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Fear Conditioning Increases NREM Sleep

Kevin Hellman and
Neuroscience Graduate Group, University of Pennsylvania;. The Department of Neurobiology, Pharmacology, and Physiology, University of Chicago

Ted Abel
Department of Biology, University of Pennsylvania

Abstract

To understand the role that sleep may play in memory storage, the authors investigated how fear conditioning affects sleep–wake states by performing electroencephalographic (EEG) and electromyographic recordings of C57BL/6J mice receiving fear conditioning, exposure to conditioning stimuli, or immediate shock treatment. This experimental design allowed us to examine the effects of associative learning, presentation of the conditioning stimuli, and presentation of the unconditioned stimuli on sleep–wake states. During the 24 hr after training, fear-conditioned mice had approximately 1 hr more of nonrapid-eye-movement (NREM) sleep and less wakefulness than mice receiving exposure to conditioning stimuli or immediate shock treatment. Mice receiving conditioning stimuli had more delta power during NREM sleep, whereas mice receiving fear conditioning had less theta power during rapid-eye-movement sleep. These results demonstrate that a single trial of fear conditioning alters sleep–wake states and EEG oscillations over a 24-hr period, supporting the idea that sleep is modified by experience and that such changes in sleep–wake states and EEG oscillations may play a role in memory consolidation.

Keywords

fear conditioning; EEG; sleep; mouse; memory

Memory consists of several stages including acquisition, consolidation, and retrieval (Abel & Lattal, 2001; Morris et al., 2003). With a single-trial learning task such as fear conditioning, it is possible to dissect these individual stages. Through the use of pharmacology, lesions, and genetics, researchers have begun to define the molecular and neural systems underlying memory consolidation for fear conditioning (Maren & Quirk, 2004). There have been many reports of sleep enhancing memory consolidation and of sleep deprivation impairing memory consolidation (Graves, Pack, & Abel, 2001; Smith, 1995; Stickgold, Hobson, Fosse, & Fosse, 2001), including recent studies showing that sleep deprivation impairs fear conditioning (Graves, Heller, Pack, & Abel, 2003; McDermott et al., 2003; Ruskin, Liu, Dunn, Bazan, & LaHoste, 2004; Silvestri, 2005). Deprivation of sleep during the first 5 hr after training selectively impairs hippocampus-dependent long-term memory for contextual fear conditioning, without altering memory for cued conditioning (a hippocampus-independent task), suggesting that sleep is important for memory consolidation of tasks mediated by the hippocampal system (Graves, Heller, et al. 2003).

Correspondence concerning this article should be addressed to Ted Abel, Department of Biology, 204G Lynch Laboratories, University of Pennsylvania, Philadelphia, PA 19104-6084. abele@sas.upenn.edu.

Two stages of sleep potentially contribute to long-term memory consolidation (Hellman & Abel, 2003; Walker & Stickgold, 2004). One stage is rapid-eye-movement (REM) sleep, when theta oscillations are predominant (Vanderwolf, 1969) and when "replay" of hippocampal neural firing patterns that occur during wakefulness might consolidate memory (Louie & Wilson, 2001; Pavlides & Winson, 1989; Poe, Nitz, McNaughton, & Barnes, 2000; Ribeiro, Goyal, Mello, & Pavlides, 1999; Ribeiro et al., 2002). During nonrapid-eye-movement (NREM) sleep, neurons are highly synchronized throughout the cortex (Steriade, Dossi, & Nunez, 1991), and this synchronized activity may induce changes at simultaneously activated synapses (Sejnowski & Destexhe, 2000). Indeed, the repeated excitation of neurons at high frequencies comparable with protocols used to induce long-term potentiation occurs in the hippocampus during NREM sleep (Buzsaki, 1998; Sirota, Csicsvari, Buhl, & Buzsaki, 2003). In addition, replay also occurs during both NREM and REM sleep (Nadasdy, Hirase, Czurko, Csicsvari, & Buzsaki, 1999; Wilson & McNaughton, 1994). Levels of many neurotransmitters, growth factors, and transcriptional regulatory factors known to affect memory consolidation change dramatically during different sleep–wake states (Hellman & Abel, 2003).

Although the role of sleep in memory consolidation is controversial (Vertes, 2004; Vertes & Siegel, 2005), reports about changes in sleep after learning are generally consistent (Walker & Stickgold, 2004). For example, REM sleep increases after two-way active avoidance (Bramham, Maho, & Laroche, 1994; Datta, 2000), but decreases in the 6-hr period after passive avoidance training (Mavanji, Siwek, Patterson, Spoley, & Datta, 2003). REM sleep increases in rats after training in the hippocampus-dependent hidden platform version of the Morris water maze but not following training for the visible platform version, suggesting that hippocampus-dependent memory involves sleep–wake changes (Smith & Rose, 1997). Correlations between performance and sleep in humans have also supported the relationship between sleep and memory (Plihal & Born, 1999; Stickgold, James, & Hobson, 2000; Walker, Brakefield, Hobson, & Stickgold, 2003). Increased EEG delta power during sleep after learning has been reported in humans (Huber, Ghilardi, Massimini, & Tononi, 2004) and rats (Miyamoto, Katagiri, & Hensch, 2003). Changes in sleep have been reported after multiple trials of fear conditioning for a model of anxiety and reductions in REM sleep have been reported after a single trial of training in BALB/cJ mice (Sanford, Fang, & Tang, 2003). Here, we examine sleep after a single trial of fear conditioning in C57BL/6J mice using controls for the presentation of conditioned stimuli and shock. Changes in sleep–wake states and EEG spectral properties after learning provide insight into the ways in which sleep may enhance memory consolidation.

Method

Subjects

Adult male C57BL/6J mice (Jackson Labs, Bar Harbor, ME) were housed in our colony under standard conditions. Food and water were provided ad libitum. Mice were maintained on a 12-hr light–dark cycle with lights on at 7 a.m. All animal care and experiments were carried out in accordance with the National Institutes of Health (1986) guidelines and were fully approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Surgery

Adult male C57BL/6J mice (12–24 weeks of age) were implanted with electroencephalographic (EEG) and electromyographic (EMG) electrodes under isofluorane anesthesia. An electric drill was used to thin the skull near the place of electrode implantation. Surface EEG electrodes were implanted 1.0 mm bilateral to midline and 1.3 mm posterior to bregma on each side. A third EEG electrode was placed 1.0 mm lateral to midline and 4.0 mm posterior to bregma. A reference EEG electrode was placed 1.5 mm posterior to lambda over

the cerebellum (Huber, Deboer, & Tobler, 2000). EMG electrodes were attached to the nuchal musculature. Electrodes were held in place with glass ionomer resin (Ketaccem, 3M, Maplewood, MN; Chemelli et al., 1999). Electrodes consisted of silver ball electrodes (Φ = 0.33 mm) insulated with Teflon, soldered to gold socket contacts (Plastics One, Roanoke, VA), and pushed into a six-pin plug (363 plug, Plastics One), which was then attached to a commutator (SLC6, Plastics One; Veasey et al., 2000). All recordings were analyzed with the right posterior EEG electrode and were referenced to the cerebellum electrode. This electrode-referencing scheme is ideal for detecting alterations in delta and theta activity in mice (Franken, Lopez-Molina, Marcacci, Schibler, & Tafti, 2000; Tafti et al., 2003). Mice were housed individually and allowed 2 weeks recovery after surgery. During the 2-week recovery period, mice remained in the recording chambers with the EEG–EMG cable attached.

Data Acquisition

EEG signals were filtered at 0.3–60.0 Hz (0.5 amplitude, 6 dB/octave), and EMG signals were filtered at 1–100 Hz with 12A5 amplifiers (Astro-Med, West Warwick, RI) and sampled at 256 Hz with 12-bit resolution (Graves, Hellman, Veasey, Pack, & Abel, 2003). The observers were blind to group conditions and scored EEG and EMG recordings (wakefulness, NREM sleep, REM sleep) according to previously established criteria (Graves, Hellman, et al., 2003; Ouyang, Hellman, Abel, & Thomas, 2004), which was similar to human criteria (Rechtschaffen & Kales, 1968). Behavioral states were scored by visual examination of 4-s EEG and EMG epochs during the 24-hr period before and after training. Samples of scored epochs are shown in Figure 1. Data acquisition and visual scoring of EEG–EMG recordings was performed with custom software. Signals were calibrated with 100-μV pulses. We performed fast Fourier transform (FFT) analysis of the EEG signals with custom software. The average FFT spectra for wakefulness, NREM sleep, and REM sleep were calculated. The normalized relative spectra were determined by calculating the relative contribution of each frequency bin for each state (Franken et al. 1999). The contributions from the delta (1–4 Hz), theta (6–8 Hz), and sigma (10–14 Hz) frequency bands were determined for relative spectra. We determined the average spectra during the 24-hr pretraining period and the 24-hr posttraining period in each of the three states (wakefulness, NREM sleep, REM sleep) as well as the average spectra in 4-hr blocks. To specifically determine whether the dominant oscillations in NREM sleep and REM sleep were altered, we identified the peak amplitude frequency within the delta and theta range within each mouse before and after training. We compared differences between the three groups of mice using two methods: mean power and time-locked comparison. In the mean power method, the power spectra for each time point was divided by the average total power of the pretraining day for each mouse. The average percent power for each group of mice for each 4-hr block was calculated by dividing the power by total mean power from the pretraining day. Time-locked comparison was carried out by calculating the percentage change in power relative to the same circadian pretraining time point. The power in each 4-hr time window was divided by the amount of power in the corresponding 4-hr time point from the previous day. Next, the average relative change at each time point for each group was calculated.

Fear Conditioning

After allowing 2 weeks for recovery from the surgical implantation of EEG and EMG electrodes, mice were handled for 5 min per day on 3 consecutive days. We performed single-trial fear conditioning (Abel et al., 1997), and control groups received either exposure to the conditioning stimuli (CS) or an immediate shock (Abel & Lattal, 2001). All mice were handled, trained, and tested at Zeitgerber (ZT, circadian time of light onset) Hour 4. The mice were trained in a rectangular chamber (16.00 in. × 6.00 in. × 8.38 in. [40.64 cm × 15.24 cm × 21.27 cm]) with a shock grid floor that was connected to a Shock-Grid Scrambler (MED Associates, East Fairfield, VT). The chamber was divided in half by a closed divider, and all of the mice

were placed in the left half of the chamber. The chamber was cleaned with 70% ethanol before the training and testing of each mouse. On the training day, each mouse was individually removed from its home cage within the recording chamber and was placed into the fear-conditioning chamber. Fear-conditioned mice (n = 16) received context exposure followed by a 30-s tone (2,800 Hz and 85 dB) at 2 min, coterminating with a 2-s 1.5-mA shock. After the shock, fear-conditioned mice were allowed to remain in the chamber for 30 s. The CS group (n = 15) received the same treatment as the fear-conditioned mice except that they did not receive a shock. A third group of mice, the immediate shock group (n = 12) was used as a control for training. Immediate shock mice received a shock and a 30-s tone initiating within the first 5 s of context exposure (Lattal & Abel, 2001). The tone and shock began at the same time in the immediate shock group. After the tone and shock, mice were allowed to remain in the context for a full 3 min. Hence, immediate shock mice received the same amount of time in the novel context as fear-conditioned mice and CS mice. After training, the mice were returned to the recording chamber and left undisturbed for the next 24 hr. Cohorts of 6–8 mice were recorded and trained simultaneously, and each cohort always included mice from each of the three training groups. Twenty-four hours after training, mice were tested in the same context by evaluating the amount of freezing over a 5-min period. Freezing, which is a complete lack of movement except for respiration, was assessed at 5-s intervals (Blanchard & Blanchard, 1969; Fanselow, 1980). The amount of freezing was calculated by counting the number of times mice exhibited freezing during each test. The percentage of freezing was calculated by the number of intervals scored as freezing during the 5-min testing period.

Statistical Analysis

Repeated measures analyses of variance (ANOVA) were performed in SigmaStat (Version 3.1, Systat Software, Richmond, CA). Post hoc Tukey tests were performed for multiple comparisons. Between-subjects comparisons were made between the different groups on the pretraining and posttraining day. Within-subject comparisons were made by comparing pretraining and posttraining day within the same mouse. The use of these two types of comparisons allowed us to evaluate the effect of the components of the fear-conditioning task on sleep–wake states as well as the differences between the groups. All results are presented as the mean plus/minus the standard error of the mean.

Results

NREM Sleep Increases Following Fear Conditioning

We analyzed sleep–wakes states in mice receiving CS exposure, immediate shock, and fear conditioning (see Figure 2). We verified that only fear-conditioned mice learned to associate the context with a shock by testing 24 hr after training. CS mice froze 2% ± 1% of the time and immediate shock mice froze 4% ± 2% of the time during the 5-min testing period. Fear-conditioned mice froze 20% ± 2% of the time, which was significantly more than CS or immediate shock mice (ps < .01). As can be seen from the reduced level of freezing (see Figure 2), the CS group provided a control for effects of novel context exposure and tone exposure. The immediate shock group received a footshock and tone immediately on context exposure and exhibited reduced conditioning when tested in the same context 24 hr after training (see Figure 2) and thus provided a control for the effect of footshock. By comparing sleep–wake states after training in these three groups, we sought to identify changes due to CS, to footshock, or to the associative learning of the relationship between the CS and US.

We found that fear-conditioned mice have more NREM sleep and less wakefulness in the 24-hr period after training (see Figure 3). We recorded from all three groups of mice prior to training to verify there were no inherent differences in the three groups. On the day prior to training, all mice were handled for 5 min and were then returned to their home cage. In the 24

hr prior to training, all three groups had similar amounts of time spent awake, in NREM sleep, and in REM sleep (see Figure 3). This distribution was altered only after training and alterations were exclusive to NREM sleep and wakefulness. During the 24-hr period after training, mice that received fear conditioning had approximately 1 more hour of NREM sleep than the mice in the control groups (*p*s < .05). During the first 24 hr after training, CS mice had 547 ± 22 min, immediate shock mice had 562 ± 23 min, and fear-conditioned mice had 625 ± 24 min of NREM sleep. Thus, mice in both control groups exhibited a 13% decrease in NREM sleep relative to the fear-conditioned mice in the 24 hr after training. In contrast, no differences were seen in the duration of REM sleep during the 24-hr period after training. CS, immediate shock, and fear-conditioned mice had 62 ± 5 min, 64 ± 6 min, and 65 ± 5 min of REM sleep, respectively, during the posttraining day. The additional NREM sleep in fear-conditioned mice was accompanied by a decrease in wakefulness. CS, immediate shock, and fear-conditioned mice were awake 827 ± 20 min, 811 ± 21 min, and 745 ± 24 min, respectively. Accordingly, fear-conditioned mice exhibit a 10% reduction in wakefulness relative to control groups (*p*s < .05). Training specifically altered sleep–wake states as demonstrated by a repeated measures ANOVA and Group × Day interactions. We found a significant effect of training with Group × Day interactions on wakefulness ($p < .05$) and NREM sleep ($p < .05$) but not for REM sleep ($p = .9$). Relative to baseline, changes in sleep were due to both an increase in NREM sleep in fear-conditioned mice and to a decrease in NREM sleep in CS and immediate shock mice (see Figure 3). We performed sleep microstructure analysis to determine the mechanism responsible for alterations in sleep time (data not shown). Although fear-conditioned mice had 17% longer average NREM sleep bouts than CS mice and had 24% longer NREM sleep bouts than immediate shock mice (over 24 hr), these differences were not significant ($p = .24$ by one-way ANOVA). Over the 24-hr period after training, fear-conditioned mice had only 1% more NREM bouts than CS mice and had 10% fewer NREM bouts than immediate shock mice. Hence, it is most likely that the longer sleep time is caused by longer bout lengths and not by a greater number of bouts. These results suggest that the experience of fear conditioning increases NREM sleep in comparison with CS exposure or immediate shock training. Thus, more NREM sleep is seen selectively in the group that experiences associative learning during conditioning.

To identify specific time periods during which sleep–wake states are altered, we examined the amount of sleep across the 24-hr period in 4-hr windows (see Figure 4). Because training occurs at ZT Hour 4, there are two 4-hr windows following training in the light period. The dark period consisted of three 4-hr windows, and there was an additional 4-hr window in the light period prior to testing. The largest difference in NREM sleep in fear-conditioned mice relative to control mice occurred 12–16 hr after training (Tukey $p < .05$). There were no significant differences in the number of NREM sleep bouts during the 12- to 16-hr period after training between the three groups (CS = 54 ± 4, immediate shock = 61 ± 9, fear conditioned = 63 ± 7). However, there was a significant difference in the NREM sleep bout length (CS = 1.41 ± 0.11, immediate shock = 1.20 ± 0.19, fear conditioned = 1.62 ± 0.16; $p < .001$). Tukey tests revealed that fear-conditioned mice had significantly larger bout lengths ($p < .05$) than did immediate shock and CS mice. During the first 5 hr after fear conditioning, which Graves, Heller, et al. (2003) demonstrated was sensitive to sleep deprivation, fear-conditioned mice had 16 min (11%) more NREM sleep than did control mice (CS = 149 ± 7, immediate shock = 149 ± 7, fear conditioned = 165 ± 9; $p = .13$). Relative to the pretraining day, significant increases in NREM sleep in fear-conditioned mice were observed 20–24 hr after training ($p < .05$). No other significant alterations in sleep (relative to the pretraining day) were observed in CS or in immediate shock mice in any of the 4-hr windows. Changes in wakefulness mirrored changes in NREM sleep (data not shown). Twelve to sixteen hours after training, fear-conditioned mice had significantly less wakefulness than CS or immediate shock mice ($p < .05$). Relative to the pretraining day, a Tukey test revealed significant decreases in wakefulness in fear-conditioned mice observed 20–24 hr after training ($p < .05$). We did not find any significant alterations in

the duration of REM sleep (when analyzed in 4-hr bins), REM bout length, REM bout number, or in the REM sleep to NREM sleep efficiency ratio (data not shown). Thus, the primary effect of fear conditioning on sleep–wake states is increased NREM sleep at the expense of wakefulness.

Increased NREM Delta Power Following CS Exposure but not Following Fear Conditioning or Immediate Shock

Behaviorally induced alterations in sleep are often accompanied by changes in EEG spectral content such as delta power (1–4 Hz; Borbely, 1982) or spindles (10–14 Hz; Gais, Molle, Helms, & Born, 2002). We examined EEG signals using a spectral analysis technique (fast Fourier transform; FFT) similar to previous studies looking for the effects of genetics on sleep oscillations (Franken, Chollet, & Tafti, 2001; Franken, Malafosse, & Tafti, 1998; Graves, Hellman, et al., 2003). Because we found an effect of fear conditioning on NREM sleep during the 24-hr period after training, we performed an FFT on the entire 24-hr period and compared the relative spectral content of the fear-conditioning group with the CS and immediate shock group (see Figure 5). Pretraining differences between the three groups were not observed. However, CS mice had a significant increase in delta power during NREM sleep relative to pretraining levels ($p < .05$). These differences are not directly caused by differences in NREM sleep time because spectral data are determined from the average power per epoch of NREM sleep. Increases in power were observed in delta frequency bands (1–4 Hz) and were not caused by shifts in the delta power due to acceleration or deceleration because the peak amplitude frequency did not change. Relative to pretraining, increased delta power was not observed in mice receiving immediate shock or fear conditioning. To establish the time course of changes in NREM sleep delta power, we performed an ANOVA on pretraining and posttraining NREM sleep delta power (see Figure 6). As expected, there was an effect of time on delta power ($p < .001$) on both pretraining and posttraining days. However, we did not observe an effect of group on delta power across the 24-hr period ($p = .29$). Nevertheless, when we compared delta power after training with pretraining power, CS mice had a significant increase in delta power 12–16 hr after training ($p < .05$). This data point merits attention because it is the same time window in which fear-conditioned mice were observed to have had more NREM sleep and less wakefulness relative to CS and immediate shock mice.

Decreased REM Theta Power Following Fear Conditioning

Although we did not observe any differences in the time spent in REM sleep after training, we examined theta oscillations underlying REM sleep (see Figures 5 and 7) as these have been the focus of much study (Bramham et al., 1994; Huerta & Lisman, 1993; Louie & Wilson, 2001; Moita, Rosis, Zhou, LeDoux, & Blair, 2003; Poe et al., 2000; Seidenbecher, Laxmi, Stork, & Pape, 2003). Theta oscillations were always prominent during REM sleep and had a peak amplitude between 7 and 8 Hz; the increased theta power in REM sleep compared with that recorded during wakefulness and NREM sleep was always observed in the FFT spectral analysis. Spectral analysis revealed a 17% ± 5% reduction in 7–8 Hz power during REM sleep in fear-conditioned mice compared with pretraining levels ($p < .01$). In contrast, reductions in 7–8 Hz power seen in CS (5% ± 3%) and immediate shock mice (1% ± 6%) were not significant. The observed reductions in theta power occur at the peak of the theta spectrum and were not a product of frequency acceleration or deceleration. To establish the time course of changes in theta power, we examined relative changes in theta power in 4-hr time windows (see Figure 7). There were no significant differences in the pretraining data between the three groups ($p = .99$), but there was a significant effect of group after training on theta power ($p < .001$). Significant decreases in theta power were observed in fear-conditioned mice throughout the posttraining day (ps $< .05$) except during the 12- to 16-hr time period after training ($p = .18$). Alterations in theta power in mice receiving CS exposure or immediate shock were not significant when compared with pretraining levels. These frequency bands and others were not

altered during wakefulness (data not shown). Our findings show that theta power is decreased during REM sleep after fear conditioning.

Discussion

Our results show that fear conditioning increases the amount of time spent in NREM sleep and decreases theta power during REM sleep during the 24-hr period after training. These changes occur within-subjects as well as relative to control groups receiving CS exposure or immediate shock. Because more NREM sleep and less theta power were seen in the groups exhibiting conditioning, our results show that these alterations are likely induced by associative learning. The most significant differences in NREM sleep in fear-conditioned mice were observed 12–16 hr after training during the dark period. In addition, the experience of CS exposure without shock produced increased delta power during NREM sleep, particularly during the 12- to 16-hr time period after training. Although recent work has implicated alterations in sleep homeostasis after learning (Huber et al., 2004; Molle, Marshall, Gais, & Born, 2004; Tononi & Cirelli, 2003), our study is the first to find a substantial difference in NREM sleep during the 24-hr period after learning.

More NREM sleep time was observed in fear-conditioned mice than in immediate shock mice, suggesting that changes in sleep were not merely due to the stress of experiencing the shock. Numerous papers have suggested that the immediate shock group controls for many aspects of associative learning that occurs during fear conditioning, particularly the effects of shock exposure (Huff et al., 2006; Kim, Rison, & Fanselow, 1993; Landeira-Fernandez, DeCola, Kim, & Fanselow, 2006; Lattal & Abel, 2001; Matus-Amat, Higgins, Barrientos, & Rudy, 2004; Rosen, Fanselow, Young, Sitcoske, & Maren, 1998; Rudy, Barrientos, & O'Reilly, 2002; Rudy & Wright-Hardesty, 2005; Wiltgen, Sanders, Anagnostaras, Sage, & Fanselow, 2006). We also included a control group in which mice were exposed only to the CS. These two control groups allow us to suggest that changes in sleep specific to the fear-conditioning group are likely the result of associative learning about the relationship between the CSs (context and tone) and the unconditioned stimulus (footshock). Fear conditioning requires the amygdala (for both tone-dependent and context-dependent freezing) and the hippocampus (primarily for context dependent freezing; Kim et al., 1993; Logue, Paylor, & Wehner, 1997; Wiltgen et al., 2006). We have previously shown that long-term memory for hippocampus-dependent contextual fear conditioning requires sleep within 5 hr after training but that amygdala-dependent tone-dependent conditioning does not-(Graves, Heller, et al., 2003). With our experimental design here, however, we cannot determine whether the observed changes in sleep are due to the hippocampus-dependent association of context and unconditioned stimulus or the amygdala-dependent association of the cue and the CS. Defining this will require researchers to conduct additional experiments incorporating context-only conditioning and context preexposure (Hall & Minor, 1984; Holt & Maren, 1999; Huff, Wright-Hardesty, Higgins, Matus-Amat, & Rudy, 2005). Future experiments could also address the potential decreases in NREM sleep following CS exposure and immediate shock that are suggested by our experiments (see Figure 3).

REM sleep was increased (albeit insignificantly) in all three groups, unlike other studies that have reported decreased REM sleep after different types of stressful experiences (Kant et al., 1995; Papale, Andersen, Antunes, Alvarenga, & Tufik, 2005; Pawlyk, Jha, Brennan, Morrison, & Ross, 2005; Rabat, Bouyer, Aran, Le Moal, & Mayo, 2005). Cage changes and exposure to novel objects also reduce REM sleep in C57BL/6J mice (Tang, Xiao, Parris, Fang, & Sanford, 2005). In our study, the overall stress was short in duration (5 min), mice were habituated to handling prior to context exposure (thus reducing overall stress), and the level of fear as measured by induced freezing was moderate. In the one published study that used a single shock protocol, an increase in NREM sleep ($p = .051$) was seen on the second posttraining day

during the light period in BALB/cJ mice (Sanford et al., 2003). Those mice received a single shock and also had an 80% reduction in REM sleep during the 1st day following training. In the same study, it was observed that multiple shocks decrease REM sleep even further. It has also been shown that repetitive cued conditioning reduces REM sleep in the C57BL/6J mice after training (Sanford et al., 2003). Therefore, it has been hypothesized that more shocks result in elevated stress, which cause greater reductions in REM sleep. It is thus unlikely that stress is responsible for the changes reported in our study.

Although changes in NREM sleep occurred throughout the 24-hr period, increased NREM sleep was most significant during the 12- to 16-hr period after training. Percentagewise, however, the most significant increase in NREM sleep within the fear-conditioned group occurred 20–24 hr after training, although fear-conditioned mice were not sleeping significantly more than mice from the other two groups during this time period. This dichotomy can be explained because apparent increases during the 12–16 hr after training is partially due to decreased sleep in CS and immediate shock mice. The baseline tendency towards increased sleep in the 12- to 16-hr window and less sleep in 20- to 24-hr window in fear-conditioned mice could amplify such effects. In any case, the most reliable comparison in this experiment is between groups because of the statistical issues associated with our experimental design and the established validity of our controls. The changes 12–16 hr after training might be a product of an enhanced siesta, a period of increased NREM sleep density that occurs in several mouse strains including the C57/B6 strain during the middle of the dark cycle (Franken, Malafosse, & Tafti, 1999; Veasey et al., 2000). Genetic deletion of the transcription factor NPAS2 specifically inhibits siesta sleep in C57BL/6J mice and also impairs long-term memory for conditioning, without affecting short-term memory or circadian parameters (Dudley et al., 2003; Garcia et al., 2000). These results support that sleep during this time period in the middle of the dark cycle may be beneficial to memory consolidation. Alteration in expression of NPAS2 after learning could affect sleep during the 12- to 16-hr time period as well as memory consolidation. Future experiments will be needed to address the issue of whether the timing of the increase in NREM sleep is affected by circadian or training time.

Increased delta power during NREM sleep was observed in mice receiving CS exposure but not in either of the groups receiving shock. Behaviorally, it has been demonstrated that CS expo sure is a form of learning because previous CS exposure facilitates future fear conditioning (Barrientos, O'Reilly, & Rudy, 2002) Exploration of a new context produces significant alterations in gene expression within the hippocampus (Guzowski, McNaugh ton, Barnes, & Worley, 1999) and results in neural activity patterns resembling prior exploration during NREM (Nadasdy et al., 1999) and REM sleep (Louie & Wilson, 2001). Only recently has it been demonstrated that learning can influence delta power (Huber et al. 2004). After learning a motor task, humans had increased delta activity localized to Brodmann Areas 40 and 7, which are responsible for processing sensory information relevant to the motor task (Huber et al., 2004). Further, there was a high correlation between postsleep performance and delta activity within these brain regions implying that slow wave activity is involved in memory consolidation.

A variety of different mechanisms could be responsible for increased NREM sleep observed in fear-conditioned mice. One candidate region mediating this process is the ventral lateral hypothalamic preoptic nucleus (VLPO), which is important for the regulation of NREM sleep (Lu, Greco, Shiromani, & Saper, 2000) Activation of the VLPO results in NREM sleep (Sherin, Shiromani, McCarley, & Saper, 1996), and lesions of the VLPO pro duce a 50%–60% reduction in NREM sleep, a 60%–70% reduction in delta power (Lu et al., 2000), and impairments in spatial memory in the Morris water maze (Ward et al., 2005). The ventral hippocampus, which is critical for expression of contextual fear (Kjelstrup et al., 2002), projects to the VLPO (Chou et al., 2002) Fear conditioning induces *c-fos* activation in the hippocampus and the entire

hypothalamus except for the suprachiasmatic nucleus (Campeau et al., 1997). Thus, projections from the ventral hippocampus to the hypothalamus might modulate levels of sleep to aid in the process of memory consolidation.

The role of sleep in memory consolidation has been contested because reductions in REM sleep in humans do not appear to affect memory tasks and because changes in task performance over time could be unrelated to sleep (Vertes, 2004). Our work challenges these assumptions because fear conditioning in this study affects NREM sleep and not REM sleep. Thus, performance on this task both requires and alters NREM sleep. The majority of animal studies have observed changes in REM sleep after learning (Vertes & Eastman, 2000). Perhaps the difficulty in controlling stimuli and environment in humans frequently precludes the ability to detect changes in sleep after learning. Human studies have found that changes in REM sleep after learning are dependent on the type of task (Smith, 2001) and that these changes may also be reflected in REM sleep density or eye movements rather than in the amount of REM sleep (Smith, Nixon, & Nader, 2004). In our study, we controlled exposure to stressful stimuli and included two control groups to define precisely the effects of associative learning on sleep. It will be interesting for future studies to explore whether fear conditioning also affects sleep in human subjects. It is worth noting that fear conditioning continues to consolidate over the first 24 hr and, indeed, over the weeks after training. As shown in Wood et al. (2005), levels of freezing increase over 24 hr (from 45% at 1 hr to 58% at 24 hr). Further, the contextual specificity of freezing varies over days of retention (Balogh & Wehner, 2003), and the neurochemical and anatomical circuits mediating retrieval vary over retention intervals ranging from days to weeks (Frankland, Bontempi, Talton, Kaczmarek, & Silva, 2004; Murchison et al., 2004). Thus, fear conditioning is not an all or none process, but it is modulated over time. Our data suggest that sleep may play a role in consolidation of memory for contextual fear conditioning.

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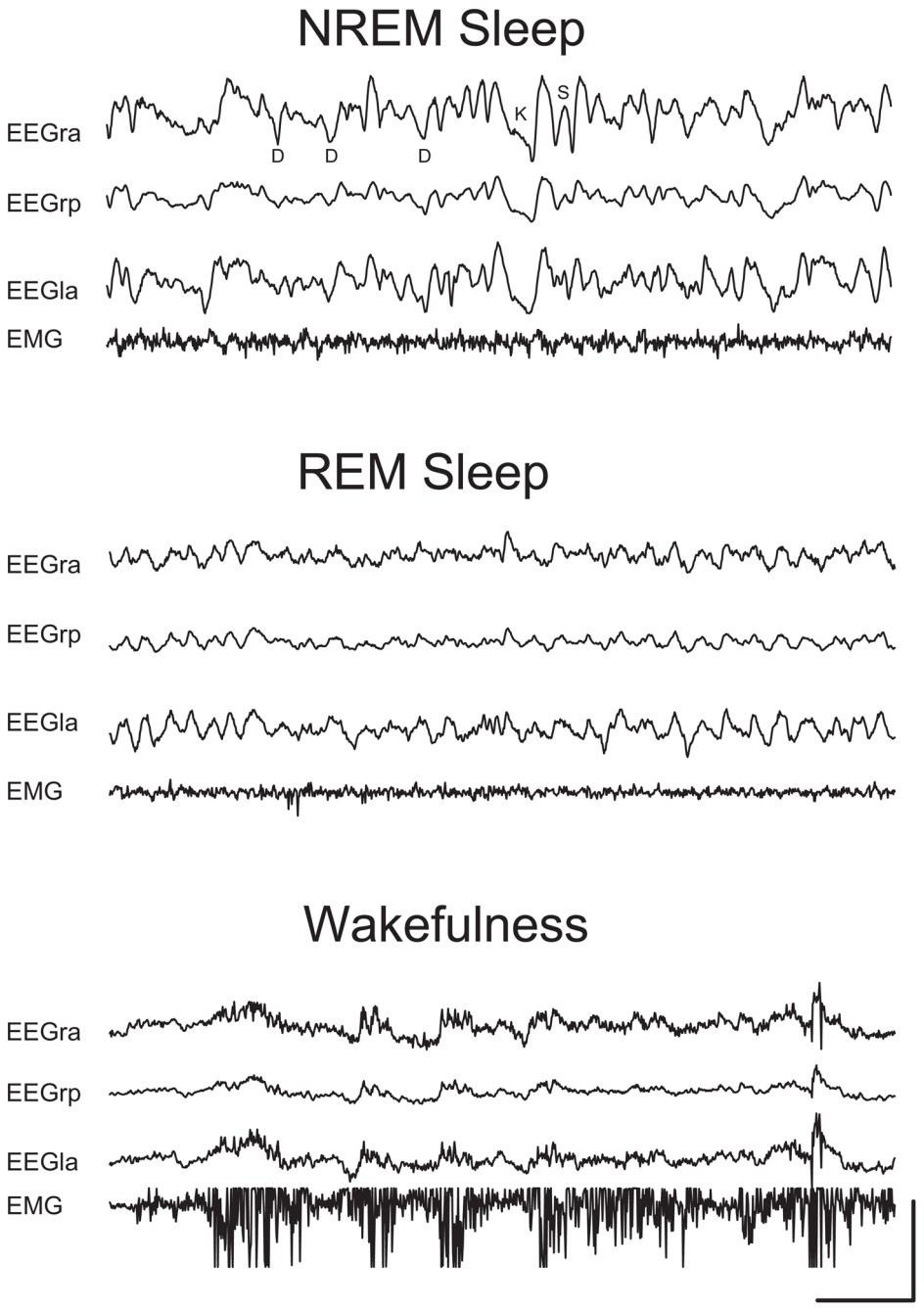

Figure 1.
Sample 4-s epochs from a single mouse during each of the three states from right anterior, right posterior, and left anterior electroencephalographic recordings (EEGra, EEGrp, and EEGla, respectively) and nuchal electromyographic recordings (EMG). During nonrapid-eye-movement (NREM) sleep, K-complexes (indicated by K), spindles (indicated by S), and delta oscillations (indicated by D) were present along with reduced EMG tone. During rapid-eye-movement (REM) sleep, there was a further reduction in EMG tone and theta oscillations dominated the EEG signal. Wakefulness was characterized by bursts of high frequency EEG activity (30+ Hz) and EMG activity. Scale bar shows 0.5 s and 100 μV.

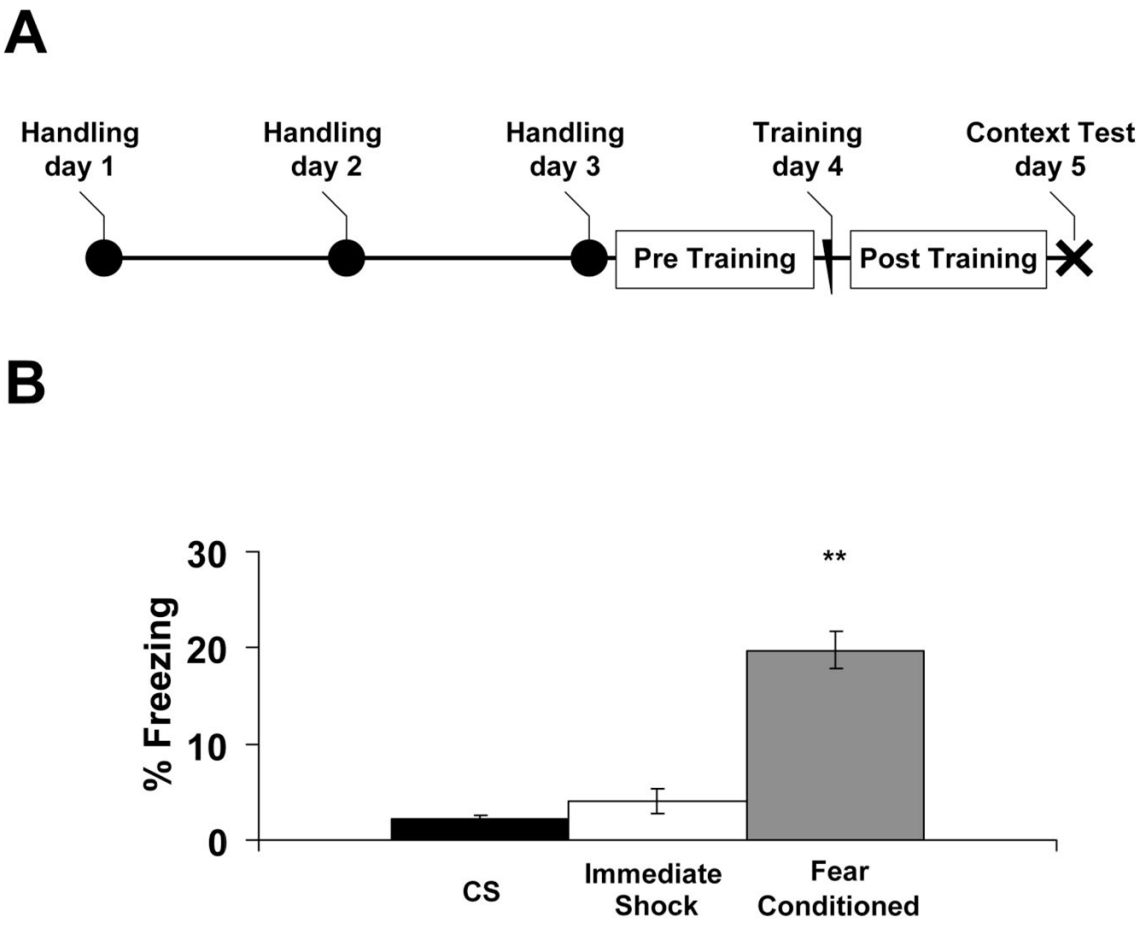

Figure 2.

Fear-conditioned mice exhibit freezing when returned to the training context 24 hr after training. A: Two weeks after implantation of electroencephalographic (EEG) and electromyographic (EMG) electrodes, mice were handled for 3 days and received either fear-conditioning training, an immediate shock, or the conditioning stimuli (CS) without receiving a shock. The 24-hr period before training is referred to as the pretraining period, and the 24-hr period after training is referred to as the posttraining period in subsequent figures. B: The day after training, mice were tested for freezing in response to the context. The percentage of time spent freezing was assessed over a 5-min period. Fear-conditioned mice (n = 16) froze about 20% of the time during the testing period in the training context. In contrast, CS (n = 15) and immediate shock (n = 12) mice froze only 2%–4% of the time during the testing period in the training context. An analysis of variance revealed an effect of group on freezing ($p < .0001$). Post hoc tests indicated that fear-conditioned mice froze significantly more than CS or immediate shock mice (ps <.01). There was no significant difference in freezing between CS and immediate shock mice ($p = .25$). Error bars indicate standard errors. $^{**}p < .01$.

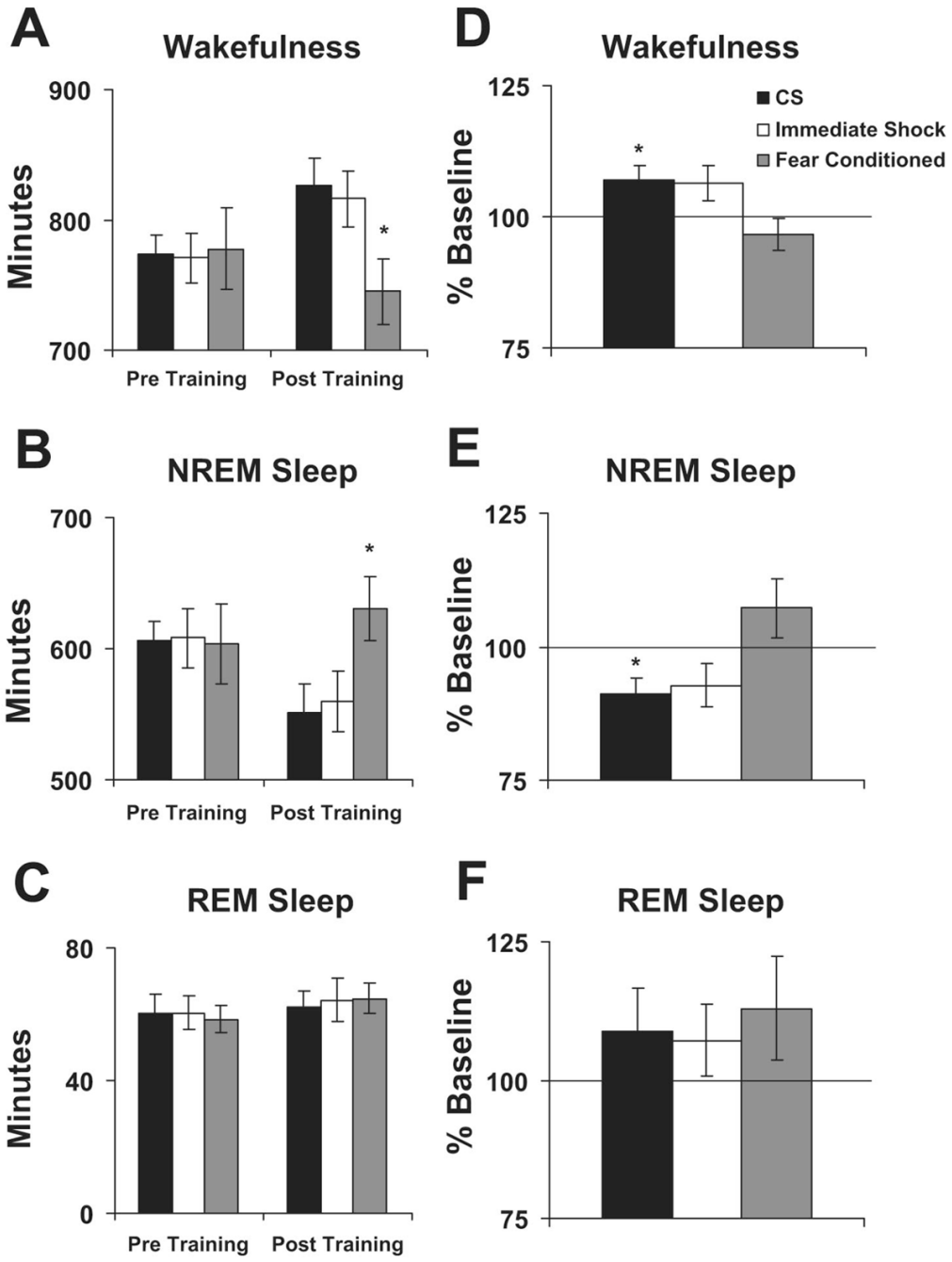

Figure 3.

Fear-conditioned mice spent approximately 1 hr more in nonrapid-eye-movement (NREM) sleep than did the conditioned stimulus (CS) and immediate shock mice in the 24 hr after training. The panels on the left show the amount of time in each state, and the panels on the right show the differences within groups between the 2 days. In the 24-hr period prior to training, mice in all three groups had comparable levels of wakefulness (A), NREM sleep (B), and rapid-eye-movement (REM) sleep (C; *p*s > .8). Training selectively altered wakefulness as revealed by a repeated measures analysis of variance Group × Day interaction ($p < .05$). Post hoc Tukey tests revealed that fear-conditioned mice ($n = 16$) had significantly less wakefulness than did CS mice ($n = 15$) or immediate shock mice ($n = 12$; *p*s < .05). This

decrease in wakefulness in fear-conditioned mice was accompanied by an increase in NREM sleep (B). Similarly, Group × Day interactions were significant for NREM sleep ($p < .05$), and fear-conditioned mice had more NREM sleep then CS or immediate shock mice (ps < .05). We also performed within-subject comparisons (D, E, F) to identify the effects of our manipulations on sleep–wake states. Within subjects, there was an overall increase in wakefulness in mice receiving CS ($p < .05$; D) and a decrease in NREM sleep ($p < .05$; E). A similar trend towards an increased wakefulness and decreased NREM sleep was observed for immediate shock mice (ps = .10). Fear-conditioned mice had decreased wakefulness and increased NREM sleep, although these relative changes were not significant (ps = .24). All three groups had similar amounts of REM sleep (CS, $p = .6$; immediate shock, $p = .4$; fear conditioned, $p = .3$; F). Error bars indicate standard errors. $^{*}p < .05$.

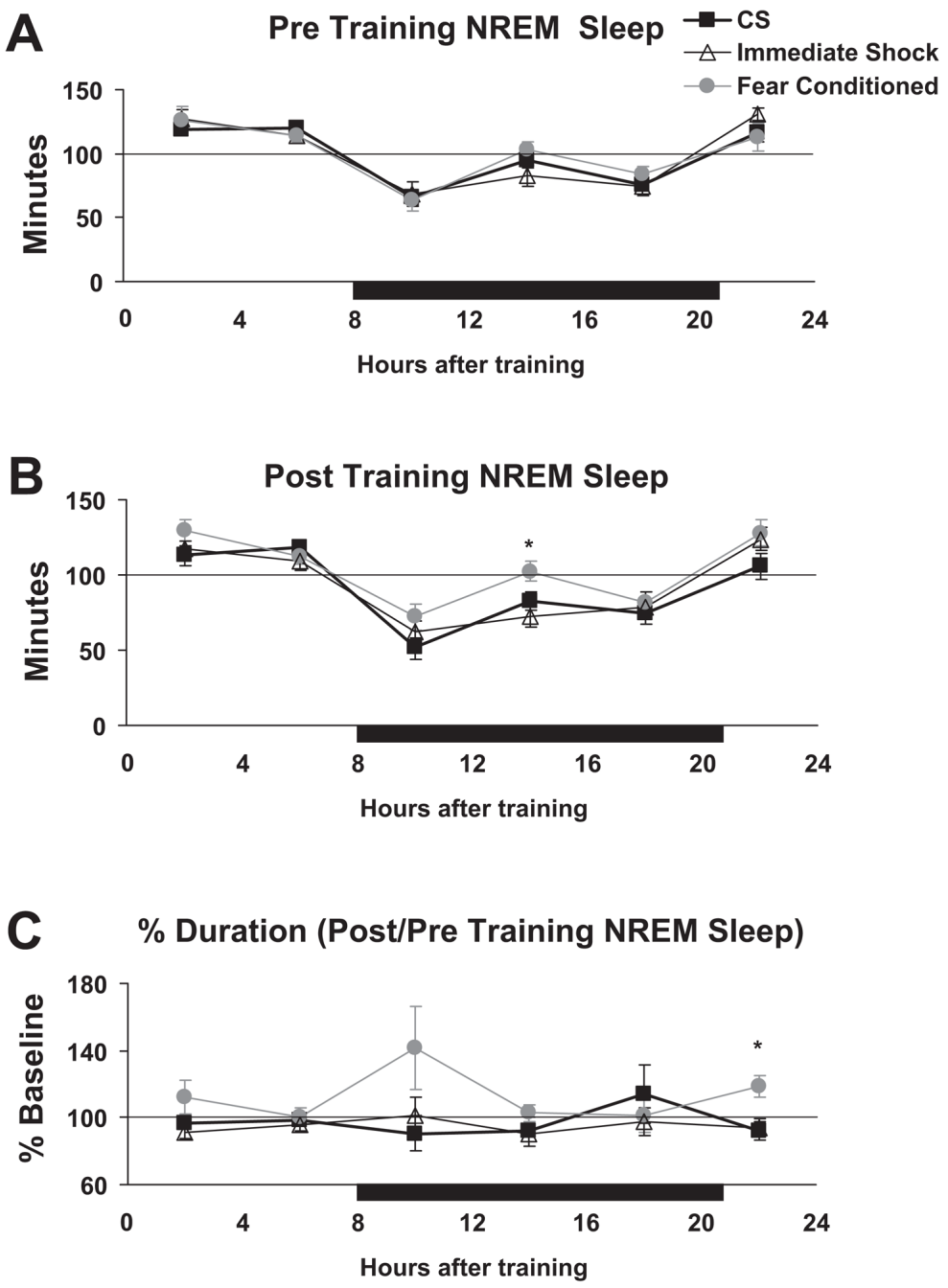

Figure 4.

The largest differences in nonrapid-eye-movement (NREM) sleep were observed during the dark period, 12–16 hr following training. The time spent in NREM sleep was tabulated in 4-hr bins for the 24-hr after training, which occurred at Zeitgerber Hour 4. The first data point represents the time spent in NREM sleep for the first 4 hr immediately after training, and the subsequent data points represent the adjacent 4-hr time windows. Lights were turned off 8 hr after training, as indicated by the black bar, and turned back on 20 hr after training. Sleep recording therefore continued for the first 4 hr of the next light period. The top two panels show the average amount of NREM sleep for each group on the pretraining (A) and posttraining (B) days. The bottom panel (C) shows the average within-subject percentage changes for each

group. There were no significant differences between the three groups in the pretraining period (A). For NREM sleep after training (B), an analysis of variance showed a main effect of time ($p < .001$) and group ($p < .01$) but not a significant Time × Group interaction ($p = .27$). Post hoc tests on individual time periods revealed that fear-conditioned mice had more NREM sleep 12–16 hours after training relative to CS or immediate shock mice ($ps < .05$). Examination of relative changes within individual mice and between groups (C) revealed overall increases in NREM sleep in fear-conditioned mice throughout the 24-hr time period relative to the pretraining day ($p < .01$). Error bars indicate standard errors. $^{*}p < .05$.

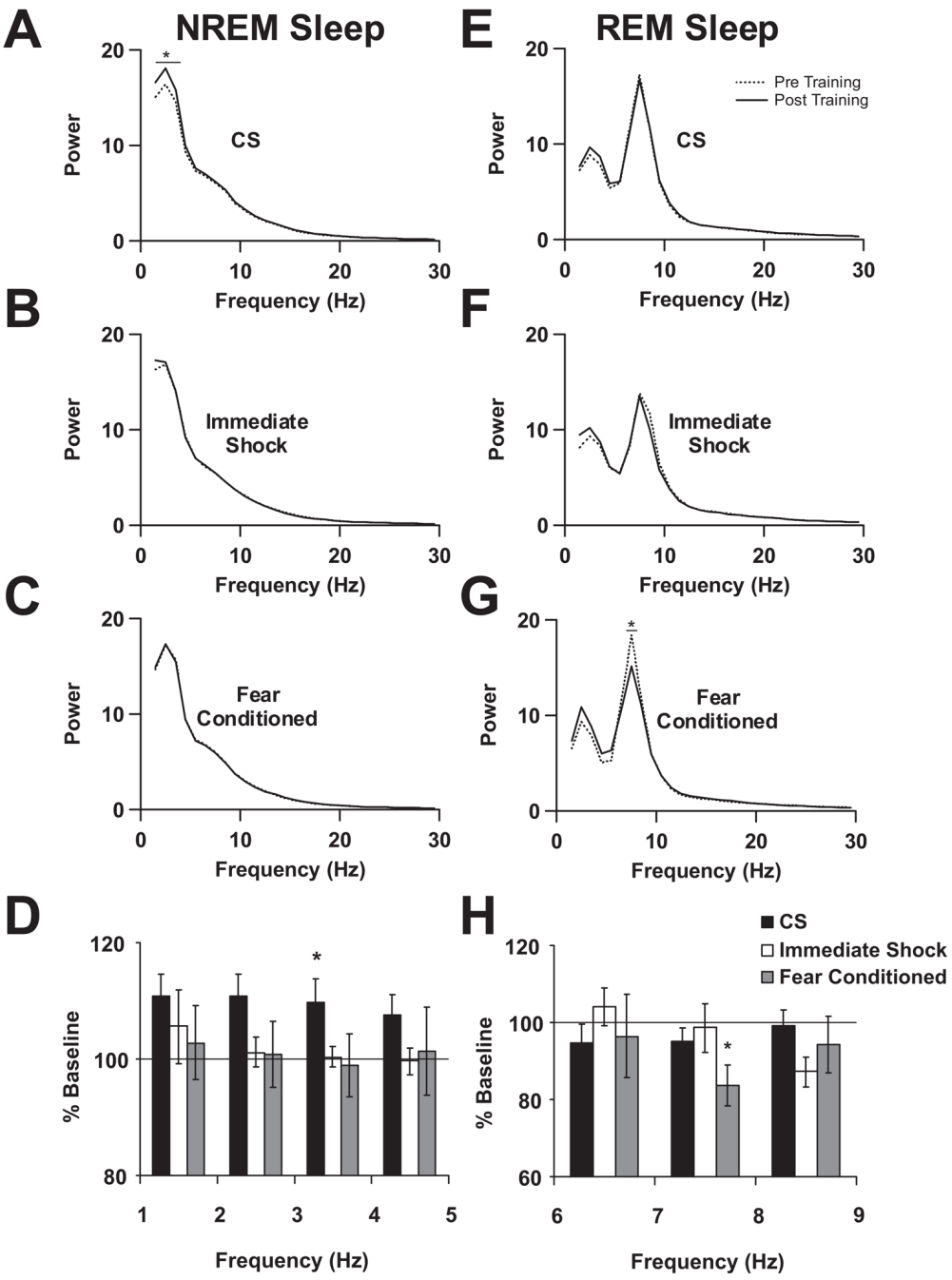

Figure 5.

Alterations in electroencephalographic spectra before and after training are selective to relevant frequency bands. The relative spectral contribution was determined by fast Fourier transform across the pretraining and posttraining day for each of the three groups. Panels A–C show the relative spectral contribution during nonrapid-eye-movement (NREM) sleep before and after training in the three groups. A relative increase in the 1–4 Hz power during NREM sleep is seen only in conditioned stimulus (CS) mice (A). Panel D shows the percentage change in 1-Hz frequency bands during NREM sleep. A comparison of the relative changes within the individual groups revealed an increase in 3–4 Hz power during NREM sleep in mice receiving CS ($p < .05$) compared with fear-conditioned mice or immediate shock mice. Panels E–H show

the relative spectral distribution during rapid-eye-movement (REM) sleep. A relative decrease in theta power during REM sleep is observed only in fear-conditioned mice (G). The relative change during REM sleep in 1-Hz frequency bands is shown in Panel H. A comparison of the relative changes within REM sleep between groups revealed a significant decrease in 7–8 Hz power in the fear-conditioned mice compared with immediate shock or CS mice ($p < .05$). Error bars indicate standard errors. $^{*}p < .05$.

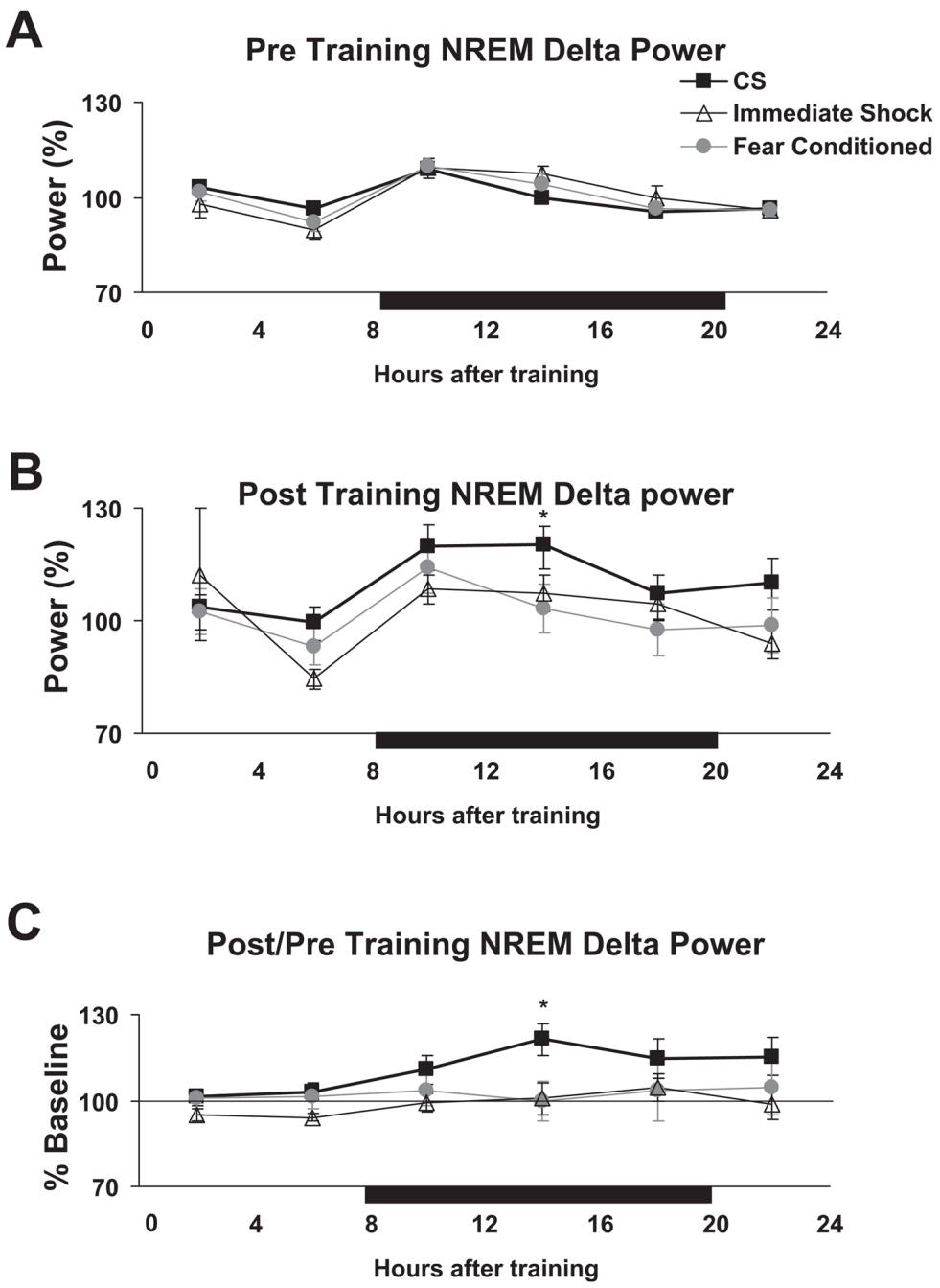

Figure 6.

Delta power during nonrapid-eye-movement (NREM) sleep was increased 12–16 hr after conditioned stimulus (CS) exposure. Delta power, normalized by the mean power on the pretraining day, was tabulated in 4-hr bins for the 24 hr before and after training. Although there was an effect of time of day ($p < .001$) on both days (Panels A and B), there was no significant effect of group on delta power across either the pretraining day ($p = .99$) or posttraining day ($p = .29$) as measured by an analysis of variance. There were no significant Time × Group interactions (pretraining, $p = .73$; posttraining, $p = .43$). Likewise, percentage change differences were not significant between the three groups ($p = .96$). We examined the 12- to 16-hr time window in which NREM sleep duration was increased for fear-conditioned

mice and increased delta power was observed 12–16 hours after training in CS mice ($p < .05$; C). Error bars indicate standard errors. $^{*}p < .05$.

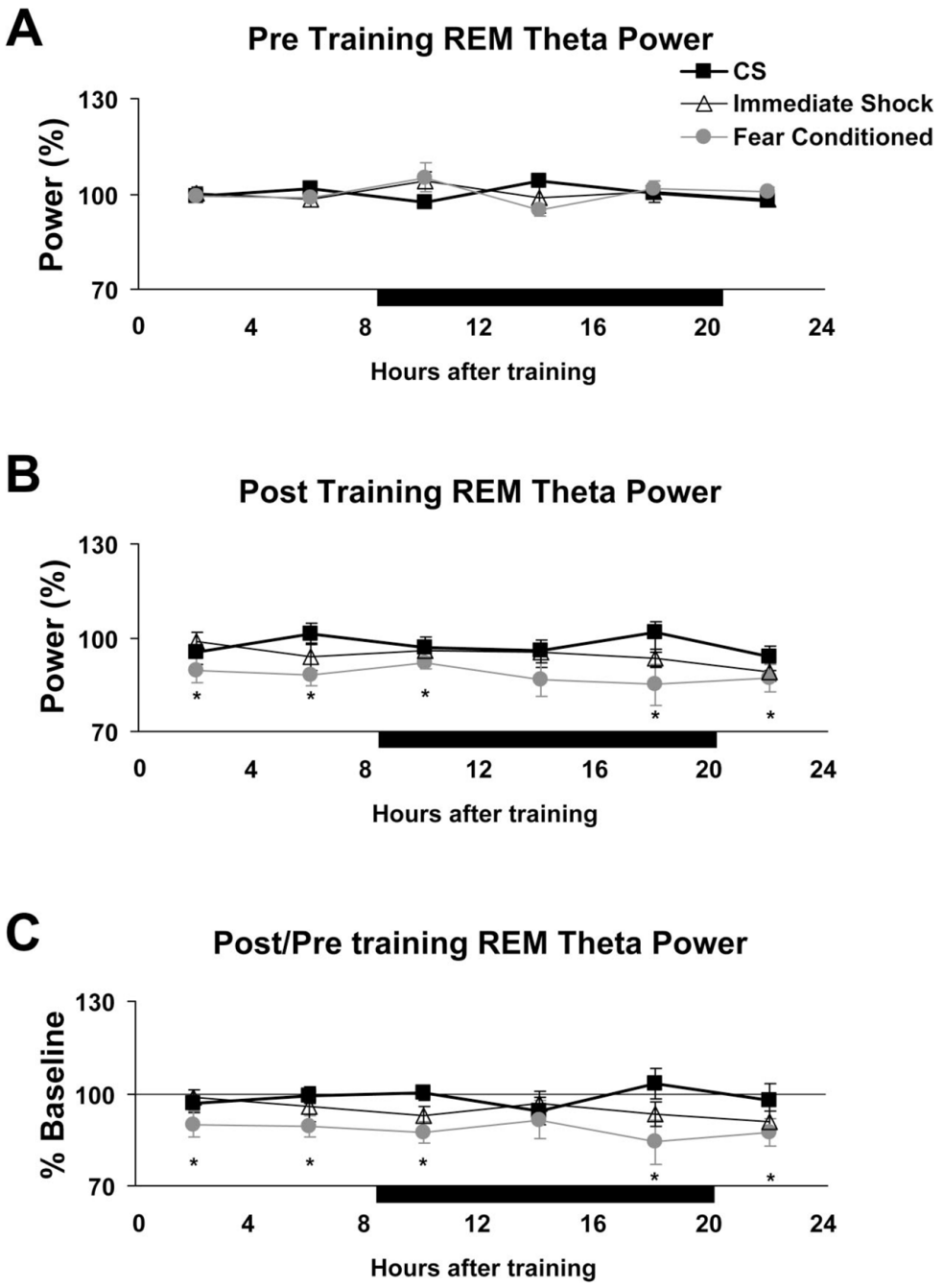

Figure 7.

Fear conditioning reduced theta power during rapid-eye-movement (REM) sleep throughout the posttraining day. Theta power, normalized by the mean power on the pretraining day, was tabulated in 4-hr bins for the 24 hr before and after training. During the pretraining day, the three groups had comparable levels ($p = .99$; A). However, after training there was a significant effect of group ($p < .001$) but no significant Group × Time interaction ($p = .78$; B). Significant decreases in theta power were observed at all time points ($p < .05$) except for 12–16 hours after training ($p = .18$). There was no significant effect of time on either day (ps > .05). Percentage changes in theta power relative to the same pretraining time period were computed for each of

the groups (C). There was a significant effect of group ($p < .001$) but no significant Group × Time interaction ($p = .86$). Error bars indicate standard errors. $^{*}p < .05$.