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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

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ABSTRACT

Aging impairs both circadian rhythms and memory, though the relationship between these impairments is not fully understood. Circadian rhythms are largely dictated by clock genes within the body's central pacemaker, the suprachiasmatic nucleus (SCN), though these genes are also expressed in local clocks throughout the body. As circadian rhythms can directly affect memory performance, one possibility is that memory deficits observed with age are downstream of global circadian rhythm disruptions stemming from the SCN. Here, we demonstrate that expression of clock gene *Period1* within a memory-relevant cortical structure, the retrosplenial cortex (RSC), is necessary for incidental learning, and that age-related disruption of *Period1* within the RSC—but not necessarily the SCN—contributes to cognitive decline. These data expand the known functions of clock genes beyond maintaining circadian rhythms and suggests that age-associated changes in clock gene expression modulates circadian rhythms and memory performance in a brain region–dependent manner.

1. Introduction

Circadian rhythms regulate many physiological and behavioral processes, including the formation of memory (Davies et al., 1973; Eckel-Mahan et al., 2008). Aging notably impairs both circadian rhythms (Renfrew et al., 1987; Weitzman et al., 1982; Witting et al., 1990) and memory (Craik and McDowd, 1987; Youngjohn and Crook, 1993), though the molecular mechanisms underlying these impairments are not well understood, nor is the relationship between them fully characterized (e.g. if age-related memory impairments are caused by age-related circadian deficits).

In mammals, circadian rhythms are generated chiefly by the body's central pacemaker, the suprachiasmatic nucleus (SCN), which is found at the base of the hypothalamus and synchronizes satellite oscillators throughout the body. Circadian oscillators, including the SCN, contain molecular clocks largely driven by a transcription-translation feedback loop (TTFL) with 4 critical components: genes Circadian Locomotor Output Cycles Kaput (*Clock*) and Brain and Muscle ARNT-like 1 (*Bmal1*) and gene families *Period* (*Per*) and Cryptochrome (*Cry*). In brief, CLOCK and BMAL heterodimerize and promote transcription of *Cry* and *Per* genes. After translation in the cytoplasm, CRY and PER proteins enter the nucleus and inhibit their own transcription, creating a negative feedback loop that lasts approximately 24 hours. The molecular clock within the SCN entrains other oscillators located throughout the rest of the brain and body, however it is worth noting that many of these peripheral oscillators cycle in the absence of SCN input (Yamazaki et al., 2000; Yoo et al., 2004), although many report that the hippocampus does not (Abe et al., 2002; Phan et al., 2011; but see also Chaudhury et al., 2005). Most of the existing research on clock genes focuses on their activity within the SCN, but these

Abbreviations: BMAL, brain and muscle ARNT-like; CLOCK, Circadian Locomotor Output Cycles Kaput; CREB, cAMP response element binding protein; Cry, Cryptochrome; DH, dorsal hippocampus; HSV, herpes simplex virus; IEG, immediate early gene; OLM, object location memory; *Per1*, *Period1*; RSC, retrosplenial cortex; SCN, suprachiasmatic nucleus; sgRNA, single guide RNA; TTFL, transcription-translation feedback loop; ZT, zeitgeber time.

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genes are also present across both the nervous system (Abe et al., 2002) and other tissues (Plautz et al., 1997). The function of clock genes outside of the TTFL is not yet fully characterized.

Recent reports have suggested that clock genes might operate in satellite brain regions (i.e. outside the SCN) to control local functions, including learning and memory (Snider et al., 2016; Woodruff et al., 2018; reviewed by Smies et al., 2022) and other behavior (McClung et al., 2005; Mukherjee et al., 2010; Spencer et al., 2013) in a brain region–dependent manner. Previous work has demonstrated that one such clock gene, *Period1* (*Per1*) modulates the phosphorylation of cAMP response element binding protein (CREB) in the dorsal hippocampus (DH; Rawashdeh et al., 2016), suggesting a possible role for *Per1* in hippocampal memory, given CREB's known role as a memory modulator (Yin et al., 1994, 1995). We recently demonstrated that bidirectional, local manipulations of *Per1* in the DH (Bellfy et al., 2022; Kwapis et al., 2018) and retrosplenial cortex (RSC; Urban et al., 2021) are sufficient to affect spatial memory. Although we reported that *Per1* expression in the DH is impaired by aging (Kwapis et al., 2018), no one has yet investigated how *Per1* expression in memory-relevant neocortical regions (e.g. the RSC) changes as a result of age and whether these changes are linked to memory performance.

The RSC (Brodmann areas 29 and 30) is a cortical brain structure integral to spatial memory and critically affected by age. This region—located immediately posterior to the corpus callosum in primates and directly dorsal to the DH in rodents—is densely connected with the DH and both the prefrontal and cingulate cortices (Shibata et al., 2004; Wang et al., 2016). Notably, expression of the immediate early gene (IEG) *Fos* is induced in the RSC following spatial learning (Vann et al., 2000), and lesions of the rodent RSC impair performance in both the Morris water maze (Harker and Whishaw, 2002) and radial arm maze (Vann and Aggleton, 2004). Further studies have suggested that RSC lesions specifically impair the ability to bind complex stimuli together, rather than the act of navigation itself (Nelson et al., 2015). Additionally, the RSC has been demonstrated to play an important role in both the retrieval (Corcoran et al., 2011) and formation (Kwapis et al., 2015; Urban et al., 2021) of contextual fear memory. Both the anterior RSC (aRSC) and the posterior RSC (pRSC) have been implicated in spatial memory, although these subregions may each support different types of information (see Discussion). Interestingly, both contextual fear memory (Moyer Jr. and Brown, 2006) and object location memory (Wimmer et al., 2012) are impaired as a result of aging, but RSC-independent modalities like delay fear conditioning (Trask et al., 2021a) and semantic memory (Wiggs et al., 1998) are resistant to the effects of aging (Craik and Grady, 2002; Moyer Jr. and Brown, 2006; reviewed by Trask and Fournier, 2022). Thus, the RSC is tightly linked to age-related memory deficits, and further investigation may elucidate the link between these memory deficits and age-related disruptions in circadian rhythms.

Here, we report that dysregulation of *Per1* within the RSC contributes to age-related memory deficits. We find that although learning induces *Per1* expression in the RSC of aging mice, this induction is smaller than in young mice. Additionally, this *Per1* induction fluctuates across the day-night cycle in both young and aging animals, although aging induces minor disruptions of this pattern. Furthermore, local downregulation of RSC *Per1* in young mice impairs the formation of spatial memory, while local upregulation of *Per1* in the RSC of aging mice is sufficient to rescue memory formation. Finally, although learning induces gene expression in the SCN of young but not aging mice, locally downregulating *Per1* in the SCN of young mice has no effect on spatial memory. Together, these data indicate that *Per1* functions within the RSC to modulate memory, whereas *Per1* in the SCN is dispensable for normal memory formation in young mice. Age-related repression of *Per1* in the RSC may therefore contribute to age-related spatial memory deficits. Although we did not directly test the role of *Per1* as a circadian regulator, these results further elucidate the relationships between clock genes, aging, and learning.

2. Methods

2.1. Subjects

A total of 233 male (after the removal of outliers) C57BL/6J mice from either Jackson Laboratories or the NIA aging rodent colony (Charles River) were used for all analyses. Mice were housed in a temperature- and humidity-controlled environment with a 12 hour light/dark cycle (lights on at 6:00 AM EST or 7:00 AM DST; off at 6:00 PM EST or 7:00 PM DST) and had *ad libitum* access to food and water. We used the moment the lights came on as Zeitgeber time (ZT) 0. Thus, ZT5 is 5 hours after the lights came on, and ZT12 is when the lights turned off each evening. Behavioral experiments were conducted between ZT5 and ZT7 unless otherwise specified, as we have previously demonstrated that young mice demonstrate both strong memory for OLM and robust hippocampal *Per1* inductions at this timepoint, while aging mice exhibit impairments in both processes at this time (Kwapis et al., 2018). Young mice were between 2 and 3 months of age at the start of experiments while aging mice were between 18 and 20 months of age at the start of experiments. In every experiment, mice were randomly assigned to groups (homecage/trained, *Per1* sgRNA/ctrl sgRNA) and all extraneous conditions (objects, boxes, etc.) were appropriately counterbalanced. As we have previously detected an effect of sex on our *Per1* manipulations within the RSC (Urban et al., 2021), we chose to only use male mice for this work. All experiments were approved by the Penn State Institutional Animal Care and Use Committee.

2.2. Surgery

Mice were anesthetized with 2% isoflurane (Patterson Veterinary, Greeley CO) dissolved in oxygen. Their heads were shaved and sterilized with betadine (Purdue Products) and 70% ethanol. Ophthalmic ointment (Dechra Veterinary Products, Portland, ME) was applied to the animals' eyes to prevent desiccation. An incision was made between the eyes with a sterile surgical blade (Aspen Surgical Products, Caledonia, MT) and the skull was dried with 70% ethanol; then, holes were bored with a surgical drill (Foredom Electric Co., Bethel, CT). For the RSC, bilateral infusers connected to injector syringes (Hamilton Co., Reno, NV) were lowered at a rate of 0.2 mm/15 sec to a final depth of 0.75 mm below the skull and allowed to rest for 5 minute. Then, 1.0 µL of viral cocktail (see below) was injected per hemisphere at 6 µL/hr. The injectors were allowed to rest for 5 minutes following injection, then were raised by 0.1 mm and allowed to rest for an additional 2 minutes. Finally, the injector was removed at a rate of 0.1mm/15 sec. For the SCN, viruses were infused with a 30-gauge Neuro Hamilton syringe directly mounted to a nanoinjector pump on the stereotaxic apparatus that was lowered at a rate of 0.3mm/15 sec to a final depth of 5.65 mm below the skull, where it was allowed to rest for 5 minutes. A bolus of 1.0 µL virus was injected at 6 µL/hr. Then, the needle rested for 5 minutes before being raised by 0.1mm and resting for an additional 2 minutes. Finally, the needle was removed at a rate of 0.2mm/15 sec and the other hemisphere was injected. Coordinates (from Bregma) were RSC (primarily targeting the anterior RSC): 1.80 mm caudal, 0.45 mm lateral, 0.75 mm ventral. SCN: 0.46 mm caudal, 0.22 mm lateral, 5.65 mm ventral.

2.3. Object location memory

OLM was conducted as previously described (Vogel-Ciernia and Wood, 2014). Briefly, mice were handled for 4 days and then habituated to polypropylene arenas (23.0 cm x 30.0 cm x 23.0 cm) for 6 days. Following the last day of habituation, mice underwent viral surgery and were allowed to rest for 72 hours to recover and promote peak HSV expression. After these 72 hours, mice were trained for 10 minutes with 2 identical objects. Twenty-four hours following training, mice underwent a test session in which one of the objects was moved. The objects were identical in appearance (100 mL beakers filled with hydraulic cement) and differed only in location. For the time course experiment investigating diurnal changes in *Per1* expression, OLM was conducted under dim red light, to prevent confounding effects of light-induced expression of *Per1*. A discrimination index was calculated using the formula $DI = (t_n - t_f) / (t_n + t_f) * 100\%$, where t_n is the time each mouse spent investigating the object in the novel location and t_f is the time each mouse spent investigating the object in the familiar location. Investigation was defined as any time the mouse spent with all 4 paws on the ground, its nose pointed directly at the object, its nose within 1 cm of the object, and not otherwise engaged in some other behavior (e.g. digging, climbing). Recorded videos were scored on a frame-by-frame basis by a blinded experimenter using the GUI of DeepEthogram (Bohnslav et al., 2021). Any animal demonstrating significant preference ($| DI | > 20$) during training was excluded from subsequent analyses (11 mice across all experiments). Additionally, any animal that spent a total of less than 3 seconds investigating or failed to explore both objects during test was also excluded from subsequent analyses (1 mouse across all experiments).

2.4. Tissue extraction

Animals were euthanized via cervical dislocation and decapitated with surgical scissors (Fine Science Tools, Foster City, CA). Brains were removed from the skull with rongeurs and a surgical spatula (Fine Science Tools, Foster City, CA) and then flash-frozen in 2-methylbutane (Fisher Scientific, Waltham, MA). Brains were stored at -80°C before being sectioned with a Leica CM150 Cryostat (Leica Biosystems, Wetzlar, Germany). Punches of 500 μm were collected from the RSC or SCN and stored at -80°C. Our punches were specifically targeting the aRSC, but we cannot rule out the inclusion of some pRSC tissue, given the lack of a definite anterior/posterior RSC boundary in mice.

2.5. qPCR

RNA was extracted from punches with RNeasy Mini Kits (Qiagen, Germantown, MD) and cDNA was generated with High-capacity cDNA Reverse Transcription Kits (ThermoFisher, Frederick, MD). PrimeTime primer/probe assays were generated with IDT PrimerQuest Design Tool (IDT, Coralville, IA) and used to quantify expression of *Per1, Fos*, and *Gapdh*. Exact sequences: *Per1* left primer: 5′-CCTGGAGGAATTGGAGCATATC-3′; *Per1* right primer: 5′- CCTGCCTGCTCCGAAATATAG3′; *Per1* probe: 5′-6-FAM/AAACCAGGA/Zen/CACCTTCTCTGTGGC/3IABkFQ-3′; *Fos* left primer: 5′-GGCACTAGAGACGGACAGAT-3′; *Fos* right primer: 5′-ACAGCCTTTCCTACTACCATTC-3′; *Fos* probe: 5′-6-FAM/CAGCCGACT/Zen/CCTTCTCCAGCATG/3IABkFQ-3′; *Gapdh* left primer: 5′- GGAGAAACCTGCCAAGTATGA-3′; *Gapdh* right primer: 5′-TCCTCAGTGTAGCCCAAGA-3′; *Gapdh* probe, 5′-HEX/TCAAGAAGG/ZEN/TGGTGAAGCAGGCAT/3IABkFQ/-3′. Analyses and statistics were performed in LightCycler 96 (Roche, Basel, Switzerland) using Roche proprietary algorithms.

2.6. Immunohistochemistry

Immunohistochemistry was performed as previously described (Kwapis et al., 2018). Briefly, 20 μm sections were mounted on slides, fixed with 4% PFA (ThermoFisher, Frederick, MD), permeabilized with 0.01% Triton-X (Fisher Scientific, Waltham, MA), and blocked for 1 hour with 8% normal goat serum (Jackson ImmunoResearch, West Grove, PA). Sections were then incubated overnight with rabbit anti-mCherry (1:500; ab167453; Abcam, Waltham, MA) and chicken anti-GFP (1:250; GFP-1010; Aves Labs, Davis, CA) primary antibodies, followed by a 1-hour incubation with goat anti-rabbit Alexa 555 (1:1000; a21430; ThermoFisher, Frederick, MD) and goat anti-chicken FITC (1:1000; ab6873; Abcam, Waltham, MA) secondary antibodies.

2.7. Viruses

All experiments used neuron-specific herpes simplex viruses (HSVs) to induce local gene expression only in neurons of the RSC or SCN. The CRISPRi virus (HSV-CRISPRi) expresses dCas9-KRAB-MeCp2 under an hSyn promoter followed by an IRES element and then mCherry. The CRISPRa virus (HSV-CRISPRa), similarly, expresses dCas9-VPR under an hSyn promoter followed by an IRES element and then mCherry. The sgRNA viruses (HSV-sgRNA) express the sgRNA under a U6 promoter and GFP under a CMV promoter. Three separate sgRNAs were used, one optimized for targeting *Per1* with the CRISPRi system (GAGTTCGACGGCTCCAGAGTA), one optimized for targeting *Per1* with the CRISPRa system (GCCCTTGTAAAGCAACCAT; Urban et al., 2021), and a nontargeting control sgRNA used for both systems (GCGAGGTATTCGGCTCCGCG; Lorsch et al., 2019). For each experiment, mice received a 1:1 viral cocktail of one of the CRISPR systems and an appropriate sgRNA (control mice received nontargeting sgRNAs). All viruses were purchased from Dr. Rachael Neve at the Gene Delivery Technology Core of Massachusetts General Hospital.

2.8. Statistics

Data are represented as Mean ± SEM in all figures. For each experiment, datapoints were dropped if they were more than 2 standard deviations away from the mean. Data were analyzed by either unpaired 2-tailed Student's t-tests (Fig. 3C, 3D, 4B, 4C, 6C, 6D) or 2-way ANOVAs followed by Sidak's multiple comparison *post hoc* tests (elsewhere). Data normality was verified by examining residuals, and no significant departures from normality were observed. Sample sizes were based on previous studies (Kwapis et al., 2018; Urban et al., 2021), but no statistical method was used to predetermine sample size. Graphpad Prism 9 (GraphPad Software, San Diego, CA) was used for all analyses.

3. Results

3.1. Learning induces Per1 and Fos expression in the RSC of young and aging mice in a circadian-dependent fashion

First, we sought to compare learning-induced expression of *Per1* between the young and aging RSC. Here, young (8-week-old) and aging (18-month-old) mice were either trained in OLM or left in their homecage at ZT5 and then sacrificed an hour later to examine *Per1* expression via qPCR. Homecage control mice were handled and habituated to the context as part of the OLM protocol but remained in their homecage on the day of training and were sacrificed in a counterbalanced manner with trained animals (Fig. 1A). We chose these ages as we have previously demonstrated that spatial learning upregulates *Per1* in the DH of 8-week-old but not

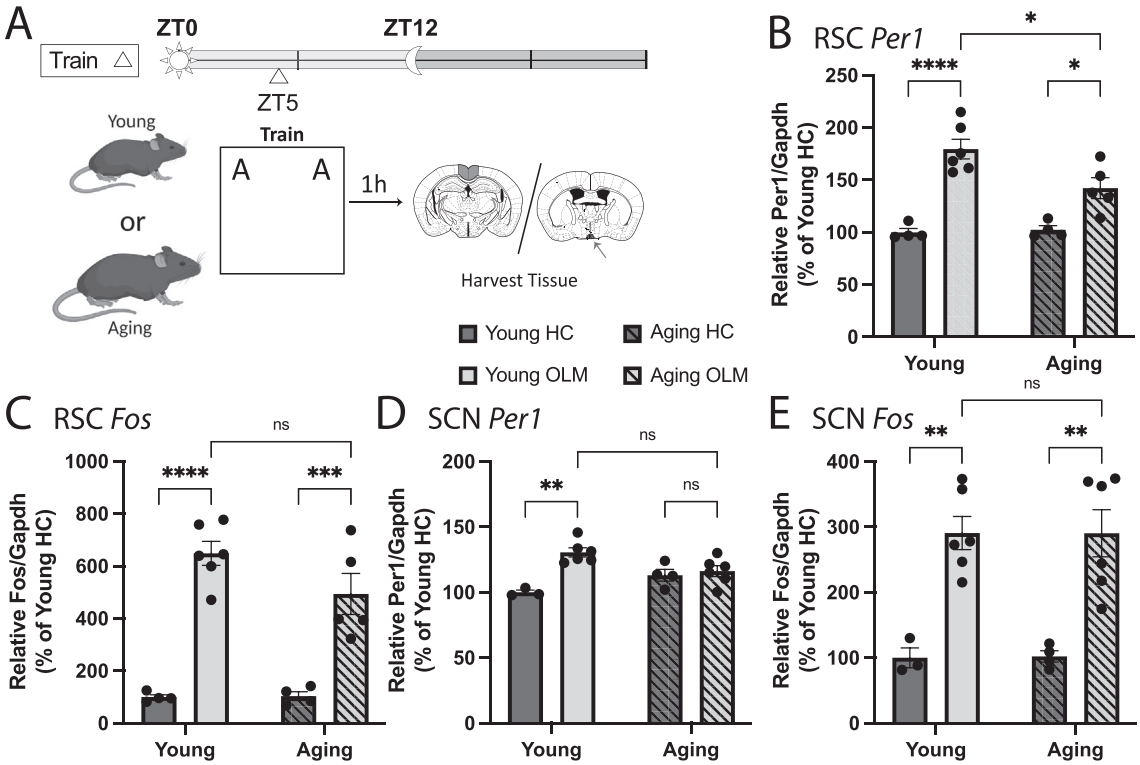

Fig. 1. Training in OLM at ZT5 induces changes in gene expression in the RSC and SCN. (A) Schematic. Young and aging mice were trained in OLM at ZT5 and sacrificed (along with homecage controls) 1 hour later. Expression of *Per1* and *Fos* in the RSC and SCN was analyzed via qPCR. (B) *Per1* expression was induced in the RSC following learning, although this induction was attenuated in aging mice (n = 4–6). (C) *Fos* expression was induced in the RSC following learning in both young and aging mice (n = 4–6). (D) *Per1* expression was induced in the SCN following learning only in young mice (n = 3–6). (E) Fos expression was induced in the SCN following learning in both young and aging mice (n = 3–6). Outliers were identified in the young trained RSC *Per1* data and in the aging trained RSC *Fos* data (1 each). * denotes $p \leq 0.05$, ** denotes $p \leq 0.01$, *** denotes $p \leq 0.001$, **** denotes $p \leq 0.0001$.

18-month-old mice around ZT5 (Kwapis et al., 2018). Furthermore, mice exhibit clear deficits in OLM at 18-months (Wimmer et al., 2012); thus, this is an ideal age to model age-related memory impairments. Based on our prior results in the DH, we anticipated that learning would fail to increase expression of *Per1* in the RSC of aging mice. We found that while learning did induce *Per1* in the aging RSC, the magnitude of this induction was smaller than in young animals (Fig. 1B; 2-way ANOVA: effect of training: $F_{(1,15)} = 49.90$, $p < 0.0001$; effect of age: $F_{(1,15)} = 4.272$, $p = 0.0565$; interaction: $F_{(1,15)} = 5.553$, $p = 0.0325$). Additionally, learning induces expression of the immediate early gene *Fos* in both the young and aging RSC (Fig. 1C; 2-way ANOVA: effect of training: $F_{(1,15)} = 80.39$, $p < 0.0001$; effect of age: $F_{(1,15)} = 2.094$, $p = 0.1684$; interaction: $F_{(1,15)} = 2.310$, $p = 0.1493$). We investigated the expression of *Per1* and *Fos* in the SCN of these mice and found that learning induces *Per1* expression in the SCN of young but not aging mice (Fig. 1D; 2-way ANOVA: effect of training: $F_{(1,15)} = 16.12$, $p = 0.0011$; effect of age: $F_{(1,15)} = 0.02490$, $p = 0.8767$; interaction: $F_{(1,15)} = 10.71$, $p = 0.0051$) while *Fos* expression is induced within the SCN regardless of age (Fig. 1E; 2-way ANOVA: effect of training: $F_{(1,15)} = 38.65$, $p < 0.0001$; effect of age: $F_{(1,15)} = 0.0009$, $p = 0.9762$; interaction: $F_{(1,15)} = 0.00164$, $p = 0.9682$). Given *Per1*'s known role as a clock gene and the documented relationship between circadian rhythms and memory, we next investigated how this learning-induced *Per1* expression was modulated by time-of-day in both young and aging animals.

To investigate the interplay between learning, time-of-day, and *Per1* expression in the aging RSC,18-month-old C57BL/6J mice were either trained in OLM or left in their homecage at 6 different timepoints (Zeitgeber Times: ZT1, ZT5, ZT9, ZT13, ZT17, ZT21), sacrificed an hour later, and then retrosplenial *Per1* and *Fos* expression was quantified with qPCR (Fig. 2A). This experiment was set up identically to a previous experiment we ran in young mice that observed robust learning-induced increases in *Per1* within the RSC during the day but not the night (Urban et al., 2021). Here, we also report that OLM training induces *Per1* expression in the aging RSC in a circadian-dependent manner (Fig. 2B; 2-way ANOVA: effect of training: $F_{(1,68)} = 336.9$, $p < 0.0001$; effect of time-of-day: $F_{(5,68)} = 17.08$, $p < 0.0001$; interaction: $F_{(5.68)} = 3.156$, $p = 0.0128$). Additionally, when we compared the *Per1* induction in the aging RSC with what we have previously published in the young RSC (Urban et al., 2021), we find minimal differences between the relative magnitude of this *Per1* induction in young and aging animals, with a significant difference only detected at ZT17 (Fig. 2C; 2-way ANOVA: effect of time-of-day: $F_{(5,66)} = 9.946$, $p < 0.0001$; effect of age: $F_{(1,66)} = 3.651$, $p = 0.0604$; interaction: $F_{(5.66)} = 2.691$, $p = 0.0283$). Notably, both young and aging animals express more learning-induced *Per1* during the day relative to night (Supplementary Fig. 1A; 2-way ANOVA: effect of day/night: $F_{(1,74)} = 5.138$, $p = 0.0263$; effect of age: $F_{(1,74)} = 31.01$, $p < 0.0001$; interaction: $F_{(1,74)} = 0.05706$, $p = 0.8119$) identical to the pattern observed in memory performance (Bellfy et al., 2022; Chaudhury and Colwell, 2002). We also investigated *Fos* expression in the same mice and found that learning induces *Fos* expression in both young (Fig. 2D; 2-way ANOVA: effect of training: $F_{(1,64)} = 336.7$, $p < 0.0001$; effect of time-of-day: $F_{(5,64)} = 18.98$, $p < 0.0001$; interaction: $F_{(1,64)} = 14.28$, $p < 0.0001$) and aging animals (Fig. 2E; 2-way ANOVA: effect of training: $F_{(1,69)} = 244.6$, $p < 0.0001$; effect of time-of-day: $F_{(5,69)} = 2.065$, $p = 0.0803$; interaction: $F_{(1,69)} = 1.729$, $p = 0.1395$), also in a circadian-dependent fashion (Fig. 2F; 2-way

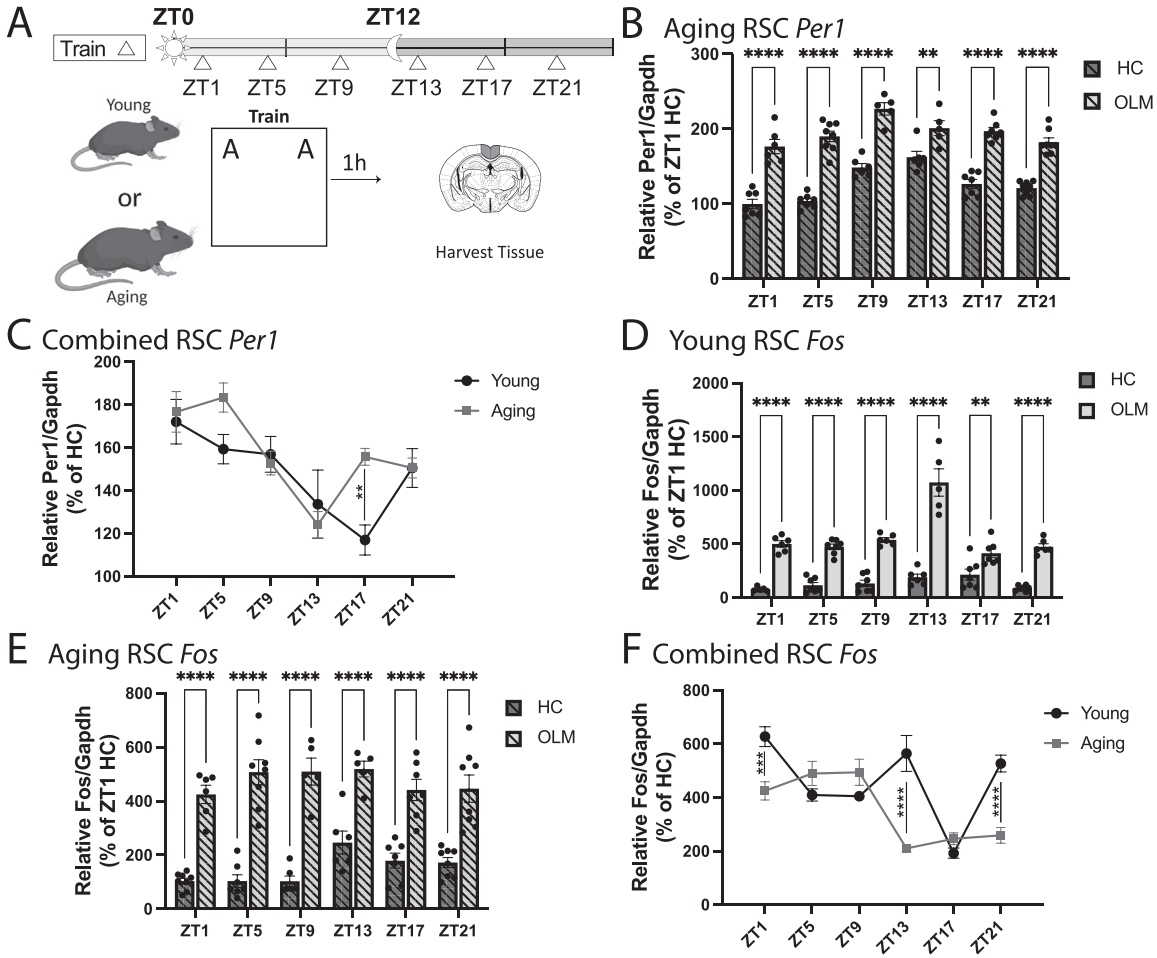

Fig. 2. Learning-induced expression of *Per1* and *Fos* within the RSC is modulated by both time-of-day and aging. (A) Schematic. Young and aging mice were trained in OLM at 6 different timepoints across the day-night cycle and then sacrified an hour later to examine gene expression in the RSC. (B) *Per1* expression in the aging RSC normalized to the mean of the ZT1 HC group (n = 5–7). (C) Relative amount of *Per1* induction for young and aging mice. Here, each timepoint's OLM values are normalized to the mean of the corresponding HC group (e.g. young ZT5 OLM mice are each normalized to the mean of the young ZT5 HC group; n = 5–7). Note that the young data presented here was previously published in the Neurobiology of Learning and Memory in 2021 as part of a different analysis (Urban et al., 2021). (D) *Fos* expression in the young RSC normalized to the mean of the ZT1 HC group (n = 5–7). (E) *Fos* expression in the aging RSC normalized to the mean of the ZT1 HC group (n = 5–8). (F) Relative amount of *Fos* induction for young and aging mice. Here, each timepoint's OLM values are normalized to the mean of the corresponding HC group (n = 5–8). Outliers were identified in the Aging RSC *Fos* ZT13 HC group, ZT13 OLM group, and ZT9 OLM group (1 each). * denotes $p \leq 0.05$, ** denotes $p \leq 0.01$, *** denotes $p \leq 0.001$, **** denotes $p \leq 0.0001$.

ANOVA: effect of time-of-day: $F_{(5,64)} = 19.37$, $p < 0.0001$; effect of age: $F_{(1,64)} = 25.23$, $p < 0.0001$; interaction: $F_{(5.64)} = 16.02$, $p < 0.0001$), though *Fos* expression was only greater during the day than the night in aging mice (Supplementary Fig. 1B; 2-way ANOVA: effect of day/night: $F_{(1,72)} = 8.156$, $p = 0.0056$; effect of age: $F_{(1,72)} = 24.63$, $p < 0.0001$; interaction: $F_{(1,72)} = 6.98$, $p = 0.0101$). These results suggest that while circadian patterns of learning-induced changes in gene expression are largely maintained in the aging RSC, the absolute amount of learning-induced *Per1* is dampened with age.

3.2. Local downregulation of Per1 expression in the RSC of young mice impairs the formation of spatial memory

Next, we investigated if direct modulation of *Per1* expression within the RSC can affect memory performance on the OLM task. Given the differences we observed in *Per1* expression between young and aging animals (Fig. 1B), we thought that age-related dysregulation (i.e. reduction) of *Per1* expression in the RSC might contribute to known age-related impairments in OLM (Wimmer et al., 2012). Additionally, previous work from our group has demonstrated that local reduction of *Per1* expression in the RSC can attenuate the formation of a contextual fear memory in young mice (Urban et al., 2021). Thus, we reasoned that locally downregulating *Per1* expression in the RSC of young mice during memory formation could impair subsequent test performance in the OLM task.

To locally downregulate *Per1* within the RSC, we used HSV-CRISPRi as previously described (Urban et al., 2021). In brief, the CRISPRi system contains a deactivated Cas9 protein (dCas9) fused to 2 transcriptional repressors (Krüppel associated box [KRAB] and methyl CpG binding protein 2 [MeCP2]). This construct is directed by a single guide RNA (sgRNA) to a gene of interest—here, *Per1*. When the dCas9 binds as specified by the sgRNA, the attached transcriptional repressors downregulate the targeted gene (Larson et al., 2013). Given the known role of the circadian rhythm in memory performance and the potential off-target effects of systemic *Per1* manipulation, we used herpes simplex viruses (HSVs) to manipulate *Per1* expression only in RSC neurons. Additionally, since HSV expression peaks only 3 days following injection and then rapidly declines (Neve et al., 2005), this method allows for

high temporal control. Given the role of gene expression in memory consolidation, we timed our viral manipulations so that peak viral expression (and, therefore, maximal inhibition of *Per1*) would occur at the time of OLM training. This HSV-CRISPRi system has been previously validated to reduce *Per1* expression in both cultured cells and the mouse RSC (Urban et al., 2021).

To investigate the effect of RSC-specific *Per1* downregulation on the formation of spatial memory, young mice were first handled and habituated at ZT5 (when memory for OLM is typically best; (Bellfy et al., 2022). Then, they were injected with the HSV-CRISPRi system with a *Per1*-targeting sgRNA, while control animals received the same CRISPRi construct but with a nontargeting sgRNA, controlling for any effect of viral surgery or CRISPRi expression. Following surgery, mice were permitted to rest for 72 hour (allowing HSV expression to peak; Neve et al., 2005) before being trained in OLM at ZT5. Twenty-four hours after training, mice were tested in OLM (Fig. 3B). We found that local downregulation of *Per1* expression in the RSC of young mice impairs spatial memory, with *Per1* knockdown mice showing significantly lower DIs at test relative to control animals (Fig. 3C; 2-tailed t-test: $t_{10} = 2.451$, $p = 0.0342$) without affecting total object exploration (Fig. 3D; 2-tailed t-test: $t_{10} = 0.1044$, $p = 0.9189$) or total distance moved (Supplementary Fig. 2A; 2-way repeated measures ANOVA: effect of day: $F_{(4.214,42.14)} = 57.17$, $p < 0.0001$; effect of virus: $F_{(1,10)} = 3.741$, $p = 0.0819$; interaction: $F_{(7,70)} = 1.605$, $p = 0.1482$; effect of subject: $F_{(10,70)} = 7.628$, $p < 0.0001$). These results demonstrate for the first time that *Per1* expression within the RSC is necessary for incidental spatial memory, confirming what we had previously shown in contextual fear conditioning (Urban et al., 2021). Additionally, these results, together with our other published work (Bellfy et al., 2022; Kwapis et al., 2018), demonstrate that *Per1* modulates a single learning task (OLM) in multiple brain regions—the RSC and the DH.

3.3. Local upregulation of Per1 expression in the RSC of aging mice restores the formation of spatial memory

In a complementary experiment, we examined whether local upregulation of *Per1* in the RSC of aging mice is sufficient to rescue memory performance in OLM. Here, we used HSV-CRISPRa, as previously described (Urban et al., 2021), to locally increase *Per1* expression within the RSC of aging mice. The CRISPRa system contains a dCas9 fused to 3 transcriptional activation domains (VP64, p65, and Rta; collectively known as VPR) that drive transcription of a gene target (here, *Per1*) specified by an sgRNA (Chavez et al., 2015). As with our *Per1* CRISPRi system, the CRISPRa construct is downstream of an hSyn promoter and validated to upregulate *Per1* expression in both cultured hippocampal neurons and the mouse RSC (Urban et al., 2021).

To elucidate how RSC-specific *Per1* upregulation affects spatial memory, we performed OLM at ZT5 with aging mice, a timepoint at which we have previously observed age-related memory impairments in 18-month-old mice (Kwapis et al., 2019, 2018). The animals were handled and habituated before receiving retrosplenial injections of the HSV-CRISPRa system with a *Per1*-targeting sgRNA or nontargeting sgRNA (control animals). Three days after injection, the mice were trained in OLM and then tested the following day, both at ZT5 (Fig. 4A). We found that local *Per1* upregulation within the RSC is sufficient to improve OLM in aging mice (Fig. 4B; 2-tailed t-test: $t_{21} = 2.562$, $p = 0.0182$). As with the *Per1* knockdown, this manipulation does not affect the total exploration of the 2 OLM objects during test (Fig. 4C; 2-tailed t-test: $t_{21} = 1.992$, $p = 0.0595$) or total distance traveled (Supplementary Fig. 2B; 2-way repeated measures ANOVA: effect of day: $F_{(3.353,73.77)} = 91.97$, $p < 0.0001$; effect of virus: $F_{(1,22)} = 0.02730$, $p = 0.8703$; interaction: $F_{(7,154)} = 0.389$, $p = 0.9077$; effect of subject: $F_{(22,154)} = 7.155$, $p < 0.0001$). Together with the previous experiment, these results reveal that bidirectional manipulation of *Per1* expression in the RSC is sufficient to modulate memory performance.

3.4. Spatial learning induces gene expression in the SCN of young and aging mice

Although our results thus far have clearly demonstrated that *Per1* in the RSC is critical for proper spatial memory formation, an open question remains as to the SCN's role in memory, with various reports describing conflicting effects of SCN lesion on memory performance (Cain et al., 2012; Fernandez et al., 2014; Mulder et al., 2014; Phan et al., 2011; Shimizu et al., 2016; Stephan and Kovacevic, 1978; briefly reviewed by Ruby, 2021). Thus far, no one has specifically investigated how local *Per1* expression in the brain's central pacemaker might control memory performance. We decided to examine how OLM training affects *Per1* expression in the SCN and test whether *Per1*'s control over spatial memory is exclusive to memory-relevant brain regions.

First, we examined learning-induced changes in *Per1* and *Fos* expression in the SCN (Fig. 5A) using tissue from the young and aging mice presented in Fig. 2. Interestingly, we found here that training in OLM modestly increases *Per1* expression within the SCN in both young (Fig. 5B; 2-way ANOVA: effect of training: $F_{(1,66)} = 27.63$, $p < 0.0001$; effect of time-of-day: $F_{(5,66)} = 2.754$, $p = 0.0255$; interaction: $F_{(5.66)} = 0.8846$, $p = 0.4965$) and aging mice (Fig. 5C; 2-way ANOVA: effect of time-of-day: $F_{(5,81)} = 3.772$, $p = 0.0040$; effect of training: $F_{(1,81)} = 18.42$, $p < 0.0001$; interaction: $F_{(5.81)} = 0.2808$, $p = 0.9224$), though this *Per1* induction is consistent across the day-night cycle and not modulated by age (Fig. 5D; 2-way ANOVA: effect of time-of-day: $F_{(5,74)} = 0.5483$, $p = 0.7391$; effect of age: $F_{(1,74)} = 2.866$, $p = 0.0947$; interaction: $F_{(5.74)} = 1.932$, $p = 0.0991$). Although we observed a main effect of training on *Per1* expression in aging animals, none of the post hoc tests revealed any pairwise differences between trained and homecage groups at each timepoint, consistent with what we reported in Fig. 1D. Additionally, there was no gross day/night differences of magnitude of *Per1* induction in either young or aging animals (Supplementary Fig. 1C; 2-way ANOVA: effect of day/night: $F_{(1,82)} = 3.216$, $p = 0.0766$; effect of age: $F_{(1,82)} = 0.1375$, $p = 0.7118$; interaction: $F_{(1,82)} = 1.119$, $p = 0.2932$) Training in OLM also induces *Fos* expression in the SCN in young (Fig. 5E; 2-way ANOVA: effect of training: $F_{(1,58)} = 154.6$, $p < 0.0001$; effect of time-of-day: $F_{(5,58)} = 0.4096$, $p = 0.8402$; interaction: $F_{(5.58)} = 0.6998$, $p = 0.6258$) and aging mice (Fig. 5F; 2-way ANOVA: effect of training: $F_{(1,79)} = 182.0$, $p < 0.0001$; effect of time-of-day: $F_{(5,79)} = 2.951$, $p = 0.0171$; interaction: $F_{(5.79)} = 0.3958$, $p = 0.0030$). Unlike with *Per1*, OLM-induced *Fos* expression in the SCN was modulated by time-of-day and varied with age (Fig. 5G; 2-way ANOVA: effect of time-of-day: $F_{(5,67)} = 6.835$, $p < 0.0001$; effect of age: $F_{(1,67)} = 0.01917$, $p = 0.8903$; interaction: $F_{(5.67)} = 3.758$, $p = 0.0047$). Additionally, there was only a significant difference of induced *Fos* expression between day and night in aging animals (Supplementary Fig. 1D; 2-way ANOVA: effect of day/night: $F_{(1,75)} < 0.001$, $p = 0.9979$; effect of age: $F_{(1,75)} = 23.77$, $p < 0.0001$; interaction: $F_{(1,75)} = 2.300$, $p = 0.1336$; post hoc Sidak's test: $p < 0.001$), although for young mice this difference approached significance (post hoc Sidak's test: $p = 0.0573$).

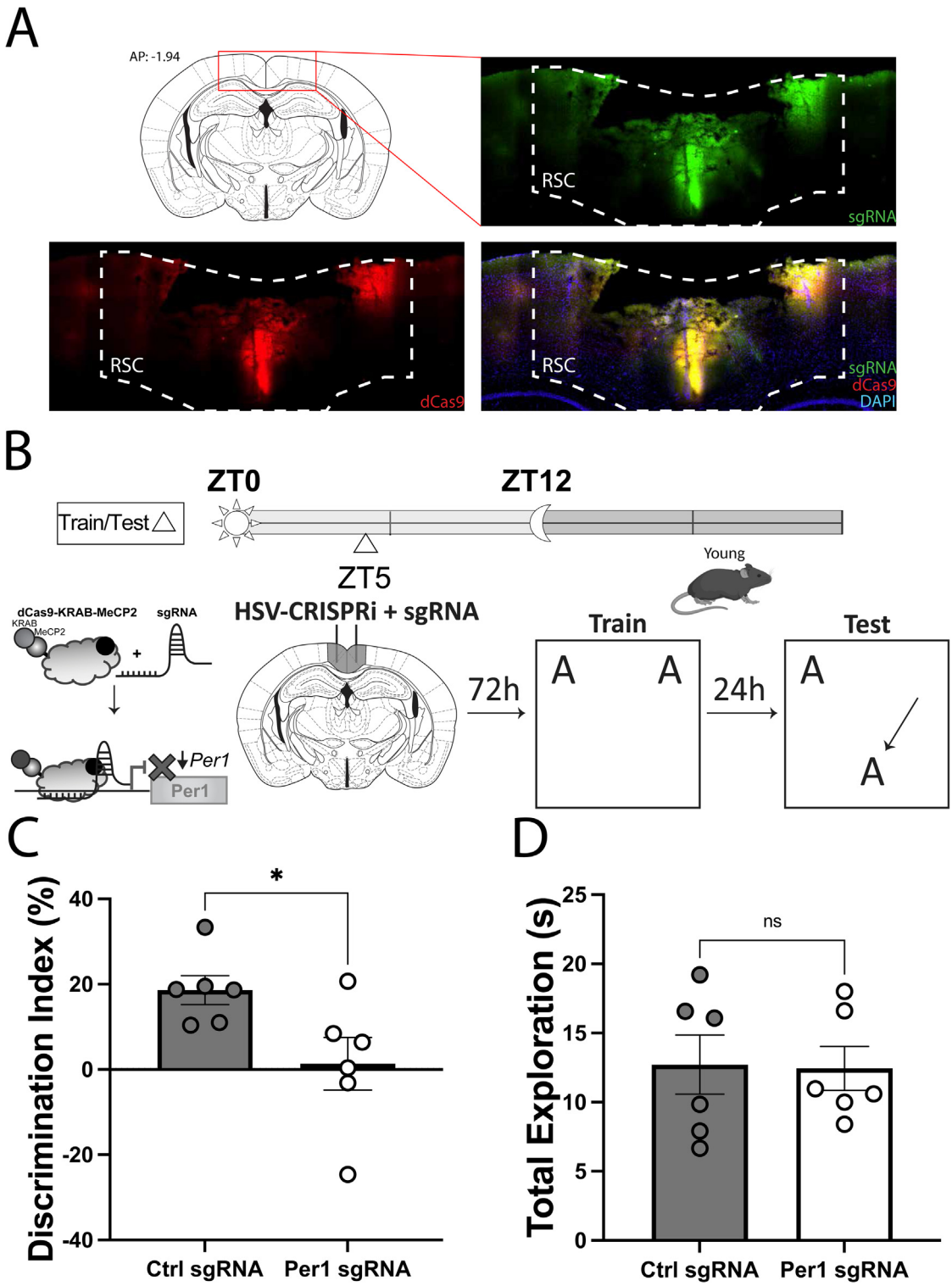

Fig. 3. Local downregulation of *Per1* within the RSC is sufficient to impair memory performance in young mice at ZT5. (A) Representative images of HSV spread in the RSC. (B) Schematic. Our two-virus HSV-CRIPRi system was infused into the RSC 72 hour prior to training in the OLM task to downregulate *Per1* expression during training. Control animals received the full CRISPRi construct but with a nontargeting sgRNA. (C) Control animals show robust memory for the original object location while animals who received the *Per1* sgRNA do not (n = 6/group). (D) RSC-specific *Per1* knockdown has no effect on total object exploration (n = 6/group). Two *Per1* downregulation mice were dropped due to demonstrating an object preference during training, and an outlier was identified in the control group and dropped. * denotes $p \leq 0.05$, ** denotes $p \leq 0.01$, *** denotes $p \leq 0.001$, **** denotes $p \leq 0.0001$.

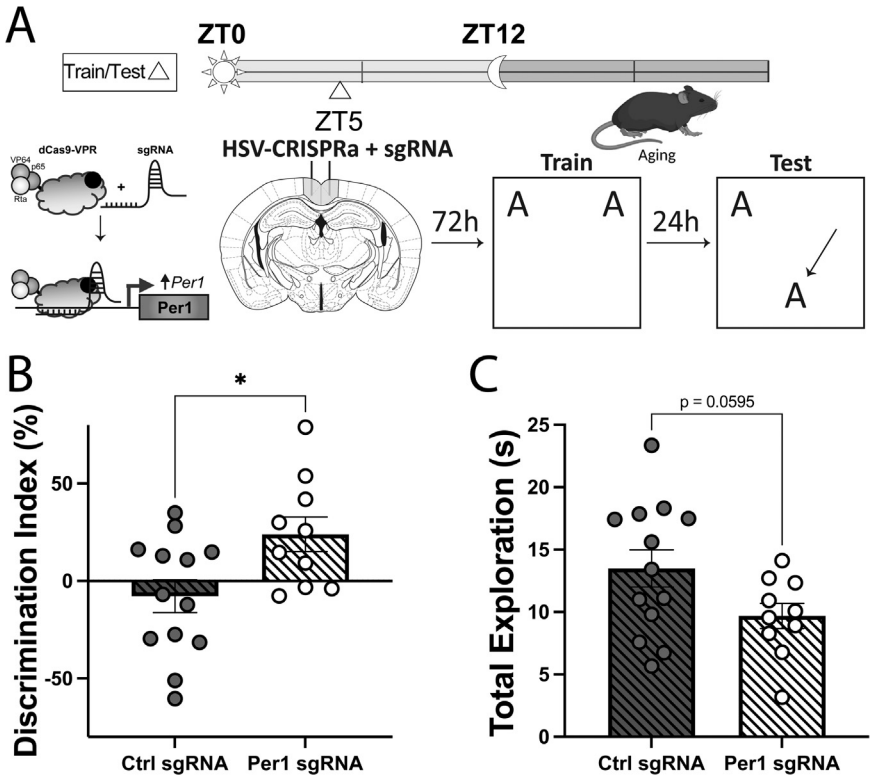

Fig. 4. Local upregulation of *Per1* within the RSC improves memory performance in aging mice at ZT5. (A) Schematic. Our two-virus HSV-CRIPRa system was infused into the RSC 72 hour prior to training in the OLM task to upregulate *Per1* expression during training. Control animals received the full CRISPRa construct but with a nontargeting sgRNA. (B) 18-month-old control animals show minimal memory for the original object location while animals who received the *Per1* sgRNA demonstrate robust memory (n = 13,10). (C) RSC-specific *Per1* knockup has no significant effect on total object exploration (n = 13,10). One control mouse was dropped due to insufficient exploration at test, while 6 mice (5 *Per1*, 1 control) were dropped due to preference during training. Due to this high attrition, this experiment was repeated and data were pooled, as no differences were detected between replications. Additionally, an outlier was identfied in the *Per1* group and dropped. * denotes $p \leq 0.05$, ** denotes $p \leq 0.01$, *** denotes $p \leq 0.001$, **** denotes $p \leq 0.0001$.

3.5. Local downregulation of Per1 expression in the SCN of young mice has no effect on the formation of spatial memory

Although we found that learning induces *Per1* expression in both the RSC and the SCN, we observed that the RSC *Per1* induction was more tightly linked to memory performance than that of the SCN—that is, learning-induced RSC *Per1* and memory performance (Bellfy et al., 2022) both peak during the day. Thus, we reasoned that *Per1* activity in the RSC is more likely to support memory formation and sought to rule out the effect of SCN *Per1* on memory performance. To do so, we used the same HSV-CRISPRi construct described previously to locally downregulate *Per1* within the SCN in young mice. As in the RSC CRISPRi experiment, mice were handled and habituated to the OLM chambers, administered HSV-CRISPRi, allowed to rest for 72 hours, and then trained and tested in OLM (Fig. 6B). Control animals received HSV-CRISPRi but with a nontargeting RNA, controlling for any effects of viral surgery or CRISPRi expression. We found that downregulation of *Per1* within the SCN has no effect on the formation of spatial memory (Fig 6C; 2-tailed t-test: $t_8 = 0.3976$, $p = 0.7013$), on total object exploration (Fig 6D; 2-tailed t-test: $t_8 = 0.1425$, $p = 0.8902$), or distance travelled (Supplementary Fig. 2C; 2-way repeated measures ANOVA: effect of day: $F_{(2.387,19.09)} = 31.00$, $p < 0.0001$; effect of virus: $F_{(1,8)} = 1.554$, $p = 0.2478$; interaction: $F_{(7,56)} = 1.961$, $p = 0.0769$; effect of subject: $F_{(8,56)} = 6.627$, $p < 0.0001$). These data support our hypothesis that the previously observed memory effects can be specifically attributed to *Per1* activity within the RSC.

4. Discussion

Here, we investigated the relationship between brain region–specific *Per1* expression, spatial memory, and aging. Our results reveal that *Per1* expression in the RSC is affected by aging and necessary for the formation of long-term spatial memory in OLM. We find that targeted downregulation of *Per1* within the RSC of young animals impairs learning, while upregulation of *Per1* within the RSC of aging mice improves learning. Finally, although learning induces *Per1* expression in the SCN of young but not aging mice, locally downregulating *Per1* expression in the SCN of young mice does not affect the formation of spatial memory. Together, these findings expand the known role of *Per1* as a memory-relevant gene in neocortical and subcortical regions and further demonstrate the diverse roles clock genes play outside of the SCN. Additionally, these data support the idea that age-related dysregulation of clock genes (namely, *Per1*) contributes to multiple symptoms of aging (circadian disruptions and memory deficits) in a brain region–dependent fashion.

Per1 has long been known to play an important role in learning and memory, but many previous studies (Abarca et al., 2002; Jilg et al., 2010; Rawashdeh et al., 2014) have relied on global *Per1* knockout mice, making it difficult to discern if the observed changes in learning are a result of decreased *Per1* activity within the SCN or within memory-relevant structures like the DH and RSC. Previous work from our group (Bellfy et al., 2022; Kwapis et al., 2018; Urban et al., 2021) has demonstrated that tar-

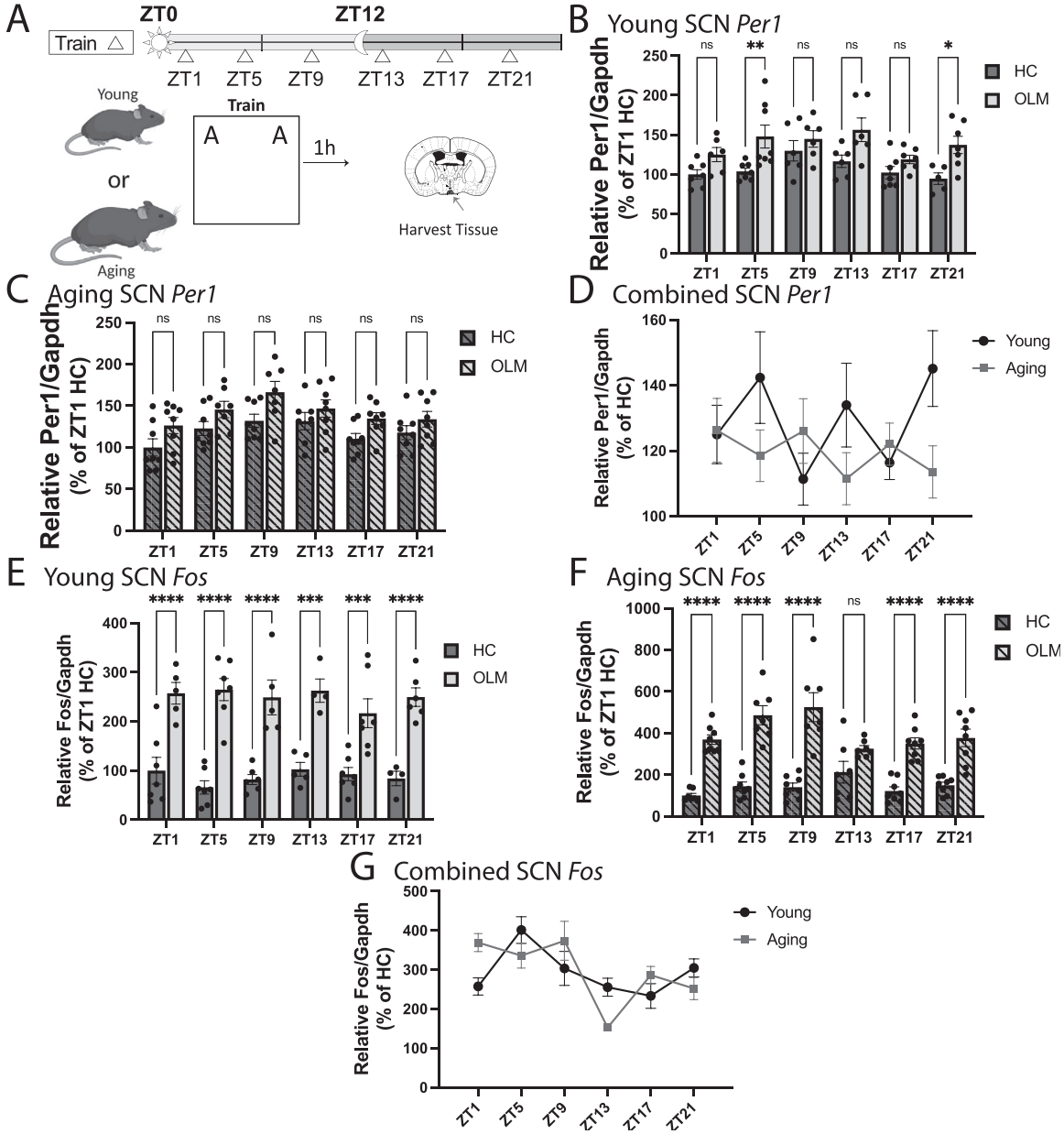

Fig. 5. Learning-induced expression of *Per1* and *Fos* within the SCN is modulated by both time-of-day and aging. (A) Schematic. Young and aging mice were trained in OLM at 6 different timepoints across the day-night cycle and then sacrified an hour later to examine gene expression in the SCN. (B) *Per1* expression in the young SCN normalized to the mean of the ZT1 HC group (n = 5–8). (C) *Per1* expression in the aging SCN normalized to the mean of the ZT1 HC group (n = 7–8). (D) Relative amount of *Per1* induction for young and aging mice. Here, each timepoint's OLM values are normalized to the mean of the corresponding HC group (e.g. young ZT5 OLM mice are normalized to the mean of the young ZT5 HC group; n = 5–8). (E) *Fos* expression in the young SCN normalized to the mean of the young ZT1 HC group; n = 5–7). (F) *Fos* expression in the aging SCN normalized to the mean of the aging ZT1 HC group; n = 7–8). (G) Relative amount of *Fos* induction for young and aging mice. Here, each timepoint's OLM values are normalized to the mean of the corresponding HC group (n = 5–7). An outlier was identified in the Aging SCN *Fos* ZT17 OLM group and removed. * denotes $p \leq 0.05$, ** denotes $p \leq 0.01$, *** denotes $p \leq 0.001$, **** denotes $p \leq 0.0001$.

geted manipulations of *Per1* expression in just the DH and RSC are sufficient to modulate memory formation, and this work builds on those studies by investigating how RSC *Per1* is changed by aging. We have previously identified that local upregulation of Per1 within the DH is sufficient to rescue age-related deficits in OLM (Kwapis et al., 2018), so it is not surprising that similar trends are observed in the RSC. Interestingly, we previously reported that DH *Per1* is not induced by learning in aging animals (Kwapis et al., 2018), but here we found that *Per1* is induced by learning within the RSC of aging mice (Fig. 1). These results suggest that some critical threshold of *Per1* expression might be necessary to consolidate memory, and that while learning does induce some *Per1* expression within the aging RSC, this induction is insufficient to reach levels necessary for proper memory formation.

It is also possible that circadian fluctuations in retrosplenial *Per1* may contribute to circadian fluctuations in memory performance. Notably, both learning-induced RSC *Per1* expression (Urban et al., 2021) and long-term memory performance (Bellfy et al., 2022; Chaudhury and Colwell, 2002; Eckel-Mahan et al., 2008) peak during the day in young animals, supporting the hypothesis that *Per1* drives memory formation in a circadian fashion. However, we have also previously revealed that

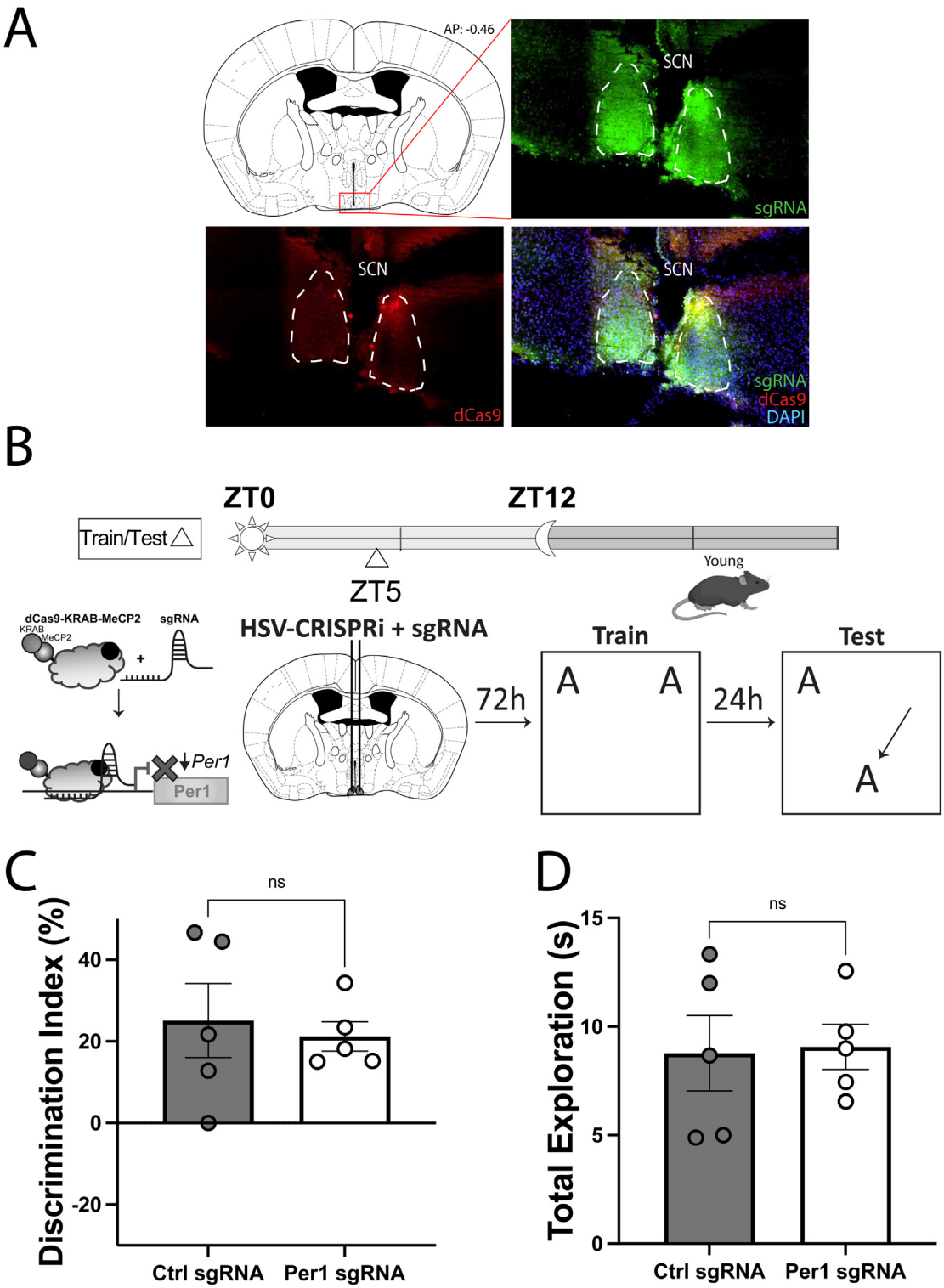

Fig. 6. Local downregulation of *Per1* within the SCN has no effect on memory in young mice trained at ZT5. (A) Representative images of HSV spread in the SCN. (B) Schematic. Our two-virus HSV-CRIPRi system was infused into the SCN 72 hour prior to training in the OLM task to downregulate *Per1* expression during training. Control animals received the full CRISPRi construct but with a nontargeting sgRNA. (C) Control animals show robust memory for the original object location, as do animals who received the *Per1* sgRNA (n = 5, 5). (D) SCN-specific *Per1* knockdown has no effect on total object exploration. (n = 5, 5). Two mice from each group were droped due to showing a preference during training. Additionally, an outlier was identified in the *Per1* group and dropped. * denotes $p \leq 0.05$, ** denotes $p \leq 0.01$, *** denotes $p \leq 0.001$, **** denotes $p \leq 0.0001$.

for context fear conditioning, locally increasing *Per1* expression within the RSC during the subjective night does not restore memory to day-time levels, and overexpression of *Per1* in the RSC during the day can impair memory performance (Urban et al., 2021). These data suggest that different mechanisms may contribute to memory formation during the day and night, and that increased *Per1* does not always improve memory. One explanation for this discrepancy is that age-related changes in memory performance and day-night changes in memory performance rely on different molecular mechanisms, such that age-related impairments can be rescued by *Per1* upregulation while night-related impairments cannot. Alternatively, *Per1* may be modulating contextual fear memory and OLM via different mechanisms. These differently motivated paradigms rely on different brain structures (e.g. the amygdala is engaged by fear conditioning but not OLM) and its possible that the mnemonic effects of *Per1* are circuit-specific. Future studies should test whether overexpression of *Per1* during the day can also negatively affect OLM performance, and whether age-related impairments in fear conditioning are restored by RSC *Per1* overexpression.

The RSC is believed to be functionally graded along its anterior-posterior axis. Prevailing theories of RSC function characterize the anterior portion (the aRSC) as more important for encoding "what" information while the posterior portion (pRSC) is more responsible for tracking "where" information (Neave et al., 1994; Trask et al., 2021b; Vann et al., 2003; Vann and Aggleton, 2004). Since OLM is a spatial memory paradigm (the only difference between the objects is their position in the arena), one might expect the pRSC to be more heavily involved than the aRSC. Nonetheless, previous work (de Landeta et al., 2020) has revealed a clear role for the aRSC in OLM and other spatial tasks (Kwapis et al., 2015). Additionally, we have previously identified *Per1* in the aRSC as important for contextual fear conditioning, so we elected to investigate *Per1*'s role in the aRSC during OLM. Our viral manipulations and punches were specifically targeting the aRSC (see Methods), but the boundary between anterior and posterior RSC is not clearly defined in mice and, as such, we cannot conclusively determine that our injection was isolated to the anterior portion. Thus, we have simply referred to our target region as the RSC throughout.

Intriguingly, we did find that learning affects *Per1* and *Fos* expression within the SCN, although *Per1* expression here is not modulated by time-of-day and is uniform across the day and night (Fig. 5; Supplemental Fig. 1). We also demonstrate here, for the first time, that targeted downregulation of *Per1* within the SCN does not affect the formation of spatial memory, consistent with prior work suggesting that SCN ablation fails to impair memory (Mulder et al., 2014; Stephan and Kovacevic, 1978; though it is worth noting there are other reports to the contrary: Phan et al., 2011; Shimizu et al., 2016). We did not perform activity monitoring on these animals to see if this manipulation affected their circadian rhythm, though the lack of detectable differences in OLM performance suggests that any possible circadian rhythm changes are insufficient to affect memory performance. Furthermore, even *Per1*$^{-/-}$ mice demonstrate largely intact circadian rhythms, suggesting this manipulation would minimally affect their circadian rhythms, if at all (Cermakian et al., 2001). Finally, these animals were housed on a 12 hour light/dark cycle, so their diurnal rhythms would have been entrained to the zeitgeber of the vivarium lights, and this entrainment is also intact in *Per1*$^{-/-}$ mice (Cermakian et al., 2001).

Given that SCN-specific *Per1* downregulation does not affect learning in young animals (Fig. 6), we suspect our observed changes in SCN gene expression are unlikely to be directly linked to learning *per se*. However, we did not directly test whether targeted upregulation in aging mice rescues long-term memory in OLM, although given that SCN-specific *Per1* manipulation had no effect on learning in young mice, we would not expect this to be the case. Additionally, there was no effect of time-of-day on induced *Per1* expression within the SCN (Fig. 5B, 5C; Supplemental Fig. 1), suggesting *Per1* here is not linking memory strength with time-of-day. However, exposure to a zeitgeber (e.g. a light pulse) induces pronounced expression of *Per1* (Shigeyoshi et al., 1997) and *Fos* (Kornhauser et al., 1990) within the SCN, and it is conceivable that our OLM paradigm constitutes a zeitgeber for trained animals, as these animals have undergone 7 consecutive days of behavior occurring at precisely the same time each day. Further, since training was conducted under dim red light and mice were housed and transported in light-protected conditions, this *Per1* induction cannot be attributed to light exposure. It is worth noting, however, that we detected day/night differences in learning-induced *Fos* expression (Supplemental Fig. 1) in the SCN of only aging animals, though the values for young animals approached significance. This is consistent with the long-known fact that the SCNs of both nocturnal and diurnal mammals tend to exhibit greater neuronal activity during the subjective day (Inouye and Kawamura, 1979). Interestingly, we only detected day/night differences in the SCN *Fos* expression of trained animals, with minimal differences between homecage groups (Fig. 5).

This study does not investigate the specific mechanism by which *Per1* is downregulated in the aging brain. However, a previous report from our group demonstrated that age-related deficits in hippocampal *Per1* expression were largely due to the repressive histone deacetylase HDAC3 (Kwapis et al., 2018). Thus, it is reasonable that the same HDAC3-induced repressive chromatin structure observed in the aging DH may also be present in the aging RSC and preventing adequate *Per1* expression following a learning event. Although we did detect an attenuated *Per1* induction in aging animals in our initial experiment (Fig. 1), our subsequent time course experiment suggests that *Per1* is induced to comparable levels between the young and aging RSC (Fig. 2). Given the fact the initial experiment was the only one to directly compare young and aging mRNA levels, we believe this best represents the true relationship between learning-induced *Per1* and *Fos* induction in the young and aging RSC. That is, it seems that learning induces some Per1 expression in the aging RSC, but not to the level seen in young animals (Fig. 1). The time course experiments (Fig. 2 and Fig. 5) were ran separately as young and aging cohorts, and then each trained mouse *Per1* value was divided by the average of its ZT-matched homecage group to produce a normalized induction value. Thus, it is possible that abnormally low homecage expression of *Per1* or *Fos* might exaggerate the apparent magnitude of induction seen in Fig. 2.

Additionally, in this study, we did not investigate which aspect of memory (i.e. acquisition, consolidation, or retrieval) was affected by *Per1* manipulation. Although we timed our HSV manipulations to ensure maximal virus expression during OLM training, there was likely still expression of our constructs during the test session 24 hours later. Thus, while these data suggest *Per1* most likely influences memory acquisition or consolidation, we cannot conclusively rule out an effect of *Per1* on memory retrieval. However, another recent report from our group suggests that diurnal changes in memory performance can be specifically attributed to memory consolidation (Bellfy et al., 2022). Given the relationship between time-of-day, memory performance, and RSC *Per1* expression, it is most likely that *Per1* specifically affects memory consolidation. Additionally, in the DH, PER1 is known to function by shuttling p90RSK into the nucleus to phosphorylate cAMP response element binding protein (CREB; Rawashdeh et al., 2016) Given CREB's known role in memory consolidation (Yin et al., 1995, 1994), this further suggests that *Per1* is also necessary for memory consolida-

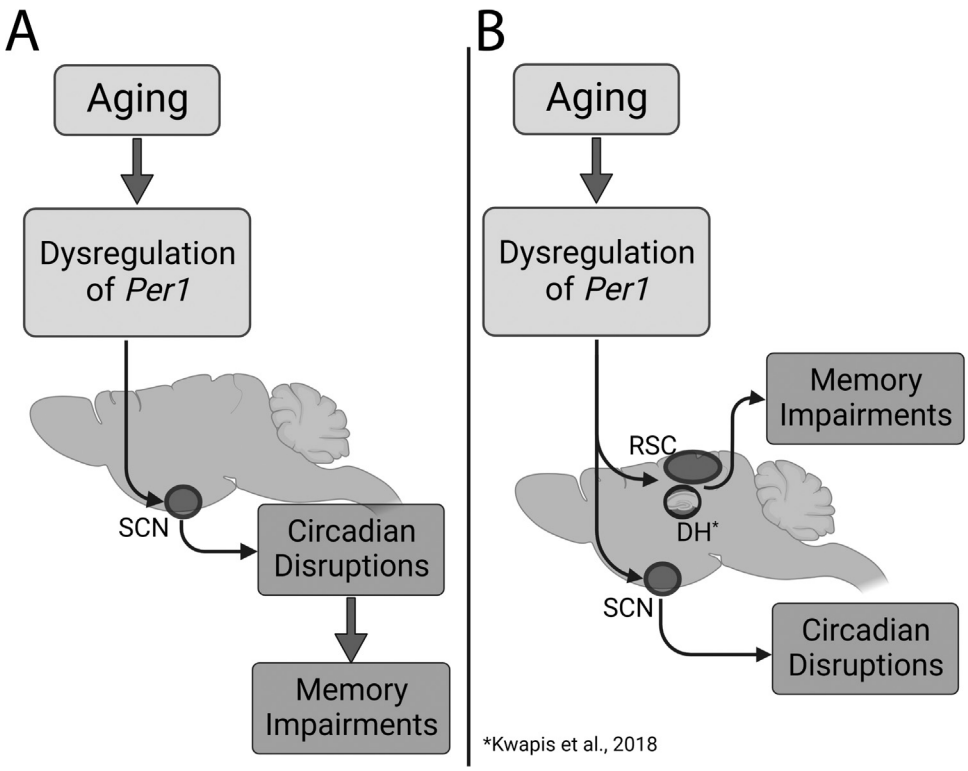

Fig. 7. Two possible models explaining the interaction between age and its associated circadian disruptions and memory impairments. (A) Model in which aging predominately disrupts the expression of *Per1* (and other clock genes) within the SCN, leading to circadian dysregulation. Then, an impaired circadian rhythm negatively affects the downstream processes of learning and results in memory deficits. (B) Model in which the local dysregulation of clock genes within various memory-relevant brain regions is directly contributing to age-related memory impairments, as supported by data here and elsewhere (Kwapis et al., 2018).

tion, possibly by controlling the allocation of individual neurons to a newly formed memory trace or engram (Zhou et al., 2009).

A notable weakness of this study is the decision to only include male subjects. Previous work from our group has noted that *Per1* modulates memory formation within the RSC in a sex-dependent manner (Urban et al., 2021). Therefore, we decided to split subsequent investigations into male and female cohorts to minimize sample sizes. Male mice were investigated first (this study) as their behavior was more readily affected by our *Per1* manipulations (Urban et al., 2021). We are currently investigating these effects in female mice in a separate series of experiments.

It has been difficult to decouple the relationship between the circadian rhythm, learning, and aging. These results expand what is known about the function of clock genes beyond the molecular clock, how the expression of these genes impacts memory formation, and how this expression is modulated by aging. Previous work has argued that age-related memory impairments are, at least partially, caused by circadian rhythm disruption stemming from the SCN (Antoniadis et al., 2000; Pang et al., 2006). In this older model (Fig. 7A), aging would result in dysregulation of the expression of *Per1* and other clock genes within the SCN, thereby disrupting circadian rhythm and subsequently affecting the downstream process of learning. However, the data presented here, in combination with previous work from our group (Bellfy et al., 2022; Kwapis et al., 2018; Urban et al., 2021) and elsewhere (Rawashdeh et al., 2014, 2016) support a model (Fig. 7B) in which age-related deficits in memory performance can be attributed to local disruption of *Per1* in memory-relevant brain regions (i.e. the RSC and the DH), while disruption of *Per1* within the SCN contributes to circadian dysfunction. This second model is further supported by other work (Kwapis et al., 2018) from our group suggesting these *Per1* disruptions stem from local epigenetic changes (i.e. excessive histone deacetylase activity in the DH) rather than disrupted communication with the SCN (an argument further supported by the negative results in Fig. 6 here). This has broad implications for clinicians interested in chronotherapy aimed at rescuing memory performance and circadian rhythms in aging populations, as therapies intended to restore circadian rhythm globally may not remedy the underlying local disruption of *Per1* within the DH, RSC, and other memory-relevant brain regions.

Data availability

The data presented here are available from the corresponding author upon reasonable request.

Authors' Contributions

C. A. B., K. K. B., and J. L. K. contributed to study design; C. A. B., D. J. B., K. K. B., A. R. M., C. Y. L., L. B., M. W. U., E. M. S., C. W. S., and J. L. K. contributed to data collection; C. A. B. performed data analysis; S. M. designed and optimized the CRISPRi/a systems for use in targeting *Per1*; C. A. B. and J. L. K. wrote and revised the manuscript.

Disclosure statement

The authors have no actual or potential conflicts of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.neurobiolaging.2023.02.009.

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