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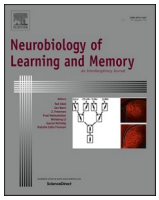

The circadian clock gene *Per1* modulates context fear memory formation within the retrosplenial cortex in a sex-specific manner

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ABSTRACT

Context memory formation is a complex process that requires transcription in many subregions of the brain including the dorsal hippocampus and retrosplenial cortex. One critical gene necessary for memory formation is the circadian gene *Period1* (*Per1*), which has been shown to function in the dorsal hippocampus to modulate spatial memory in addition to its well-documented role in regulating the diurnal clock within the suprachiasmatic nucleus (SCN). We recently found that alterations in *Per1* expression in the dorsal hippocampus can modulate spatial memory formation, with reduced hippocampal *Per1* impairing memory and overexpression of *Per1* ameliorating age-related impairments in spatial memory. Whether *Per1* similarly functions within other memory-relevant brain regions is currently unknown. Here, to test whether *Per1* is a general mechanism that modulates memory across the brain, we tested the role of *Per1* in the retrosplenial cortex (RSC), a brain region necessary for context memory formation. First, we demonstrate that context fear conditioning drives a transient increase in *Per1* mRNA expression within the anterior RSC that peaks 60 m after training. Next, using HSV-CRISPRi-mediated knockdown of *Per1*, we show that reducing *Per1* within the anterior RSC before context fear acquisition impairs memory in both male and female mice. In contrast, overexpressing *Per1* with either HSV-CRISPRa or HSV-*Per1* before context fear acquisition drives a sex-specific memory impairment; males show impaired context fear memory whereas females are not affected by *Per1* overexpression. Finally, as *Per1* levels are known to rhythmically oscillate across the day/night cycle, we tested the possibility that *Per1* overexpression might have different effects on memory depending on the time of day. In contrast to the impairment in memory we observed during the daytime, *Per1* overexpression has no effect on context fear memory during the night in either male or female mice. Together, our results indicate that *Per1* modulates memory in the anterior retrosplenial cortex in addition to its documented role in regulating memory within the dorsal hippocampus, although this role may differ between males and females.

1. Introduction

The ability to form long-term memory is a critical survival tool for nearly every species, as it allows an animal to use past experience to predict future events. Previous studies have shown that for many species, the time of day can have a significant impact on how well a memory is formed (Rawashdeh, Parsons, & Maronde, 2018), although the mechanism through which this occurs has not been fully established. Most species have an internal circaid circadian clock that drives the rhythmic cycling of biological processes, including memory, across the 24 h day. The molecular mechanism that sets the diurnal rhythm is a transcription-translation negative feedback loop largely controlled by four gene families: *Circadian Locomotor Output Cycles Kaput* (*Clock*), *Brain and Muscle ARNT-Like* (*BMAL*), *Period* (*Per*), and *Cryptochrome* (*Cry*). The canonical role of these clock genes has been well-established within the suprachiasmatic nucleus (SCN), the master timekeeper,

Abbreviations: BMAL, brain and muscle ARNT-like; Clock, circadian locomotor output cycles kaput; CMV, cytomegalovirus; Cry, cryptochrome; dCas9, cas9 endonuclease dead; eGFP, green fluorescent protein; FBXL3, F-box/LRR-repeat protein 3; IE4/5, immediate-early 4/5 promoter; KD, knockdown; KRAB, krüppel associated box; MeCP2, methyl CpG binding protein 2; OE, overexpression; Per1, period 1; NLS, nuclear localization signal; pP90RSK, phosphorylated P90 ribosomal s6 kinase; RSC, retrosplenial cortex; SCN, suprachiasmatic nucleus; sgRNA, single guide ribonucleic acid (RNA); U6, U6 promoter; ZT, Zeitgeber Time.

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where these genes interact in a ~24 h negative feedback loop that constitutively maintains time-of-day information (Sollars & Pickard, 2015). Most of the work characterizing these genes has focused on their roles within the SCN, yet these genes also oscillate in peripheral tissues throughout the brain and body. It has only recently been appreciated that these satellite clocks play a functional role in regulating peripheral functions like blood pressure (Paschos & FitzGerald, 2010), renal function (Stow & Gumz, 2011), metabolism (Marcheva et al., 2013), and behavior (Albrecht, 2011; Rawashdeh et al., 2018; Snider, Sullivan, & Obrietan, 2018) across the day/night cycle. Little is known about how these genes function in memory-relevant brain regions.

It is possible that clock genes function locally within memory-relevant brain regions to modulate memory performance across the 24 h day. Clock genes are expressed in subregions of the brain that are critical for memory encoding, storage, and recall, including the hippocampus and retrosplenial cortex (Cermakian, Lamont, Boudreau, & Boivin, 2011; Coogan et al., 2011). Further, research has specifically identified an important role for the circadian gene *Period1* (*Per1*) in hippocampal memory formation (Kwapis et al., 2018; Rawashdeh, Jilg, Maronde, Fahrenkrug, & Stehle, 2016). We recently demonstrated that bidirectional modulation of *Per1* expression directly in the dorsal hippocampus modulates spatial memory formation; reducing *Per1* levels in the hippocampus impaired object location memory in young mice whereas local overexpression of *Per1* in the hippocampus of old mice ameliorates age-related memory impairments (Kwapis et al., 2018). *Per1* may therefore function within the dorsal hippocampus to regulate spatial memory across the day/night cycle. This is consistent with work from Rawashdeh and colleagues (2016), who demonstrated that PER1 protein in the hippocampus regulates the activity of CREB (Ca^{2+}/cAMP response element-binding protein), a major transcription factor necessary for memory formation. Thus, hippocampal *Per1* may modulate memory across the day/night cycle by regulating CREB activity. Whether *Per1* plays a similar role in memory-relevant brain regions beyond the hippocampus is currently unknown.

To test the role of *Per1* in memory formation outside of the dorsal hippocampus, we turned to the retrosplenial cortex (RSC), a critical brain region that contributes to context memory (Corcoran et al., 2011), cue-specific memory (Todd, Fournier, & Bucci, 2019), spatial navigation (Cooper & Mizumori, 2001), and associative learning (Keene & Bucci, 2008). The RSC seems to be particularly important for spatial and contextual learning and plays a key role in context fear memory, in which a neutral context is associated with an aversive footshock. Immediate early genes such as *cFos* and *Arc* are elevated in the RSC following contextual fear conditioning, suggesting that RSC neurons are actively involved in context fear acquisition (Minatohara, Akiyoshi, & Okuno, 2015; Robinson, Poorman, Marder, & Bucci, 2012). Further, one study using simultaneous electrophysiological recordings of RSC and hippocampus demonstrated that RSC neurons fire in phase with hippocampal neurons during REM sleep, indicating that hippocampal-RSC interactions in sleep may be necessary for memory consolidation (Koike et al., 2017). The retrosplenial cortex is important for both the acquisition and retrieval of context fear, as blocking NMDA receptors within the RSC disrupts the retrieval of both recent and remote context fear memory (Corcoran et al., 2011) and blocking protein synthesis in the RSC or silencing the RSC during training impairs the acquisition of contextual fear memory (Kwapis, Jarome, Lee, & Helmstetter, 2015; Trask, Pullins, Ferrara, & Helmstetter, 2021). Finally, lesioning the RSC causes both retrograde and anterograde amnesia for context fear conditioning (Fournier, Eddy, DeAngeli, Huszár, & Bucci, 2019; Keene & Bucci, 2009; Todd, DeAngeli, Jiang, & Bucci, 2017; Todd, Mehlman, Keene, DeAngeli, & Bucci, 2016). Thus, the RSC plays a key role in context fear memory that is similar but complementary to that of the dorsal hippocampus. Whether *Per1* plays a role in memory formation within the RSC is unknown.

Here, we show that *Per1* functions within the retrosplenial cortex to modulate context fear memory formation. While knockdown of *Per1* impairs context fear memory in both males and females, we were surprised to find that overexpression of *Per1* also impairs context fear memory, but only in males. Finally, we show that overexpression of *Per1* at night (when *Per1* levels are lowest) has no effect on context fear memory in either male or female mice, indicating that intra-RSC *Per1* may function differently during the day and night. *Per1* in the RSC therefore regulates context fear memory formation in addition to its well-documented role in generating the diurnal rhythm in the SCN. *Per1* levels may need to be tightly regulated to promote memory formation, however, and this role may differ between male and female mice.

2. Methods

2.1. Mice

All studies used C57BL/6J mice from Jackson Laboratories at 8 weeks of age. Mice were kept in temperature and humidity-controlled environments under a 12 h light/dark cycle (lights on at 7am, lights off at 7pm). Some experiments required mice to be maintained on a reverse light cycle (lights off at 7am, lights on at 7pm). These mice were housed in a separate unit and allowed 2 weeks to acclimate to the new light cycle prior to any experimentation. All behavior conducted during the dark portion of the cycle was conducted under red light. Mice had free access to food and water. All experiments were conducted 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 Pennsylvania State University.

2.2. Viruses

We used neuron-specific herpes simplex viruses (HSVs) to express each CRISPRi (CRISPR inhibition) or CRISPRa (CRISPR activation) system along with the appropriate single guide RNA (sgRNA) targeting *Per1* (or a non-targeting control sgRNA). All viruses were purchased from Dr. Rachael Neve at the Gene Delivery Technology Core (GDTC) at Massachusetts General Hospital. Both CRISPRi and CRISPRa were expressed in bicistronic p1005 + vectors that express mCherry under the CMV promoter and the target gene (CRISPRi: dCas9-KRAB-MeCp2 or CRISPRa: dCas9-VPR) under the IE4/5 promoter. The *Per1* sgRNAs were designed and tested in HT22 cells in the Kwapis lab. After identifying the most effective sgRNAs for *Per1* CRISPRi and CRISPRa, these sequences were cloned into separate donor vectors (pDonr221, ThermoFisher) to create entry clones. Each clone consists of the U6 promoter, a seed region to target *Per1* (or a nontargeting control sequence), and the gRNA scaffold sequence. These entry clones were then sent to Dr. Neve who subcloned the inserts into a second HSV vector that also expresses GFP under a CMV promoter. The seed region of each sgRNA was as follows: CRISPRi targeting *Per1*: GAGTTCGACGGCTCCAGAGTA; CRISPRa targeting *Per1*: AGCCCTTGTAAAGCAACCAT; non-targeting control for both CRISPRi and CRISPRa: GCGAGGTATTCGGCTCCGCG. This non-targeting control sgRNA has been previously validated and used *in vivo* with similar CRISPR systems (Lorsch et al., 2019). All context fear conditioning sessions took place 3 days after HSV injection, when HSV expression peaks (Neve, Neve, Nestler, & Carlezon, 2005; Sarno & Robison, 2018).

2.3. Cell culture verification of CRISPRi and CRISPRa targeting Per1

To verify that our CRISPRi and CRISPRa systems effectively reduce and increase *Per1* mRNA in neurons, respectively, mouse HT22 cells were transfected with CRISPRi (dCas9-KRAB-MeCP2) or CRISPRa (dCas9-VPR) along with the appropriate *Per1*-targeting sgRNA or non-targeting control sgRNA using Lipofectamine LTX (Invitrogen). For CRISPRa, cells were harvested 48 h after transfection without stimulation, as we wanted to determine whether CRISPRa alone could drive expression of *Per1*. For CRISPRi, 48 h after transfection, cells were

stimulated with 50 mM KCl and harvested 1 h later to test whether CRISPRi can prevent stimulation-induced increases in *Per1*. For both experiments, after harvesting, cells were lysed and the mRNA was isolated as described below. RT-qPCR was performed as described below using the same *Per1* primers/probe combination listed below.

2.4. In vivo verification of CRISPRi and CRISPRa targeting Per1

To verify that our HSV-CRISPRi and HSV-CRISPRa systems effectively reduce or increase *Per1* mRNA *in vivo*, separate cohorts of mice were given stereotaxic intra-RSC injections (described below) of either HSV-CRISPRi (dCas9-KRAB-MeCP2) or HSV-CRISPRa (dCas9-VPR) along with the appropriate HSV-sgRNA (*Per1*-targeting or non-targeting control). The appropriate HSV-sgRNA and HSV-CRISPR system were combined before injection in a 1:1 ratio. Mice were sacrificed 3 days after injection, when HSV expression peaks, and RT-qPCR was performed as described below using the same *Per1* primers/probe combination listed below.

2.5. RSC injection

-Mice were anesthetized with 2% isoflurane (Patterson Veterinary, Greeley, CO) dissolved in oxygen, the head was shaved and cleaned with betadine (Purdue Products), and eye gel was applied to the eyes to prevent drying. Animals were placed in a stereotaxic apparatus (Stoelting Co, Wood Dale, IL), the head was secured using ear bars and a tooth bar, and each mouse was injected with the analgesic Ketoprofen. An approximate halfinch incision was made with a sterile surgical blade (Aspen Surgical Products, Caledonia, MT) into the skin starting between the eyes and run caudally to the occipital bone. The skull was dried using 70% ethanol and the bregma was located. The anterior retrosplenial cortex was targeted using the coordinates: 1.80 mm caudal, 0.45 mm lateral, 0.75 mm ventral. The skull was bored using a surgical drill (Foredom Electric Co., Bethel CT) with 0.7 mm burr (Fine Science Tools, Foster City, CA) at this region and the needles were placed on the surface of the skull. Bilateral injection needles (P1 Technologies, Roanoke, VA) were connected to the injector syringes (Hamilton Co., Reno, NV) via PE-50 thin wall 10′ tubing (Plastics One Inc., Torrington, CT). The needles were lowered at a rate of 0.20 mm/15 sec to a final depth of −0.75 mm at which point a 2 min rest period was done prior to injection. The mice were then injected with vector at a rate of 6 μl/hr using a micro pump injector (Harvard Apparatus, Holliston, MA) to a total volume of 1 μl per hemisphere. After injection, the needle was unmoved for 5 min, then removed 0.1 mm followed by another 5 min wait. After this wait, the needles were removed at a rate of 0.1 mm/15 sec. The skin was washed with sterile saline and sealed with vetbond (3 M, St. Paul, MN). For post-surgical recovery, mice were placed in a clean cage on a heating pad and were monitored until they awoke from the anesthesia, then returned to the vivarium. All behavior was conducted 3 days after HSV infusion.

2.6. Fear conditioning apparatus

Context fear conditioning and retention testing were conducted in a set of 4 identical chambers housed within sound-attenuating boxes (Ugo Basile, Gemonio (VA) Italy). Each chamber was made of plastic with dimensions of 17.5 cm × 17.5 cm × 25.0 cm (length × width × height). The floor was raised 4.0 cm above the bottom of the chamber and consisted of evenly spaced metal bars capable of delivering footshocks. Each context was illuminated by both dim white light and infrared light and low background white noise (~58 dB) was played for the duration of each session. Chambers were cleaned with 70% ethanol followed by Windex between trials.

2.7. Fear conditioning procedure

Mice were individually housed for at least 5 days before beginning the fear conditioning protocol. Handling, fear conditioning, and testing were all conducted at Zeitgeber time (ZT) 5 (Figs. 1–3) or ZT17 (Fig. 4). For experiments conducted at ZT5, mice were housed in normal light conditions (lights on at 7am and off at 7pm, so ZT0 = 7am) and mice were trained at 12 pm (ZT5). For experiments conducted at ZT17, mice were housed in reverse light cycle conditions (lights on at 7pm/off at 7am, so ZT0 = 7pm) and mice were trained at 12 pm (ZT17). Each mouse was handled for 1 min daily for 5 days prior to fear conditioning. During the context fear conditioning acquisition session, mice were placed into a chamber for 2 m, 28 s followed by a 2 s (1 mA) footshock. Mice remained in the context for an additional 30 s before being removed and transported back to the vivarium. 24 h later, mice were given a context retention test. Mice were placed back into the training chamber and activity was recorded for 5 min with no shock. After the test, mice were immediately removed from chamber and escorted back to their normal housing unit. Each trial was recorded and analyzed with Ethovision software (Noldus, Leesburg, VA) to evaluate activity and all mice were handled by an experimenter blind to the group assignments. For qPCR experiments, mice were killed and the brain was extracted 30 min, 60 min, or 2 h after acquisition. Homecage control mice were sacrificed between behavior groups in a counterbalanced manner. We also included an immediate shock (IS) control condition that received a single 2 s (1 mA) shock immediately after being placed in the conditioning chamber. IS mice remained in the chamber for 3 min total and were sacrificed 60 min after acquisition.

2.8. Object location memory (OLM)

OLM was used to investigate how *Per1* mRNA levels fluctuate in the RSC across the day/night cycle. All behavior was conducted under dim red lighting to prevent resetting of the diurnal clock. Each mouse was handled for 2 min/day for 4 days and then habituated to the context for 5 min/day for six consecutive days in the absence of objects. During training, mice were placed individually into one of four arenas each containing two identical objects (100 mL beakers filled with cement) and were allowed to explore for 10 min. Mice were sacrificed 60 m after training and brains were extracted to assess learning-induced *Per1* levels in the RSC across the 24 h day. Time-matched homecage control mice were handled and habituated normally but were sacrificed from their homecages without training along with the appropriate time-matched trained group.

2.9. Tissue extraction

Animals were euthanized via cervical dislocation and immediately decapitated with surgical scissors (Fine Science Tools, Foster City, CA). The skull was retracted using rongeurs and the brain was carefully removed using a surgical spatula (Fine Science Tools, Foster City, CA) and flash frozen with 2-methylbutane (Fisher Scientific, Waltham, MA) chilled on dry ice. Brains were kept at −80 °C until sectioned with a cryostat (Leica Biosystems, Wetzlar, Germany). 500 μm punches were collected from the anterior retrosplenial cortex and stored at −80 °C. Tissue punches were used for quantitative RNA analysis immediately or kept at −80 °C for long-term storage.

2.10. RT-qPCR

Tissue punches were collected from anterior RSC (described above) and frozen at −80 °C until processing. RNA was isolated using RNeasy Mini Kits (Qiagen, Germantown, MD) according to the manufacturer's instructions and cDNA was created using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher). PrimeTime primer/probe assays were designed with the IDT PrimerQuest Design tool and were

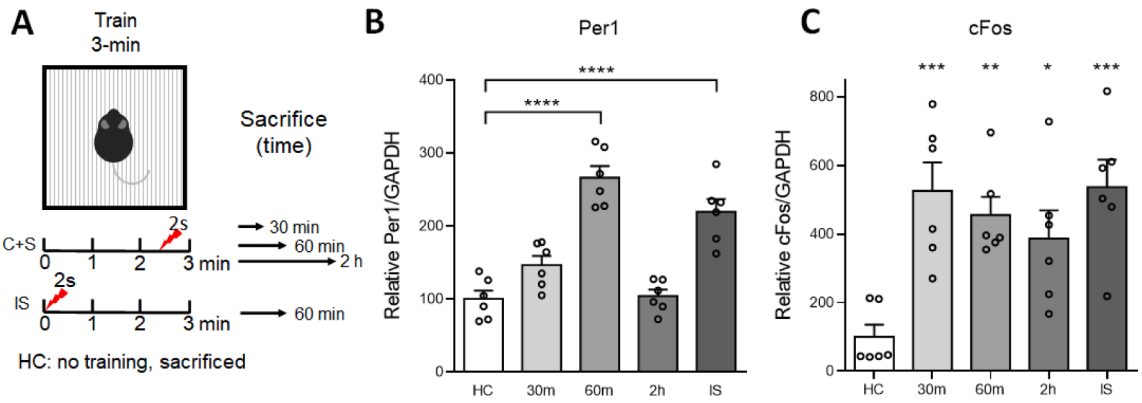

Fig. 1. *Per1* expression is induced 60 min after context fear conditioning in the RSC. A. Experimental design. Mice were sacrificed 30 m, 60 m, or 2 h after context fear conditioning along with homecage and immediate shock mice. C + S: context + shock; IS: immediate shock; HC: home cage. B. *Per1* mRNA is increased in the RSC 60 m after context fear conditioning and returns to baseline levels by 2 h after acquisition. Immediate shock mice sacrificed 60 min after behavior also showed increased *Per1* relative to homecage controls. C. *cFos* mRNA expression in the RSC is increased in a rapid and sustained manner following context fear conditioning. Immediate shock control animals also show increased *Per1* relative to homecage controls. Data are presented as mean ± SEM. *indicates $p < 0.05$, **indicates $p < 0.01$, ***indicates $p < 0.001$, ****indicates $p < 0.0001$, all relative to the HC control group.

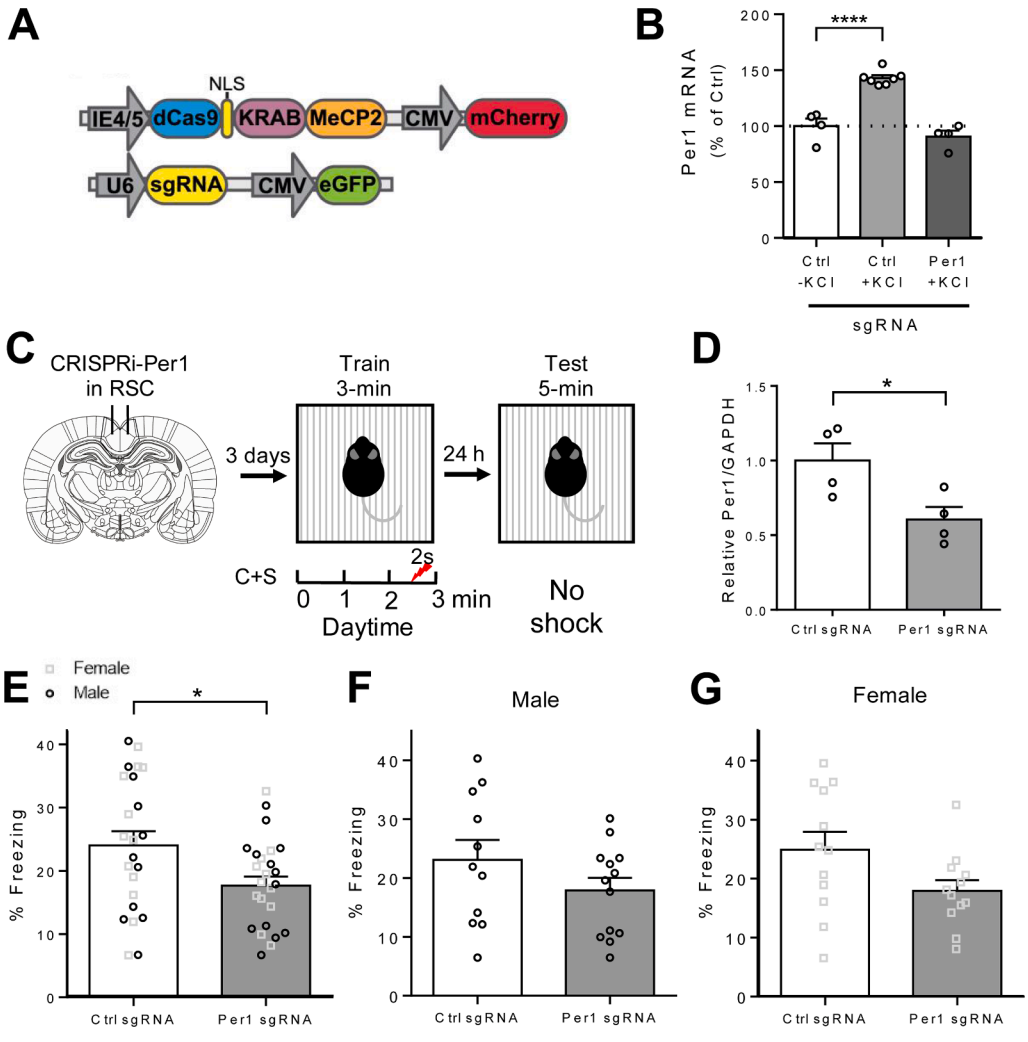

Fig. 2. RSC *Per1* knockdown during the day impairs context fear memory. A. Dual HSV strategy for CRISPRi. Separate HSVs deliver CRISPR-dCas9-KRAB-MeCP2 (top) and the sgRNA (bottom). IE4/5: immediate-early 4/5 promoter; dCas9: nuclease-dead cas9; NLS: nuclear localization signal; KRAB: krüppel associated box; MeCP2: methyl CpG binding protein 2; CMV: cytomegalovirus; U6: U6 promoter; sgRNA: single guide ribonucleic acid (RNA); eGFP: green fluorescent protein. B. Quantification of *Per1* mRNA in HT22 cells transfected with dCas9-KRAB-MeCP2 and either the *Per1*-targeting sgRNA or a nontargeting control sgRNA. 48 h after transfection, cells were stimulated with 50 mM of KCl to drive *Per1* expression and then harvested 1 h later. C. Schematic of context fear conditioning procedure. D. *In vivo* quantification of dCas9-KRAB-MeCP2 driven knockdown of *Per1*. Mice were injected with 1 μl per hemisphere of dCas9-KRAB-MeCP2 and either nontargeting control sgRNA or *Per1*-targeting sgRNA into the anterior RSC. Tissue was collected 3 days after injection and RT-PCR analysis was performed to analyze *Per1* mRNA expression. *Per1* mRNA was significantly reduced by *Per1*-targeting sgRNA. E-G. Quantification of percent time spent freezing during the 5 min context retention test. E. Combined freezing behavior for both males and females at test. Knockdown of *Per1* in the RSC impaired context fear memory. F. Freezing behavior at test for the males shown in Fig. 2E. *Per1* knockdown in the RSC produced a nonsignificant impairment in context freezing. G. Freezing behavior at test for the females shown in Fig. 2E. *Per1* knockdown in the RSC produced a similar nonsignificant impairment in context freezing. Data are presented as mean ± SEM; black circles, males; gray squares, females. *indicates $p < 0.05$, ****indicates $p < 0.0001$, all relative to the corresponding control group.

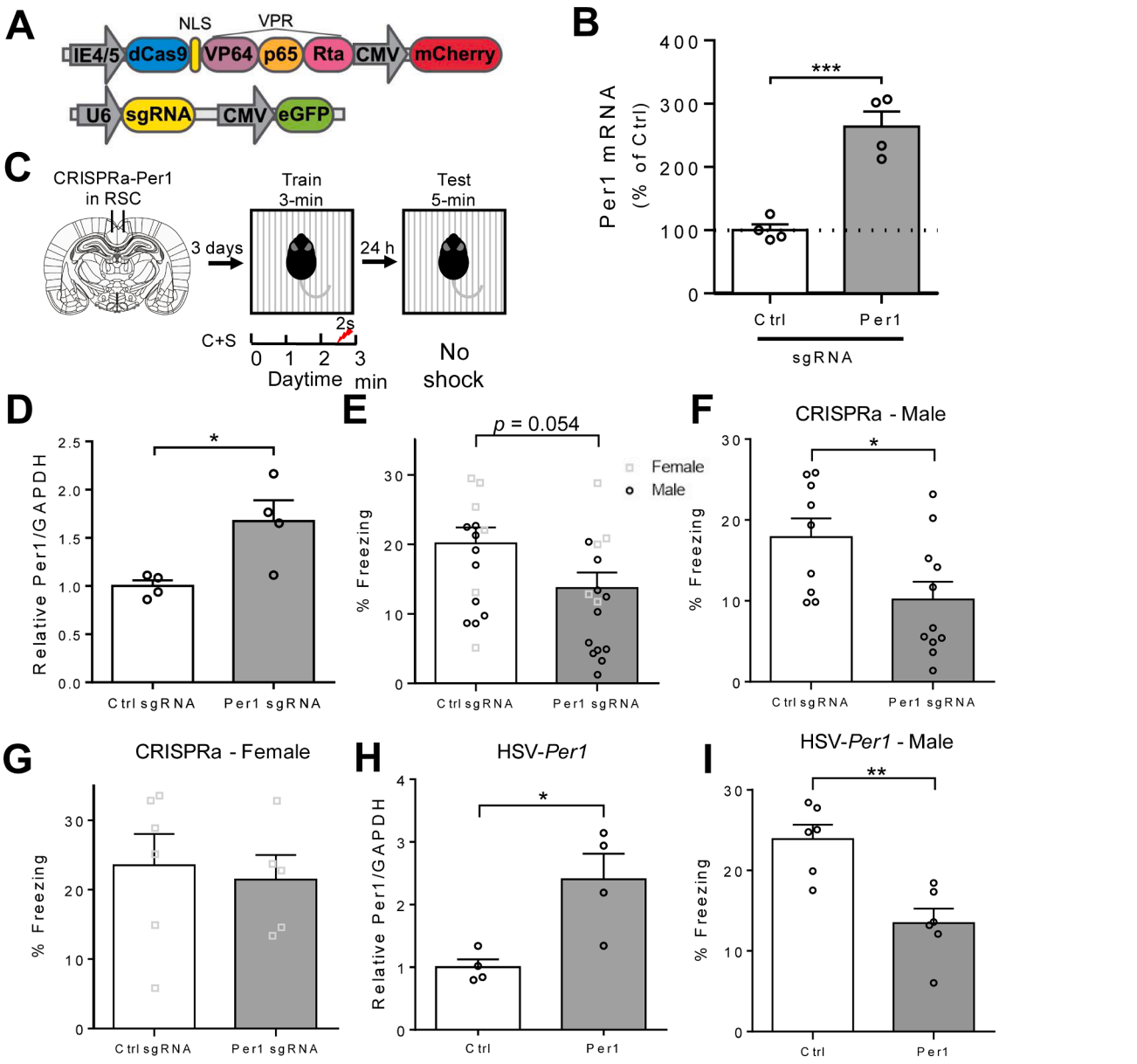

Fig. 3. RSC *Per1* overexpression during the day impairs fear memory in male mice. A. Dual HSV strategy for CRISPRa. Separate vectors deliver CRISPR-VPR (top) and the sgRNA (bottom). IE4/5: immediate-early 4/5 promoter; dCas9: cas9 endonuclease dead; NLS: nuclear localization signal; VP64: Vp64 transcriptional activator; p65: p65 transcriptional activator; CMV: cytomegalovirus; U6: U6 promoter; sgRNA: single guide ribonucleic acid (RNA); eGFP: green fluorescent protein. B. Quantification of *Per1* mRNA of HT22 cells transfected with dCas9-VPR and either the *Per1*-targeting sgRNA or a nontargeting control sgRNA. C. Schematic of context fear conditioning procedure. D. *In vivo* quantification of dCas9-VPR driven overexpression of *Per1*. Mice were injected with 1 μl per hemisphere of dCas9-VPR and either nontargeting control sgRNA or *Per1*-targeting sgRNA into the anterior RSC. Tissue was collected 3 days after injection and RT-PCR analysis was performed to analyze *Per1*mRNA expression. *Per1* mRNA was significantly increased by *Per1*-targeting sgRNA. E-I. Quantification of percent time spent freezing during the context retention test. E. Combined freezing behavior for both males and females at test. Overexpression of *Per1* had no effect when the sexes were collapsed. F. Freezing behavior at test for the males shown in Fig. 3E. *Per1* overexpression in the RSC significantly impaired context fear memory in males. G. Freezing behavior at test for the females shown in Fig. 3E. *Per1* overexpression in the RSC had no effect on context freezing in female mice. H. *In vivo* quantification of *Per1* mRNA 3 days after injection of either HSV-*Per1* or HSV-EV in a separate cohort of male mice. *Per1* mRNA was significantly increased in HSV-*Per1* mice relative to HSV-EV controls. I. Freezing behavior at test for males injected with HSV-EV or HSV-*Per1*. *Per1* overexpression with HSV-*Per1* significantly impaired freezing behavior at test. Data are presented as mean ± SEM; black circles, males; gray squares, females. One control sgRNA male mouse, one *Per1* sgRNA mouse, and one control sgRNA female mouse were removed as outliers from the CRISPRa context fear conditioning experiments. *indicates $p < 0.05$, **indicates $p < 0.01$, ***indicates $p < 0.001$, all relative to the corresponding control group.

used for multiplexing in the Roche LightCycler 96 machine. The following primers were used: *Per1* left primer, 5′-CCTGGAGGAATTGGAGCATATC-3′; *Per1* right primer, 5′- CCTGCCTGCTCCGAAATATAG-3′; probe, AAACCAGGACACCTTCTCTGTGGC; *cFos* left primer, 5′-GGCACTAGAGACGGACAGAT-3′; *cFos* right primer, 5′-ACAGCCTTTCCTACTACCATTC-3′; probe, CAGCCGACTCCTTCTCCAGCATG. All target probes were conjugated to the dye FAM. All values were normalized to *Gapdh* expression, which used the following primers: left primer, 5′- GGAGAAACCTGCCAAGTATGA-3′; right primer: 5′-TCCTCAGTGTAGCCCAAGA-3′ probe, TCAAGAAGGTGGTGAAGCAGGCAT. The *Gapdh* probe was conjugated to the dye HEX. Analyses and statistics were performed using the Roche proprietary algorithms based on the Pfaffl method (Pfaffl, 2001; Pfaffl, Horgan, & Dempfle, 2002).

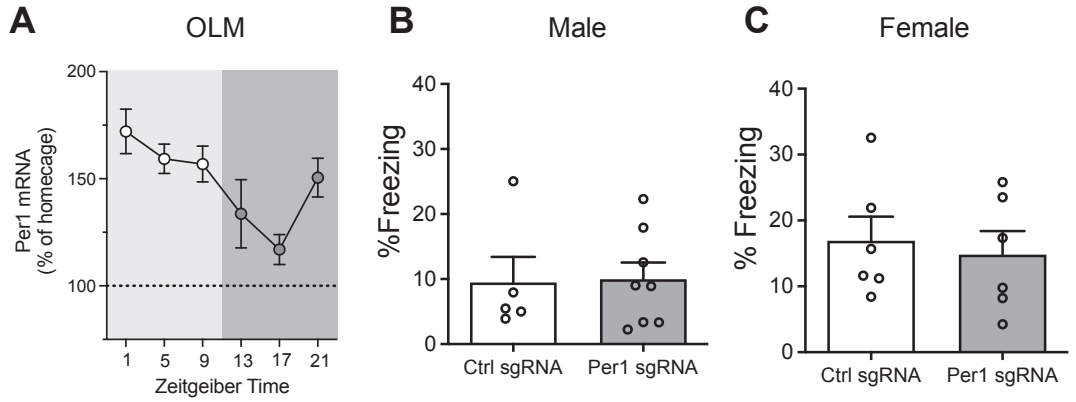

Fig. 4. RSC *Per1* over-expression at night (ZT 17) does not affect fear memory. A. Quantification of RSC *Per1* mRNA following object location memory acquisition across the day/night cycle. Values are expressed as the percent change in OLM-trained mice compared to each time-locked homecage control; B-C. Mice were given HSV-CRISPRa-mediated knockdown of *Per1* in the RSC at night and trained in context fear conditioning. Behavior during the context retention test 24 h later is shown. B. Freezing behavior at test in males given control- or *Per1*-targeting sgRNA at night (ZT17). Nighttime *Per1* overexpression in the RSC had no effect on males. C. Freezing behavior at test in females given control- or *Per1*-targeting sgRNA at night (ZT17). Nighttime *Per1* overexpression in the RSC had no effect on females. Data are presented as mean ± SEM; one control male mouse was removed as an outlier in the dCas9-VPR fear conditioning experiment.

2.11. Statistics

The average percent time freezing was calculated in real-time with Ethovision XT14. The computer scoring parameters were chosen to closely match handscoring methods previous used to measure freezing behavior (Kwapis, Jarome, Ferrara, & Helmstetter, 2017; Kwapis, Jarome, Lee, Gilmartin, & Helmstetter, 2014). Data are expressed as mean ± standard error of the mean (SEM). Statistical significance was assessed using Student's *t*-test, one-way ANOVAs, or two-way ANOVAs followed by Sidak's multiple comparisons *post hoc* tests. Graphpad Prism 6 (Graphpad Software Inc.) was used to calculate all analyses and $p < 0.05$ was considered significant. For each experiment values ± 2SD from the group mean were considered outliers and were removed from analyses.

3. Results

3.1. Per1 expression is transiently induced by context fear conditioning in the RSC

To test whether context fear conditioning alters *Per1* expression in the RSC, mice were trained in context fear conditioning during the early afternoon (Zeitgeber time (ZT) 5–7, where ZT0 = 7am, lights on) and were sacrificed 30 m (n = 6), 60 m (n = 6), or 2 h (n = 6) after the end of the conditioning session (Fig. 1A). Homecage (HC) mice (n = 6) were treated identically except that they received no training session and were sacrificed between behavior groups. We also included an immediate shock (IS) control group (n = 6) that received a 1.0 mA shock immediately after being placed in the chamber and was sacrificed 60 m later. qPCR of RSC tissue punches revealed a transient increase in *Per1* in response to context fear conditioning, with *Per1* levels peaking 60 min after training and returning to baseline levels by 2 h post-training (Fig. 1B; one-way ANOVA: $F_{(4,25)} = 28.7$, $p < 0.0001$; *post-hoc* tests: HC vs 30 m: $p = 0.08$, HC vs 60 m: $p < 0.0001$, HC vs 2 h: p = 0.99, HC vs IS: $p < 0.0001$; n = 6 per group). Interestingly, the immediate shock condition (sacrificed 60 m after IS) also drove an increase in *Per1* of approximately the same magnitude as standard context fear conditioning. We also measured expression of the immediate early gene *cFos*, which is typically used as a marker of activity following learning (Kubik, Miyashita, & Guzowski, 2007; Lehner et al., 2009; Milanovic et al., 1998; Swank, Ellis, & Cochran, 1996). *cFos* also showed a significant upregulation at all timepoints and in the IS group compared to HC controls (Fig. 1C; one-way ANOVA: $F_{(4,25)} = 6.6$, $p < 0.001$; *post-hoc* tests: HC vs 30 m: $p < 0.001$, HC vs 60 m: $p < 0.01$, HC vs 2 h: $p < 0.05$, HC vs IS: $p < 0.001$; n = 6 per group). Together, these results suggest that context fear conditioning activates gene expression within the anterior RSC, including both experience-induced IEG expression and expression of the memory-relevant circadian gene *Per1*.

3.2. Knockdown of Per1 in the RSC during the day impairs context fear memory

Next, we wanted to test whether *Per1* is critical for the formation of long-term contextual fear memory. To reduce *Per1* mRNA expression before conditioning, we used HSV-CRISPRi (CRISPR inhibition) to rapidly and locally repress transcription of the endogenous *Per1* gene in the RSC (Fig. 2A) (Navabpour, Kwapis, & Jarome, 2020). In CRISPRi, nuclease dead Cas9 (dCas9) is fused to two transcriptional repressors: KRAB (Krüppel associated box) and MeCP2 (methyl CpG binding protein 2), which synergistically provide transcriptional repression of the target gene (Yeo et al., 2018). This CRISPRi system is directed to *Per1* with a single guide RNA (sgRNA) to provide gene-specific transcriptional repression. The CRISPRi and sgRNA plasmids were separately packaged into replication-deficient herpes simplex viruses (HSVs), which show peak expression 3 days after injection, to provide rapid, neuron-specific repression of *Per1* within the RSC. To verify that our CRISPRi system effectively represses *Per1* mRNA in neurons, we transfected HT22 cells with dCas9-KRAB-MeCP2 and either the *Per1*-targeting sgRNA or a nontargeting control sgRNA. 48 h after transfection, cells were stimulated with 50 mM of KCl to drive *Per1* expression and then harvested 1 h later. RT-qPCR revealed that KCl stimulation drove a significant increase in *Per1* mRNA in cells treated with the control sgRNA, but this increase was prevented when dCas9-KRAB-MeCP2 was targeted to *Per1* (Fig. 2B; One-way ANOVA: $F_{(2,12)} = 46.26$, $p < 0.0001$; *post hoc* comparing -KCl to + KCl for the control sgRNA: $p < 0.0001$; comparing control to *Per1* sgRNA for the + KCl condition, $p < 0.0001$). CRISPRi therefore prevents stimulation-induced increases in *Per1* mRNA within neurons.

We then used this CRISPRi system *in vivo* to reduce *Per1* within the RSC before context fear conditioning during the daytime (ZT5/12 pm). We injected the RSC with a 1:1 mixture of HSV-dCas9-KRAB-MeCP2 and HSV-sgRNA targeting either *Per1* or a non-targeting control (1 µl total HSV/hemisphere). Three days after injection, a subset of mice (n = 4/group) were sacrificed and anterior RSC tissue was collected for RT-qPCR. Quantification of RSC *Per1* mRNA showed a significant reduction of *Per1* expression in mice treated with *Per1* sgRNA compared to control sgRNA mice (Fig. 2D; $t_{(6)} = 2.77$ $p = 0.032$).

The remainder of the mice were trained in context fear conditioning 3 days after HSV infusion (Fig. 2C). 24 h after acquisition, mice were placed back into the conditioning chambers for a context retention test.

During the context test, CRISPRi-mediated knockdown of *Per1* significantly reduced total freezing to the context compared to nontargeting controls (Fig. 2E; $t_{(45)} = 2.43$ $p = 0.019$; *Per1* sgRNA n = 24, control sgRNA n = 23). As we used both male and female mice, we also assessed whether this effect was sex-specific. Both males (Fig. 2F; $t_{(22)} = 1.34$, $p = 0.193$; *Per1* sgRNA n = 13, control sgRNA n = 11) and females (Fig. 2G; $t_{(22)} = 1.96$, $p = 0.063$; *Per1* sgRNA n = 12, control sgRNA n = 12) showed a similar reduction in freezing due to *Per*1 KD; however, neither group reached significance compared to controls. These results indicate that successful formation of context fear memory during the daytime requires *Per1* expression within the RSC for both male and female mice.

3.3. Overexpression of Per1 in the RSC during the day impairs context fear memory in male mice

We next wanted to test whether overexpression of *Per1* within the RSC would have the opposite effect, improving contextual fear memory during the daytime. To overexpress *Per1*, we used HSV-CRISPRa (CRISPR activation) to rapidly and locally drive transcription of the endogenous *Per1* gene (Fig. 3A). In CRISPRa, dCas9 is fused to a trio of transcriptional activation domains (VP64, p65, and Rta, collectively called the VPR) to drive transcription of the target gene (Chavez et al., 2015). This CRISPRa system is directed to *Per1* with an sgRNA to provide gene-specific transcriptional activation. As with CRISPRi, both the CRISPRa and sgRNA plasmids are separately packaged into replication-deficient HSVs to provide local control of *Per1* within the RSC. To verify that our CRISPRa system effectively drives *Per1* transcription in neurons, we transfected HT22 cells with dCas9-VPR and either the *Per1* targeting sgRNA or a nontargeting control sgRNA. 48 h after transfection, cells were harvested (without stimulation). RT-qPCR revealed that *Per1*-targeted dCas9-VPR drove a significant increase in *Per1* mRNA relative to the nontargeting control sgRNA (Fig. 3B; *t*-test: $t_{(6)} = 6.416$, $p < 0.0007$). CRISPRa therefore drives an increase in *Per1* mRNA within neurons in cell culture.

We then used this CRISPRa system *in vivo* to overexpress *Per1* within the RSC before context fear conditioning during the daytime (ZT5/12 pm). We ran identical protocols for RSC injections and context fear conditioning paradigm as previously with the exception of using the dCas9-VPR vector to overexpress *Per1* and using a slightly different *Per1*-targeting sgRNA optimized for CRISPRa (Fig. 3A, C). As before, a subset of mice was sacrificed 3 days after injection and RSC tissue was collected for RT-qPCR analysis. Quantification of RSC *Per1* mRNA shows the *Per1*-targeting group had significantly increased *Per1* mRNA relative to the non-targeting control group (Fig. 3D; $t_{(6)} = 3.0$, $p = 0.024$).

The rest of the mice were trained and tested in context fear conditioning 3 days after HSV infusion, as before. At test, *Per1* overexpression did not increase freezing behavior as expected, instead producing a nonsignificant trend towards reduced freezing behavior (Fig. 3E; $t_{(29)} = 2.01$, $p = 0.054$; *Per1* sgRNA n = 16, control sgRNA n = 15). Furthermore, when we analyzed context freezing by sex, we found that *Per1* overexpression significantly impaired context fear memory selectively in males (Fig. 3F; $t_{(18)} = 2.43$, $p = 0.026$; *Per1* sgRNA n = 11, control sgRNA n = 9). We saw no effect of *Per1* overexpression in female mice (Fig. 3G; $t_{(9)} = 0.35$, $p = 0.734$; *Per1* sgRNA n = 5, control sgRNA n = 6).

To ensure that the observed effect in males was due to *Per1* overexpression, rather than an unintended side effect of the HSV-CRISPRa system, we used a complementary approach to overexpress *Per1* in a separate cohort of male mice: overexpressing the full-length *Per1* cDNA with HSV-*Per1* or HSV-EV, an empty vector control that contains no *Per1* coding sequence. We first confirmed that HSV-*Per1* also increases *Per1* mRNA expression after injection in the RSC (Fig. 3H; $t_{(6)} = 3.29$ $p = 0.017$; n = 4 per group). Next, we injected HSV-*Per1* directly into the RSC 3 days before context fear conditioning during the daytime (ZT5/12 pm), as before. As with our CRISPRa system, overexpressing *Per1* with HSV-Per1 significantly impaired context fear memory during the daytime (Fig. 3I; $t_{(10)} = 4.14$, $p = 0.002$; n = 6 per group) replicating our finding that *Per1* overexpression in the RSC during the daytime impairs context fear memory in male mice. Together, these results indicate that daytime overexpression of *Per1* within the RSC impairs context fear memory selectively in male mice.

RSC *Per1* overexpression at night (ZT 17) does not affect fear memory. Because *Per1* levels oscillate across the day/night cycle throughout the brain (Chun, Woodruff, Morton, Hinds, & Spencer, 2015), we next wanted to test whether overexpression of *Per1* during the night would have the same effect on memory as *Per1* overexpression during the daytime. First, to determine when endogenous *Per1* levels peak and trough in the RSC, we used RT-qPCR to measure *Per1* mRNA expression across the 24 h day in both naïve mice and mice trained in a spatial object location memory (OLM) task. We chose to use OLM for this experiment as *Per1* induction following OLM has already been well-characterized in the mouse brain and is known to be tightly linked to memory formation. Importantly, our previous work has demonstrated that *Per1* is increased in the dorsal hippocampus 60 m after OLM and bidirectional manipulation of *Per1* levels in the hippocampus before learning modulates memory for OLM. *Per1* expression therefore plays a critical role in OLM acquisition (Kwapis et al., 2018). As neural activity within the anterior RSC is also critical for OLM (de Landeta, Pereyra, Medina, & Katche, 2020), this task is ideal for assessing learning-induced changes in *Per1* across the day/night cycle. Here, to understand how learning-induced *Per1* expression in the RSC oscillates across the diurnal cycle, we trained mice at six diurnal timepoints: ZT1, ZT5, ZT9, ZT13, ZT17, and ZT21 (where ZT0 = 7am, lights on; ZT12 = 7pm, lights off) and sacrificed each group 60 m after training. Each timepoint had a time-matched homecage control, allowing us to determine how spatial learning alters *Per1* expression in the RSC across the 24 h day. We found that learning-induced *Per1* peaks during the early morning, at approximately ZT1 (8am), and troughs at approximately ZT17 (12am) (Fig. 4A; one-way ANOVA: $F_{(5,33)} = 4.613$, $p < 0.01$; *post hoc* comparing each group revealed significant differences between ZT17 vs ZT1 ($p < 0.01$), ZT17 vs ZT5 ($p < 0.05$), and ZT17 vs ZT9 ($p < 0.05$), with no other significant comparisons). Further, learning-induced *Per1* expression was significantly higher in the RSC during the daytime than at night (two-way ANOVA, main effect of Day/Night ($F_{(1,33)} = 14.05$, $p < 0.001$) with no main effect of Timepoint or Interaction)). Learning-induced *Per1* therefore oscillates across the day/night cycle, peaking during the day and showing a trough at night. Notably, context fear memory is known to oscillate across the 24 h day and has been reported to peak during the day and trough at night (Chaudhury & Colwell, 2002), in tandem with the oscillation in retrosplenial *Per1* reported here.

As we conducted all of our previous context fear conditioning experiments at ZT5, when *Per1* levels are high, we reasoned that overexpression of *Per1* during the daytime might impair memory in male mice because we exceeded the ideal amount of *Per1* needed to support memory. If *Per1* in the RSC is tightly controlled by the circadian system, it is possible that both low and high levels of *Per1* can restrict memory formation. To test this idea, we assessed whether overexpressing *Per1* during the dark cycle (at ZT17), when endogenous *Per1* levels are lowest, would restore "daytime" levels of *Per1* and improve memory formation. Here, we injected HSV-CRISPRa into the RSC before training mice in context fear conditioning at ZT17. At test, we found that overall context freezing in control mice was slightly (but not significantly) reduced compared to daytime freezing levels (for males, compare controls in Fig. 3F with Fig. 4B; $t_{(d12} = 1.99$ $p = 0.07$; for females, compare controls in Fig. 3G with Fig. 4C; $t_{(10)} = 1.14$ $p = 0.28$), consistent with previous reports of reduced context fear conditioning during the dark cycle (Chaudhury & Colwell, 2002). We saw no effect of *Per1* overexpression on memory in either males (Fig. 4B; $t_{(11)} = 0.11$, $p = 0.91$; *Per1* sgRNA n = 8, control sgRNA n = 5) or females (Fig. 4C; $t_{(10)} = 0.41$ $p = 0.69$; *Per1* sgRNA n = 6, control sgRNA n = 6). This indicates that, contrary to our hypothesis, overexpressing *Per1* at night was unable to improve memory in either male or female mice.

4. Discussion

We tested the effect of *Per1* knockdown and overexpression within the anterior RSC on context fear memory in mice. Our results show that *Per1* is transiently upregulated in the RSC in response to a fear learning event. Further, we found that locally reducing *Per1* within the anterior RSC during the light cycle impairs context fear memory for both male and female mice. Surprisingly, we found that overexpression of *Per1* within the anterior RSC during the day also impairs context fear memory, but in a sex-dependent manner; only male mice showed impaired memory following *Per1* overexpression. Finally, we found that overexpressing *Per1* at night did not affect context fear memory in either male or female mice. Together, our findings demonstrate for the first time that *Per1* functions within the anterior RSC to regulate context fear memory, although its role appears to be more complex than in the dorsal hippocampus, where knockdown of *Per1* impairs spatial memory but overexpression improves memory (Kwapis et al., 2018). Further, our results suggest a sex-specific function of *Per1* within the RSC that should be explored in future studies.

Per1 expression has been positively linked with memory performance in the dorsal hippocampus, with increased hippocampal *Per1* improving memory in aging mice and reduced hippocampal *Per1* impairing memory in young mice (Kwapis et al., 2018). It is therefore not surprising that fear conditioning induces *Per1* expression within the RSC, a region critical for context fear memory (Kwapis et al., 2015; Todd et al., 2017), or that *Per1* knockdown within the RSC impairs fear memory formation. It was surprising, however, that overexpression of *Per1* within the RSC impaired memory formation in male mice. As *Per1* is a tightly regulated gene throughout many regions of the brain due to its critical role in establishing the circadian rhythm, any alteration (up or down) in its expression could potentially impair context fear memory. Our previous work has shown that overexpression of *Per1* within the hippocampus is sufficient to improve memory in aging mice (Kwapis et al., 2018), however, indicating that *Per1* may be more tightly regulated within RSC. Alternatively, it is possible that the aging brain benefited from *Per1* overexpression due to the aberrantly low endogenous levels of *Per1* during the daytime in the old hippocampus, although increasing *Per1* levels at night (when endogenous *Per1* is lowest) was still unable to improve memory in the current study. Finally, it is possible that *Per1* regulates memory through unique mechanisms in the RSC and hippocampus. In the hippocampus, *Per1* is known to shuttle p90RSK into the nucleus to enable CREB phosphorylation (Rawashdeh et al., 2016), potentially allowing diurnal oscillations in *Per1* to exert control over memory formation. In contrast, the mechanism through which *Per1* regulates memory formation within the RSC is not currently characterized. Although it is likely that *Per1* functions in a similar manner within the RSC, regulating CREB activity across the day/night cycle, this has yet to be demonstrated. It is possible that *Per1* plays unique roles in the hippocampus and RSC by regulating unique mechanisms in each structure. Future work should therefore aim to identify the specific pathways and transcripts regulated by *Per1* in the hippocampus, RSC, and other memory-relevant structures.

Our study also revealed sex differences in the requirement for retrosplenial *Per1* in context fear memory. While both males and females showed similar impairments in context fear memory following *Per1* knockdown, only males showed impairments following daytime *Per1* overexpression. Females, in comparison, were not affected by *Per1* overexpression in the RSC during either the day or night. This suggests that the *Per1* may function differently or may have different requirements in the RSC of male and female mice. Previous work has shown sex differences in *Per1* expression in response to different tasks, including the cold swim stress test (Bohacek, Manuella, Roszkowski, & Mansuy, 2015), food-anticipatory activity (FAA) (Li, Xu, Chen, Duan, & Zhao, 2015), and social interactions (Sonker & Singaravel, 2021). For example, Bohacek and colleagues (2015) show that *Per1* induction after the cold swim stress test is higher in females than in males and that this is independent of corticosterone and *Crh*, two mediators of the stress response. Females are also more susceptible to pathological fear responses (Ramikie & Ressler, 2018), suggesting that different mechanisms control fear and stress memories in male and female rodents. Our results suggest that male mice are more susceptible than females to alterations in *Per1* expression within the RSC, with overexpression impairing context fear memory in males, but not females. Identifying the differences in *Per1* function between sexes therefore remains an important topic for further study.

Most of the work on *Per1* in memory formation relies on global manipulations of *Per1* function, such as *Per1* knockout mice, which lack *Per1* within the brain's central pacemaker (the SCN) in addition to the hippocampus, RSC, and other brain and body tissue (Abarca, Albrecht, & Spanagel, 2002; Jilg et al., 2010; Rawashdeh et al., 2014; Rawashdeh et al., 2016; Sakai, Tamura, Kitamoto, & Kidokoro, 2004). Here, our viruses were targeted specifically to the RSC, without affecting *Per1* expression in the SCN. We can therefore be confident that our context fear memory impairments were specifically driven by altered *Per1* levels within RSC. Our previous work has shown that similar manipulations restricted to the dorsal hippocampus do not affect circadian sleep/wake activity patterns, which are largely regulated by the SCN. As our virus-based *Per1* manipulations here are restricted to the RSC, which does not play a known role in generating or regulating the circadian rhythm, it is unlikely that our effects on memory are due to circadian clock disruption, although this cannot be conclusively ruled out.

As the RSC is highly interconnected with other subregions of the brain, it would be interesting to examine whether alterations in *Per1* levels within one brain region affect the function of other brain regions and whether interregional crosstalk is affected. For example, hippocampal learning is dependent upon RSC efferents to influence other subregions of the brain (Bassett & Berger, 1982), suggesting that manipulations of *Per1* within the hippocampus could affect the downstream function of the RSC. Further elucidating the crosstalk between brain subregions during context fear learning and determining what role *Per1* might play in regulating these interactions could be an interesting avenue of study.

Interestingly, we found that both *Per1* and *cFos* levels were increased following either context fear conditioning or an immediate shock procedure intended to mimic shock exposure while minimizing learning of the context-shock association (Fig. 1B) (Blanchard, Fukunaga, & Blanchard, 1976; Jarome et al., 2021; Landeira-Fernandez, DeCola, Kim, & Fanselow, 2006). This indicates that the RSC might respond similarly to both associative context fear conditioning and shock exposure. As we sacrificed these animals for molecular studies, however, we were unable to test fear memory in these animals and cannot therefore rule out the possibility that our immediate shock condition did produce some learning of the context-shock association. Nonetheless, it is clear that exposure to a fearful event rapidly increases *cFos* mRNA expression and transiently increases *Per1* mRNA expression within the RSC.

In conclusion, our work demonstrates that the circadian gene *Per1* regulates context fear memory formation within the retrosplenial cortex in both male and female mice. Local knockdown of *Per1* within the RSC during the daytime impairs fear memory in both males and females whereas *Per1* overexpression during the day selectively impairs memory in male mice. Overexpression of retrosplenial *Per1* at night, however, had no effect on context fear memory. Together, our results demonstrate that *Per1* levels affect memory formation in the RSC in addition to its known role in regulating hippocampal memory formation, suggesting that *Per1* may play a broad role in regulating memory formation in memory-relevant structures across the brain.

CRediT authorship contribution statement

Mark W. Urban: Conceptualization, Methodology, Formal analysis, Investigation, Validation, Visualization, Writing – original draft, Writing – review & editing. **Chenyu Lo:** Conceptualization, Validation,

Visualization, Methodology, Formal analysis, Investigation. **Kasuni K. Bodinayake:** Conceptualization, Methodology, Formal analysis. **Chad A. Brunswick:** Formal analysis, Investigation. **Shoko Murakami:** Methodology, Formal analysis, Investigation. **Ashley C. Heimann:** Formal analysis, Investigation. **Janine L. Kwapis:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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