American College of Neuropsychopharmacology

www.nature.com/npp

ARTICLE

The clock gene *Per1* may exert diurnal control over hippocampal memory consolidation

Lauren Bellfy[1,2], Chad W. Smies[1], Alicia R. Bernhardt[1], Kasuni K. Bodinayake[1], Aswathy Sebastian[2], Emily M. Stuart[1], Destiny S. Wright[1], Chen-Yu Lo[1], Shoko Murakami[1], Hannah M. Boyd[1,2], Megan J. von Abo[1], Istvan Albert[2,3] and Janine L. Kwapis[1,2]✉

© The Author(s), under exclusive licence to American College of Neuropsychopharmacology 2023

The circadian system influences many different biological processes, including memory performance. While the suprachiasmatic nucleus (SCN) functions as the brain's central pacemaker, downstream "satellite clocks" may also regulate local functions based on the time of day. Within the dorsal hippocampus (DH), for example, local molecular oscillations may contribute to time-of-day effects on memory. Here, we used the hippocampus-dependent Object Location Memory task to determine how memory is regulated across the day/night cycle in mice. First, we systematically determined which phase of memory (acquisition, consolidation, or retrieval) is modulated across the 24 h day. We found that mice show better long-term memory performance during the day than at night, an effect that was specifically attributed to diurnal changes in memory consolidation, as neither memory acquisition nor memory retrieval fluctuated across the day/night cycle. Using RNA-sequencing we identified the circadian clock gene *Period1* (*Per1*) as a key mechanism capable of supporting this diurnal fluctuation in memory consolidation, as learning-induced *Per1* oscillates in tandem with memory performance in the hippocampus. We then show that local knockdown of *Per1* within the DH impairs spatial memory without affecting either the circadian rhythm or sleep behavior. Thus, *Per1* may independently function within the DH to regulate memory in addition to its known role in regulating the circadian system within the SCN. *Per1* may therefore exert local diurnal control over memory consolidation within the DH.

Neuropsychopharmacology; https://doi.org/10.1038/s41386-023-01616-1

INTRODUCTION

Circadian rhythms are responsible for regulating integral physiological processes across the 24 h day in most organisms [1, 2]. The circadian system is primarily regulated by the suprachiasmatic nucleus (SCN) in the hypothalamus [3], but local molecular oscillations occur across the brain and body, including within memory-relevant regions like the dorsal hippocampus (DH) [4]. Although it is clear that memory performance oscillates across the day/night cycle, the mechanisms that modulate this fluctuation are unknown. Recent work has suggested that clock genes function within memory-relevant brain regions to exert local diurnal control over memory [5–10], but it is unclear which clock genes regulate memory and which phase of memory (acquisition, consolidation, or retrieval) is impacted by the time of day.

The mammalian molecular clock begins with a CLOCK-BMAL1 heterodimer binding to E-box motifs upstream of two gene families (*Period* (*Per*) and *Cryptochrome* (*Cry*)) to induce their transcription [11]. *Per* and *Cry* are then translated in the cytoplasm and these proteins heterodimerize before returning to the nucleus to inhibit the CLOCK-BMAL1 complex, blocking subsequent transcription of *Per* and *Cry* [12–14]. Proteolytic decay of PER and CRY proteins frees up the CLOCK-BMAL1 complex, which restarts the feedback loop, enabling a new round of transcription of *Per1* and *Cry* [15]. This entire transcription/translation feedback loop takes ~24 h and is roughly aligned with the natural light/dark cycle. Although this molecular clock has been well-characterized within the SCN, it also operates within memory-relevant brain regions like the DH [4], providing a potential mechanism through which the circadian system modulates memory; clock genes could function within specific brain structures to exert local diurnal control over memory and other region-specific functions [5, 6]. In particular, previous work has shown that the core clock gene *Per1* is important for memory formation [7, 9, 16]. Bidirectional manipulation of *Per1* within the DH modulates memory; local knockdown of *Per1* impairs spatial memory whereas local overexpression improves memory in aging mice [7]. Thus, *Per1* functions locally within the DH to regulate memory, although it is not clear what phase of memory is modulated via this mechanism.

Successful long-term memory formation requires several phases: acquisition, consolidation, and retrieval. During acquisition, or training, the information is initially learned. Following acquisition, the information can be stored as either short- or long-term memory. Short-term memories, created in the absence of transcription, retain the information only transiently (typically a few hours). For long-term memory to form, transcription needs to occur around the time of learning [17–19], presumably to drive the cellular and synaptic modifications needed for long-term storage through a process termed consolidation. Finally, to behaviorally express the memory at a subsequent test, the

[1]Department of Biology, Pennsylvania State University, University Park, PA 16802, USA. [2]Huck Institutes of the Life Sciences, The Pennsylvania State University, University Park, PA 16802, USA. [3]Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, PA 16802, USA. ✉email: jlk855@psu.edu

Received: 17 January 2023 Revised: 17 May 2023 Accepted: 19 May 2023
Published online: 01 June 2023

memory must be properly retrieved. Long-term memory is typically tested 24 h or longer after acquisition, after the consolidation process is complete, and can be very long lasting, up to the lifespan of the animal [20]. Although memory performance is clearly affected by the time of day [2, 21, 22], because most studies use the same diurnal timepoint for both training and testing (i.e. training and testing are separated by 24 h), it is unknown which phase of memory is specifically impacted across the day/night cycle.

In this study, we tested the hypothesis that memory consolidation, rather than acquisition or retrieval, is altered across the diurnal cycle. Specifically, we hypothesized that circadian clock genes function locally within the DH to exert diurnal control over spatial memory consolidation. To test this, first, we used Object Location Memory (OLM), to test how hippocampus-dependent spatial memory performance in mice is affected by the time of day and found that memory is better during the day than at night. Next, we ran a series of experiments to determine which phase of memory (acquisition, consolidation, or retrieval) is specifically affected by the day/night cycle. We found that nighttime memory deficits are specifically due to altered consolidation, suggesting that diurnal changes in transcription might underlie daily fluctuations in memory. Using unbiased RNA-seq, we then identified the clock gene *Per1* as a key mechanism that may regulate the consolidation process in a time-of-day dependent manner in the DH, consistent with previous work from our lab and others [7–9, 16]. Finally, we show that reducing local *Per1* in the DH impairs long-term memory for OLM without affecting circadian activity patterns or sleep behavior. This demonstrates, for the first time, that *Per1* exerts local diurnal control over memory consolidation within the hippocampus in addition to its well-documented role in modulating the circadian system within the SCN.

MATERIALS AND METHODS

Extended materials and methods are available in the supplemental materials.

Mice

Mice were young (2–4 months) adult male C57BL/6 J mice. Food and water were accessible *ad libitum*, and all mice were housed on a 12 h light/dark cycle, with lights turning on at 6am (7am during Daylight Saving Time). Mice trained during the day (ZT1, 5, and 9) were entrained to a standard light cycle whereas mice trained at night (ZT13, ZT17, and ZT21) were entrained to a reverse light cycle. All experiments were performed according to US National Institutes of Health guidelines for animal care and use and were approved by the Institutional Animal Care and Use Committee of the Pennsylvania State University.

Object location memory (OLM)

OLM was conducted as previously described [7, 23] (see Supplement). Briefly, mice were exposed to two identical objects in specific locations and later tested with one object moved to a new location. Preference for the moved object was quantified as an index of memory.

RT-qPCR and RNA-Sequencing

Tissue was harvested from DH punches and RNA extraction was followed by cDNA synthesis and RT-qPCR for *Per1* expression [8] and RNA-sequencing [7] as previously described.

HSV production and stereotaxic infusion

The CRISPR-interference (CRISPRi) system and the single guide RNA (sgRNA) targeting either *Per1* or a non-targeting control were packaged into separate herpes simplex viruses as previously described [8]. An equal mixture of HSV-CRISPRi and HSV-sgRNA (HSV-sgRNA-*Per1* or HSV-sgRNA-ctrl) was bilaterally infused into the DH at a rate of 10 µL/h to a total volume of 2 µl/hemisphere.

Circadian rhythm and sleep time analysis

After a 2-week entrainment period, activity was monitored with infrared activity monitors under 12 h light/dark (LD) conditions for 2 weeks before infusion of HSV-CRISPRi in the DH. Following 2 days of recovery, mice were continuously monitored under constant darkness (DD) in the absence of zeitgebers to assess their endogenous circadian activity pattern. Sleep behavior was assessed using a modified COMPASS system as previously described [24].

Statistical analysis

Differences in memory performance were analyzed using one sample *t*-tests (to compare each group's object preference to zero), unpaired *t*-tests, or one- or two-way ANOVAs followed by Sidak's multiple comparison *post-hoc* tests. For RT-qPCR, each group was normalized to the ZT1 homecage or to its time-matched homecage group. Sleep behavior and habituation movement data were analyzed with mixed-model ANOVAs with sleep behavior or movement treated as a repeated measures variable. For all analyses, significance was indicated by an α value of 0.05.

RESULTS

Memory performance oscillates over the diurnal cycle

Memory performance is known to oscillate over the 24 h day [2, 21, 22], but the literature is conflicted on when memory is best and worst. To determine how spatial memory oscillates across the day/night cycle, mice were trained in DH-dependent object location memory (OLM) at 6 distinct Zeitgeber Times (ZTs): ZT1, ZT5, ZT9, ZT13, ZT17, and ZT21, where ZT0 = lights on and ZT12 = lights off (Fig. 1A). Mice were tested 24 h after training to specifically assess their long-term memory (LTM) performance at the same diurnal timepoint (Fig. 1B). Consistent with many previous reports [2, 8, 16, 25, 26], we found that memory was best during the day and worst at night (Fig. 1C–D). Specifically, we found that mice showed significantly better memory for OLM when trained and tested during the day (ZT1, 5, and 9) than at night (ZT13, 17, 21; Fig. 1C; one-way ANOVA: $F_{(5,63)} = 4.143$, $p < 0.01$, Sidak's *post-hoc* tests: ZT5 significantly higher than ZT13 and ZT17, $p < 0.05$, no other comparisons significant). Robust memory was observed at all of the day timepoints, ZT1, ZT5, and ZT9, (one-sample *t*-tests comparing each group to 0, ZT1: $t_{(9)} = 4.728$, $p < 0.01$, ZT5: $t_{(10)} = 4.214$, $p < 0.01$, ZT9: $t_{(10)} = 5.713$, $p < 0.001$), but poor memory was observed at night, with DIs near 0, indicating no preference for the moved object (one-sample *t*-tests comparing each group to 0, ZT13: $t_{(12)} = 0.8414$, $p = 0.4166$, ZT17:$t_{(10)} = 0.4014$, $p = 0.5356$, ZT21 $t_{(12)} = 1.290$, $p = 0.2214$). Overall, memory peaked at ZT5, in the middle of the light cycle, and showed a trough at ZT17, in the middle of the dark cycle. When all the day and night timepoints were collapsed, we found that memory was significantly better during the day compared to at night (Fig. 1D; Unpaired *t*-test, $t_{(65)} = 4.025$, $p < 0.001$). No significant differences were seen in total object exploration time across the timepoints (Fig. S1A; One-way ANOVA: $F_{(5,63)} = 1.085$, $p = 0.2176$) indicating memory performance is not dependent on exploration time. In addition, we did not see any significant differences in movement speed (Fig. S2A; Mixed-effects ANOVA, significant effect of Habituation Day $F_{(5,181)} = 19.34$, $p < 0.0001$, but no effect of Day/Night ($F_{(1,37)} = 0.49$, $p > 0.05$), or Interaction ($F_{(5,181)} = 0.36$, $p > 0.05$) and no significant difference between Day and Night animals on any individual day) or in distance traveled (Fig. S2B; Mixed-effects ANOVA, significant effect of Habituation Day $F_{(5,181)} = 20.92$, $p < 0.0001$, but no effect of Day/Night ($F_{(1,37)} = 0.09$, $p > 0.05$), or Interaction ($F_{(5,181)} = 0.39$, $p > 0.05$) and no significant difference between Day and Night animals on any individual day) between Day and Night animals across the 6-day habituation period, suggesting that movement is similar whether mice are trained during the day or night. Together, this suggests that spatial memory performance oscillates over the day/night cycle, with better memory performance occurring during the day and worse memory at night.

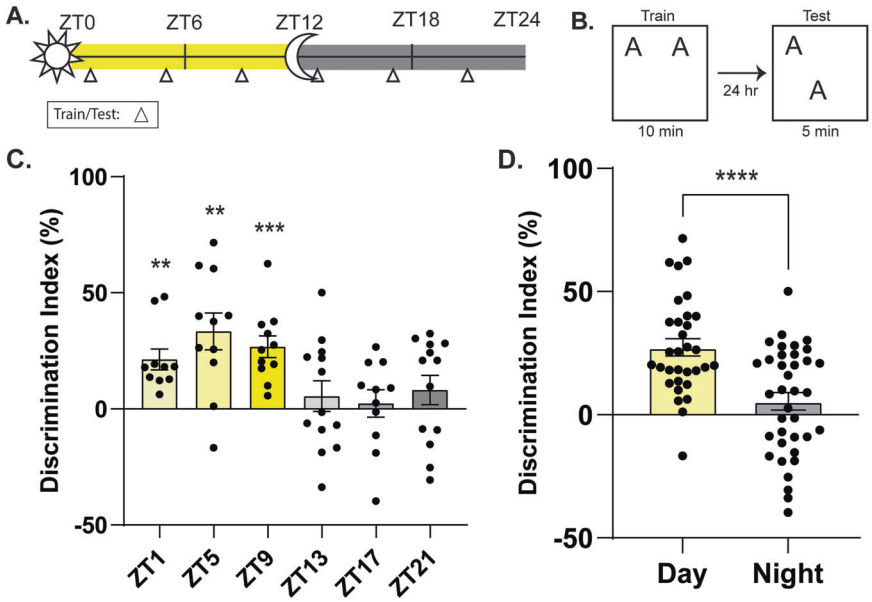

Fig. 1 Memory performance oscillates over the diurnal cycle with better memory performance during the day. A Schematic for behavioral time points. Triangle indicates time of training and testing. **B** Object location memory experimental design. **C** Memory performance oscillates over 24 h with best memory observed at ZT5 and worst at ZT17. Yellow bars indicate day timepoints while grey bars indicate night timepoints (n = 10–13 timepoint). **D** Memory performance is significantly better during the day than at night (n = 32–38/timepoint). ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ compared to zero (**C**) or between groups (**D**). ZT Zeitgeber Time, where ZT0 = 6am (7am DST), lights on, ZT12 = 6 pm (7 pm DST), lights off.

OLM acquisition is intact across the day/night cycle

After confirming that memory oscillates over the 24 h day, we next wanted to determine which phase of memory is specifically regulated by the day/night cycle: acquisition, consolidation, or retrieval. First, to test whether acquisition changes across the 24 h day, we measured short-term memory (STM) for OLM during the day and night. We trained a cohort of mice in OLM at the peak of memory, ZT5, or the trough of memory, ZT17 (Fig. 2A), as identified in the previous experiment (Fig. 1C). Here, however, mice were tested 60 min after training (Fig. 2B) during the transcription-independent STM phase [17, 27]. We found that all mice showed intact short-term memory regardless of the time of acquisition (Fig. 2C; Unpaired *t*-test comparing ZT5 to ZT17: $t_{(12)} = 0.3749$, $p > 0.05$; one-sample *t*-test comparing each group to 0: ZT5: $t_{(6)} = 4.801$, $p < 0.01$; ZT17: $t_{(6)} = 11.77$, $p < 0.0001$). Even mice trained at ZT17 that show drastic impairments in long-term memory (Fig. 1C) showed intact memory when tested 60 m after acquisition, indicating they successfully learned OLM. Interestingly, nighttime mice showed significantly more total exploration of the objects compared to the daytime-tested mice (Fig. S1B; unpaired *t*-test comparing ZT5 to ZT17: $t_{(12)} = 5.498$, $p = 0.0001$), a difference that was not observed in the long-term memory test (Fig. S1A). As the nighttime-tested mice were in their active phase, the short interval between training and testing may have contributed to this enhancement in exploratory activity. Notably, even despite their lower exploration time, daytime-tested mice showed robust short-term memory, indicating they sufficiently explored the objects during acquisition (Fig. 2C). Together, this demonstrates that mice successfully learn OLM at night, even when long-term memory fails. Therefore, acquisition is not affected by the time of day and the observed nighttime deficits in long-term memory are not due to an acquisition deficit but are more likely due to a consolidation or retrieval deficit.

OLM retrieval is also stable across the day/night cycle

After ruling out acquisition as driving time-of-day effects on memory performance, we next tested whether memory retrieval is altered across the day/night cycle. It is possible that mice tested at night have intact long-term memory for OLM but have difficulty retrieving that stored information during the dark cycle. To rule out a retrieval deficit, we again trained animals at the peak and trough of memory and tested them either 24 h (at the same ZT) or 36 h later (at the opposite ZT) to separate the acquisition time from the retrieval time (Fig. 2D–E). We found that the time of acquisition, and not the time of retrieval, drove memory performance in OLM. Regardless of when they were tested, mice trained during the daytime (ZT5) showed strong memory whereas mice trained at night (ZT17) showed weak object location memory (Fig. 2F). We saw a significant difference in memory performance between the groups trained during the day compared to those trained at night (Two-way ANOVA, significant effect of Training Time ($F_{(1,22)} = 43.57$, $p < 0.0001$), no significant effect of Retrieval Time or Interaction), but observed no significant differences between groups tested during the day or night within either training ZT cohort (Sidak's *post-hoc*, $p > 0.05$), indicating that groups performed similarly at the 24 h and 36 h tests. Finally, there was no significant difference in exploration times for any condition (Fig. S1C; Two-way ANOVA, no significant effect of Training Time or Retrieval Time, and no significant Interaction) indicating memory performance is not dependent on exploration time. Thus, if memory acquisition occurred during the day, memory was successfully retrieved at test and if memory acquisition occurred at night, retrieval was impaired at test, regardless of when that test occurred. This suggests that retrieval itself is not altered across the day/night cycle. Together with the results of the short-term memory test, our work suggests that hippocampal memory consolidation, rather than memory acquisition or retrieval, oscillates across the 24 h day.

Daytime learning drives major changes in gene expression

Our behavioral findings suggest that the observed nighttime memory deficits are specifically due to a consolidation error, as both memory acquisition and memory retrieval were intact at night, when long-term memory fails. As de novo transcription is critically important for the memory consolidation process [18, 20, 28], we reasoned that changes in learning-induced gene

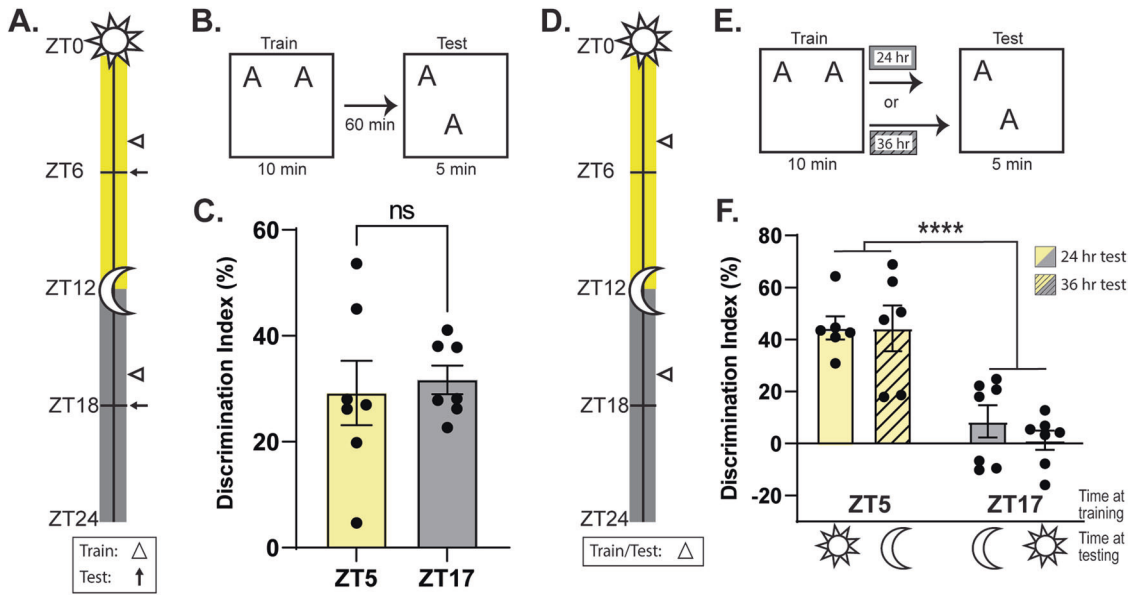

Fig. 2 Memory acquisition and memory retrieval are unaffected by the time of day. A Schematic for behavioral time points testing short-term memory. Triangle indicates time of training. Arrow indicates time of test. **B** Short-term object location memory experimental design. **C** No difference is seen in short term memory performance between the day (yellow bar) and the night (grey bar; n = 7/timepoint). **D** Schematic for behavioral time points testing memory retrieval. Triangle indicates time of training and/or testing. **E** Object location memory retrieval experimental design. Testing was performed 24 h (solid bars) or 36 h (striped bars) post-training. **F** Mice trained during the day (yellow bars) showed strong memory performance whether tested 24 h (day) or 36 h (night) later. Mice trained at night (grey bars) showed weak memory performance whether tested 24 h (night) or 36 h (day) later (n = 6–7/timepoint). ns not significant, **** = $p < 0.0001$, ZT Zeitgeber Time, where ZT0 = 6am (7am DST), lights on, ZT12 = 6 pm (7 pm DST), lights off.

expression could underlie these nighttime memory deficits. Specifically, we hypothesized that a subset of learning-induced genes that support memory during the daytime are not properly expressed at night, leading to the observed impairments in long-term memory consolidation. Although previous work has suggested that clock genes (notably *Per1*) function within the hippocampus to regulate memory [7], we wanted to use an unbiased method to identify potential genes capable of exerting diurnal control over hippocampal memory consolidation. We therefore used RNA sequencing (RNA-seq) to identify genes that oscillate in tandem with memory consolidation across the day/night cycle. To this end we trained mice in OLM at 6 timepoints across the diurnal cycle, as in Fig. 1A and then sacrificed them 60 min later (along with time-matched homecage controls) to assess RNA expression in the DH (Fig. 3A). During training, all groups showed similar object exploration times (Fig. S1D; One-way ANOVA: $F_{(5,31)} = 1.1119$, $p = 0.3706$). After collecting punches from the DH (area CA1) and isolating RNA, we created libraries and ran RNA-seq to identify genes capable of enabling robust memory during the day but poor memory at night.

First, we used JTK-Cycle to identify genes that oscillate across the day/night cycle [29]. JTK-Cycle is an algorithm that identifies transcripts exhibiting a rhythmic oscillation pattern across the diurnal cycle in large-scale datasets. To see how learning affects oscillatory gene expression, we first identified genes that oscillate under homecage (HC) conditions (Fig. 3B, left column) and then plotted the same genes for the OLM trained group (right column). We found that during the day (ZT1, ZT5, ZT9), OLM drives dramatic changes in gene expression whereas during the night (ZT13, ZT17, ZT21) fewer genes were affected by the same training event, with most genes continuing to oscillate normally even after OLM (Fig. 3B). This shows that learning is massively disruptive to baseline gene oscillations during the day, but not at night. This is consistent with our hypothesis that some genes fail to respond to learning at night, potentially contributing to nighttime memory consolidation impairments.

Next, we aimed to identify individual genes capable of modulating memory across the day/night cycle. A number of genes are known to be induced by learning and necessary for memory consolidation [18, 28, 30], but there is very little known about how these learning-induced genes oscillate across the 24 h day. As we specifically wanted to identify genes that might exert diurnal control over memory (supporting robust memory during the day but only weak memory at night), we aimed to identify genes that show learning-induced increases during the daytime that are reduced or eliminated at night in tandem with memory performance. We therefore used differential gene expression analyses to identify genes induced by learning (i.e. genes expressed at significantly higher levels in mice trained with OLM compared to time-matched homecage controls) at each ZT and then compared these learning-induced genes across the day and night to specifically identify genes capable of supporting robust learning during the day but not at night.

First, we compared genes induced by OLM during the day (at ZT1, 5, or 9) to those induced by OLM during the night (ZT13, 17, or 21). We considered a gene to be upregulated if the FDR was <0.05 and had a positive log2fold-change in the OLM group when directly comparing OLM and homecage groups. With these criteria we identified 757 genes upregulated only during the day, 35 genes upregulated only at night, and 74 that were upregulated by learning during both the day and the night (Fig. S3A; File S1). This is consistent with our JTK-cycle results, indicating that OLM training drives massive changes in gene expression during the daytime (when memory is robust) that are muted at night (when memory is weak). Next, to understand the functional relevance of the genes that might support diurnal oscillations in memory, we ran pathway analyses on genes exclusively upregulated during the day using the KEGG database (Fig. S3B). During the daytime, when memory is robust, this analysis identified pathways involved in RNA processing, transport, and degradation, which was unsurprising, considering that de novo gene expression is critical to long-term memory formation [18, 20, 28].

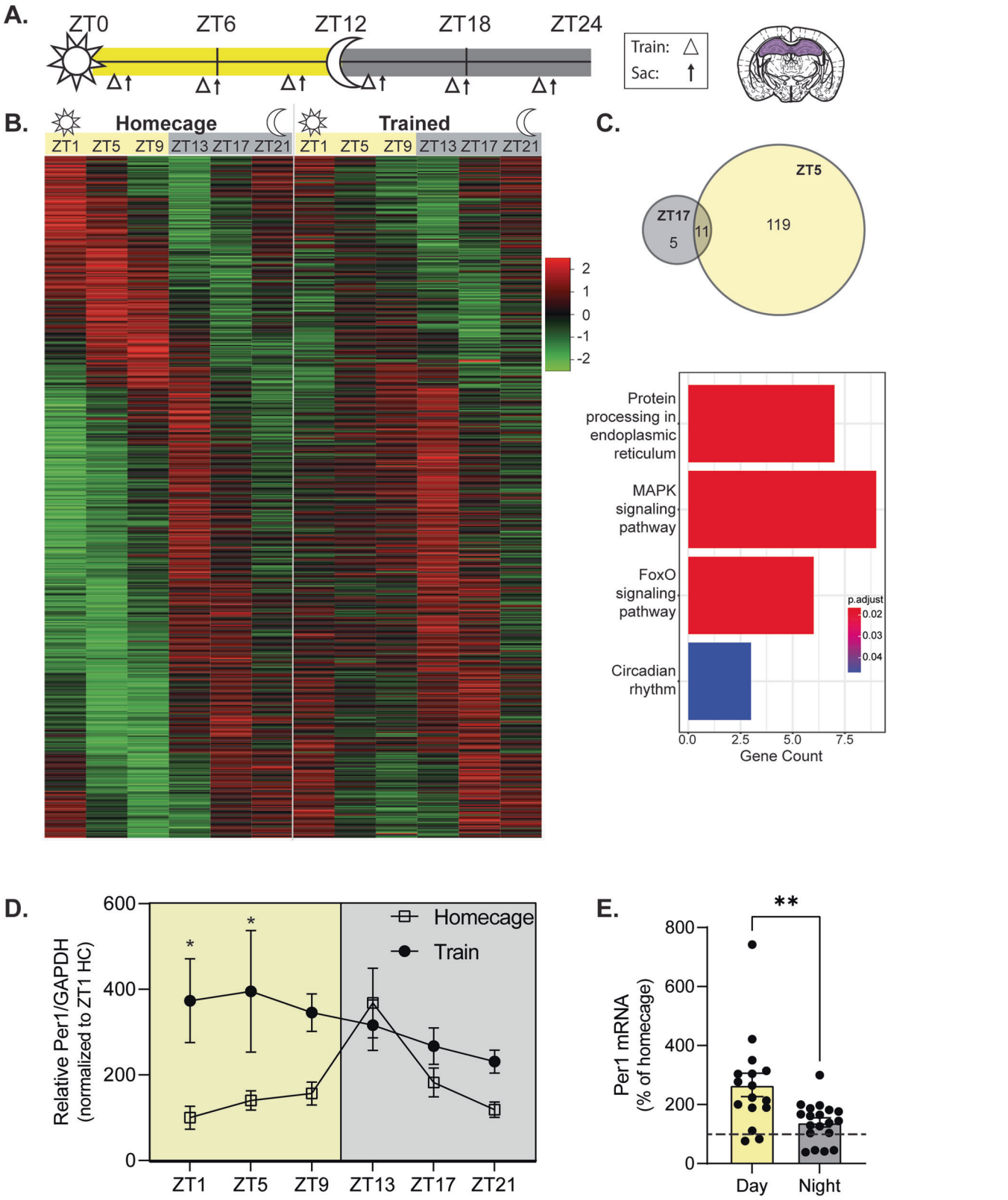

Fig. 3 Learning during the day drives major changes in gene expression that do not occur at night. A Schematic for behavioral and sacrificing timepoints. Triangle: training time; arrow: sacrifice time. **B** Oscillatory genes in homecage mice (n = 6/timepoint; left) show drastic learning-induced changes during the day (n = 6/timepoint; right, yellow columns) but not at night (gray columns). **C** Top: genes upregulated in response to learning at ZT5 (yellow), ZT17 (gray), or both. Bottom: top Kegg pathways upregulated at ZT5 but not at ZT17. **D** *Per1* mRNA is increased by learning during the day (ZT1 and ZT5) but not at any night timepoint (n = 4–8/timepoint). **E** During the day (yellow bar) learning-induced *Per1* expression is higher than the night (grey bar). Dotted line indicates no induction of *Per1* expression. * = p<0.05, ** = p<0.01 ZT Zeitgeber Time, where ZT0 = 6am (7am DST), lights on, ZT12 = 6pm (7pm DST), lights off.

To narrow down this list, we decided to restrict our analyses to directly compare learning-induced genes when memory is best (ZT5) and worst (ZT17). We identified 119 genes upregulated in response to learning exclusively at ZT5, 5 upregulated only at ZT17, and 11 upregulated at both times (Fig. 3C; File S2). Again, to determine the functional identities of these transcripts, we ran Kegg pathway analyses on genes that were upregulated exclusively at ZT5. The top pathways identified genes involved in protein processing in the endoplasmic reticulum, MAPK signaling, FoxO signaling, and circadian rhythm genes (Fig. 3C). We were particularly intrigued to see that circadian rhythm genes were induced by learning during the day but not at night, as this would suggest that clock genes might function locally in the hippocampus to exert circadian control over memory, as previously hypothesized [5, 6, 31]. Of these, one gene in particular stood out: *Period1* (*Per1*). *Per1* plays a well-established role in the circadian rhythm within the brain's central pacemaker, the suprachiasmatic nucleus [12–14], but has more recently been implicated in learning and memory as well [7–10, 16]. *Per1* has specifically been hypothesized to play a role in "gating" memory formation across the diurnal cycle [31], although the precise role that local *Per1* plays in the DH is unclear. In addition to *Per1*, there were two other circadian rhythm genes differentially regulated in response to learning during the day but not the night: *Protein Kinase AMP-Activated Non-Catalytic Subunit Beta 1* (*Prkab1*) and *Basic Helix-Loop-Helix Family Member E40* (*Bhlhe40*). The roles of these genes in learning have yet to be explored.

To get a better understanding of how *Per1* oscillates across the day/night cycle within the DH, we ran RT-qPCR on these samples to measure *Per1* mRNA across the day/night cycle in both homecage and trained mice (Fig. 3D). In homecage controls, we observed rhythmic oscillations in hippocampal *Per1* that peaked at the beginning of the night (one-way ANOVA just on homecage group: $F_{(5,39)} = 5.321$, $p < 0.001$, Sidak's *post hoc* test comparing ZT1 to ZT13,***$p < 0.001$; no other timepoints different). Following learning, *Per1* was increased during the daytime but this induction was dampened at night. Comparing the homecage and trained groups, we found a significant increase in *Per1* response to OLM (two-way ANOVA, significant effect of Training ($F_{(1,69)} = 15.58$, $p = 0.0002$), but no effect of ZT Time or Interaction). Sidak's *post hoc* tests comparing homecage and trained groups within each timepoint revealed that *Per1* was significantly upregulated by OLM at ZT1 and ZT5 ($p < 0.05$) but not at any other timepoint (Fig. 3D; Sidak's *post-hoc*, $p > 0.05$). To further investigate the relationship between learning-induced *Per1* and time-of-day, we expressed each trained group as a percent of its time-locked homecage control and collapsed all day and night data points (Fig. 3E).

Here, we observed that OLM drives a significantly larger induction of *Per1* during the day compared to the night (unpaired *t*-test comparing day to night: $t_{(33)} = 3.181$, $p = 0.0032$). Thus, hippocampal *Per1* is induced by learning during the day, but this induction largely fails at night, as indicated by our RNA-seq (Fig. 3C). Together with our behavioral data, this demonstrates that *Per1* oscillates in tandem with spatial memory consolidation; both memory performance and hippocampal *Per1* peak during the daytime and trough at night. As previous work has shown that manipulating *Per1* levels in the mouse hippocampus modulates long-term memory [7], *Per1* may be capable of exerting local diurnal control over hippocampal memory, with nighttime reductions in learning-induced *Per1* potentially limiting memory formation.

Knocking down *Per1* expression in the dorsal hippocampus during the day disrupts memory formation

Hippocampal *Per1* oscillates in tandem with memory performance, providing a potential mechanism through which the circadian system could regulate memory across the diurnal cycle. Indeed, previous studies have suggested that *Per1* levels may modulate memory formation [7–9, 32]. Here, to directly test whether local knockdown of *Per1* in the DH disrupts memory formation during the day, we locally knocked down *Per1* with a viral CRISPR interference (HSV-CRISPRi) system before OLM. CRISPRi consists of a dead Cas9 (dCas9) fused to two transcriptional repressors: KRAB and MeCP2 (Fig. 4A) to synergistically repress transcription of the target gene (here, *Per1*). We packaged this CRISPRi system into HSV to drive neuron-specific knockdown of *Per1* in vivo [33]. HSV-CRISPRi was infused directly into the CA1 region of the DH along with either *Per1* sgRNA or non-targeting control sgRNA before training the mice in OLM.

First, to confirm that HSV-CRISPRi reduces hippocampal *Per1* expression, a group of mice was sacrificed three days after HSV-CRISPRi infusion (when HSV expression peaks [34, 35]) for immunofluorescence and qPCR (Fig. S4A). We observed high colocalization of the sgRNA (green) and dCas9-KRAB-MeCP2 (red) in neurons of the DH (Fig. S4B). Further, in punches collected from this region, we found that hippocampal *Per1* was significantly reduced in mice given the *Per1*-targeting sgRNA compared to mice given the non-targeting control sgRNA (Fig. S4C; Unpaired t-test $t_{(13)} = 3.422$, $p = 0.0045$). Therefore, our HSV-CRISPRi system appropriately reduces *Per1* expression within the DH.

Next, we tested whether memory formation is disrupted by hippocampal *Per1* knockdown during the day (ZT5, the peak of memory performance (Fig. 1C)). 24 h after the final day of habituation, mice were given intra-hippocampal infusions of HSV-CRISPRi (targeting *Per1* or a control, nontargeting system). Mice were trained in OLM 3d later (when HSV peaks) at ZT5 and were tested 24 h after training (Fig. 4B). *Per1* knockdown mice showed significantly impaired memory performance compared to

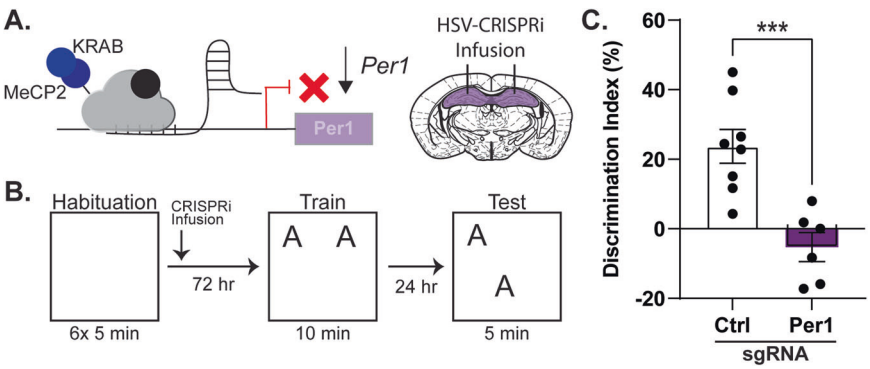

Fig. 4 Knockdown of *Per1* in the dorsal hippocampus disrupts long-term spatial memory formation. A Schematic of CRISPRi system. dCas9 is fused with two repressive elements that reduce the transcription of endogenous *Per1*. **B** Object location memory experimental design. **C** Mice that received an infusion of the control sgRNA (white bar) showed significantly better memory for OLM than mice that received an infusion of the *Per1* sgRNA (purple bar; n = 6–8/condition). *** = $p < 0.001$.

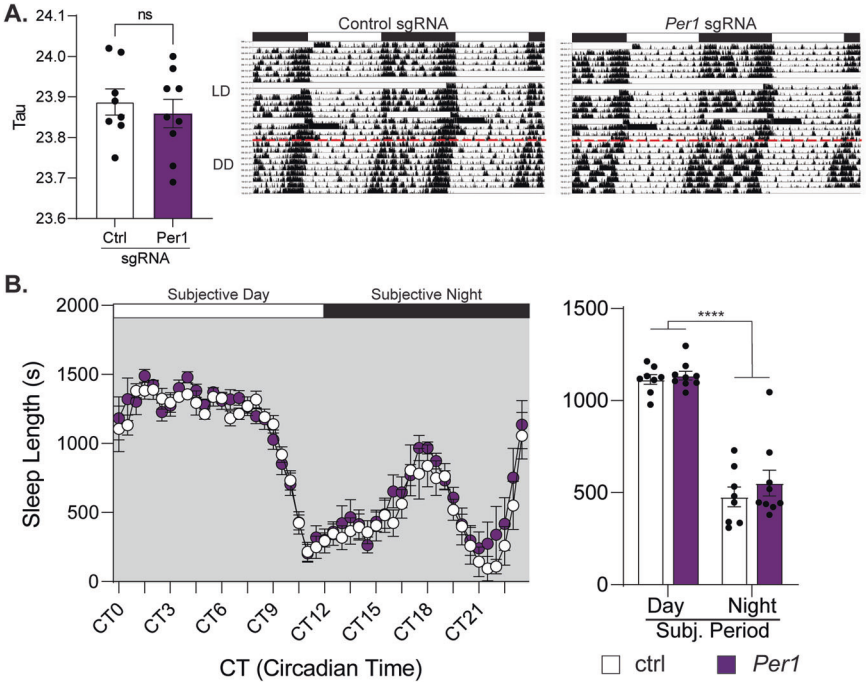

Fig. 5 Knocking down *Per1* in the dorsal hippocampus does not affect circadian rhythms or sleep behavior. A Knocking down *Per1* in the DH does not affect free-running tau under dark-dark conditions. Right: representative actograms for control sgRNA (middle panel) and *Per1* sgRNA (right panel). **B** Sleep length was not affected by *Per1* knockdown. Right: average sleep length for the subjective day and night. Mice showed longer sleep bouts during the subjective day, as expected, but there was no effect of *Per1* knockdown (n = 8–9/condition). **** = $p < 0.0001$, ns not significant, LD light/dark, DD dark/dark, CT Circadian Time, ZT Zeitgeber Time, where ZT0 = 6am (7am DST), lights on, ZT12 = 6 pm (7 pm DST), lights off.

controls (unpaired *t*-test comparing control and *Per1* knockdown: $t_{(12)} = 4.336$, $p = 0.001$; Fig. 4C). In addition, control sgRNA mice showed robust long-term memory, while *Per1* knockdown mice showed little evidence of intact memory, preferring both objects equally (One-sample *t*-test comparing each group to 0, control: $t_{(7)} = 4.8828$, $p = 0.0018$, *Per1* knockdown: $t_{(5)} = 1.270$, $p = 0.2599$). Our results corroborate previous work [7], demonstrating that local expression of *Per1* in the dorsal hippocampus is critical for long-term spatial memory formation.

Manipulation of *Per1* expression in the dorsal hippocampus does not affect circadian rhythmicity or sleep patterns

Given that *Per1* plays a key role in the central circadian system, which itself can modulate memory [2, 25, 36–41], we wanted to ensure that our local knockdown of *Per1* in the DH does not indirectly affect memory by disrupting the central clock in the SCN. Specifically, we wanted to ensure that hippocampal *Per1* knockdown with our HSV-CRISPRi system (Fig. 4A) does not affect either the circadian rhythm or sleep behavior of these mice.

To test whether hippocampal *Per1* knockdown affects the animals' circadian activity pattern, a cohort of mice underwent activity monitoring in LD conditions followed by DD conditions. Mice were acclimated to the standard LD cycle for 2 weeks prior to infusion of HSV-CRISPRi into the DH. Three days post-infusion, at the peak of viral expression, the lights were turned off and activity and sleep behavior were monitored in constant darkness for 10 days (for the duration of HSV expression) in the absence of external time cues. We found that local *Per1* knockdown within the DH had no significant effect on circadian activity patterns. There was no significant difference in free-running τ between groups (*Per1* knockdown mice: 23.86, control mice: 23.91 (Fig. 5A; Unpaired *t*-test $t_{(15)} = 0.5955$, $p = 0.5604$), indicating that the circadian rhythm was intact even in mice with hippocampal *Per1* knockdown. Therefore, hippocampus-specific knockdown of *Per1* does not appear to affect the circadian activity pattern.

We also assessed sleep behavior in these mice. Briefly, we used our infrared monitors to identify bouts of inactivity lasting 40 s or longer as a behavioral correlate of sleep. This immobility-defined sleep has previously been shown to tightly correlate with sleep defined via EEG records [24]. We observed no differences in sleep behavior (including both sleep duration and sleep bout length) between *Per1* knockdown and control mice in either the LD or the DD phase (Fig. 5B; LD: Two-way mixed-model ANOVA, effect of Time ($F_{(1,15)} = 289.6$, $p < 0.0001$), no effect of Infusion or Time x Infusion interaction). Therefore, knocking down *Per1* in the DH does not affect sleep behavior (Fig. 5B) or circadian activity patterns (Fig. 5A). Along with previous research showing that hippocampus-specific manipulations of HDAC3 (a major epigenetic regulator of *Per1*) [7] and even electrolytic lesions of the DH [42] have no effect on the circadian rhythm, this work strongly suggests that the sleep/wake cycle is not affected by site-specific manipulations in the dorsal hippocampus.

DISCUSSION

Memory is carefully regulated by the circadian system, but the mechanisms that control memory across the day/night cycle are largely unclear. Here, we show that hippocampal memory oscillates across the diurnal cycle, with memory peaking during the daytime (specifically at ZT5) and showing a trough at night (ZT17). Next, through a series of experiments, we determined that memory consolidation, not memory acquisition or retrieval, is impacted by the time of day. Using RNA-seq, we next determined that learning drastically affects oscillating gene patterns during the daytime and identified the clock gene *Per1* as a key player potentially capable of exerting diurnal control over memory. Finally, we verified that *Per1* manipulations restricted to the DH impair long-term memory during the daytime but have no effect on either circadian activity patterns or sleep behavior. Together, these data suggest that *Per1* may play a local, autonomous role in

the DH to exert diurnal control over memory consolidation in addition to its well-documented role in regulating the circadian system within the SCN.

Our work suggests that hippocampal *Per1* could regulate memory based on the time of day, a modulatory role that may not be specific to the hippocampus. Our lab has recently shown that *Per1* levels increase in response to learning in another memory-relevant structure, the anterior retrosplenial cortex (aRSC) [8, 32] and local knockdown of *Per1* within the aRSC before learning (in this case, context fear conditioning) impairs memory. This suggests that *Per1* modulates multiple forms of memory consolidation across different memory-relevant brain regions. Interestingly, our previous work found that retrosplenial *Per1* may modulate memory in a sex-specific manner [8], with overexpression of *Per1* having different effects in male and female mice. Here, to achieve the necessary power (particularly in circadian experiments with 6–12 groups), we used only male mice. We are currently investigating these effects in female mice in a parallel set of experiments.

Here, in a systematic and controlled experiment, we found that mice showed better memory consolidation for OLM during the day than at night. Similarly, in a previous study, we found that mice trained with context fear conditioning during the daytime also showed better memory than mice trained at night [8], suggesting that memory for multiple hippocampus-based memory tasks is best during the daytime. Other groups have identified similar diurnal memory patterns [2, 8, 16, 25, 26], but some studies have shown that mice have better memory at night in some tasks [43–45]. Although it is not clear why this variability exists, it may be due to differences in either the memory task or experimental procedures. For example, memory tasks that require the participation of other brain structures with different oscillatory patterns might change when memory is best and worst. Further, procedural differences, such as the use of overhead lights during the day but dim red lighting at night might affect the peak performance of the animals. Here, we carefully controlled the conditions to be able to directly compare performance in hippocampus-dependent OLM across the day/night cycle and found that memory was much better during the day than at night. This was somewhat surprising, as mice are nocturnal, but many species similarly perform better during the day than at night regardless of their active time [2]. Further, we observed similar levels of movement and similar total object exploration times (5–10 s) between mice trained during the day versus those trained at night, which are similar to the levels we have observed in previous OLM experiments [7, 23, 32], indicating that differences in activity are not responsible for driving this oscillation in memory. Overall, this suggests that a species' diurnal activity pattern is not a reliable predictor of memory performance across the 24 h day. A better predictor of memory performance might be something happening at the cellular or molecular level, like local *Per1* induction or even the spontaneous activity of SCN cells, which are more responsive during the day in both nocturnal and diurnal animals [46]. Future work should therefore systematically determine whether other forms of memory, including those that do not require the hippocampus, show a similar oscillation, peaking during the day.

Our experiments demonstrate that memory consolidation is specifically modulated across the diurnal cycle, as short-term memory is intact even at night (Fig. 2C) and memory retrieval itself did not oscillate across the day/night cycle (Fig. 2F). Notably, in our retrieval experiment (Fig. 2D–F), both cohorts tested at 36 h had a full sleep cycle between acquiring the memory and retrieving it, but only the daytime-trained mice were able to successfully remember the object locations. Further, these results also suggest that the training event does not simply serve as a zeitgeber that selectively improves memory at that specific timepoint; mice trained during the daytime showed good memory even when tested 36 h later in the middle of the night. This, along with the observation that memory is better during the day (when mice are normally asleep) suggests that our memory effects do not occur simply because the behavioral task disrupts the animals' sleep.

Together, our work suggests that *Per1* plays two key roles in the brain: the canonical role of regulating the circadian system within the SCN and a noncanonical role in exerting diurnal control over memory consolidation in the DH. We have previously identified *Per1* as an important mechanism that contributes to age-related hippocampal memory impairments in aging, 18-month-old mice [7]. Here, we show that *Per1* may specifically function within the dorsal hippocampus to exert local circadian control over memory consolidation, independent of its canonical role in regulating the circadian system within the SCN.

REFERENCES

1. Richards J, Gumz ML. Advances in understanding the peripheral circadian clocks. FASEB J. 2012;26:3602–13.
2. Gerstner JR, Yin JCP. Circadian rhythms and memory formation. Nat Rev Neurosci. 2010;11:577–88.
3. Reppert SM, Weaver DR. Coordination of circadian timing in mammals. Nature. 2002;418:6901. 2002;418:935–941
4. Jilg A, Lesny S, Peruzki N, Schwegler H, Selbach O, Dehghani F, et al. Temporal dynamics of mouse hippocampal clock gene expression support memory processing. Hippocampus. 2010;20:377–88.
5. Hartsock MJ, Spencer RL. Memory and the circadian system: Identifying candidate mechanisms by which local clocks in the brain may regulate synaptic plasticity. Neurosci Biobehav Rev. 2020;118:134–62.
6. Smies CW, Bodinayake KK, Kwapis JL. Time to learn: The role of the molecular circadian clock in learning and memory. Neurobiol Learn Mem. 2022;193:107651.
7. Kwapis JL, Alaghband Y, Kramár EA, López AJ, Vogel Ciernia A, White AO, et al. Epigenetic regulation of the circadian gene Per1 contributes to age-related changes in hippocampal memory. Nat Commun. 2018;9:3323.
8. Urban MW, Lo C, Bodinayake KK, Brunswick CA, Murakami S, Heimann AC, et al. The circadian clock gene Per1 modulates context fear memory formation within the retrosplenial cortex in a sex-specific manner. Neurobiol Learn Mem. 2021;185:107535.
9. Rawashdeh O, Jilg A, Maronde E, Fahrenkrug J, Stehle JH. Period1 gates the circadian modulation of memory-relevant signaling in mouse hippocampus by regulating the nuclear shuttling of the CREB kinase pP90RSK. J Neurochem. 2016;138:731–45.
10. Woodruff ER, Chun LE, Hinds LR, Varra NM, Tirado D, Morton SJ, et al. Coordination between prefrontal cortex clock gene expression and corticosterone contributes to enhanced conditioned fear extinction recall. ENeuro. 2018;5:455–73.
11. Gekakis N, Staknis D, Nguyen HB, Davis FC, Wilsbacner LD, King DP, et al. Role of the CLOCK protein in the mammalian circadian mechanism. Science. 1998;280:1564–9.
12. Kume K, Zylka MJ, Sriram S, Shearman LP, Weaver DR, Jin X, et al. mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. Cell. 1999;98:193–205.
13. Griffin EA, Staknis D, Weitz CJ. Light-independent role of CRY1 and CRY2 in the mammalian circadian clock. Science. 1999;286:768–71.
14. Sato TK, Yamada RG, Ukai H, Baggs JE, Miraglia LJ, Kobayashi TJ, et al. Feedback repression is required for mammalian circadian clock function. Nat Genet. 2006;38:312–9.
15. Albrecht U, Sun ZS, Eichele G, Lee CC. A differential response of two putative mammalian circadian regulators, mper1 and mper2, to light. Cell. 1997;91:1055–64.
16. Rawashdeh O, Jilg A, Jedlicka P, Slawska J, Thomas L, Saade A, et al. PERIOD1 coordinates hippocampal rhythms and memory processing with daytime. Hippocampus. 2014;24:712–23.
17. Alberini CM. Transcription factors in long-term memory and synaptic plasticity. Physiol Rev. 2009;89:121–45.
18. Alberini CM, Kandel ER. The regulation of transcription in memory consolidation. Cold Spring Harb Perspect Biol. 2015;7:a021741.
19. Chatterjee S, Angelakos CC, Bahl E, Hawk JD, Gaine ME, Poplawski SG, et al. The CBP KIX domain regulates long-term memory and circadian activity. BMC Biollogy. 2020;18:155.
20. Nadel L, Hupbach A, Gomez R, Newman-Smith K. Memory formation, consolidation and transformation. Neurosci Biobehav Rev. 2012;36:1640–5.
21. Tapp WN, Holloway FA. Phase shifting circadian rhythms produces retrograde amnesia. Science. 1981;211:1056–8.

22. Davies JA, Navaratnam V, Redfern PH. A 24-h rhythm in passive-avoidance behaviour in rats. Psychopharmacologia. 1973;32:211–4.
23. Vogel-Ciernia A, Wood MA. Examining object location and object recognition memory in mice. Curr Protoc Neurosci. 2014;2014:8.31.1–8.31.17.
24. Brown LA, Hasan S, Foster RG, Peirson SN. COMPASS: Continuous open mouse phenotyping of activity and sleep status. Wellcome Open Res. 2017;1:1–18.
25. Chaudhury D, Colwell CS. Circadian modulation of learning and memory in fear-conditioned mice. Behavioural Brain Research. 2002;133:95–108.
26. Eckel-Mahan KL, Phan T, Han S, Wang H, Chan GCK, Scheiner ZS, et al. Circadian oscillation of hippocampal MAPK activity and cAMP: Implications for memory persistence. Nat Neurosci. 2008;11:1074–82.
27. Davis HP, Squire LR. Protein synthesis and memory: A review. Psychol Bull. 1984;96:518–59.
28. Igaz LM, Bekinschtein P, Vianna MMR, Izquierdo I, Medina JH. Gene expression during memory formation. Neurotox Res. 2004;6:189–203.
29. Hughes ME, Hogenesch JB, Kornacker K. JTK-CYCLE: An efficient nonparametric algorithm for detecting rhythmic components in genome-scale data sets. J Biol Rhythms. 2010;25:372–80.
30. Rao-Ruiz P, Couey JJ, Marcelo IM, Bouwkamp CG, Slump DE, Matos MR, et al. Engram-specific transcriptome profiling of contextual memory consolidation. Nat Commun. 2019;10:1–14.
31. Rawashdeh O, Parsons R, Maronde E. Clocking in time to gate memory processes: The circadian clock is part of the Ins and outs of memory. Neural Plast. 2018;2018:6238989.
32. Brunswick CA, Baldwin DJ, Bodinayake KK, McKenna AR, Lo C-Y, Bellfy L, et al. The clock gene Per1 is necessary in the retrosplenial cortex—but not in the suprachiasmatic nucleus—for incidental learning in young and aging male mice. Neurobiol Aging. 2023. https://doi.org/10.1016/j.neurobiolaging.2023.02.009.
33. Yeo NC, Chavez A, Lance-Byrne A, Chan Y, Menn D, Milanova D, et al. An enhanced CRISPR repressor for targeted mammalian gene regulation. Nat Methods. 2018;15:611–6.
34. Neve RL, Neve KA, Nestler EJ, Carlezon WA. Use of herpes virus amplicon vectors to study brain disorders. Biotechniques. 2005;39:381–9.
35. Sarno E, Robison AJ. Emerging role of viral vectors for circuit-specific gene interrogation and manipulation in rodent brain. Pharmacol Biochem Behav. 2018;174:2–8.
36. Krishnan HC, Lyons LC. Synchrony and desynchrony in circadian clocks: Impacts on learning and memory. Learning and Memory. 2015;22:426–37.
37. Cho K. Chronic 'jet lag' produces temporal lobe atrophy and spatial cognitive deficits. Nat Neurosci. 2001;4:567–8.
38. Marquié JC, Tucker P, Folkard S, Gentil C, Ansiau D. Chronic effects of shift work on cognition: Findings from the VISAT longitudinal study. Occup Environ Med. 2015;72:258–64.
39. Horsey EA, Maletta T, Turner H, Cole C, Lehmann H, Fournier NM. Chronic jet lag simulation Decreases Hippocampal Neurogenesis and Enhances Depressive Behaviors and Cognitive Deficits in Adult Male Rats. Front Behav Neurosci. 2020;13:272.
40. Valentinuzzi VS, Neto SPD, Carneiro BTS, Santana KS, Araújo JF, Ralph MR. Memory for time of training modulates performance on a place conditioning task in marmosets. Neurobiol Learn Mem. 2008;89:604–7.
41. Valentinuzzi VS, Menna-Barreto L, Xavier GF. Effect of circadian phase on performance of rats in the Morris water maze task. J Biol Rhythms. 2004;19:312–24.
42. Phan TH, Chan GCK, Sindreu CB, Eckel-Mahan KL, Storm DR. The diurnal oscillation of MAP (Mitogen-Activated Protein) kinase and adenylyl cyclase activities in the hippocampus depends on the suprachiasmatic nucleus. J Neurosci. 2011;31:10640–7.
43. Liu D, Li J, Lin H, Lorsung E, Le N, Singla R, et al. Circadian activities of the brain MNK-eIF4E signalling axis contribute to diurnal rhythms of some cognitive functions. Eur J Neurosci. 2022;56:1–17.
44. Loss CM, Binder LB, Muccini E, Martins WC, de Oliveira PA, Vandresen-Filho S, et al. Influence of environmental enrichment vs. time-of-day on behavioral repertoire of male albino Swiss mice. Neurobiol Learn Mem. 2015;125:63–72.
45. Tsao CH, Flint J, Huang GJ. Influence of diurnal phase on behavioral tests of sensorimotor performance, anxiety, learning and memory in mice. Sci Rep. 2022;12:1–10.
46. Colwell CS. Linking neural activity and molecular oscillations in the SCN. Nat Rev Neurosci. 2011;12:553–69.

ACKNOWLEDGEMENTS

We would like to thank Dr. Rachael Neve and the Gene Delivery Technology Core at Massachusetts General Hospital for help designing and packaging all HSV viruses described here. We would also like to thank the Huck Institutes of the Life Sciences at Penn State for funding, Dr. Craig Praul and the Huck Genomics Core Facility for library construction, Dr. Yuka Imamura and the Penn State Hershey Genome Sciences Facility for sequencing, and the Penn State Bioinformatics Consulting Center for assistance with the RNA-seq.

AUTHOR CONTRIBUTIONS

Study concept and design: LB and JLK. Acquisition of data: LB, CWS, ARB, KKB, EMS, DSW, CL, SM, HMB, and MJV. Analysis of data: LB, ARB, KKB, MJV, AS, and IA. Drafting of manuscript: LB and JLK. Final approval of manuscript: All authors.

FUNDING

This research was funded by NIH grants R01AG074041 (JLK), K99/R00AG056586 (JLK) and R21AG068444 (JLK), Whitehall Foundation Grant #2020-05-06 (JLK), American Federation for Aging Research Grant #A21105 (JLK), startup funds from the Eberly College of Science and Department of Biology at Pennsylvania State University (JLK) and the National Institute on Aging under Grant T32 AG049676 to The Pennsylvania State University (LB).

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41386-023-01616-1.

Correspondence and requests for materials should be addressed to Janine L. Kwapis.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor (e.g. a society or other partner) holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.