

SPECIAL ISSUE: BEHAVIORAL NEUROSCIENCE OF SLEEP

Short-Term Total Sleep Deprivation Alters Delay-Conditioned Memory in the Rat

Shweta Tripathi and Sushil K. Jha
Jawaharlal Nehru University

Short-term sleep deprivation soon after training may impair memory consolidation. Also, a particular sleep stage or its components increase after learning some tasks, such as negative and positive reinforcement tasks, avoidance tasks, and spatial learning tasks, and so forth. It suggests that discrete memory types may require specific sleep stage or its components for their optimal processing. The classical conditioning paradigms are widely used to study learning and memory but the role of sleep in a complex conditioned learning is unclear. Here, we have investigated the effects of short-term sleep deprivation on the consolidation of delay-conditioned memory and the changes in sleep architecture after conditioning. Rats were trained for the delay-conditioned task (for conditioning, house-light [conditioned stimulus] was paired with fruit juice [unconditioned stimulus]). Animals were divided into 3 groups: (a) sleep deprived (SD); (b) nonsleep deprived (NSD); and (c) stress control (SC) groups. Two-way ANOVA revealed a significant interaction between groups and days (training and testing) during the conditioned stimulus–unconditioned stimulus presentation. Further, Tukey post hoc comparison revealed that the NSD and SC animals exhibited significant increase in performances during testing. The SD animals, however, performed significantly less during testing. Further, we observed that wakefulness and NREM sleep did not change after training and testing. Interestingly, REM sleep increased significantly on both days compared to baseline more specifically during the initial 4-hr time window after conditioning. Our results suggest that the consolidation of delay-conditioned memory is sleep-dependent and requires augmented REM sleep during an explicit time window soon after training.

Keywords: amygdala, associative memory, learning, REM sleep, sleep deprivation

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There is increasing evidence suggesting that sleep helps in memory consolidation. The role of sleep in memory consolidation has been investigated by performing sleep deprivation and polysomnographic recordings (to characterize changes in sleep architecture) after learning, both in rodents and humans (for review see Stickgold, 2005; Walker & Stickgold, 2004). In several studies, it has been demonstrated that short-term deprivation of either total sleep or REM sleep alone, soon after training, induces memory deficit. For example, total sleep deprivation soon after training impairs memory of object recognition task (Palchykova et al., 2006), five-choice serial reaction time task (an animal model of

attention deficit disorder; Cordova et al., 2006), spatial learning task (Alzoubi et al., 2012), and associative learning tasks (Chowdhury et al., 2011; Graves et al., 2003; Kumar & Jha, 2012). REM sleep deprivation alone, however, impairs place and reference memories but not visual cue and working memories in the Morris water and radial arm maze tasks, respectively (Smith et al., 1998; Smith & Rose, 1996). Interestingly, it has been observed in human studies that procedural learning is improved only across a night of sleep and not over an equivalent period of wake (Walker, 2005; Walker et al., 2003). In addition, the degree of overnight improvement correlated with specific sleep stages and stage selective sleep disruption during a specific time frame (either early or late night primarily dominated by NREM sleep and REM sleep, respectively) impaired the gain in performances of different motor learning tasks (Karni et al., 1994; Walker, 2005; Walker et al., 2003). These studies suggest that consolidation based enhancement does not merely depend on time, rather requires time spent in specific sleep states (either NREM or REM sleep) after learning. Similarly, it has also been observed in rodent studies that sleep (either total or REM sleep) deprivation performed soon after training induces memory deficit but not after 4–6 hr post-training (Bjorness et al., 2005; Graves et al., 2003; Smith et al., 1998; Smith & Rose, 1996). All these suggest that sleep facilitates and optimizes memory

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Shweta Tripathi and Sushil K. Jha, School of Life Sciences, Jawaharlal Nehru University.

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Correspondence concerning this article should be addressed to Sushil K. Jha, School of Life Sciences, Jawaharlal Nehru University, New Delhi 110067, India. E-mail: sushilkjha@mail.jnu.ac.in or sushil_1000@yahoo.com

formation of some tasks during an explicit time window soon after training.

Quantitative and qualitative changes in sleep architecture after learning different training tasks further suggests that discrete memory types may require specific sleep stage for optimal memory consolidation. For example, non-REM (NREM) sleep, slow wave activity, and sleep spindle density during NREM sleep increase after fear conditioning, odor–reward association, and motor learning tasks, in rodents (Eschenko et al., 2006; Hanlon et al., 2009; Hellman & Abel, 2007; Kumar & Jha, 2012). Similar results have also been reported in humans after learning the verbal memory retention and motor-skill learning tasks (Clemens et al., 2005; Gais & Born, 2004). A selective increase in REM sleep has been reported after learning a variety of tasks such as spatial learning tasks, negative and positive reinforcement tasks, avoidance tasks, classic, aversive, and appetitive conditioning task, and so forth (Datta, 2000; Hennevin et al., 1995; Smith & Rose, 1997; and for review see Rasch & Born, 2013; Walker, 2009). Besides these, REM sleep also helps strengthen emotional memory (for review see Rasch & Born, 2013; Walker, 2009). REM sleep and theta power during REM sleep significantly positively correlated with the amount of emotional memory improvement after learning about the emotionally arousing stimuli (Nishida et al., 2009; Wagner et al., 2001). These studies suggest that a selective sleep state may be involved in the processing of learning information of some specific tasks.

Although, studies suggest that sleep plays an important role in processing of different memories but its role in a complex conditioned-learning paradigm is not clear. Total sleep deprivation soon after conditioning impairs fear-memory in the rat (Graves et al., 2003; Kumar & Jha, 2012) but REM sleep and REM sleep theta activity significantly decrease during the consolidation period of fearful memory (Hellman & Abel, 2007; Jha et al., 2005; Kumar & Jha, 2012; Madan et al., 2008; Pawlyk et al., 2005). On the other hand, Menz et al. (2013) have reported that the recall-efficiency of learned fear memory positively correlates with the preceding time spent in REM sleep, suggesting that REM sleep helps in the consolidation of fearful memory in humans (Menz et al., 2013). Thus, the role of sleep in the consolidation of conditioned memory is contradictory.

Two distinct paradigms of classical conditioning, trace and delay conditioning, are widely used to study learning and memory. Total sleep deprivation impairs trace conditioned memory (Chowdhury et al., 2011) and reexposure of the conditioned stimulus during NREM sleep significantly increases evoked EEG's K-complexes after trace conditioning (Wamsley & Antrobus, 2009), suggesting the role of sleep in the consolidation of trace conditioned-memory. Similarly, REM sleep deprivation alters cerebellum mediated delay-conditioned eyeblink reflex in humans (Ohno et al., 2002), suggesting its role in the consolidation of delay-conditioned memory. The effect of short-term total sleep deprivation on the consolidation of delay-conditioned memory and changes in sleep architecture after delay conditioning, however, is not known. Here, we have studied (a) the effect of short-term total sleep deprivation on delay-conditioned memory, and (b) changes in sleep architecture after delay conditioning in the rat. We have used classical appetitive conditioning paradigm to avoid any confounding effects of fear or defensive responses on sleep, which generally remains associated with classical fear or eyeblink-conditioning.

Method and Materials

Male Wistar rats (250–300 gm; $n = 52$) were used in this study. Animals were obtained from the University's animal house facility. They were kept in the institutional in-house animal facility for one week before experiments started. Animals were maintained on 12:12 hr light:dark cycle (lights on at 7:00 a.m.) in a temperature controlled (24 °C) room. Food and water were given ad lib. All procedures and protocols were approved by the Institutional Animal Ethical Committee of the Jawaharlal Nehru University, New Delhi, India.

We performed two sets of experiments; Experiment 1 to evaluate the effects of short-term total sleep deprivation on delay-conditioned memory and Experiment 2 to study the changes in sleep architecture after delay conditioning.

Experiment 1: The Effect of Short-Term Sleep Deprivation on Delay-Conditioned Memory

Rats were trained for delay conditioning task using the standard protocol (see Figure 1). Soon after training, animals were randomly divided into three groups (a) sleep-deprived (SD) group ($n = 10$); (b) nonsleep-deprived (NSD) group ($n = 10$); and (c) stress control (SC) group ($n = 5$).

Delay Conditioning

Animals were trained for the delay conditioning task in a small behavioral chamber (12" × 12" × 11"), which was placed inside a sound dampened experimental chamber (48" × 24" × 24"). Diffused light (20 Lux) was always maintained in the experimental chamber during the experiments. For delay conditioning, house light (an additional light in the behavioral chamber) was used as a conditioned stimulus (CS), while mango fruit juice (Tropicana Product, PepsiCo India, Gurgaon, India) was used as an unconditioned stimulus (US). The paired CS and US were delivered to the animal through a computer using the Graphic State software (Coulbourn, Inc., Whitehall, PA). The light source for the CS was fixed above the juice dispensing unit close to the roof of the behavioral chamber. Fruit juice was delivered through the liquid dispensing unit (Coulbourn, Inc., Whitehall, PA). One end of the lever of the liquid dispensing unit was connected to a computer-controlled motor while a small cup was attached at the other end. The liquid dispenser cup carried approximately 100 μ l juice from the tank to the dispensing window of the behavioral chamber. The animal had to poke his head into the window to obtain the juice. Number of head entries to obtain juice (as an outcome measure of learning) was registered on a computer through the Graphic State software (Coulbourn, Inc., Whitehall, PA) using photo-sensors attached on both sides of the walls of the juice dispensing window.

Animals were first habituated to the behavioral conditioning chamber for 1 hr each on three consecutive days between 10:30 a.m.–11:30 a.m. On Day 1, the animal was simply kept in the conditioning chamber for 1 hr but no fruit juice was given. On Day 2, fruit juice (mango juice, Tropicana Product, PepsiCo India, Gurgaon, India) was introduced intermittently to the animal during habituation through a small bottle by the experimenter. It was done

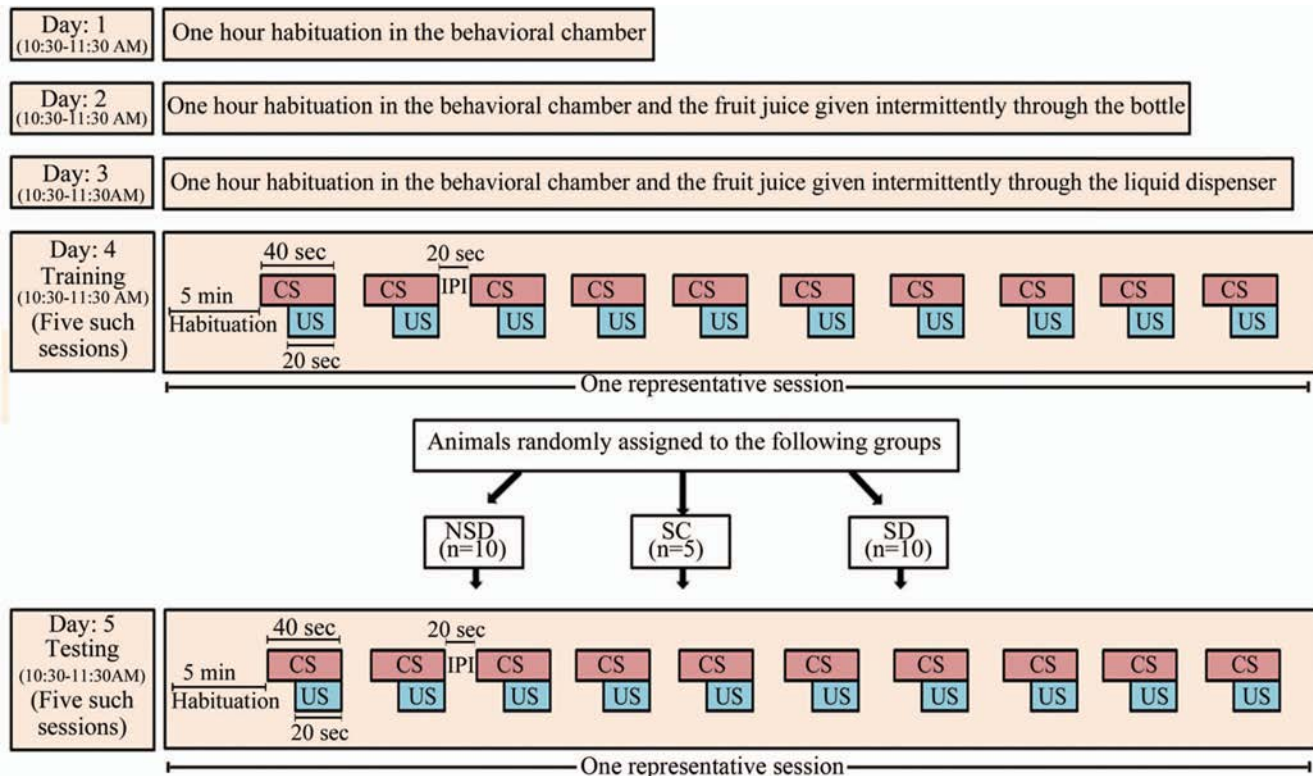


Figure 1. Delay-conditioning protocol: On Day 1, animals were habituated to the conditioning chamber for 1 hr (10:30 a.m.–11:30 a.m.). Animals were again habituated to the conditioning chamber for 1 hr on Day 2, during which mango fruit juice was given manually by the experimenter through the nozzle fitted small bottle. It was done so that animals develop the taste for the juice. On Day 3, mango juice was introduced to the animal several times through the liquid dispenser of the juice dispensing unit over a 1-hr period. The experimenter manually operated the juice dispensing unit and guided the animal toward the juice dispensing window. Animals were then trained for the delay-conditioned task on Day 4. The conditioned stimulus (CS; house light) and unconditioned stimulus (US; mango fruit juice) were presented through a computer using the Graphic State software (Coulbourn Inc., Whitehall, PA). After 5 min of initial habituation period, the CS was delivered for 40 sec, which coterminated with a 20-sec US presentation followed by a 20-sec interpresentation interval. The paired CS–US was presented in five sessions (10 paired CS–US presentation/session) with 2-min intersession interval. Animals were then randomly divided into three groups: nonsleep deprived, stress control, and sleep deprived groups. All animals were tested for the delay conditioning task on the next day (Day 5). Abbreviations: CS = conditioned stimulus; IPI = interpresentation interval; NSD = nonsleep-deprived group; SC = stress control group; SD = sleep-deprived group; US = unconditioned stimulus. See the online article for the color version of this figure.

to familiarize the animal with the taste and aroma of the juice. On Day 3, the juice was given to the animal through the liquid dispenser of the juice dispensing unit. The experimenter manually lifted the lever of the juice-filled cup and guided the animal toward the juice dispensing window for approximately 15–20 times. The majority animals learned the location of the juice dispensing site after this small training. Animals, which did not learn to approach the window on their own with this small training, were retrained the next day during the same period. Out of a total of 25 animals, only three animals failed to learn about the juice delivery site during their first trial and were retrained the next day. Out of these three animals, one was assigned to the SD group, and two were assigned to the NSD group randomly.

Animals were trained for the delay conditioning task on Day 4 between 10:30 a.m.–11:30 a.m. The protocol of Graphic State software was written in such a way that after 5 min of initial

habituation period, it delivered the CS (house light) for 40 s, which coterminated with 20 s US presentation followed by 20 s interpresentation interval (see Figure 1). Fifty CS–US paired stimuli were presented (10 paired CS–US per session) in five sessions over a period of 1 hr with a 2-min inter-session interval. Animals were then taken out from the behavioral chamber 5 min after the completion of training trials. They were then randomly divided into three groups: SD ($n = 10$); NSD ($n = 10$), and SC ($n = 5$) groups. The SD animals were total sleep deprived with gentle handling, cage shaking, and gentle prodding, continuously for 6 hr from 11:30 a.m.–5:30 p.m. immediately after training. They were then returned to the animal colony and left undisturbed till next day for recovery. Animals in the NSD group were brought back to the animal colony soon after delay-conditioned training. The SC animals were kept confined in a small, transparent, and well-ventilated barrel-shaped chamber (9" long and 3" diameter) for 6

hr from 11:30 a.m.–5:30 p.m. soon after training. These animals were returned to the animal colony after completion of a 6-hr confinement period and were kept undisturbed till the next day.

All animals were tested for the delay-conditioned task on Day 5 between time-matched hours. For testing, the animal was kept in the conditioning chamber and allowed to explore the behavior chamber for 5 min before presenting the CS and US. After 5 min, the CS and US were presented in a similar way as these were presented during the training period (see Figure 1). It was done because we observed that animals did not poke into the dispensing window without the juice (US) presentation. We evaluated the conditioned response in an additional group ($n = 10$) with the CS alone (no US) during the testing. It was found that animals did not frequently poke their heads to obtain juice (Supplementary Figure 1). All animals, however, clearly demonstrated the conditioned response as they made more head entries during Session 1 with a successive decrease in each subsequent session (Supplementary Figure 1). These animals might have learned that there was no juice available, and hence made little attempt to obtain the juice through the dispensing window during the later sessions. For this reason, we presented paired CS–US stimuli during the testing period as well.

Data Analysis

The grouped data (number of head entries in NSD, SD, and SC animals on the training and testing days) were statistically analyzed using Sigma Stat 3.5 software (Systat, Inc., Chicago, IL). The data were analyzed using two-factor analysis of variance (ANOVA) followed by Tukey post hoc test implementing factors between and within experimental groups and days (training and testing days).

Experiment 2: The Changes in Sleep Architecture After Delay Conditioning

In another set of experiments, we characterized the changes in sleep architecture in the same animals after training and testing of the delay-conditioned task ($n = 12$).

Surgical Procedures and Polysomnographic Recordings

Animals were prepared for chronic sleep–wake (S–W) recordings using the similar procedure as has been reported earlier (Kumar & Jha, 2012). In brief, the animal was anesthetized using isoflurane inhalation anesthesia. The animal was fixed in the stereotaxy and the skull was exposed. Two pairs of small, stainless steel screw electrodes were implanted on the frontal and parietal cortices to record electroencephalogram (EEG). One screw was implanted in the nasal bone for reference. Three flexible insulated wires (except at the tip) were implanted in the neck muscle to record electromyogram (EMG; third EMG was implanted as a safeguard). The free ends of the EEG, EMG, and reference electrodes were soldered in a nine pin connector, which was secured on the skull with dental acrylic. The neck skin was then sutured and the animal was removed from the stereotaxy. Postoperatively, dexamethasone and nebaself powder (antibiotic) were used to reduce brain inflammation and infection.

After 1 week recovery, the animal was engaged in experiments. The animal was first habituated to the behavioral conditioning chamber between 10:30–11:30 a.m. and to the sleep recording chamber between 11:30 a.m.–5:30 p.m. on Day 1 (as per Figure 1). For conditioning, we followed the same protocol as was used in Experiment 1. S–W was recorded daily for 6 hr (for 4 consecutive days starting on Day 2) beginning at 11:30 a.m. after the conditioning protocol of each day got over. The animal was tethered to the recording cable and polysomnographic recording setup through a commutator. S–W was recorded in a computer through Spike2 software (CED, Cambridge, United Kingdom) and 15 LT physio data (Astro Med Inc., West Warwick, RI). Because at habituation days (Days 2 and 3), animals were exposed to the fruit juice, and they also therefore learned some procedural task, it is likely that such novelty and procedural learning may also affect sleep architecture. To ensure an exclusive delay-conditioned task dependent alteration in sleep architecture, habituation Days 2 and 3 were purposely considered as Baseline 1 and Baseline 2 for S–W recordings. EEG signals were processed with a high pass 0.1 Hz and a low pass 40 Hz while EMG signal was processed with a high pass 10 Hz and a low pass 100 Hz at 100 Hz sampling rate. Recordings were stored for off-line analysis.

Additionally, we characterized the changes in sleep architecture in the stress control rats ($n = 5$). Animals were prepared for chronic S–W recordings and sleep was recorded using similar procedure as mentioned above. Baseline sleep was recorded for two consecutive days in the sleep recording chamber for 6 hr (11:30 a.m.–5:30 p.m.). Next day, the animal was placed inside a well-ventilated barrel-shaped stress chamber (9" long and 3" diameter), the stress chamber was kept inside the same sleep recording chamber and S–W was recorded for 6 hr. The recordings were stored for off-line analysis.

Data Analysis

Delay-conditioned task. The overall changes in average head entries on training and testing days were compared using one-way RM-ANOVA followed by Tukey post hoc test.

Sleep–wakefulness. Offline, polysomnographic Spike2 records were converted into the European data format (EDF) and were opened in the Somnologica Science software (Medcare Flaga, Iceland) for scoring. Computerized polysomnographic records were visually displayed in Somnologica Science software, divided into 4-sec epochs and manually scored, employing the standard criteria used for the rat. Low-voltage and high-frequency EEG waves associated with increased motor activity were analyzed as wakefulness. High-voltage, low-frequency EEG waves with prominent delta waves (0.5–4 Hz) and decreased motor activity were analyzed as NREM sleep. Whereas, low-voltage, high-frequency EEG waves with a prominent theta peak (5–9 Hz) and nuchal muscle atonia were analyzed as REM sleep. The total time spent in wake, NREM and REM sleep were scored. These values were expressed at every 2 hr and total mean percent of the total recording time. Further, NREM and REM sleep onset periods (latency), episode number, and episode length on the baseline, training, and testing days were computed. The differences in the vigilant states and its parameters on the training and testing days were statistically compared with baseline days using one-way RM-ANOVA followed by Tukey post hoc test. The changes in

sleep architecture in stress control group were compared with baseline days using one-way RM-ANOVA followed by Tukey post hoc test. Also, we compared the changes in sleep architecture at Baseline 1 and Baseline 2 with the baseline of stress control animals using two-way ANOVA followed by Tukey post hoc test implementing factors between experimental groups and days. It was done to determine the effects of novel fruit juice exposure and some procedural learning on sleep.

Results

Experiment 1: The Effect of Short-Term Total Sleep Deprivation on Delay Conditioning

Short-term sleep deprivation and stress did not induce any gross behavioral changes in the SD and SC animals. The food and water intake in the SD and SC animals in the post-SD and poststress period and before testing were comparable with the NSD animals. On the testing day, the SC, SD, and NSD animals explored the behavioral chamber as usual during the initial 5-min habituation period.

During testing, the NSD and SC animals showed significant increase in the performance of delay-conditioned task. The sleep deprived animals, however, exhibited a learning deficit. Two-way ANOVA revealed a significant interaction between groups and days (training and testing) during the CS-US presentation, $F(2, 44) = 39.48$; $p < 0.001$. Animals in all three groups made an almost similar number of head entries during the training and no statistical significant changes were observed between groups (Figure 2A). This suggests that all these animals were equally trained. Further, Tukey post hoc comparison revealed that the NSD and SC animals exhibited significantly increased head poking (NSD animals: Tukey $p < .001$; SC animals: Tukey $p < .001$) during the CS-US presentation on the testing day compared with the training day (Figure 2A). However, the SD animals exhibited significantly less head entries on the testing day during the CS-US presentation compared with the training day (Tukey $p < .001$) (Figure 2A). Further, a between-groups comparison on the testing day showed that SD animals made significantly less number of head entries during the CS-US presentation compared with the NSD (Tukey $p < .001$) and SC (Tukey $p < .001$) animals (Figure 2A). Two-way ANOVA revealed no significant interaction between groups and days (training and testing) during the interpresentation interval ($p = .43$; Figure 2B).

Two-way ANOVA also revealed a significant interaction between groups and days across different sessions (Session 1: $F(2, 44) = 23.93$, $p < 0.001$; Session 2: $F(2, 44) = 41.47$, $p < 0.001$; Session 3: $F(2, 44) = 71.88$; $p < 0.001$; Session 4: $F(2, 44) = 39.13$; $p < 0.001$; Session 5: $F(2, 44) = 15.38$; $p < 0.001$). Further, Tukey post hoc comparison demonstrated that the number of head entries during different training sessions was comparable (Figure 2C), however, it was significantly less in the SD animals in all five testing sessions compared with NSD and SC animals (Session 1 NSD: Tukey $p < .001$, and SC: Tukey $p < .001$; Session 2 NSD: Tukey $p < .001$ and SC: Tukey $p < .001$; Session 3 NSD: Tukey $p < .001$ and SC: Tukey $p < .001$; Session 4 NSD: Tukey $p < .001$ and SC: Tukey $p < .001$; Session 5 NSD: Tukey $p < .001$ and SC: Tukey $p < .001$; Figure 2D).

Experiment 2: The Effect of Delay Conditioned Task on Sleep Architecture

All animals trained for the delay-conditioned task performed well during the testing. The number of head entries during the CS-US paired presentation period on the testing day was significantly more compared with the training day ($p < .001$, $F(1, 23) = 158.35$; one-way RM-ANOVA; see Figure 3). These results demonstrate that animals had properly learned the delay-conditioned task.

Before baseline sleep recording, animals were exposed to the fruit juice and also obtained hand-guided training for the juice delivery location, which might have altered their sleep architecture. Therefore, we compared wakefulness, NREM sleep, and REM sleep amount on baseline days of these delay-conditioned animals with the baseline of the stress-control group (see Table 1). The percent wakefulness, NREM sleep, and REM sleep amounts were comparable between groups. Two-way ANOVA revealed no significant interaction between groups and days suggesting that such novelty intervention did not alter sleep architecture in the delay-conditioned animals (see Table 1).

After delay conditioning, wakefulness, and NREM sleep amount did not change, but REM sleep amount significantly increased on both training and testing days compared with the baseline days. Animals spent comparable time in wakefulness (Figure 4A) and NREM sleep (Figure 4B) on the baseline, training, and testing days. The percent REM sleep amount, however, significantly increased ($p < .001$, $F(3,47) = 34.98$; one-way RM-ANOVA) on training and testing days compared to the baseline days (Figure 4C). It increased by 104% compared with Baseline 1 (Tukey $p < .001$) and 59.24% compared with Baseline 2 (Tukey $p < .001$) on the training day. Similarly, on the testing day, the percent REM sleep amount significantly increased by 91.94% compared with Baseline 1 (Tukey $p < .001$) and 49.82% compared with Baseline 2 (Tukey $p < .001$; Figure 4C). Further, at every 2-hr analysis, it was observed that percent REM sleep amount was significantly high at 0–2 hr ($p < .01$, $F(3,47) = 6.32$) and 2–4 hr ($p < .01$, $F(3,47) = 4.73$) periods on the training and testing days. The changes at the 4–6-hr period were not significant. Tukey post hoc comparison demonstrated a significant increase at the 0–2-hour period on the training day compared with Baseline 1 (Tukey $p < .05$) and Baseline 2 (Tukey $p < .05$) and the 2–4-hr period compared with Baseline 1 (Tukey $p < .05$), but it was not significant compared with Baseline 2 (Figure 4D). On the testing day, percent REM sleep amount was significantly high only at the 0–2-hr period (compared with Baseline 1: Tukey $p < .05$ and compared with Baseline 2: Tukey $p < .05$). The changes at the 2–4-hr and the 4–6-hr period were not significant (Figure 4D).

The delay-conditioned animals exhibited a significant increase in REM sleep episode numbers on the training and testing days compared with Baseline 1 ($p < .001$, $F(3,47) = 9.60$) (one-way RM-ANOVA; Figure 5D). Nevertheless, other sleep parameters such as NREM sleep episode numbers, NREM, and REM sleep episode length, and NREM, and REM sleep onset (latency) did not change (see Figure 5). Tukey post hoc comparisons demonstrated a significant increase in REM sleep episode numbers on the training days compared with Baseline 1 (Tukey $p < .001$) and Baseline 2 (Tukey $p < .05$). REM sleep episode number was also

Average head entries during the training and testing of delay conditioning task in NSD, SC and SD groups

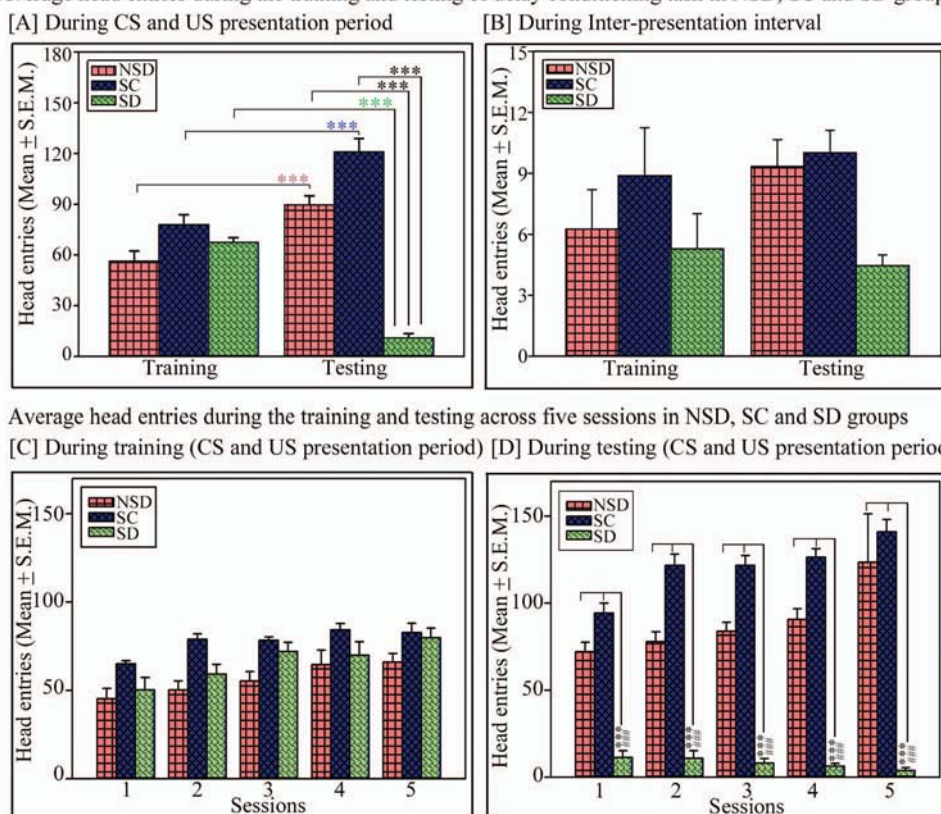


Figure 2. The average head entries in the NSD, SC, and SD animals during the CS–US presentation, interpresentation interval, and across five sessions on the training and testing days. Two-way ANOVA revealed a significant interaction between groups and days during the CS–US presentation, $F(2, 44) = 39.48$; $p < 0.001$. (A) NSD, SC, and SD animals made a comparable number of head entries in the juice dispensing window on the training day. The NSD and SC animals, however, exhibited significantly more number of head entries (NSD animals: Tukey $p < .001$; SC animals: Tukey $p < .001$) during the CS–US presentation period on the testing day compared with the training day. On the other hand, the SD animals made significantly less number of head entries in CS–US paired presentation period on the testing day compared with their training day (Tukey $p < .001$). Between groups comparison on the testing day also demonstrated that the SD animals exhibited less number of head entries compared with the NSD (Tukey $p < .001$) and SC (Tukey $p < .001$) animals. (B) Number of head entries in NSD, SC, and SD animals during the interpresentation interval. Two-way ANOVA revealed no significant interaction between groups and within groups and also between groups and days (training and testing) during this period ($p = .43$). (C) The number of head entries in each five sessions during the training was comparable in NSD, SC, and SD animals and it demonstrates consistency in learning across all five sessions in all groups. (D) During the testing, the NSD and SC animals exhibited comparable number of head entries in each session while the SD animals made significantly less number of head entries in all five sessions (Session 1 compared with NSD: Tukey $p < .001$, SC: Tukey $p < .001$; Session 2 compared with NSD: Tukey $p < .001$, SC: Tukey $p < .001$; Session 3 compared with NSD: Tukey $p < .001$, SC: Tukey $p < .001$; Session 4 compared with NSD: Tukey $p < .001$, SC: Tukey $p < .001$; Session 5 compared with NSD: Tukey $p < .001$, SC: Tukey $p < .001$). SD animals poked significantly less from the beginning to the last sessions, suggesting that these animals had difficulty in remembering the learnt task. *** = $p < .001$. In Fig. D *** = $p < .001$ (comparison between NSD and SD groups) and #### = $p < .001$ (comparison between SC and SD groups). Abbreviations: CS = conditioned stimulus; NSD = nonsleep-deprived group; SC = stress control group; SD = sleep-deprived group; US = unconditioned stimulus. See the online article for the color version of this figure.

significantly increased on the testing day but only compared with Baseline 1 (Tukey $p < .01$).

Discussion

In this study, we observed that (a) the SD animals exhibited learning deficit in delay-conditioned task compared with NSD and

SC groups; and (b) after successful learning of the delay-conditioned task, the animals experienced significant increase in REM sleep. During the training, animals in all groups exhibited comparable head entries in the juice dispensing window (see Figure 2), suggesting a comparable learning in all groups. The NSD and SC animals exhibited significant increases in the perfor-

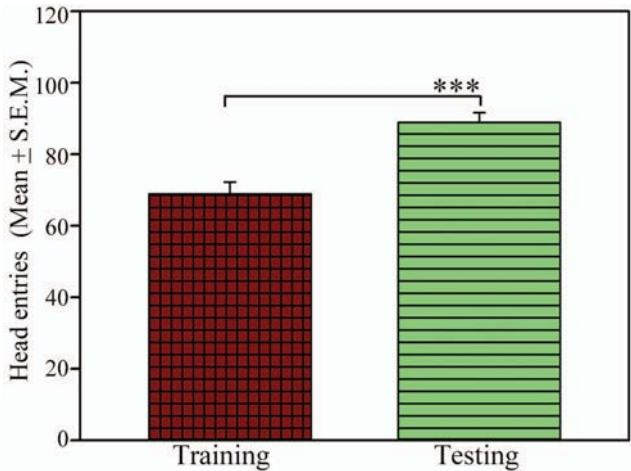


Figure 3. The average head entries during the training and testing days. The number of head entries significantly increased during the testing compared with the training day ($p < .001$; $F(1, 23) = 158.35$; one-way RM-ANOVA followed by Tukey post hoc test). *** = $p < .001$. Abbreviations: CS = conditioned stimulus; US = unconditioned stimulus. See the online article for the color version of this figure.

mances, but interestingly, the SD animals did not perform well during testing. The learning deficit in the SD animals can be attributed to sleep loss and not to any associated stress because SC animals also performed better during testing. There are studies suggesting that exposure to certain stressors such as social defeat, short-term immobilization stress, and learned helplessness may accelerate the build-up of sleep debt and more sleep in the rat (Adrien et al., 1991; Akerstedt, 2006; Cespuglio et al., 1995; Meerlo et al., 1997), while exposure to some another stressors may reduce sleep (Pawlyk et al., 2008). Here, we observed that confinement stress in a restricted area did not cause sleep loss in the SC animals; rather they spent marginally more time in NREM sleep (Supplementary Figures 2A and 2B). Our results therefore suggest that the learning deficit in the SD animals was primarily due to sleep loss.

The memory remains labile for a few minutes to few hours after training, and the labile state of memory is transformed into a stable form during the consolidation (Abel & Lattal, 2001). Brain regions, which get activated during training, are activated again during subsequent sleep periods and memory transformation from a labile to stabilized state possibly occurs during these sleep

periods (Graves et al., 2001). Reexposure of the cue during subsequent sleep period after training induces memory reactivation and facilitates memory consolidation (Rasch et al., 2007). Interestingly, it has also been observed that memory reactivation by reexposing cue during wakefulness and sleep has opposite effects on the memory consolidation. The memory reactivation during wake destabilizes while during sleep stabilizes the memory (Diekelmann et al., 2011). These reports suggest that the consolidation of some specific memories would require sleep soon after training. Because acquired memories are transferred with time from a labile to a stabilized mode, sleep loss during such period may induce a detrimental effect on its stabilization processes.

In the conditioning paradigm, animals learn about (a) the effectiveness of the CS and US; and (b) the temporal contingency of the CS-US presentation during the conditioning (Bouton, 2007). Repeated presentations of CS alone decreased the conditioned response, so we used the CS-US pairing during testing as well. It was done because animals have shown less number of head entries in the absence of US (Supplementary Figure 1). The fewer number of head entries, during testing in the absence of the US, could be as a result of a decrease in the effectiveness of the CS to predict US. The animals might have relearned quickly that the fruit juice is no more paired with the CS, and hence have made very less number of head entries in the juice dispensing window (Supplementary Figure 1). However, animals clearly demonstrated the conditioning response in the absence of US but only during the first session (there were more head entries during the first session) and it gradually decreased in the subsequent sessions (Supplementary Figure 1). We have used both the CS and US during testing to get an optimal learning response of the delay-conditioned task.

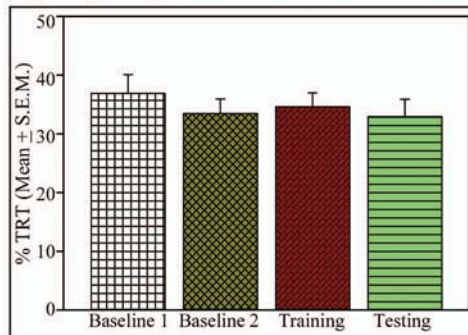
The NSD and SC animals have exhibited better learning during testing while the SD animals have exhibited learning deficit. The learning impairment after sleep deprivation in the SD animals could be attributed to either of the two facts: (a) sleep deprivation may have altered the memory associated with the CS-US temporal contingency, or (b) animals might have difficulty in recognizing the site of juice delivery in the behavioral chamber. It is important to note that the experimenter has trained all animals manually to obtain juice from the juice dispenser a day before conditioning (Day 3, Figure 1). Then again animals were trained to obtained juice from the juice dispensing unit during the delay conditioning along with the CS (Day 4, Figure 1). Thus, all animals were trained to localize the site of the juice delivery for 2 days. The animals in all groups (NSD, SC, and SD) have exhibited a comparable number of head entries on the training day (see Figure 2). Because all

Table 1
Percent Wakefulness, NREM Sleep, and REM Sleep on Baseline Days in the Stress-Control and Delay-Conditioned Animals

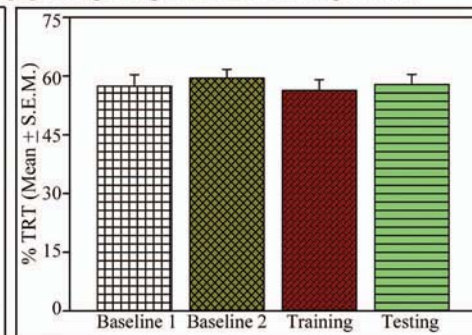
% Total recording time	Baseline (Mean ± S. E. M.) (stress control group)	Baseline 1 (Mean ± S. E. M.) (delay-conditioned group)	Baseline 2 (Mean ± S. E. M.) (delay-conditioned group)
Wake	39.67 ± 4.90	36.90 ± 3.19	33.48 ± 2.45
p value	p = .95 (no significant interaction between groups and days)		
NREM sleep	53.94 ± 4.42	57.41 ± 2.92	59.52 ± 2.18
p value	p = .94 (no significant interaction between groups and days)		
REM sleep	6.61 ± .59	5.42 ± .65	6.95 ± .70
p value	p = .12 (no significant interaction between groups and days)		

Changes in sleep architecture after delay conditioning

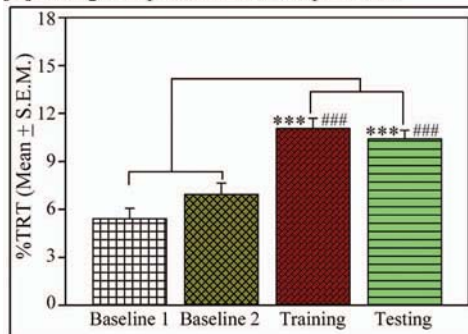
[A] Changes in percent Wakefulness amount



[B] Changes in percent NREM sleep amount



[C] Changes in percent REM sleep amount



[D] Every two-hourly changes in percent REM sleep

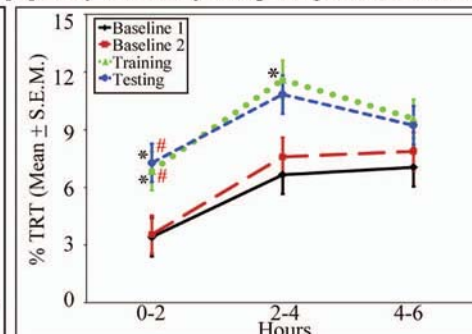


Figure 4. Changes in sleep architecture after training and testing of the delay-conditioning task. Animals spent comparable time in wakefulness (A) and NREM sleep (B) on the baseline, training, and testing days. (C) The percent REM sleep amount, however, significantly increased on the training and testing days, $p < .001$, $F(3, 47) = 34.98$; one-way RM-ANOVA followed by Tukey post hoc test; on the training day, compared with Baseline 1 (Tukey $p < .001$) and Baseline 2 (Tukey $p < .001$); on the testing day, compared with Baseline 1 (Tukey $p < .001$) and Baseline 2 (Tukey $p < .001$). (D) Percent REM sleep amount at every 2-hr period on the baseline, training, and testing days. At every 2-hr analysis, percent REM sleep amount was significantly high on the training day at the 0–2-hr ($p < .01$, $F(3, 47) = 6.32$) and the 2–4-hr ($p < .01$, $F(3, 47) = 4.73$) periods. The changes on the training and testing days at the 4–6-hr period were not significant. Tukey post hoc comparisons demonstrated significant increase on the training day at the 0–2-hr period compared with Baseline 1 (Tukey $p < .05$) and Baseline 2 (Tukey $p < .05$) and also at the 2–4-hr period compared with Baseline 1 (Tukey $p < .05$). Similarly, on the testing day, REM sleep was significantly high at the 0–2-hr period compared with Baseline 1 (Tukey $p < .05$) and Baseline 2 (Tukey $p < .05$). It was, however, not significant at the 2–4-hr period. * = $p < .05$ (compared with Baseline 1); # = $p < .05$ (compared with Baseline 2) *** = $p < .001$ (compared with Baseline 1); #### = $p < .001$ (compared with Baseline 2). Abbreviations: TRT = Total recording time. See the online article for the color version of this figure.

animals have been trained to localize the juice delivery site on 2 consecutive days, and the SD animals were sleep deprived only after the delay-conditioned training. It is, therefore, unlikely that SD animals would have difficulty in recognizing the site of juice delivery on the postconditioning day.

Further, in Experiment 2, we observed that NREM sleep or wakefulness amount did not change after training and testing. However, REM sleep increased significantly after successful learning of the delay-conditioned task. Interestingly, REM sleep significantly increased during an initial 4-hr window (Figure 4D) and returned to the basal level at the end of the 6-hr period. It has been reported earlier that REM sleep increases within a similar 1–4-hr window after the spatial learning in the Morris water maze (Smith & Rose, 1997). The number of REM sleep episodes significantly increased during the training and testing days compared

with Baseline 1; REM sleep episode length and latency (REM sleep onset), however, did not change. It demonstrates that animals were frequently going into REM sleep after learning the delay-conditioned task.

Learning a different task may augment various sleep stages (Huber et al., 2004; Smith & Rose, 1996; Smith & Rose, 1997; Stickgold et al., 2000). Some reports have demonstrated that NREM sleep increases after learning the hippocampal-dependent task while, REM sleep increases after learning the nonhippocampal dependent task (Marshall & Born, 2007). However, in some other studies, opposite results were observed (Smith & Rose, 1996; Smith & Rose, 1997). Learning a task does not necessarily require the activation of an isolated circuit; rather specific neuronal groups, may be from more than one brain areas, get activated (Kim & Jung, 2006). Therefore, possibly the different sleep stages or

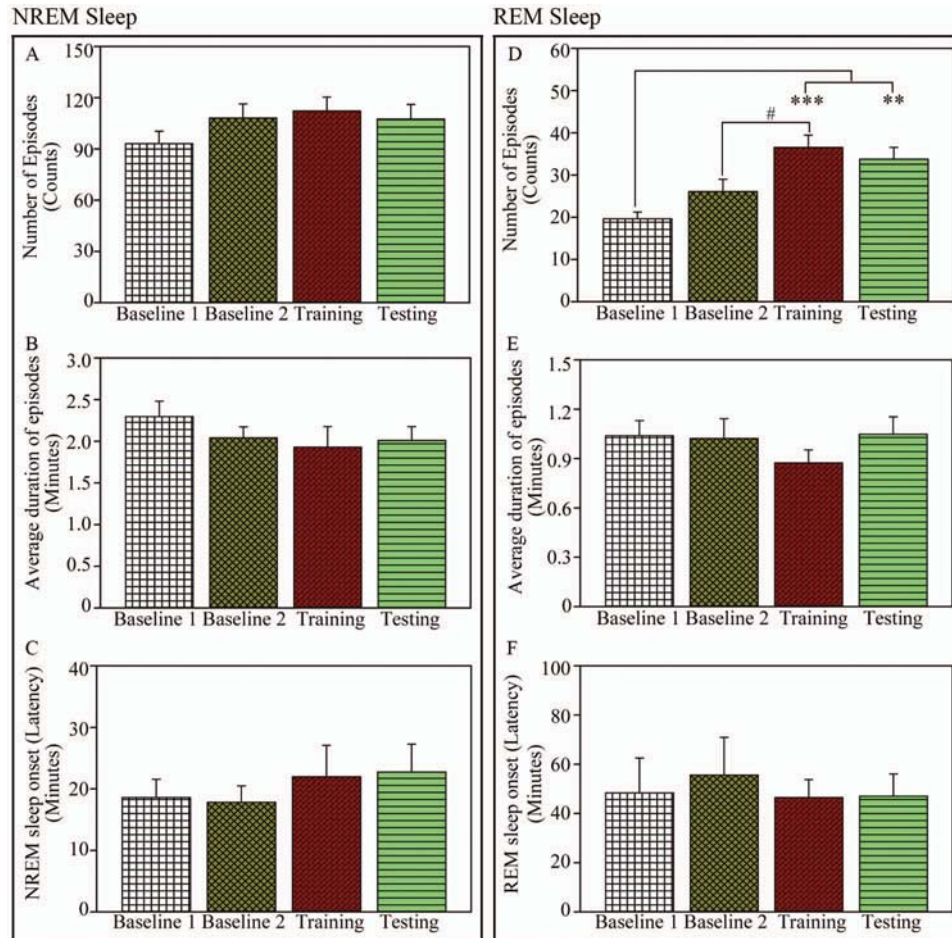


Figure 5. NREM and REM sleep episode numbers, episode length, and latency (NREM and REM sleep onset) on the baseline, training, and testing days. NREM sleep episode numbers (A), episode length (B), and latency (NREM sleep onset; C) did not change. However, REM sleep episode numbers (D) significantly increased on the training and testing days compared to the baseline days ($p < .001$, $F(3,47) = 9.59$; one-way RM-ANOVA followed by Tukey post hoc test). Tukey post hoc comparisons demonstrated a significant increase in REM sleep episode numbers on the training day compared to Baseline 1 (Tukey $p < .001$) and Baseline 2 (Tukey $p < .05$). During testing, REM sleep episode number significantly increased compared with Baseline 1 only (Tukey $p < .01$). It was, however, not significant with Baseline 2. REM sleep episode duration (E) and latency (REM sleep onset; F) did not change on the training and testing days. ** = $p < .01$. *** = $p < .001$. (compared with Baseline 1) # = $p < .05$ (compared with Baseline 2). See the online article for the color version of this figure.

their components (such as sleep spindles, delta waves, and hippocampal theta waves) may act in coherence with the activated circuitries to help enhance memory consolidation and synaptic plasticity (Aton et al., 2009; Frank et al., 2006; Marshall & Born, 2007). However, in this study, we have observed that REM sleep is augmented after training and testing, suggesting that it may be playing a distinctive role in the consolidation of delay-conditioned memory.

The brain areas involved in the acquisition and consolidation of this type of appetitive delay-conditioned task is not clearly known. Majority reports on delay eyeblink conditioning task suggest that the brainstem-cerebellar network plays an important role in the consolidation of delay eyeblink conditioning (Clark et al., 1984; Mauk & Thompson, 1987). Nevertheless, the amygdala lesion and stimulation studies have shown that the amygdala also plays a

significant role in the acquisition of delay eyeblink conditioning (Canli & Brown, 1996; Whalen & Kapp, 1991). Studies suggest that the amygdala helps in acquiring the delay-conditioned memory by increasing the saliency of the behavioral events associated with *motivated-conditioning paradigms* (Chau & Galvez, 2012). Interestingly, some neuronal population in the amygdala manifests necessary plastic changes during postlearning sleep (Blair et al., 2001; Hennevin et al., 1998; Rogan et al., 1997). Such changes could reinforce neural encoding in the amygdala. Also, it has been found that sleep deprivation induces a hyperlimbic response to the negative emotional stimuli in the amygdala (Yoo et al., 2007). The activated hyperlimbic response is possibly because of the loss of inhibitory connectivity between the amygdala and medial prefrontal cortex after prolonged sleep deprivation (Yoo et al., 2007). It seems that sleep loss alters the functions of the

amygdala neurons, which could in turn impair encoding and/or consolidation of memory information. Nevertheless, it needs to be studied in detail if our paradigm of delay conditioning is amygdala-dependent and induces similar plastic changes in the amygdala neurons or not.

Conclusions

In summary, we observed that short-term total sleep loss can perturb delay-conditioned memory, and REM sleep is significantly increased after the delay conditioning. These results suggest that sleep plays a significant role in the consolidation of delay-conditioned memory. However, there are a few questions that remain unclear and would await future research. For example, (a) How does sleep loss affect neural circuitries underlying delay-conditioning? (b) How does REM sleep contribute to the memory consolidation of conditioned memory? (c) Why does REM sleep increase after appetitive delay conditioning, but decreases after fear delay-conditioning? A detailed knowledge would provide insights into the precise role of sleep in learning and memory.

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