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Sleep loss immediately after fear memory reactivation attenuates fear memory reconsolidation

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Abstract

Permanently stored memories become labile through a process called reactivation. Once reactivated, these memories need reconsolidation to become permanent. Sleep is critical for memory consolidation. Is sleep necessary for memory reconsolidation? We hypothesized that sleep loss immediately after fear reactivation (FR) will prevent memory reconsolidation. To test our hypothesis, two experiments were performed in adult male C57BL/6J mice exposed to contextual fear conditioning paradigm with inescapable foot shock as unconditional stimulus (US) and contextual cage as conditional stimulus (CS). Sleep loss was achieved either by 5 hours of sleep deprivation (SD; Experiment 1) or by systemic infusion of modafinil (200 mg/Kg, ip), an FDA approved wake-promoting agent (Experiment 2). One hour after light-onset, fear memory acquisition (FMA) was performed on Day 1. Mice were allowed to explore CS for 5 min followed by presentation of US (7 foot-shocks; 0.5 mA, 2.0 s duration) at pseudorandom intervals. Controls were exposed to similar CS but no shocks were delivered. On Day 2, mice were exposed to CS for 2 min (without US; for FR) followed by either sleep loss or no sleep loss. On Day 3, Fear memory recall (FMR) was performed by exposing mice to CS (without US) for 12 min. Percent time spent in freezing was monitored during FC, FR and FMR. Our results suggested that as compared to sleeping controls, mice with sleep loss immediately after FR displayed a significant reduction in percent time freezing during FMR. These results suggest that sleep loss may prevent memory reconsolidation.

Keywords

Sleep deprivation; Fear reactivation; Contextual conditioning; Modafinil; Foot-shock

INTRODUCTION

When memories are newly formed, they are labile and can be easily disrupted. However, over a period time, through the process of consolidation, these newly formed memories enter

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“permanent storage” where they become resistant to disruption or interference and can remain stable for many years (McGaugh, 2000). Evidence suggests that these permanently stored memories can be reactivated and brought back to labile state, when retrieved in the right conditions, making them vulnerable to manipulation (Dudai and Eisenberg, 2004; Lewis et al., 1968; Nader, 2003; Nader et al., 2000; Przybyslawski and Sara, 1997). These memories can be reinserted into permanent storage by a process of reconsolidation (Dudai, 2006; Tronson and Taylor, 2007).

Strong evidence exists implicating the criticality of sleep in consolidation of all memories including hippocampal based declarative/contextual and amygdala-based fear/emotional memories (Born et al., 2006; Graves et al., 2001; Sara, 2017). Once memories are encoded, consolidation occurs during sleep (Abel et al., 2013; Diekelmann and Born, 2010; Fischer et al., 2002; Graves et al., 2003; Korman, 2007; Nishida et al., 2009; Plihal and Born, 1997; Walker, 2010; Walker and Stickgold, 2004). Although the underlying neuronal mechanisms responsible for these processes are unclear, sufficient evidence suggests that the process of active systems consolidation is responsible for memory consolidation (Diekelmann and Born, 2010; Dudai, 2004; Menz et al., 2016). In this process, prior experiences are replayed during sleep resulting in reactivation and strengthening of hippocampal memory traces followed by integration of information into the neocortical long-term memory networks (Euston et al., 2007; Ji and Wilson, 2007; Louie and Wilson, 2001; Nadasdy et al., 1999; Peyrache et al., 2009; Ribeiro, 2004; Wilson and McNaughton, 1994).

Human studies have also demonstrated that contextual hippocampal activity is re-expressed during post-learning sleep and the amount of reactivation predicts later memory performance (Peigneux, 2004). Furthermore, it is also possible to enhance later memory recall of declarative information by presenting cues of the learning task during sleep (Rasch and Born, 2007; Rasch et al., 2007; Rudoy et al., 2009). According to the ‘sequential hypothesis’, optimum benefits of sleep on the consolidation of both, declarative and non-declarative memory occur only when NREM and REM sleep occur in succession (Giuditta, 1995). Indeed, even though the time spent in NREM and REM sleep remains unchanged, memory consolidation is impaired by disruption of sleep architecture (Diekelmann and Born, 2010).

As described above, strong evidence exists implicating sleep in memory consolidation. However, the role of sleep in reconsolidation of reactivated memory is unclear. Is sleep necessary for reconsolidation of contextual fear memory? It was hypothesized sleep loss immediately after fear memory reactivation will result in attenuation of fear memory reconsolidation as evident by reduction in freezing behavior during fear memory recall testing.

We used two approaches to achieve sleep loss to test our hypothesis. In the first approach (Experiment 1) sleep loss was achieved by sleep deprivation (SD; 5 hours; by minimally stressful gentle handling technique), performed immediately after FR. In the second approach (Experiment 2) sleep loss was achieved by systemic administration of modafinil (FDA approved wake promoting agent), immediately after FR.

MATERIALS AND METHODS

Animals:

Adult male C57BL/6J mice (7–8 weeks old; 21 to 26 g; Jackson Laboratories, Bar Harbor, ME) were housed in the animal home room and habituated to new environment for at least two weeks before any experiment. Ambient room temperature ($25\pm 2^{\circ}\text{C}$) with 12:12 hour light-dark cycle [light onset at 8:00 AM] and *ad libitum* access to standard laboratory chow and water was provided throughout the experiment.

All experimental procedures met NIH guidelines for appropriate care and use of animals in research. All protocols were approved by local committees at Harry S. Truman Memorial Veterans' Hospital.

Based on data (percent time spent in freezing) obtained during preliminary study ($N = 3/\text{group}$), effect and sample size was calculated by performing *a priori* power analysis ($\alpha = 0.05$; power = 0.9; G*Power (Faul et al., 2007)).

All group assignments were performed randomly, using [Online GraphPad randomization calculator](#), just before the experiment.

Contextual Fear Conditioning:

Contextual fear conditioning (FC) was performed by using the NIR Video FC System (MED-VFC-SCT-M; Med Associates Inc., Fairfax, VT). This system consists of four FC contextual cages [CS; shock chambers with white acrylic back; stainless steel sides, grid floor, encased in a white sound-attenuated box equipped with a mono video camera (Basler, Germany) and a ventilation fan that provides background noise].

All FC sessions began after three days of habituation to the FC room. The habituation protocol was as follows: One hour after light onset, each mouse was brought from the animal home room to the adjoining FC room, housed singly in his cage. The mouse was removed from the cage and handled by placing it on the table for 15 min. On completion, the mouse was put back into his home cage and transferred back to the animal home room. Thus, all mice were housed singly after first day of habituation.

Fear Memory Acquisition (FMA):

FMA training session was conducted on Day 1, one hour after the light onset and lasted for 12 min. Each CS was wiped clean with alcohol (70%; Fisher Scientific; Pittsburgh, PA). Subsequently, four mice in their individual cages, were brought to the FC room and each mouse was placed in each CS. Mice were allowed to explore the chamber for 5 min. Subsequently, 7 foot-shocks (0.5 mA, 2.0 s duration) were delivered at pseudorandom intervals [average inter-trial interval = 52 sec; Range = 25–120 sec]. On completion, the animals were allowed to remain undisturbed for additional 56 sec (Total time = 12 min) before removing them from CS.

Controls underwent the same FMA procedure except no foot-shocks were delivered. Percent time spent in freezing was monitored for the entire 12 min period. After completion of

FMA, each animal was removed from the CS and returned to home cage and taken back to the animal home room after approximately 10 min. Once in the animal home room, mice were left undisturbed for 24 hours except for regular check for food and water and video monitoring of their behavior. This same procedure was followed during remaining two days of the experiment.

Fear Reactivation (FR):

FR was performed on Day 2, beginning one hour after the light onset. CS was wiped clean with 70% alcohol. Individual mouse was placed in CS and allowed to explore the CS for 2 min. No shock was delivered. Percent time spent in freezing was monitored during FR.

Fear Memory Recall (FMR):

On Day 3, FMR testing was performed. Each mouse was placed in the CS as described above and left undisturbed for 12 min. No shock was delivered. Between each mouse, CS was wiped clean with 70% alcohol. On completion of the experiment, mice were returned back to their home cage.

Experiment 1: Effect of sleep loss achieved by 5 hours of SD, immediately after fear reactivation, on fear memory reconsolidation.

Mice were divided into 3 groups [Controls (N=5); SD (N=5) and NSD (N=5)] and exposed to FMA on Day 1 and FR on Day 2 as described above. Immediately after the completion of FR session (within 30 min), mice in the NSD group (N = 5) were returned to the animal home room in the respective cages and allowed to sleep. Mice in the Control (N = 5) and SD (N = 5) groups were taken to an adjoining room for SD. SD was performed for 5 hours by gentle handling (gentle cage tapping, gentle prodding, introducing novel objects) as described previously (Sharma et al., 2010; Thakkar et al., 2008; Thakkar et al., 2003). On completion, mice were returned back to the vivarium and left undisturbed until FMR session on the following day.

Experiment 2: Effects of systemic modafinil administration, immediately after fear reactivation, on fear memory reconsolidation.

Mice were divided into 3 groups [Controls (N=5); Modafinil (N=5) and Vehicle (N=5)] and exposed to FMA on Day 1 and FR on Day 2 as described above. Immediately (within 30 min) after FR session, mice in the Control (N = 5) and Modafinil (N = 5) groups were transferred to the adjacent room and modafinil (Sigma-Aldrich, St. Louis, MO), dissolved in saline (0.9%) containing 0.5% DMSO, was administered intraperitoneally (200 mg/kg; 0.2ml) and then transferred to the animal home room. Whereas the mice in the vehicle group (N = 5) were transferred to the animal home room after FR and saline containing 0.5% DMSO (0.2 mL) was administered.

Data and Statistical Analysis:

On completion of the experiment, percent time spent in freezing during FMA (Day 1), FR (Day 2) and FMR (Day 3) were analyzed separately for both the experiments. One-Way ANOVA followed by Newman-Keuls *post-hoc* test was performed to examine freezing

behavior during FMA, FR and FMR. All statistical analysis was performed by Prism software (Graphpad Software, Inc., La Jolla, CA). $p < 0.05$ was considered level of significance.

RESULTS

Effect of 5 hours of SD, immediately after fear reactivation, on fear memory reconsolidation.

G*Power analysis (F test; $\alpha = 0.05$; power = 0.99) conducted after preliminary experiment suggested a total of 12 mice with effect size = 1.6.

a) During FMA: There was a significant [$F_{2, 14} = 8.35$, $p < 0.01$; $N = 5/\text{group}$; One-Way ANOVA] change in percent time spent in freezing during FMA. Subsequent Newman-Keuls *post-hoc* analysis showed that as compared to the Controls, mice in SD and NSD groups displayed a significant ($p < 0.01$) increase in the percent time spent in freezing. However, percent time spent in freezing during FMR was comparable between SD and NSD groups (Figure 1A).

b) During FR: On Day 2, FR was examined by monitoring percent time spent in freezing during 2 min exposure of CS. A significant [$F_{2, 14} = 31.40$, $p < 0.001$; $N = 5/\text{group}$; One-Way ANOVA] difference in the percent time spent in freezing during FR was observed. Subsequent *post-hoc* analysis showed that as compared to Controls, mice in the SD and NSD groups displayed a significant ($p < 0.001$) increase in the percent time spent in freezing. However, percent time freezing was comparable between SD and NSD groups (Figure 1B).

c) During FMR: A significant [$F_{2, 14} = 13.66$, $p < 0.001$; $N = 5/\text{group}$; One-Way ANOVA] effect was observed in the percent time spent in freezing behavior during FMR. *Post-hoc* analysis showed that as compared to the mice in the NSD group, mice in the SD group displayed a significant ($p < 0.001$) reduction in the percent time spent in freezing. No significant difference was observed in the percent time freezing in the Control and SD groups (Figure 1C).

Effects of systemic modafinil administration, immediately after fear reactivation, on fear memory reconsolidation.

G*Power analysis (F test; $\alpha = 0.05$; power = 0.99) conducted after preliminary experiment suggested a total of 12 mice with effect size = 1.7.

a) During FMA: A significant [$F_{2, 14} = 16.71$, $p < 0.01$; $N = 5/\text{group}$, One-Way ANOVA] difference in percent time spent in freezing during FMA. *Post-hoc* analysis showed that while mice in Vehicle and Modafinil groups displayed comparable time freezing during FMA, as compared to the Controls, mice in the Vehicle ($p < 0.01$) and Modafinil ($p < 0.01$) groups displayed a significant increase in percent time spent in freezing (Figure 2A).

b) During FR: A significant [$F_{2, 14} = 554.4$, $p < 0.001$; $N = 5/\text{group}$; One-Way ANOVA] difference was observed in percent time spent in freezing during FR. Subsequent analysis showed that as compared to controls, mice in the Vehicle ($p < 0.001$) and Modafinil ($p < 0.001$)

groups displayed a significant increase in the percent time spent in freezing in mice (Figure 2B) suggesting reactivation of fear in both vehicle and Modafinil groups animals. The percent time freezing was comparable between Vehicle and Modafinil groups.

c) During FMR: There was a significant [$F_{2, 14} = 35.7$, $p < 0.001$; $N = 5/\text{group}$; One-Way ANOVA] change in the percent time spent in freezing during FMR. Subsequent *post-hoc* analysis showed that as compared to mice in the Vehicle group, mice in the Modafinil group displayed a significant ($p < 0.001$) reduction in the percent time spent in freezing, while percent time spent in freezing during FMR was comparable in Control and Modafinil groups (Figure 2C).

DISCUSSION

In this study, we examined the role of sleep in reconsolidation of fear memory. The major finding of our study is that sleep have a critical role in memory reconsolidation. Sleep loss, achieved either by behavioral (sleep deprivation) or pharmacological (modafinil) intervention, immediately after FR, attenuates memory reconsolidation and causes amnesia. We believe our study is the first to demonstrate that sleep has a pivotal role in reconsolidation of fear memories.

We used the standard contextual fear-conditioning paradigm to examine the role of sleep in the reconsolidation of fear memory. To avoid for any non-specific effects, mice were singly housed and habituated to the FC room, before the experiment. Single housing also allowed us to perform video monitoring of the animal's behavior throughout the experimental period.

FMA was performed on Day 1. To rule out any non-specific effects Controls were exposed to same environment (CS), but no US was presented. As expected, during FMA training, while experimental groups showed freezing behavior, no such behavior was observed in controls. On completion of FMA training, mice were left undisturbed until the following day.

Fear reactivation was performed on Day 2. Mice were re-exposing to CS for a brief period (2 min), sufficient to reactivate fear memories but not enough to cause extinction (Debiec et al., 2002; Pedreira and Maldonado, 2003). As expected, as compared to Controls, all mice in the experimental group spent significantly more time in freezing suggesting that fear was reactivated in all the mice equally.

Sleep in mammals is a cyclic occurrence of two distinct states: Non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep. While REM sleep is implicated in consolidation of non-declarative memories, NREM sleep consolidates all major memory forms (Born and Gais, 2000; Diekelmann and Born, 2010; Ficca and Salzarulo, 2004; Giuditta, 1995; Maquet, 2001; Menz, et al., 2016; Rolls et al., 2013; Rudoy, et al., 2009). Thus, we examined the effect of total sleep (NREM and REM) loss on fear memory reconsolidation.

We used two different approaches to achieve sleep loss: behavioral and pharmacological. Behavioral intervention was achieved by performing total sleep deprivation for 5 hours,

using the gentle handling paradigm. The gentle handling sleep deprivation protocol is minimally stressful and is used extensively to examine the effects of total sleep deprivation on several behaviors including learning and memory (Blanco-Centurion et al., 2006; Cordeira et al., 2017; Graves, et al., 2003; Kalinchuk et al., 2008; Palchykova et al., 2006; Sharma, et al., 2010; Thakkar, et al., 2008; Thakkar, et al., 2003). Sleep deprivation was performed in the adjoining room to avoid disturbing other animals. We also performed total sleep deprivation in control mice (exposed to the CS but no shock) to rule out any non-specific effects.

Sleep loss by pharmacological approach was achieved by systemic administration of modafinil (200 mg/kg), a FDA approved wake promoting agent. The 200 mg/kg dose was chosen because several studies suggest that this dose of modafinil is sufficient to cause 3 to 5 hours of sleep loss (Kopp et al., 2002; Parmentier et al., 2007). Furthermore, to rule out any non-specific effects, the same dose of modafinil was administered to controls.

Our results suggest that irrespective of the intervention used, mice exposed to sleep loss (SD and Modafinil groups) displayed a significant reduction in time spent in freezing behavior during FMR implicating the importance of sleep in fear memory reconsolidation.

Our data are congruent with a previous study where the effect of total sleep deprivation on reactivation of morphine reward memory was examined (Shi et al., 2011). Six hours of sleep deprivation immediately after reactivation resulted in disruption of morphine reward memory (Shi, et al., 2011). However, selective REM sleep deprivation, using highly stressful flowerpot technique, had no effect on fear memory reconsolidation in rats (Thakkar and Mallick, 1991; Tian et al., 2009). The results of this study suggest that NREM sleep and/or intact sleep architecture may have a prominent role in fear memory reconsolidation.

It is well recognized that consolidated memories can be brought back to vulnerable state by the process of reactivation (Tronson and Taylor, 2007). Reactivation process is observed during retrieval as well as during any operation that enlarges or modifies consolidated memories. This is followed by the process of reconsolidation. The process of reconsolidation maintains and/or updates memories (Hardt et al., 2010; Lee, 2010; Morris et al., 2006). Both consolidation and reconsolidation require protein synthesis and/or synaptic consolidation. Sleep has a critical role in memory consolidation. Our results suggest that sleep may have a major role in memory reconsolidation. Thus, sleep is important for both, memory consolidation and reconsolidation.

CONCLUSION

In summary, we have examined the effect of sleep loss on reconsolidation of fear memories in mice. We used behavioral (sleep deprivation) and pharmacological (Modafinil) approaches to achieve sleep loss. The result of our studies suggests that sleep loss after FR resulted in attenuation of fear memories implicating a role of sleep in fear memory reconsolidation.

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Highlights:

- Permanently stored memories become labile through a process of reactivation.
- Reactivated memories need reconsolidation to become permanent.
- Sleep deprivation, after memory reactivation, blocks memory reconsolidation.
- Modafinil treatment, after memory reactivation, attenuates memory reconsolidation.

Figure 1:

Effects of SD on fear memory reconsolidation. **Panel A:** On Day 1, mice in the NSD and SD groups, exposed to US in the CS, spent significantly more time in freezing as compared to mice in the Control group (no US) during FMA. **Panel B:** On Day 2, mice were exposed to CS for a brief period (2 min). As compared to Controls, mice in NSD and SD groups displayed a significant increase in the percent time spent in freezing suggesting reactivation of fear memories. **Panel C:** On Day 3, mice were exposed to CS for 12 min to test memory recall. While mice in the SD and Control group displayed comparable freezing behavior, mice in the SD group showed a significant reduction in time spent in freezing as compared to mice in the NSD group suggesting that SD after FR prevented reconsolidation of fear memories. Individual data points (red triangles) are superimposed on all bar graphs.

** $p < 0.01$ and *** $p < 0.001$ vs Control. §§ $p < 0.01$ vs NSD.

Figure 2:

Effects of modafinil (a wake promoting agent) on fear memory reconsolidation. **Panel A:** On Day 1, mice in Vehicle and Modafinil groups, received US in the CS, displayed a significant increase in the percent time spent in freezing as compared to mice in the Control group during FMA **Panel B:** On Day 2, mice were briefly (2 min) exposed to CS alone during FR. As compared to the Controls, mice in the Vehicle and Modafinil group displayed a significant increase in the percent time spent in freezing suggestive of reactivation of fear memories. **Panel C:** On Day 3, mice in all the groups were exposed to CS for 12 min to determine fear memory recall. While mice in the Modafinil and the Control group displayed a comparable freezing behavior, mice in the Modafinil group displayed a significant reduction in the percent time spent in freezing as compared to mice in the Vehicle group suggesting that modafinil after FR leads to erasure of the fearful memories. Individual data points (red triangles) are superimposed on all bar graphs. ** $p < 0.01$ and *** $p < 0.001$ vs Control. §§ $p < 0.01$ vs Vehicle.