<0.05$ for all comparisons). *per^L* flies, however, showed a performance curve that essentially matched wild-type with a peak in the early night ($p<0.005$ for all

comparisons). When we examined *per⁰* flies, we saw severe deficits in memory performance compared to YW at all time points ($p < 0.001$ for all comparisons) and levels did not vary across the day ($p > 0.12$ for all comparisons).

To test if these differences in memory capacity are due to altered task acquisition in our paradigm, we trained flies at these same time points and tested the flies immediately after the end of training (Figure 1B). *per^S* and *per^L* flies showed intact acquisition at all time points, with no values significantly different from YW at any point ($p > 0.05$ for all comparisons). Within genotype, YW, *per^S*, and *per^L* flies also displayed no TOD effects on performance levels immediately after the end of training ($p > 0.05$ for all comparisons). While *per⁰* mutants also showed no TOD effects on performance across time points ($p > 0.05$ for all comparisons), they had severe acquisition deficits compared to other genotypes at all time points ($p < 0.001$ for all comparisons).

These behavioral analyses when Period is manipulated highlight the importance of TOD- or arousal-dependent effects on molecular manipulations of memory. *period* mutations have differential effects on memory capacity across the day: *per^S* flies have a shifted peak and trough in performance compared to wild-type, while *per⁰* flies show memory deficits at all time points and fail to show any TOD effects on performance levels. The behavioral data sets also indicate that there are specific alterations in memory processing occurring in *period* null mutant flies and that the role of Period that supports task acquisition may be dissociable from the role supporting the TOD effects on 24-hour memory.

Period mutants have specific alterations in sleep

Given that *period* mutants have alterations in specific aspects of memory, we hypothesized that dissociable features of sleep may also be different in the mutants. We first examined baseline sleep in YW flies and *period* mutants under LD entrainment and when flies are shifted to dark/dark (DD) conditions (Figure 2A). Under free-running conditions, wild-type flies showed the expected 24-hour oscillations in sleep behavior. Also as expected based on genotype, *period* mutant flies showed shorter (*per^S*), longer (*per^L*), or arrhythmic (*per⁰*) sleep oscillations under DD conditions (Figure 2C).

A LD-entrained wild-type sleep curve is shown in blue with very low sleep around dawn and dusk, some sleep during the middle of the day, and high, consolidated sleep throughout the night. While LD entrained, *period* mutant flies show altered sleep levels but normal rhythmicity since activity peaks, or sleep troughs, occur at dawn and dusk in both mutants and wild-type flies. However, compared to wild-type, *per^L* and *per⁰* had elevated and *per^S* had reduced daytime sleep ($p < 0.001$ for all comparisons) (Figure 2B). These baseline sleep levels didn't match with alterations in memory capacity, as *per^L* and *per⁰* had very different memory abilities while LD entrained but had similar sleep changes compared to wild-type. We next examined another aspect of sleep, post-learning sleep plasticity, to determine if that measure correlates better with memory performance.

In flies, social enrichment and learning homeostatically regulate sleep, so we examined changes in sleep after memory formation between different genotypes. Animals were trained in the olfactory conditioning task at ZT14, loaded into the sleep apparatus, and sleep levels

were measured for the following day (schematic in Figure 3A). For each genotype, sleep was measured in two different groups: one group was trained in a forward-paired protocol (FS) which induces long-term memory formation, while the other group was trained with a backward-paired protocol (BS) which changes the timing of stimuli and should result in no learning or memory. In both wild-type and *per^S* flies, sleep during the day period following training was higher in flies trained in the FS protocol when compared to flies trained with BS pairing (Figure 3B, daytime increases quantified in Figure 3C; $p < 0.005$ for all comparisons). Post-training sleep in *per^O* mutant flies, however, showed no daytime sleep increase following FS compared to BS training (Figure 3B, 3C; $p = 0.94$), consistent with their observed acquisition and memory deficits.

While relative sleep amounts at baseline don't correlate with memory performance, post-training sleep plasticity tracks more closely with the TOD-independent ability to acquire the olfactory conditioning task, as *per^S* has a similar effect to YW while *per^O* show no effects. We therefore hypothesize that this facet of sleep regulation is important to maintain brain circuitry that supports early memory processing in olfactory conditioning. We further hypothesize that the distinct aspects of Period's involvement in sleep and memory alterations may be anatomically dissociable in flies. Thus, we decided to test sleep and memory after specific Period expression in different brain cells. To interrogate both time-of-day and baseline effects of Period manipulation, we decided to express the *per^S* gene within restricted cell types of the *per^O* mutant fly. Using this design, we sought to detect brain networks involved in regulating the TOD effects on memory as well as baseline sleep, sleep plasticity, olfactory conditioning acquisition, and long-term memory.

Restricted *period* manipulation alters specific aspects of sleep and memory

In both flies and mammals, glial cells are known to be important in memory formation and processing. Likewise, specific glial events are required for intact sleep behavior. The Period protein is highly expressed in glia, but any role for it within glia for these two behaviors is unknown

To solely express *per^S* in glia, we combined UAS-*per^S* with Repo-Gal4 within the *per^O* background (orange). As genetic controls, we used UAS-*per^S* (purple) or Repo-Gal4 (green) within the *per^O* background. In these genotypes, we tested baseline sleep, sleep plasticity, and learning and memory. At baseline, flies with *per^S* expressed in glia showed a decrease in daytime sleep, returning closer to wild-type levels (Figure 4A, compare to Figure 2B). When flies were moved to dark-dark free-running conditions, there was no effect of *per^S* expression within glia (Figure 4A). This dataset shows that Period manipulations can affect baseline sleep/behavioral excitability under light/dark entrainment without affecting rhythmicity under free-running conditions. We posit these results may indicate a new role for *period* in excitability that is distinct from its role in circadian rhythms.

We next examined sleep plasticity, again comparing flies trained in a forward-paired, memory-inducing protocol (FS) to flies trained in backward-paired protocol that does not produce learning or memory (BS). In these experiments, the expression of *per^S* in glia did not rescue the lack of post-training sleep plasticity in *per^O* mutant flies (Figure 4B).

Likewise, *per^S* expression in glia did not improve either learning or memory when flies were trained in olfactory conditioning and tested immediately or 24 hours later (Figure 4C).

Collectively, these results support the indication given by our *period* mutant sleep data, namely that baseline sleep does not correlate with memory performance. Instead, sleep plasticity is a better measure for memory performance. Additionally, we believe that the baseline sleep data with glial expression of *per^S* indicate a role for *period* in sleep and arousal that is dissociable from its role in regulating the circadian aspect of sleep.

The Fan-shaped Body (FB) has recently been shown to be involved in sleep regulation in flies. Furthermore, Period expression within the fan-shaped body has been shown to be important in the courtship conditioning memory task, an effect hypothesized to result from altered sleep. Therefore, if Period expression in the FB plays a role in memory processing via sleep, we might be able to dissect which aspects of sleep and memory the *period* gene modulates.

To express *per^S* in the FB, we combined UAS-*per^S* with 104Y-Gal4 driver within the *per⁰* background (green). As genetic controls, we introduced UAS-*per^S* (grey) with no Gal4 driver into the *per⁰* background. We compared baseline sleep, sleep plasticity, and learning and memory in these two genotypes. There was no difference in baseline sleep between genotypes either when flies were light/dark entrained or when flies were placed under dark-dark conditions (Figure 5A). When the flies with *per^S* in the FSB underwent olfactory conditioning, we saw a trend toward a training-induced increase in sleep (Figure 5B, 5C), an effect that was absent in genetic controls (data not shown).

When post-training sleep was more closely examined in these flies, we did see a significant increase in daytime bout length, an increase that was absent in control genotypes (Figure 5D). The longer average length of a sleep bout after training indicates a stronger sleep drive in animals undergoing memory formation. Based on our hypothesis, a partial restoration of sleep plasticity could indicate that *per^S* expression in the FSB may be sufficient to support some memory olfactory conditioning. When we tested learning in these flies we saw a significant increase in performance when *per^S* was expressed in FSB (Figure 5E), although no rescue of 24-hour memory (Figure 5F). Taken together with the effects of *per^S* expression in glial, these data again indicate that Period is involved in distinct aspects of sleep regulation mediated through specific cell types of the brain. These anatomically specific roles of Period function may also indicate specific roles for sleep in different aspects of memory formation.

The Lateral Neurons (LNs) are well-characterized circadian pacemaker cells in the central clock of the *Drosophila* brain. Period expression in these neurons is both necessary and sufficient to drive behavioral circadian rhythmicity, so we wondered what effects Period manipulation might have on the specific aspects of sleep and memory we have tested.

We used the pdf-Gal4 driver to express *per^S* in a subset of LNs within the *per⁰* background (red), with UAS-*per^S* with no Gal4 driver (grey) as a genetic control. We compared baseline sleep, sleep plasticity, and learning and memory in these two genotypes. Unlike *per^S* expression in glia or the FSB, *per^S* expression in LNs resulted in a dramatic change in

baseline sleep in both light/dark and dark/dark conditions (Figure 6A). In addition to LN expression of *per^S* altering overall sleep levels, rhythmicity was restored in the free-running conditions (Figure 6F). When we looked at post-training sleep, we saw a small but significant increase in rebound sleep after forward-paired training (Figure 6B), indicating that this genetic rescue may be sufficient to rescue memory. When we tested 24-hour memory in these flies, we saw a significant increase compared to control genotypes (Figure 6C).

Unlike *per^S* expression in glia and the FSB, expression within the LNs altered baseline sleep in both LD and DD conditions, reinstated sleep plasticity induced by memory training, and improved performance at a 24-hour memory test. Given these results, we wondered if *per^S* expression in these clock neurons is involved in the TOD effects on long-term memory. We first studied the effects of LN expression of *per^S* within a WT background and found that *per^S* expression did alter baseline sleep under both LD and DD conditions (Figure 6D). Next, we trained and tested flies at ZT8, which is the peak performance time for *per^S* mutants, or ZT14, which is the best training time for YW flies. Memory was significantly higher at ZT14, a result expected for YW flies (Figure 6F). Based on these experiments, we conclude that lateral neuron expression of *per^S* does not shift the TOD peak and trough in memory performance.

The Dorsal Anterior Lateral (DAL) neurons, a pair of cells that highly interact with the Mushroom Body, are interesting possible candidates to be involved in TOD effects on memory. DAL activity is necessary for intact 24-hour memory and *period* transcription is altered in the DAL after olfactory conditioning.

To test for a functional role of Period in memory processing within the DAL, we next examined memory performance in flies with *per^S* expressed in the two DAL neurons using the G0431 Gal4 driver. We first tested whether *per^S* expression within DAL could rescue baseline memory in *per⁰* mutant flies. As shown in Figure 7A, DAL-specific expression of *per^S* does not rescue 24-hour memory at either ZT8 or ZT14. While these results indicate that *per^S* is not sufficient to rescue memory in the *per⁰* mutant background, we still wondered whether Period function in the DAL neurons contributes to the TOD effects on 24-hour memory. We next tested the effects when *per^S* is expressed within the DAL within a wild-type fly, and found that this genetic manipulation resulted in an altered peak in memory performance, with optimal memory levels at ZT8 as opposed to the peak in performance at ZT14 for wild-type flies (Figure 7B, compare to Figure 1A). The significantly higher memory performance at ZT8 compared to ZT14 clearly shows a role for Period within the DAL to regulate the TOD effects on memory.

Discussion

Rhythmic behaviors are common to all animals, and our current understanding of how the brain controls these phenomena is incomplete. Because the circadian system is conserved across species, and well characterized at the molecular level, it is instinctual to suspect that it might contribute to regulating these behaviors. The discovery that peripheral clocks exist, in which circadian activity is maintained in many cells outside of those that constitute the

central clock, has greatly expanded our view on how some rhythmic phenomena might be regulated. Whether core circadian genes always function within the context of other clock components, or indeed whether they have consistent mechanisms across different peripheral clocks, is unclear. These issues motivated us to examine the role of *period* in complex fly behavior.

In the present study, we examined the results of *period* manipulation on sleep and memory. For memory, we focused on TOD effects on 24-hour memory as well as immediate memory after training (acquisition). The features of sleep that we documented were changes in baseline sleep under either light-dark (entrained) or dark-dark (free-running) conditions, as well as alterations in sleep plasticity after memory formation. We initially assessed these distinct aspects of behavior in *period* mutants, which have mutations that shorten, lengthen, or abolish behavioral rhythmicity under constant conditions.

When we examined memory in the *period* mutants, we found significant, but distinct, changes to 24-hour memory and acquisition. Compared to wild-type (YW), *per^S* mutants show a shifted peak and trough in 24-hour memory performance across the day, while *per^L* mutants show the same TOD effects as YW. In contrast, *per^O* flies have severe memory deficits at all time points. When we tested memory immediately after acquisition of the task, we found that *per^S* and *per^L* flies have the same memory performance as YW, *per^O* mutants have significant deficits, and no genotypes show TOD effects on immediate memory. The divergent results between immediate and 24-hour memory suggest that *period* may be differentially involved in these phenomena.

There are well-documented TOD changes in gene expression and protein localization, and future work examining if and how these factors may be altered in *period* mutants could provide valuable insight into how *period* affects molecular mechanisms underlying behavior. One potential downstream target of *period* transcriptional regulation is the transcription factor dCREB2, which has published TOD changes in protein localization across the day (Fropf et al., 2014). Further, dCREB2 manipulations have differential effects on memory if performed at different times. It would be interesting to see if oscillations in dCREB2 localization are altered in *period* mutants and whether dCREB2 manipulations are effective at different times in *period* mutants. Interestingly, the differing TOD effects on 24-hour memory in the *per^S* mutant flies occurs even while animals are LD entrained, which may indicate altered molecular signaling in these flies even when behavioral rhythmicity appears normal.

The severe memory deficits we see in *per^O* mutants at all time points, both immediately and 24 hours after training, add to a complex set of previously published behavioral data showing intact or deficient memory capacities depending on the task. *per^O* mutants have intact memory after appetitive conditioning and Anesthesia Resistant Memory (ARM) after olfactory avoidance conditioning (Chouhan et al., 2015, Chen et al., 2012, Glou et al., 2012). However, *per^O* mutants show memory deficits in the aversive courtship conditioning task, in time-of-day specific memory in appetitive conditioning, and in long term memory following spaced olfactory avoidance training (Sakai et al., 2004, Chouhan et al., 2015, Chen et al., 2012). Together, our data support this body of work addressing aversive conditioning, which

indicate that our sleep and anatomical findings could be appropriately applied to these other behavioral paradigms. However, some care may need to be exercised when applying our results to appetitive conditioning, as it seems that baseline memory is intact in *per⁰* mutants for appetitive memory.

While sleep rhythms under free-running conditions match expected rhythms based on circadian phenotypes, sleep amount, though not rhythmicity, is altered when flies are entrained on a LD schedule. This result is again consistent with the possibility that molecular signaling may be altered even while animals are light/dark entrained. Further, this suggests that *period* may contribute to both the timing and homeostatic regulation of sleep. Given this intriguing potential role for Period in sleep need, we decided to test changes in sleep after memory formation in *period* mutants. After training in an olfactory memory task, we found that YW and *per^S* flies show similar increases in daytime sleep when flies are trained at times that have differential memory peaks for these animals (ZT=14). However, *per⁰* mutants show no increase in daytime sleep regardless of when the animals are trained. Based on our collective results with *period* mutants, we hypothesize that distinct aspects of memory may be related to specific aspects of sleep.

Given that *per⁰* flies were the only mutants that showed acquisition deficits and the absence of TOD variation in acquisition across genotypes, immediate memory may be most closely correlated to sleep plasticity. While it makes sense that memory-based alterations in sleep rely on acquisition of the memory task, we hypothesize that the brain mechanisms that support sleep plasticity may be crucial for the ability to acquire a task. Because *per^S* flies show a shifted peak and trough in 24 hour memory performance across the day and *per⁰* flies show no TOD variation, this phenotype most closely resembles the rhythmicity of sleep behavior when flies are under free-running conditions. Though the longer sleep oscillations displayed by *per^L* flies are not reflected in the TOD variation in 24-hour memory that matches YW, it is possible that further manipulations of light/dark schedule entrainment could uncover TOD distinctions between the genotypes. Though our data do not establish causality between any sleep or memory phenomena, we do find a correlation between these specific aspects of behavior and we were interested to see if that correlation persisted when we manipulated *period* expression in restricted numbers of cells.

When we used Gal4 drivers to express a *per^S* transgene in different brain cells of a *per⁰* mutant, we found distinct behavioral differences when compared to background controls. Expressing *per^S* within glia altered baseline sleep under LD entrainment but did not affect immediate or 24-hour memory, free-running sleep, or sleep plasticity. Restricting *per^S* expression to the FB partially restored sleep plasticity in *per⁰* flies and improved immediate memory, but had no effect on baseline sleep or 24-hour memory. Driving *per^S* in pdf-containing neurons altered baseline sleep in LD entrained and free-running conditions, reinstated sleep plasticity after memory training, improved 24-hour memory, but did not change TOD alterations in memory. While *per^S* expression within the DAL did not rescue memory in the *per⁰* mutant, DAL-driven *per^S* expression did alter TOD effects on memory in a wild-type background. To our knowledge, these results are the first indication of any particular cellular population serving as the substrate for TOD effects on memory in *Drosophila*. These results suggest that Period plays a role in maintaining some level of

baseline memory that is distinct from Period's role within the DAL mediating the TOD effects on 24-hour memory. Though no role for DAL in sleep has yet been reported, it would be interesting to test specific aspects of sleep during Period manipulation as well. On the other hand, phenomena such as the TOD effect on memory formation could easily involve other core circadian molecules functioning in the DAL. Further experiments would need to be done to determine if DAL might, or might not be, an example of a "peripheral clock".

Taken together, these results present a comprehensive examination of sleep and memory behavior alterations after Period manipulation. While different brain cells have been implicated in specific aspects of sleep and memory, these results synthesize and compare multiple behavioral outcomes of Period alteration across multiple cell types. This could help uncover diverse roles of Period in different cell types as well as find potential mechanistic connections between these different behaviors.

The mechanism of *period* regulation as part of the core molecular circadian clock has been extensively studied in *Drosophila*. As a dimer with Timeless (*tim*), Period acts as a transcriptional repressor of the Clock/Cycle dimer that activates transcription of circadian-related genes. Because Clock/Cycle activate *period* transcription, Period acts in a negative feedback loop to maintain rhythmic oscillations in gene expression. Although these core circadian clock proteins have differential expression in the fly brain, it is unknown whether Period can have molecular interactions outside this canonical circadian pathway. Since some of the behavioral phenotypes we uncovered in the current study seem to be distinct from circadian rhythmicity, it would be interesting to investigate the molecular mechanisms occurring in the relevant brain areas. Experiments aimed at tagging and specifically measuring Period interactions in particular cells could address questions of novel molecular mechanisms underlying the *period* regulation of behaviors outside of circadian rhythms.

Finally, all the memory experiments we presented in this study involve testing either immediately after training or at 24 hours. It is well known that long-term processing of memories is closely tied to sleep and circadian regulation, so it would be interesting to see if Period is involved in this long-term regulation. Adding temporal as well as spatial restrictions on Period manipulations, and testing memory at time points longer than 24 hours, may help uncover if and how Period could be involved in this long-term process. This spatiotemporal information, combined with a fuller understanding of the molecular mechanism underlying the Period-based regulation of sleep and memory, could provide important insight about how sleep and circadian rhythms contribute to memory processing over time. This could be invaluable information, as more details about when and where particular molecules are involved in memory consolidation will help develop a fuller picture of how memories are continually processed and maintained throughout time.

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Significance

While it is increasingly clear that brain-wide neural processes such as sleep and circadian rhythms are crucial for appropriate long-term processing of memories, the specific molecular bases for these interactions remain unclear. One candidate molecular effector in these systems-level processes is the clock gene *period*, which has a well established role in circadian rhythms and a known involvement in regulating memory and sleep. However, it remains unclear what aspects of sleep and memory *period* is specifically affecting. Here, we describe behavioral effects of global and spatially restricted manipulation of *period* gene function. These results demonstrate connections between distinct aspects of sleep and memory and provide an avenue to study the dissociable anatomic and molecular substrates of these complex behaviors.

Highlights

- Period mutants have differential time-of-day effects on memory
- Baseline sleep changes in period mutants do not predict memory alterations
- Training-induced sleep plasticity predicts subsequent memory performance
- Manipulating period in different subsets of cells results in distinct phenotypes

Figure 1. Wild type and *period* mutant flies have differential time-of-day variations in memory capacity but no variation in acquisition

Wild-type (YW) and *period* mutant flies were trained using 5× Spaced Training in aversive olfactory conditioning. Flies were trained at four different Zeitgeber times (ZTs) throughout the day and tested for memory 24 hours later (**A**) or immediately after training for acquisition (**B**). YW flies do not show memory variation during the light period ($p=0.385$), but compared to ZT2 PI values show enhanced memory at ZT14 ($p<0.001$) and diminished memory at ZT20 ($p<0.05$). *per^S* mutants, however, have a significant memory peak at ZT8 compared to any other time point ($p<0.05$). Compared to YW, *per^S* mutants have similar memory at ZT2 ($p=0.34$), higher PI scores at ZT8 and ZT20 ($p<0.001$), and lower performance scores at ZT14 ($p<0.001$). *per^L* mutants have a memory peak at ZT14 compared to all other time points ($p<0.005$) and PIs that are not significantly different from YW at any time point ($p>0.05$ for all comparisons). *per^O* mutants display 24-hour memory deficits compared to YW ($p<0.001$) that do not vary across the day ($p>0.05$ for all comparisons). YW, *per^S* and *per^L* flies show acquisition values that do not vary across the day nor are any PI values different from the other two genotypes at any time point. *per^O* mutants also show no TOD variation in acquisition, but have severe acquisition deficits at all time points compared to YW, *per^S* and *per^L* flies ($p<0.001$). Data are presented as means, error bars reflect the standard error of the mean. Comparisons were made using a two sample t-test. $n=8-23$ for all conditions.

Figure 2. Baseline sleep architecture is altered in period mutant flies under free-running and light/dark entrained conditions

YW and period mutant flies were entrained on a 12:12 light:dark (LD) schedule and baseline sleep was recorded for 8 days (A). Two days of sleep were measured while flies remained entrained on the 12:12 LD cycle before flies were switched to dark:dark (DD) conditions for six days. Bars underneath graph indicate light (white) dark (black) or relative light (grey) conditions. While on LD entrainment, all flies maintain rhythms in sleep cycles that sync with light cues, as seen by troughs at 12 and 24 hours. However, daytime sleep levels vary between mutants and wild-type (B). *per^S* flies sleep less during the day and *Per^L* and *Per^O* flies sleep more during the day compared to YW ($p < 0.001$). In DD conditions, YW flies maintain approximately 24-hour sleep cycling, while the cycle is shorter (*per^S*), longer (*per^L*), or arrhythmic (*per^O*) in period mutants (C). Data are presented as means, error bars reflect the standard error of the mean. Comparisons were made using a two sample t-test. $n = 29-32$ for all conditions. Significant differences between groups are indicated as $*p < 0.001$.

Figure 3. Sleep plasticity after olfactory conditioning is intact in *per^S* but not in *per⁰* flies
 Flies were trained with 5xSpaced Training at ZT14 and sleep was measured during the following light/dark period (A shows schematic of experiment). Forward-trained (FS) flies were compared to flies trained in a backward conditioning paradigm (BS) after which no memory formation occurs. Sleep per hour the day following training is shown in B and quantified total daytime sleep is shown in C. After FS training, YW flies show a daytime-specific increase in overall sleep compared to backward-paired training ($p < 0.005$). *per^S* flies show a similar daytime-specific increase in sleep following training ($p < 0.005$), while *per⁰* mutants do not exhibit similar increases ($p = 0.94$). Data are presented as means, error bars reflect the standard error of the mean. Comparisons were made using a two sample t-test. $n = 29-32$ for all conditions. Significant differences between groups are indicated as $*p < 0.005$. Data are presented as means, error bars reflect the standard error of the mean. Comparisons were made using a two sample t test. Significant differences between groups are indicated as $*p < 0.005$. $n = 32$ for all conditions.

Figure 4. Glial expression of *per^S* in *per⁰* mutant flies alters sleep under light/dark entrainment *per⁰* mutants with a UAS-*per^S* and/or a Repo-Gal4 transgene were assessed for baseline sleep, post-memory sleep changes, and learning and memory. **(A)** Baseline sleep is lower in the daytime when UAS-*per^S* is expressed in glia, but not different when flies are shifted to a dark:dark schedule. *per^S* expression in glia did not rescue post-training sleep plasticity when flies were trained in olfactory conditioning and assessed for sleep the following day **(B)**. Glial expression of *per^S* in *per⁰* mutants did not improve PI scores for immediate acquisition or 24-hour memory ($p > 0.05$ for all comparisons). Data are presented as means, error bars reflect the standard error of the mean. Comparisons were made using a two sample t-test. Significant differences between groups are indicated as * $p < 0.05$, ** $p < 0.01$. $n = 32$ for sleep, 8–15 for olfactory conditioning.

Figure 5. Expression of *per*^S within the FB alters specific aspects of sleep and memory *per*⁰ mutant flies with *per*^S expression driven by the 104Y Gal4 driver (*per*⁰;104Y-Gal4;UAS-*per*^S) were assessed for different aspects of sleep and memory. *per*^S expression within FSB-projecting neurons does not alter baseline sleep in either LD or DD conditions compared to flies with no Gal4 driving expression (A). When *per*⁰;104Y-Gal4;UAS-*per*^S flies were trained in olfactory conditioning that produces memory, sleep in the following day trends toward increasing compared to olfactory training that does not produce memory ($p=0.052$, B, C). Further examination of post-training sleep revealed a significant increase in daytime sleep bout length in FS compared to BS flies with *per*^S expressed in the FSB ($p<0.05$), an effect that is absent in flies with no *per*^S in the FSB ($p=0.17$, D). When expressed in the FSB, *per*^S rescues *per*⁰ mutant deficits in learning ($p<0.01$, E) but not memory ($p=0.9$, F). Data are presented as means, error bars reflect the standard error of the mean. Comparisons were made using a two sample t-test. Significant differences between groups are indicated as * $p<0.05$, ** $p<0.01$. $n=25-31$ for sleep, 7-8 for olfactory conditioning.

Figure 6. Restricted expression of *per*^S within LNs alters specific aspects of sleep and memory
 LN expression of *per*^S in *per*⁰ mutant flies (*per*⁰;pdf-Gal4;UAS-*per*^S) alters baseline sleep, sleep plasticity, and memory. *per*^S expression within LNs alters baseline sleep in both LD or DD conditions (A). *per*⁰ mutant flies show no sleep oscillations under DD conditions but

when *per^S* is expressed in LNs flies have 20–24 hour rhythms in sleep behavior, as shown by the sleep periodogram calculated from baseline sleep (**E**). After memory formation, *per⁰*;104Y-Gal4;UAS-*per^S* flies show daytime increases in sleep during the following day ($p < 0.05$, **B**). LN expression of *per^S* also improves performance on a 24 hour memory task after olfactory conditioning ($p < 0.05$, **C**). In YW flies, LN expression of *per^S* affects baseline sleep in both LD and DD conditions (**D**). When sleep under DD conditions is analyzed for frequency power, YW flies show a clear 24 rhythm in sleep behavior while YW flies with LN-driven *per^S* have oscillations with 20–24 hour frequency (**E**). When *per⁰*;pdf-Gal4;UAS-*per^S* flies were trained in olfactory conditioning at ZT8 or ZT14 and tested 24 hours later, PI scores were higher at ZT14 (**F**). Data are presented as means, errors bars indicate standard error of the mean. Comparisons were made using a two sample t-test. Significant differences between groups are indicated as $*p < 0.005$.

Figure 7. Expression of PerS within DAL Neurons does not rescue memory in *Per⁰* mutants but does affect TOD memory performance in wild-type flies

Per⁰ mutant (A) or wild-type (B) flies were combined with UAS-*per^S* and G0431 Gal4 transgenes and tested for 24 hour memory. (A) In the *per⁰* mutant background, compared to a condition with no Gal4 driver, expression of *per^S* within DAL neurons does not improve memory scores at ZT8 ($p=0.87$) or ZT14 ($p=0.36$) ($n=7-8$ for all groups). (B) In a wild-type background, *per^S* expression within DAL neurons alters the TOD effects on memory performance. Memory no longer peaks at ZT14, but rather is significantly higher at ZT8 ($n=9$). Data are presented as experimental means, error bars reflect the standard error of the mean. Comparisons were made using a two-sample t-test. Significant differences between groups are indicated as $*p<0.01$.