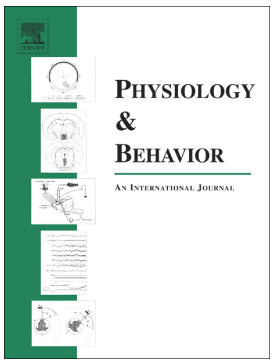

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Modulation of learning and memory by the genetic disruption of circadian oscillator populations

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Abstract

While a rich literature has documented that the efficiency of learning and memory varies across circadian time, a close survey of that literature reveals extensive heterogeneity in the time of day (TOD) when peak cognitive performance occurs. Moreover, most previous experiments in rodents have not focused on the question of discriminating which memory processes (e.g., working memory, memory acquisition, or retrieval) are modulated by the TOD. Here, we use assays of contextual fear conditioning and spontaneous alternation in WT (C57Bl/6J) mice to survey circadian modulation of hippocampal-dependent memory at multiple timescales – including working memory (seconds to a few minutes), intermediate-term memory (a delay of thirty minutes), and acquisition and retrieval of long-term memory (a delay of two days). Further, in order to test the relative contributions of circadian timing mechanisms to the modulation of memory, a parallel set of studies were performed in mice lacking clock timing mechanisms. These transgenic mice lacked the essential circadian gene *Bmal1*, either globally (*Bmal1* null) or locally (floxed *Bmal1* mice which lack *Bmal1* in excitatory forebrain neurons, e.g. cortical and hippocampal neurons). Here, we show that in WT mice, retrieval (but not working memory, intermediate-term memory, or acquisition of long-term memory) is modulated by TOD. However, transgenic mouse models lacking *Bmal1* – both globally, and only in forebrain excitatory neurons – show deficits regardless of the memory process tested (and lack circadian modulation of retrieval). These results provide new clarity regarding the impact of TOD on hippocampal-dependent memory and support the key role of hippocampal and cortical circadian oscillations in circadian gating of cognition.

Keywords

Bmal1, hippocampus, learning, memory, circadian

1. Introduction

Over the last half a century, it has become increasingly clear that circadian rhythms – approximately 24-hour biological oscillations – exert a profound influence on nearly every aspect of mammalian physiology and behavior. Memory is no exception; numerous studies have documented circadian modulation of learning and memory in organisms ranging from the sea slug *Aplysia* [1,2] to *Homo sapiens* [3,4], and in tasks ranging from working memory [5–8] and passive avoidance [9,10] to hippocampal-dependent visuospatial memory [5,11–14] and novel object recognition [14–16]. However, as documented in our recent review [17], considerable variability in the TOD of peak performance has been reported in various memory assays. For example, of the cited studies here that used nocturnal rodents, seven reported peak performance during the night [5,6,8,13–16] while four reported peak performance during the day [7,9,11,12]. Inter-study variability, such as the animal models used, assay methods, and lighting conditions, make it difficult to formulate a general hypothesis as to when circadian modulation gates memory performance. Moreover, the various models available to disrupt circadian rhythms (e.g., lesioning of the suprachiasmatic nucleus (SCN), a region responsible for setting the circadian phase of the organism, or KO of circadian genes like *Bmal1* or *Per1*) also result in varying effects on learning and memory [17], with some models and assays resulting in substantial deficits in memory [13,16,18–20] and other model-assay combinations resulting in WT-like memory performance [19–22].

Here, we combine a memory-process-specific test of two well-validated hippocampal-dependent memory assays, spontaneous alternation and contextual fear conditioning, with transgenic conditional and germline *Bmal1* knockout mouse models. Of note, BMAL1 is a critical component of the core clock transcription / translation feedback loop, upon which the circadian timing system is built. As such, the deletion of *Bmal1* results in a loss of circadian time keeping capacity [16,23–25]. *Bmal1* germline KO (gKO) animals are completely arrhythmic [20]. By contrast, *Bmal1* conditional KO (cKO) animals lack *Bmal1* only in excitatory forebrain neurons (e.g. cortical and hippocampal neurons); they maintain both *Bmal1* expression in the SCN and WT-like locomotor rhythms [13]. As such, if we were to observe deficits in the *Bmal1* gKO (but not the *Bmal1* cKO), that would indicate that the circadian clock in forebrain excitatory neurons is not necessary for that particular memory process. However, deficits in the *Bmal1* cKO that are similar to those seen in *Bmal1* gKO animals indicate that cell-autonomous circadian rhythms in forebrain excitatory neurons are necessary for effective memory performance. Moreover, by

using multiple memory-process-specific assays within the same set of models and testing conditions, we are able to draw conclusions about the relative contribution of the circadian timing system to the modulation of these specific memory processes. Thus, a key goal of this study was to further our understanding of the interaction of circadian time, circadian clocks in forebrain excitatory neurons, and the neurobiological processes that underlie memory.

2. Materials and Methods

2.1 Mice

Behavioral assays compared WT (C57Bl6/J mice) to *Bmal1* conditional knockout (a.k.a. forebrain knockout) mice [13] and to *Bmal1* germline knockout mice [23]. Briefly, while the *Bmal1* germline knockout (gKO) mouse model has total knockout of *Bmal1*, the *Bmal1* conditional knockout (cKO) mouse model utilized a Cre-Lox system where a *CaMKII-Cre* transgene targeted loxP sites to delete the bHLH exon of the *Bmal1* gene in excitatory forebrain neurons (including the hippocampus, prefrontal cortex, and amygdala, but sparing the hypothalamus and SCN). All assays used adult mice (at least 7 weeks old) of both sexes (sex distributed evenly in all groups). All procedures were approved by the Ohio State University IACUC.

2.2 Lighting and timing conditions

Animals were entrained to a 12 hour light: 12 hour dark (LD) cycle for at least 21 days before undergoing behavioral assays. For all experiments, zeitgeber time (ZT) is used to refer to LD conditions (such that lights on time is ZT0) and circadian time (CT) is used to refer to conditions of total darkness (DD, such that CT0 is equivalent to the animal's most recent ZT0). For all behavioral assays, mice were dark-adapted (moved to total darkness at ZT0) two days before beginning the assay. Testing was conducted under 1 lux dim red light to avoid both the phase-shifting effects of white light on the circadian clock [26,27] as well as the masking effect wherein white light reduces the activity of nocturnal rodents [28,29]. Animals were moved to the testing room in a light-tight shuttle box.

2.3 Overall behavioral design

In order to identify the memory processes that are regulated by TOD, we developed a between-subjects design that explicitly tested the impact of early day (CT4) vs early night (CT16) on working memory, intermediate-term

memory, long-term memory acquisition, and long-term memory retrieval (Fig. 1). First, all animals were entrained for at least 21 days to a 12 h light / 12 h dark cycle. On day 1 of testing, animals were transitioned to total darkness (DD) (Fig. 1B-D). To test working memory (on the order of several seconds (Barak and Tsodyks, 2014)) and intermediate-term memory (here, memory with a training – testing delay of 30 minutes, sometimes called short-term memory [30–32]), animals were trained and tested on day 2, at either CT4 or CT16 (Fig. 1B). To test the TOD effect of acquisition (i.e. how well the memory is initially stored), animals were trained at either CT4 or CT16 on day 2, and then tested at CT10 on day 4 (Fig. 1C). Finally, to test the TOD effect of memory retrieval, animals were trained at CT10 on day 2, and then tested at either CT4 or CT16 on day 4 (Fig. 1D). These time points resulted in long-term memory delays of either 42 hours (CT16 train to CT10 test, or CT10 train to CT4 test) or 54 hours (CT4 train to CT10 test, or CT10 train to CT16 test). The disparity in delays was necessary in order to clearly separate effects due to TOD of acquisition vs TOD of retrieval.

Additionally, we compared these same memory-process specific assays in WT animals to both the *Bmal1* cKO (a model that lacks circadian rhythms in excitatory forebrain neurons, including the cortex and hippocampus, but maintains typical locomotor rhythms [13,16]) and the *Bmal1* gKO (a model that lacks any circadian rhythm [23]). Thus, we are able to distinguish the impact of the circadian rhythm, both globally and locally (within forebrain excitatory neurons), on learning and memory.

2.3.1 Spontaneous Alternation

This procedure was adapted from [33]. Mice were placed in the Y maze (a maze with three equally-sized arms, each 8 cm wide and 40 cm long) for 8 minutes. Location of the mouse was recorded and scored by Noldus Ethovision XT 11.5 software (Noldus Ethovision Technology, The Netherlands). An alternation was considered any three entries with no repeats of the same arm (e.g. A, B, C or C, A, B; but not C, A, C or C, B, B). Percent alternation was scored as the number of alternations divided by the total possible alternations; total possible alternations was the total number of entries minus two.

2.3.2 Contextual Fear Conditioning

Mice were gently handled for three days before fear conditioning. For conditioning, mice were first placed in a fear conditioning arena with shapes on the walls and a wire floor. Mouse movement and freezing was recorded

and scored by Noldus Ethovision XT 11.5 software. After three minutes of habituation, the mice received one, 0.1 mAmp, electric shock for 1.0 s. The mice were removed from the box and returned to their home cages after 4 minutes had passed in total (3 minutes before the shock and 1 minute after). The intermediate-term memory test occurred after a 30-minute delay; mice were placed in the conditioning box and their movement was recorded for five minutes. At the end of the five minute test, the mice received two 0.2 mA, 2 second, shocks with inter-shock delays of 20s; after a total of six minutes (five minutes before the shock and one minute after), the mice were returned to their home cages. This second conditioning was used to prevent the intermediate-term memory test from producing any extinction effect [34]. The long-term memory test occurred two days after the initial fear conditioning. Here, mice were placed in the conditioning box for five minutes and their movement was recorded. All mice undergoing long-term memory tests had gone through the initial fear conditioning, the intermediate-term memory test, and the second conditioning.

2.4 Statistics

All graphs show data as mean ± standard error of the mean (SEM). Statistical analyses were conducted in Prism 3.0 (with the exception of two-way ANOVAs, which were conducted in IBM SPSS Statistics 24). We determined the following analyses between groups *a priori*: in behavioral assays with the genotype x TOD design, we would analyze by two-way ANOVA with predetermined sets of Bonferroni multiple-comparison tests (each to answer a different question) based on the ANOVA output. If a significant interaction was found (or effect of both genotype and TOD without an interaction), three sets of Bonferroni multiple comparisons would determine effects of genotype during day, effects of genotype during night, and effects of TOD within genotype. If only genotype was significant, then only one set of Bonferroni post hocs on genotype difference would be done. If only TOD was significant, then only the one Bonferroni post hoc set on TOD differences within genotype would be done. Outliers were identified by Grubbs test.

3. Results

3.1 Spontaneous Alternation

We first measured working memory (memory on the order of seconds to a few minutes). Here, we used the y-maze, a Y-shaped maze with three arms, and measured hippocampal-dependent spontaneous alternation (the preference of

the mouse to enter the most novel arm and minimize repeat entries) [35]. As in Fig. 1B, animals were tested at either CT4 or CT16 following two days of dark adaptation. While we found no differences in spontaneous alternation by TOD or interaction of genotype with TOD (Fig. 2A; two-way ANOVA, respectively $F_{(1,111)} = 0.003$, $p = 0.955$; $F_{(2,111)} = 0.388$, $p = 0.679$), we did find an effect of genotype (two-way ANOVA, $F_{(2,111)} = 7.470$, $p = 0.001$). By Bonferroni post-hoc test, we found that *Bmal1* gKO animals showed significantly decreased spontaneous alternation compared to WT animals ($t_{(92)} = 3.829$, $p = 0.0006$), while *Bmal1* cKO animals showed intermediate spontaneous alternation and were not significantly different from either WT ($t_{(83)} = 1.984$, $p = 0.1518$) or *Bmal1* gKO animals ($t_{(53)} = 1.277$, $p = 0.6216$). Of note, while there were no effects of TOD or interactions of genotype and TOD on total entries (Fig. 2B; two-way ANOVA, respectively $F_{(1,111)} = 0.421$, $p = 0.518$; $F_{(2,111)} = 1.438$, $p = 0.242$), there was an effect of genotype ($F_{(2,111)} = 31.736$, $p < 0.001$). Here, *Bmal1* gKO animals showed significantly more entries than both WT (Bonferroni correction, $t_{(92)} = 8.04$, $p = 0.0003$) and *Bmal1* cKO animals ($t_{(53)} = 4.474$, $p = 0.0003$), while WT and *Bmal1* cKO animals had similar numbers of entries ($t_{(83)} = 2.159$, $p = 0.1011$).

3.2 Contextual Fear Conditioning

First, we measured baseline freezing during the three minutes animals were in the context before the initial shock (Fig. 1B). Baseline freezing (Fig. 3A) was not significantly different between groups (two-way ANOVA, no significant effect of TOD, $F_{(1,64)} = 2.3933$, $p = 0.127$, genotype, $F_{(2,64)} = 2.782$, $p = 0.069$, or interaction, $F_{(2,64)} = 1.333$, $p = 0.271$). Next, to test the effect of TOD on intermediate-term memory, animals underwent fear conditioning (a single 0.1mA electric footshock following three minutes of habituation to the context) on day 2 at CT4 or CT16, followed by retrieval 30 minutes later (Fig. 1B). This minimally aversive paradigm was used to maximize any potential circadian differences in intermediate-term memory efficiency. Freezing during the retrieval test 30 minutes later (Fig. 3B) differed significantly by genotype (two-way ANOVA $F_{(2,74)} = 73086$, $p = 0.002$) but not by TOD or interaction of TOD with genotype (respectively, $F_{(1,74)} = 0.160$, $p = 0.691$; $F_{(2,74)} = 0.675$, $p = 0.512$). Here we found that *Bmal1* cKO and *Bmal1* gKO animals had a reduction of freezing of about 11% of the total trial time when compared to WT animals (Bonferroni correction, respectively $t_{(61)} = 3.459$, $p = 0.003$ and $t_{(47)} = 5.259$, $p = 0.003$). No difference was seen between *Bmal1* cKO and *Bmal1* gKO animals (Bonferonni correction, $t_{(41)} = 0.03046$, $p = 1.000$). Finally, given that our *Bmal1* cKO mouse model utilizes a Cre-lox system, and that some Cre transgenes have been shown to cause deleterious effects due to Cre toxicity [36], we also tested WT mice versus

sibling controls with only the *Cre-CaMKII* transgene (lacking the floxed *Bmal1* allele) at a single TOD (CT4). We found no effect of the *Cre-CaMKII* transgene alone on intermediate-term memory (Fig. 3C, $t_{(16)} = 0.3141$, $p = 0.7575$), indicating that memory deficits in the *Bmal1* cKO model are due to conditional loss of *Bmal1* and not Cre toxicity.

Next, we tested for effects of the TOD of acquisition on long-term memory. Animals underwent contextual fear conditioning at either CT4 or CT16 (Fig. 1C); two days later, they were all tested for fear memory at CT10 (a timepoint equidistant between CT4 and CT16). We hypothesized that if memory encoding is enhanced at either CT4 or CT16, we would observe differences in freezing by the TOD at which the animal was trained. Similarly to our results in intermediate-term memory, we found that differences in freezing were not due to any difference by TOD or interaction of genotype with TOD (Fig. 4A, two-way ANOVA, respectively $F_{(1,70)} = 0.001$, $p = 0.972$; $F_{(2,70)} = 0.480$, $p = 0.621$). However, we did find strong differences due to genotype ($F_{(2,70)} = 13.953$, $p < 0.001$); WT freezing overall was significantly higher when compared to both *Bmal1* cKO animals (Bonferroni correction, $t_{(58)} = 3.471$, $p = 0.003$) and *Bmal1* gKO animals (Bonferroni correction, $t_{(47)} = 5.259$, $p = 0.0003$), but not significantly different between *Bmal1* cKO animals and *Bmal1* gKO animals (Bonferroni correction, $t_{(41)} = 2.223$, $p = 0.0954$).

Finally, we tested for effects of the TOD of retrieval on long-term memory. Animals underwent contextual fear conditioning at CT10 (Fig. 1D) then were tested for long-term memory two days later at either CT4 or CT16. This resulted in an interaction of TOD and genotype in freezing (Fig. 4B, two-way ANOVA, effect of interaction $F_{(2,67)} = 4.023$, $p = 0.022$; effects of genotype alone $F_{(2,67)} = 14.309$, $p < 0.001$, effects of TOD alone $F_{(1,67)} = 1.370$, $p = 0.246$). We observed a TOD difference in WT animals with high freezing when retrieval occurred at CT4 compared to CT16 (Bonferonni correction, $t_{(23)} = 3.135$, $p = 0.0138$) but no TOD difference in *Bmal1* cKO animals (Bonferonni correction, $t_{(30)} = 0.3775$, $p = 1.000$) or *Bmal1* gKO animals (Bonferonni correction, $t_{(14)} = 0.4536$, $p = 1.000$). We then performed planned comparisons of genotype within each TOD. Notably, during the day, WT animals displayed significantly more freezing than *Bmal1* cKO animals (Bonferonni correction, $t_{(20)} = 3.922$, $p = 0.0003$) or *Bmal1* gKO animals (Bonferonni correction, $t_{(19)} = 5.327$, $p = 0.0003$). There was no daytime difference between *Bmal1* cKO animals and *Bmal1* gKO animals (Bonferonni correction, $t_{(15)} = 1.426$, $p = 0.5229$)). However, at night, all genotypes showed similar levels of freezing (Bonferroni correction, WT vs *Bmal1* cKO $t_{(33)} = 0.8359$, $p = 1.000$; WT vs *Bmal1* gKO $t_{(18)} = 1.998$, $p = 0.1833$, *Bmal1* cKO vs *Bmal1* gKO $t_{(29)} = 1.497$, $p = 0.4356$).

4. Discussion

The data reported here indicate that the gating of contextual fear conditioning in WT mice is limited to retrieval; working memory, intermediate-term memory, and acquisition showed no difference between CT4 and CT16. Notably, previous studies have shown circadian gating of contextual fear conditioning memory [11,12,20]. However, our design furthered the differentiation of TOD effects on different memory processes. Importantly, rodents can learn to discriminate between contexts based on the time of day [37], as well as show a peak in retrieval 24 hours following training [10]. Thus, training 24 hours before a retrieval timepoint (as in [12,20]) can confound identification of retrieval effects as distinct from acquisition effects. Moreover, we avoided repeated testing of fear conditioning [11]. As a test constitutes exposure to the context without a footshock, repeated tests complicate the potential interpretation of retrieval results with extinction, a process that has also been observed to be gated by circadian rhythms [11,38].

Further, our data suggest that a circadian clock in the hippocampus and other forebrain excitatory regions is necessary to display circadian modulation of CFC retrieval, as conditional *Bmal1* KO animals showed a complete abrogation of circadian modulation, even for an assay where WT mice displayed very strong circadian modulation (Fig. 3B). The lack of circadian modulation we observed in germline *Bmal1* KO animals (similarly to what we observed in conditional *Bmal1* KO animals) is consistent with previous studies in this model [20]. Moreover, our data also suggest that the molecular circadian clock plays roles in learning and memory that are necessary for non-TOD dependent tasks (given the deficits on spontaneous alternation, intermediate-term memory, and acquisition in *Bmal1* knockout models). The deficits in all behavioral tasks except spontaneous alternation were very similar between conditional and germline *Bmal1* KO mice, suggesting that the molecular circadian clock local to cortical and subcortical regions is a necessary link; loss of *Bmal1* in these regions is sufficient to cause deficits comparable to germline KO. This is seen despite the fact that conditional *Bmal1* KO mice are phenotypically normal in regards to physiology and affect [13,16] while the germline *Bmal1* KO model displays widespread deleterious effects (such as early mortality, small size, and an inability to reproduce [39–41]).

Moreover, our experimental protocol follows a design that is optimized for detecting the impact of circadian rhythms on cognition. Importantly, we utilized dim red light (1 lux) during all behavioral tests, and maintained animals in total darkness from two days before testing until the conclusion of testing. White light, even

dim white light, contains blue/green wavelengths that can activate intrinsically-photosensitive retinal ganglion cells and disrupt or phase-shift the circadian clock [26,27,43,44]. Thus, using dim red light allowed animals to learn the task without disruption of circadian rhythms by white light. Further, as using white light during day and dim red light during night could lead to differences in activity due to masking [28,29], we used dim red light at all timepoints.

Additionally, while caution should always be used when considering a negative finding (here, a lack of TOD differences in working memory, intermediate-term memory, and acquisition of long-term memory), our design maximized our ability to detect a TOD effect by using a minimally aversive design. We used low footshock stimulus, a 0.1 mA, 1s shock for the intermediate-term conditioning and 0.2 mA, 2s shocks for the long-term conditioning. This was minimal enough that ~24% of WT animals in the intermediate-term memory test and ~21% of WT animals in the test of acquisition of long-term memory displayed freezing less than 15% (a level commonly seen in animals that are repeatedly returned to a context without any aversive stimulus [45,46]). This is important because circadian modulation of memory in WT animals is often a relatively small effect, constituting only a fractional influence on memory performance. Thus, we posited that designing this task to be difficult enough that a portion of the animals "fail" the task (displaying low levels of freezing) would enhance the likelihood of observing small effects and avoid a "ceiling" effect where the stimulus is so strong that all animals learn (as a ceiling effect could preclude the detection of relatively small clock-gated components of memory). It is worth noting that while we found no effect of TOD on the spontaneous alternation assay, Ruby et al. [8] have previously shown enhanced spontaneous alternation on the T-maze at night in hamsters. Here, our negative finding is likely not due to ceiling effects, as only 22% of our WT animals showed spontaneous alternations of 40-60% (or about chance performance). More likely, we observed different results due to differences in experimental design (Ruby et al. used white light, and tested each animal twice, first during day and then during night; Ruby et al. used a T-maze while we used a Y-maze) or species differences (Ruby et al. used hamsters).

It is possible, especially in regards to the *Bmal1* gKO animals, that the lack of circadian modulation is in part due to a lack of acquisition (i.e., an animal that has no memory whatsoever for a task cannot show circadian modulation of memory). Notably, *Bmal1* gKO animals demonstrated higher total entries on the spontaneous alternation task (Fig. 2B). This is consistent with a previous study that found *Bmal1* gKO animals continued to

show active exploration of an open field even when WT animals had reduced exploratory activity due to habituation [42]. This, together with the very low levels of freezing of *Bmal1* gKO animals on all of the fear conditioning assays, may indicate that they are unable to learn these tasks under our conditions. However, ~45% of the *Bmal1* cKO animals demonsrated freezing of greater than 15% on the long-term memory task, indicating that a substantial subset of animals had some memory of the task. Thus, we expect that the lack of TOD modulation of *Bmal1* cKO mice is not primarily due to a lack of memory.

Despite the strengths of our behavioral testing paradigm, several limitations in our approach are worth noting. Our tests were conducted at two times of day (CT4 and CT16), which, while commonly found to be peaks and troughs of circadian effects on learning and memory [5,12,13,16], are not sufficient to rule out a circadian rhythm. In the assays where we did not detect any TOD differences, it is conceivable that this was simply due to our assays being out of phase with peaks and troughs of memory performance. If, for example, a peak in memory efficiency was present at CT10 and a trough at CT22, CT4 and CT16 could be similar in memory performance and a clock effect could simply have gone undetected. Thus, our results should be interpreted strictly as pertaining to these two circadian times in our model. Additionally, sleep is a process that is well-known to impact learning and memory [47,48], and by the very nature of circadian experiments, one circadian time has a different place in the sleep-wake cycle than another circadian time. (e.g., CT4 is early in the sleep phase for nocturnal rodents, and CT16 is early in the wake phase). However, we would predict that disruption of sleep by the training protocol was unlikely to alter our TOD results; all of our long-term memory paradigms allowed mice at least one uninterrupted sleep period between the initial training and the test. It is perhaps more difficult to disentangle sleep-related deficits in the transgenic models. Along these lines, sleep in the germline *Bmal1* KO mouse has been shown to be less consolidated than that of WT mice [40]. The sleep phenotype of *Bmal1* cKO has not been examined; however, these mice display consolidated activity rhythms in constant dark [13,16], indicating likely consolidation of sleep to the inactive period. Nonetheless, given the widespread impact of the circadian clock on cellular excitability (Barnes et al., 1977; Besing et al., 2017; Chaudhury et al., 2005; Eckel-Mahan et al., 2008; Nakatsuka and Natsume, 2014; Phan et al., 2011; Shimizu et al., 2016), we would anticipate that the memory deficits in these transgenic models are not solely or primarily due to sleep disruption.

Together, this dataset indicates that circadian-gated cellular events modulate the efficiency of learning and memory, especially intermediate- and long-term memory. Moreover, it highlights the importance of using memory assays that will differentiate between circadian effects that modulate acquisition and/or retrieval. The tested memory processes – working memory, intermediate-term memory, acquisition of long-term memory, and retrieval - utilize fairly disparate molecular mechanisms [49,50]. As such, these findings speak to the powerful and far reaching effects that the circadian timing system has on cognition. There has been important progress on our understanding of the role that the circadian clock plays in learning and memory; to further these studies, work comparing novelty-motivated vs. fear-motivated learning assays, and the circadian oscillations of cellular excitability that may modulate these behaviors in the hippocampus and amygdala, is highly merited.

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Declarations of interest: none

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Figure Legends:

Fig. 1. Experimental design of behavioral assays. A) Key: white and black rectangles indicate, respectively, light and dark conditions under a 12 hour light / 12 hour dark light schedule. Light gray and dark gray rectangles indicate, respectively, subjective day and subjective night under total darkness (DD). Arrows indicate training times, while triangles indicate testing times. B) Working memory (spontaneous alternation) and intermediate-term memory (CFC) assays. Animals were dark adapted on day 1. On day 2, they underwent either the spontaneous alternation test, or fear conditioning with testing following a 30-minute delay. C) Assay to test TOD impact on memory acquisition of the long-term memory. Animals were dark adapted on day 1. On day 2, they underwent fear conditioning at either CT4 or CT16. Two days later (day 4), memory was tested at CT10. D) Timeline to test TOD effects on memory retrieval of the long-term memory. Animals were dark adapted on day 1. On day 2, they underwent fear conditioning at CT10. Two days later (day 4), memory was tested at either CT4 or CT16.

Fig. 2. Spontaneous alternation. A) Spontaneous alternation of WT, Bmal1 cKO, and Bmal1 gKO mice at CT4 or CT16 on the y-maze (N, respectively, = 31, 31, 11, 12, 17, 15). B) Total entries of WT, Bmal1 cKO, and Bmal1 gKO mice at CT4 or CT16 on the y-maze (N, respectively, = 31, 31, 11, 12, 17, 15). *, $p < 0.05$; n.s., $p > 0.05$; bars indicate genotype comparisons; no TOD differences found.

Fig. 3. Contextual fear conditioning – baseline freezing and intermediate-term memory. A) Baseline freezing of WT, Bmal1 cKO, and Bmal1 gKO mice in the context before fear conditioning at CT4 or CT16 (N = 14, 14, 13, 14, 5, 10). B) Freezing response of WT, Bmal1 cKO, and Bmal1 gKO mice following conditioning at CT4 or CT16, with a 30-minute delay between training and retrieval (N = 18, 16, 14, 15, 7, 10). C) Freezing response of WT mice with or without the Cre-CaMKII gene following conditioning at CT4 with a 30-minute delay between training and retrieval (N = 9, 9). *, $p < 0.05$; n.s., $p > 0.05$; bars indicate genotype comparisons; no TOD differences found.

Fig. 4. Contextual fear conditioning – long-term memory. A) Freezing response of WT, Bmal1 cKO, and Bmal1 gKO mice (here, CT indicates time of acquisition; all animals tested at CT10; N = 17, 16, 13, 14, 6, 10). B) Freezing response of WT, Bmal1 cKO, and Bmal1 gKO mice (here, CT indicates time of retrieval; all animals underwent fear

conditioning at CT10; N = 13, 12, 9, 23, 8, 8). *, $p < 0.05$; n.s., $p > 0.05$; long bars indicate genotype comparisons, short bars indicate TOD comparisons; no TOD difference seen in panel A; for panel B, the letters A, B indicate genotype differences at CT4, and C indicates no genotype differences at CT16.

Highlights:

- Circadian modulation of learning and memory was assayed over multiple time periods.
- In WT mice, clock time modulated long-term memory retrieval.
- Disruption of hippocampal oscillators blocked the circadian modulation of memory retrieval.

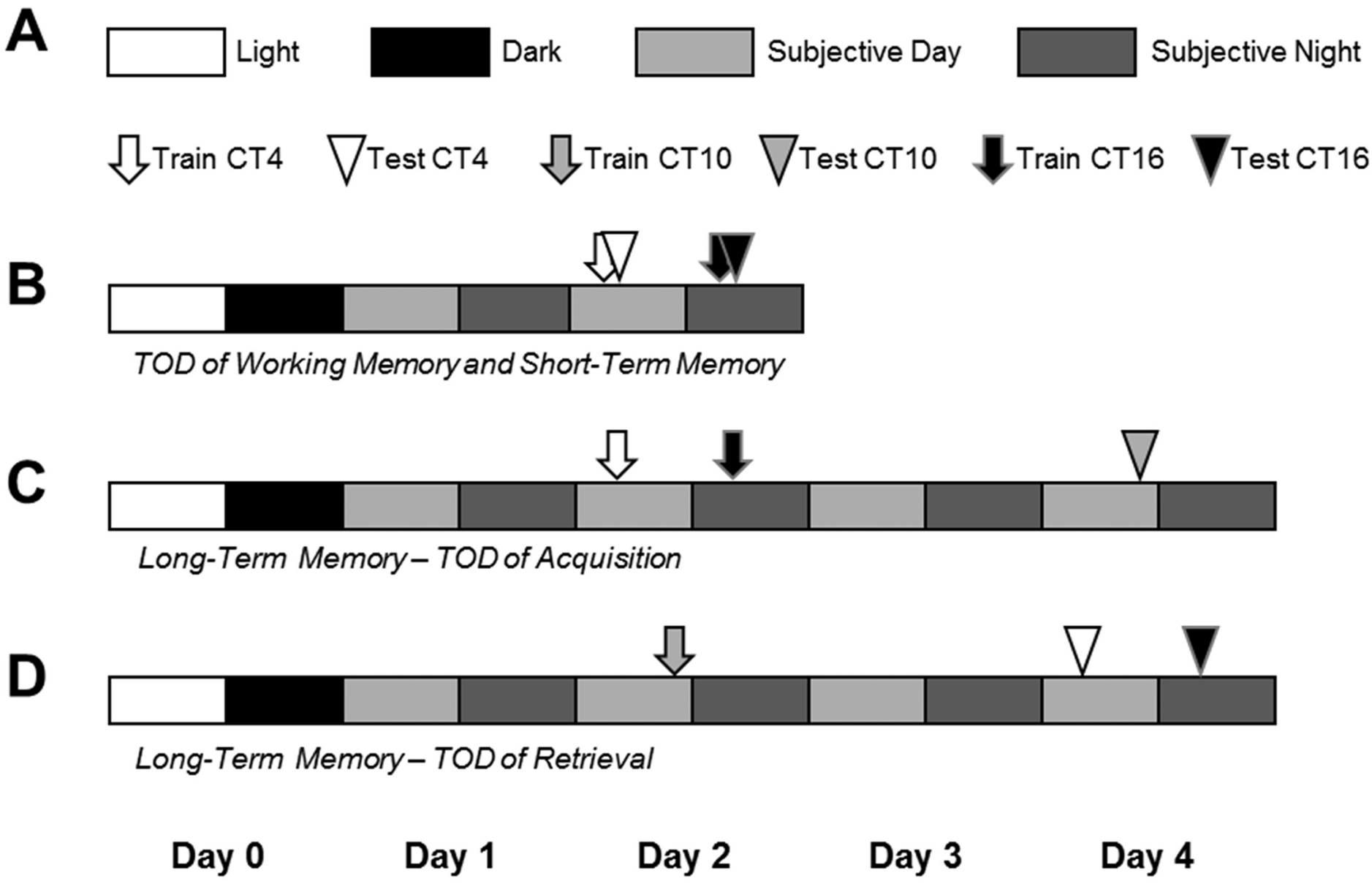
A
Light
Dark
Subjective Day
Subjective Night
Train CT4
Test CT4
Train CT10
Test CT10
Train CT16
Test CT16
B
TOD of Working Memory and Short-Term Memory
C
Long-Term Memory – TOD of Acquisition
D
Long-Term Memory – TOD of Retrieval
Day 0
Day 1
Day 2
Day 3
Day 4

Figure 1

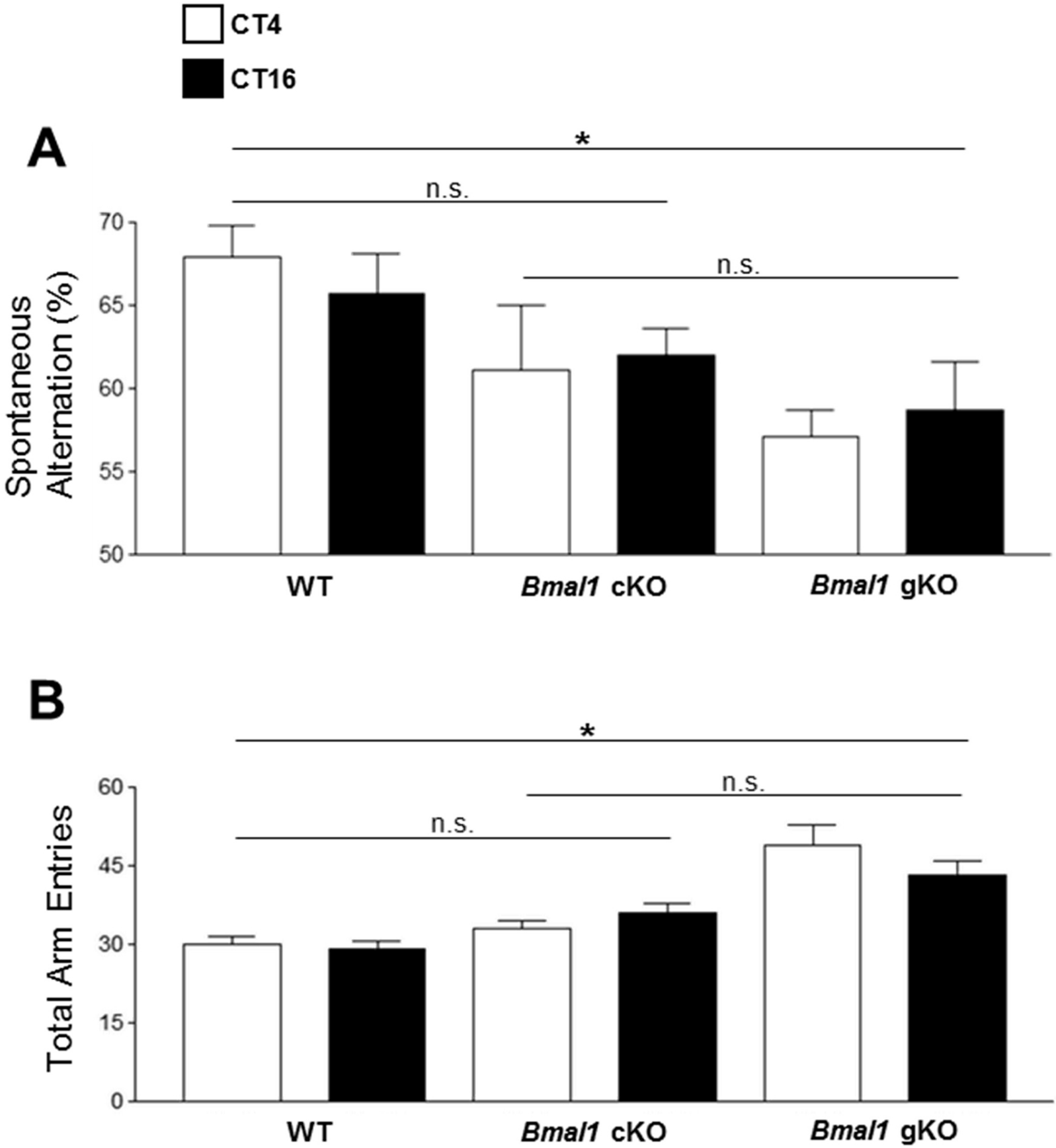
CT4
CT16
A
*
n.s.
n.s.
70
65
60
55
50
Spontaneous Alternation (%)
WT
Bmal1 cKO
Bmal1 gKO
B
*
n.s.
n.s.
60
45
30
15
0
Total Arm Entries
WT
Bmal1 cKO
Bmal1 gKO

Figure 2

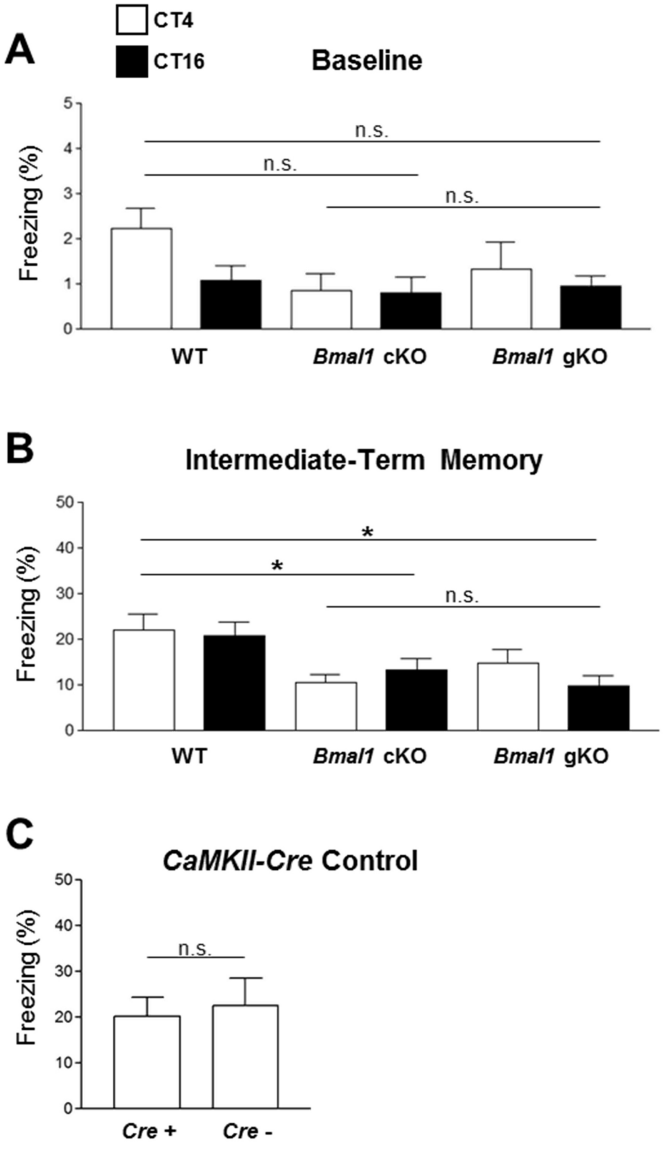
A
CT4
CT16
Baseline
Freezing (%)
n.s.
n.s.
n.s.
WT
Bmal1 cKO
Bmal1 gKO
B
Intermediate-Term Memory
Freezing (%)
*
*
n.s.
WT
Bmal1 cKO
Bmal1 gKO
C
CaMKII-Cre Control
Freezing (%)
n.s.
Cre +
Cre -

Figure 3

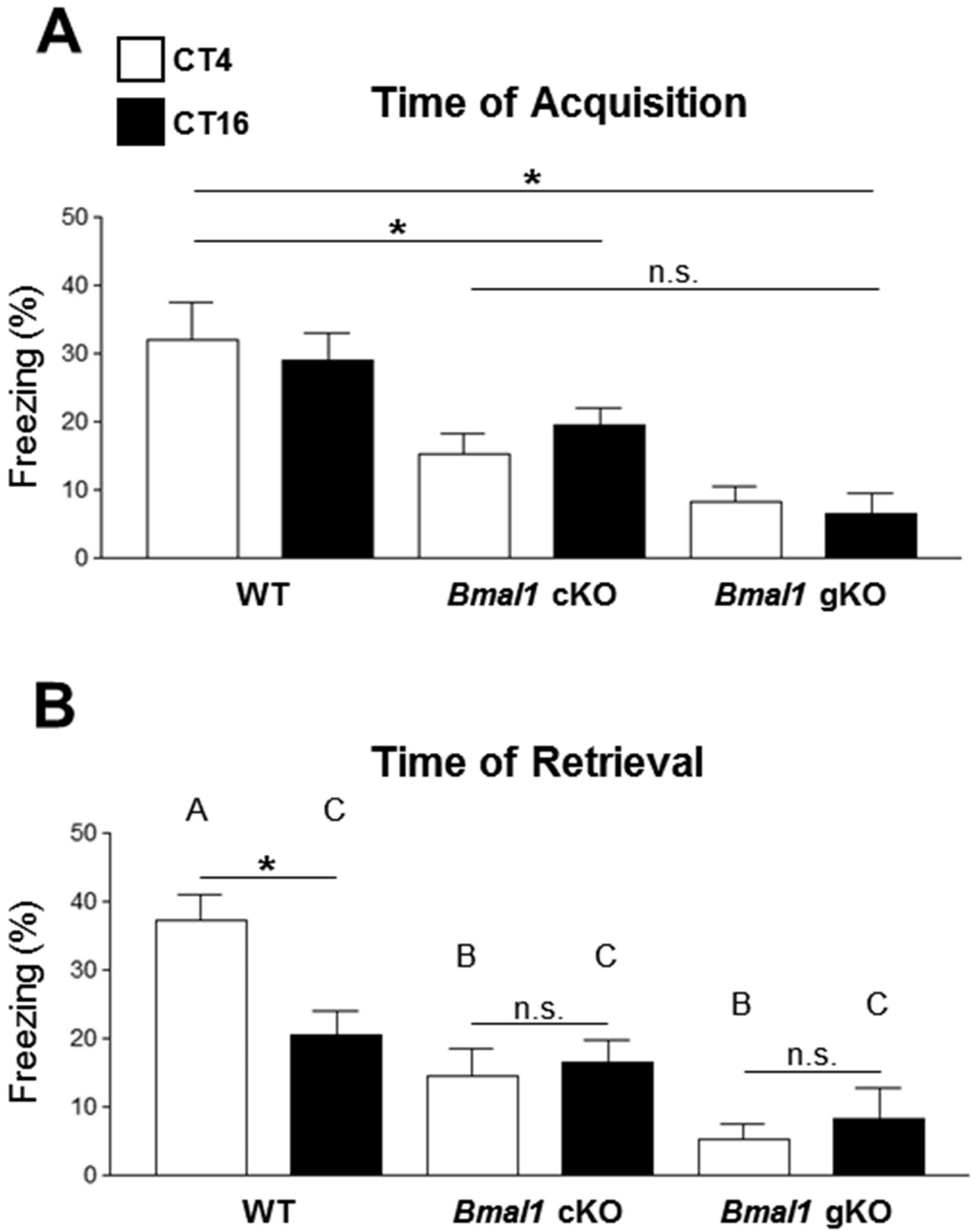
A
CT4
CT16
Time of Acquisition
*
*
n.s.
Freezing (%)
50
40
30
20
10
0
WT
Bmal1 cKO
Bmal1 gKO
B
Time of Retrieval
A
C
*
B
C
n.s.
B
C
n.s.
Freezing (%)
50
40
30
20
10
0
WT
Bmal1 cKO
Bmal1 gKO

Figure 4