Fear memory in humans is consolidated over time independent of sleep

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Abstract

Background

Many pathological fear memories rely on processes of fear conditioning upon an aversive event. These fear memories may continue to change after acquisition, but the factors contributing to these changes are not well understood. Sleep is known to consolidate, strengthen and change newly acquired declarative and procedural memories. However, evidence on the role of time and sleep in the consolidation of fear memories is inconclusive. Here, we used highly sensitive electrophysiological measures to examine the development of fear-conditioned responses over time and sleep in humans.

Methods

We assessed event-related brain potentials (ERP) in 18 healthy young individuals during fear conditioning before and after a 2-hour afternoon nap or a corresponding wake interval in a counterbalanced within-subject design. The procedure involved pairing a neutral tone (CS+) with a highly unpleasant sound (US). As a control, another neutral tone (CS-) was paired with a neutral sound. Fear responses were examined before the interval during a habituation phase and an acquisition phase as well as after the interval during an extinction phase and a re-acquisition phase.

Results

Differential fear conditioning during acquisition was evidenced by a more negative slow ERP component preceding the CS+ compared to the CS- (stimulus-preceding negativity). This differential fear response was even stronger after the interval during re-acquisition compared to initial acquisition, but this effect was similarly pronounced after sleep and wakefulness.

Conclusions

These findings suggest that fear memories are consolidated over time, with this effect being independent of intervening sleep.

Introduction

Fear-related disorders, such as panic disorder, specific phobias, and posttraumatic stress disorder (PTSD) are characterized by pathological fear responses as a result of exposure to strong fear-evoking events. The mechanism underlying the formation of many fear memories is a process of fear conditioning. Fear conditioning is a type of associative learning where an initially neutral stimulus (e.g., sound) – conditioned stimulus (CS) – is repeatedly paired with an aversive stimulus (e.g., electric shock or noise burst) – unconditioned stimulus (US). After multiple pairings, the CS starts eliciting the conditioned response, a physiological and/or behavioral reaction similar to the one observed previously to the US. In differential fear conditioning paradigms, one stimulus (CS-) typically represents a safety signal that is never paired with the aversive US, while another stimulus is predictive of an aversive event (CS+). Extinction learning is achieved by repeated presentation of the CS+ without the US, leading to attenuation of the conditioned response. Extinction learning is considered a central mechanism for changing maladaptive fear memories in the therapeutic process, e.g., as a part of exposure therapy. Understanding how different factors affect fear memory formation, consolidation and extinction is essential for the advancement of targeted therapeutic interventions for the treatment of anxiety disorders.

Sleep plays an essential role in memory formation, particularly in the consolidation of new memories, i.e., strengthening and stabilization of memory traces after initial acquisition. Beneficial sleep effects are especially evident for declarative memory – the memory for facts and personal experiences, as well as procedural memory – memory for skills such as how to ride a bicycle (Diekelmann & Born, 2010; Schimke et al., 2021; Schmid et al., 2020). For emotionally charged memories, the role of sleep in memory consolidation is less clear. Two recent meta-analyses showed no overall effect for preferential sleep-dependent consolidation of emotional over neutral material (Lipinska et al., 2019; Schäfer et al., 2020), and studies on sleep-related consolidation of conditioned fear memories in humans have yielded highly conflicting results. Some studies found enhanced consolidation of conditioned fear memories during sleep (Menz et al., 2013), whereas others observed no difference between sleep and a period of wakefulness (Davidson et al., 2016; Menz et al., 2016; Zenses et al., 2020) or even stronger differential fear responses after wakefulness (Davidson et al., 2018; Lerner et al., 2021). Thus, the question of whether and how sleep affects the consolidation of fear memories remains largely open. Taking into account that overconsolidation of fear memories after sleep can contribute to the development of anxiety disorders (Davidson & Pace-Schott, 2020), understanding whether sleep strengthens fear memories and to what degree is particularly important for the development of adaptive interventions.

The research on fear memories has employed a number of different measures to characterize fear. Most studies assessed behavioral and/or peripheral physiology measures of freezing, avoidance, and hyperarousal, which, however, do not necessarily correlate with the mental state of fear (LeDoux & Hofmann, 2018; Mobbs et al., 2019). On the other hand, the brain mechanisms directly related to mental states are less frequently investigated. In humans, neural indices of fear learning have been studied by means of fMRI (Fullana et al., 2016) and

EEG/MEG (Miskovic & Keil, 2012). As an advantage, EEG can track fast-changing cortical responses corresponding to different stages of fear processing. Previous fear conditioning research employing EEG identified several event-related potential (ERP) components that can serve as conditioned responses. ERP components such as P1 (Pizzagalli et al., 2003), N1 or P2 (Kluge et al., 2011), and P3 (Kotchoubey & Pavlov, 2017; Pavlov & Kotchoubey, 2019; Rothemund et al., 2012) have been reported to produce a differential response in the contrast between conditioned stimuli (CS+) and safety signals (CS-). Several recent studies suggest that the late positive potential (LPP), i.e. a slow ERP component that typically appears after 400 ms with a maximum at the posterior electrodes, is a particularly reliable conditioning index (Bacigalupo & Luck, 2018; Panitz et al., 2015; Pavlov & Kotchoubey, 2019; Sperl et al., 2021). Other studies found the stimulus preceding negativity (SPN), i.e., a slow ERP component that typically occurs between 200 and 500 ms before the presentation of emotionally relevant stimuli (Ferreira de Sá et al., 2019; Tanovic et al., 2018; Tanovic & Joormann, 2019), to be a suitable conditioning index by showing a greater amplitude to cues predicting threat than safety events (Baas et al., 2002; Böcker et al., 2001; Ferreira de Sá et al., 2019; Hellwig et al., 2008; Regan & Howard, 1995). The earlier ERP components, such as P1, N1, and P2, can manifest changes in sensory processing of conditioned stimuli (Miskovic & Keil, 2012), while P3 may represent attention capture (Pavlov & Kotchoubey, 2019). The LPP may be related to attention allocation to emotionally salient information (Schupp et al., 2006), while the SPN could reflect affective anticipation of the US (Baas et al., 2002; Böcker et al., 2001). The processes reflected in these different ERP components can help to unveil the mechanisms underlying the consolidation of fear memories.

In the current study, we examined the effects of a post-learning retention interval of sleep or wakefulness on the consolidation of fear memories, assessed with behavioral measures (subjective ratings) and neural correlates (ERP) of fear-conditioned responses. In a within-subject design, subjects acquired auditory-conditioned fear memory and were tested on extinction and re-acquisition following a 2-hour afternoon nap or a respective wake period. We hypothesized that (1) a reliable correlate of classical fear conditioning is observed in ERP components, (2) the retention interval results in fear memory consolidation manifested in stronger ERP conditioned responses (and potentially subjective ratings) after the retention interval compared to before (time effect), and (3) fear memory consolidation is further enhanced by sleep, which is evidenced by stronger brain responses (and potentially subjective ratings) to fear conditioned sounds after sleep than wakefulness during extinction and reacquisition (sleep effect).

Methods and Materials

Participants

The study included 18 participants (8 females) with an average age of 24.7 ± 3.18 years. All but 2 participants were students at the University of Tübingen. No participants reported having

neurological or psychiatric diseases or taking any medication that could have impaired test performance. All participants were nonsmokers. All participants but one were right-handed. All participants were German native speakers. Participants gave their informed written consent and were paid for their participation in the study. The study was approved by the local Ethics Committee of the Medical Faculty of the University of Tübingen.

Procedure and task

The general design of the experiment is depicted in Figure 1a. Each participant visited the laboratory for a total of three afternoons. During the first "adaptation" day, participants had a two-hour nap to adapt to sleeping in the lab environment. Separated by at least 13 days (mean±SD, 27.9±16.8), the second and third days then took place, which consisted of a "sleep" and a "wake" day, respectively. The order of these two days was counterbalanced between participants. The tests performed on these days were identical; the only difference was the intervention: on sleep days, participants went to bed and had a 2-hour sleep opportunity between the conditioning sessions. On wake days, participants stayed awake for the same time sitting in a comfortable chair whilst changing activities every 30 min between watching a silent nature film and playing a computer game ("bubble shooter"). After both interventions, to give them time to fully awake after the nap, the participants spent additional 30 minutes filling out questionnaires unrelated to the purpose of the experiment. On both days, participants arrived at 1:00 pm at the lab. Before and after the sleep/wake period the participants underwent a fear conditioning paradigm. For the duration of this paradigm, they were asked to stay awake, sit still, and attentively listen to the stimuli with closed eyes; they were not required to perform any actions (i.e., a passive auditory oddball paradigm). To facilitate the probability of falling asleep on the sleep day, before each of the three days, participants were asked to wake up 1 hour earlier than usual.

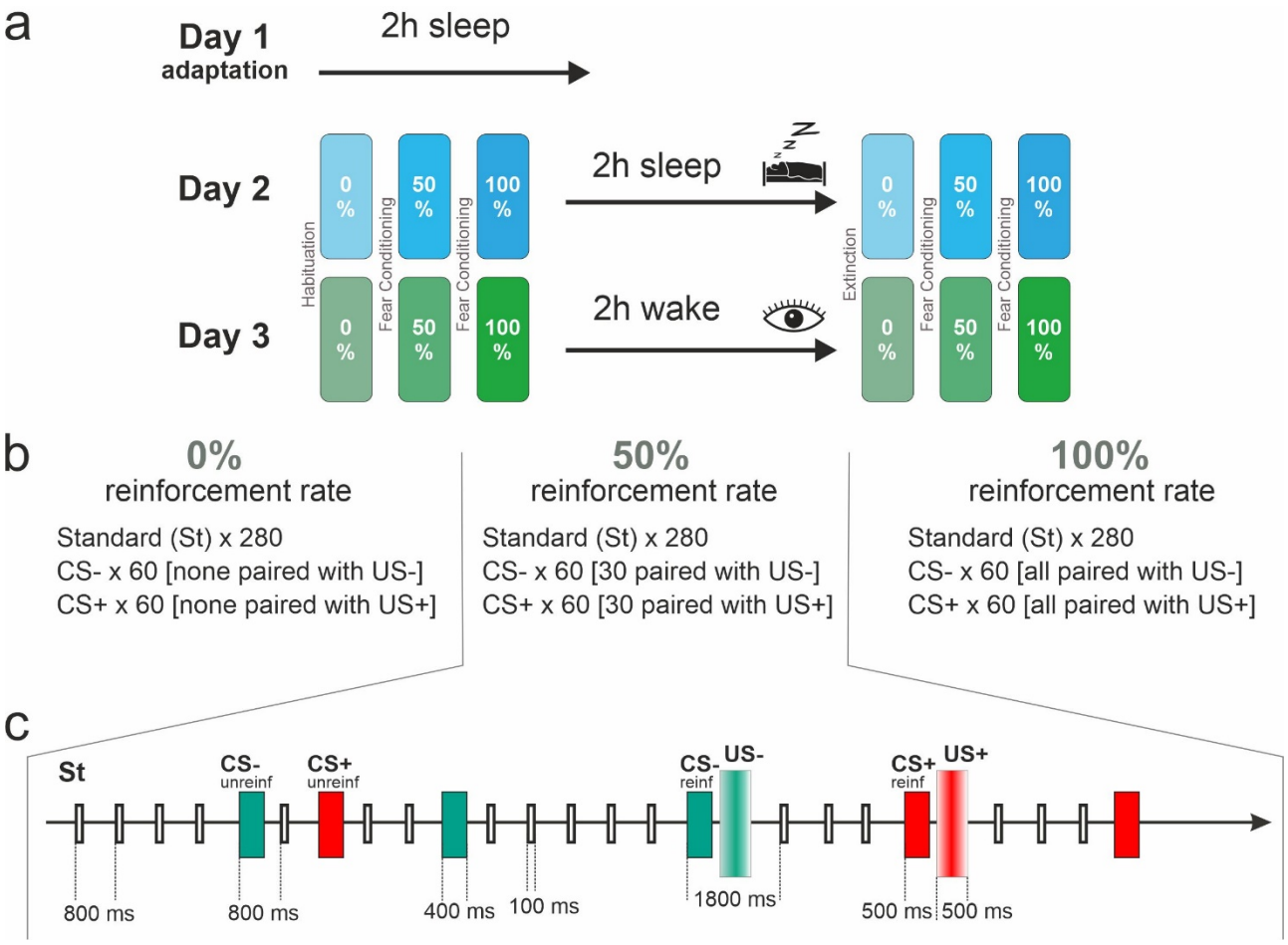

Figure 1 – (a) General design. The participants came to the lab three times on three separate days. On the first (adaptation) day the participants went to bed and had a 2-hours sleep opportunity. On the second and third days, the participants either went to bed for 2 hours or remained awake. Before and after the period of sleep or wakefulness the participants underwent a fear conditioning experiment with three blocks of trials. (b) Content of the fear conditioning experiment. 0, 50, and 100% reinforcement rate blocks contained 0, 30, and 60 reinforced conditioned stimuli, respectively, and 280 Standard stimuli. (c) An exemplary sequence of several trials corresponding to a 50% reinforcement rate block with all stimulus types shown. St – standard stimulus, CS+ and CS- – conditioned stimuli, US+ and US- – unconditioned stimuli (US+ – loud noise burst, US- – mildly pleasant sound), reinf – reinforced, unreinf – unreinforced.

All the stimuli were presented auditorily via pneumatic earphones (3M E-A-RTONE). One of them (Standard) consisted of five frequencies with the fundamental frequency (f1) randomly chosen between 320 and 500 Hz, and f2 = f1*2, f3 = f2*2, f4 = f3*2, and f5 = f4*2. The other two tones were referred to as CS+ and CS-. One of those tones had the main frequency 25% higher, and the other one 25% lower than the fundamental frequency of the standard tone. The frequency assignment was random. The timbre of the CS tones also varied between experimental days including either three or five harmonics per tone. One of the CS tones, randomly selected, was presented to the left ear and the other one was presented to the right ear while the standards were presented to both ears. The duration of the standard tone and the CS tones was 100 ms and 400 ms respectively, with 5 ms fade-in and fade-out. The loudness of the CS tones was 70 dB SPL, and for the standards it was 65 dB SPL. On each of the two

experimental days, the stimuli were generated anew assuring no repetition of the previous day's stimuli but remained the same within the day.

The CS tones were paired with either an aversive unconditioned stimulus (US+) or a neutral sound (US-). The stimulus-onset-asynchrony (SOA) between CS and US was fixed to 500 ms. SOA for unpaired CS was 800±50 ms and SOA for US was 1300±50 ms (see Figure 1c). The aversive stimulus (US+) was a 1000 Hz sine wave embedded into a burst of white noise (92 dB, 500 ms). The US- was a mildly pleasant sound that consisted of mixed frequencies (70 dB, 500 ms) (the stimuli are available on OSF: https://osf.io/xph69/).

In each fear conditioning session (before and after the sleep/wake interval), participants were presented with three blocks of 400 stimuli each, where the rare CSs were interspersed with frequent standard stimuli (Figure 1c). In each block, 280 Standard, 60 CS+ and 60 CS- stimuli were presented with different reinforcement rates: 0%, 50% and 100% (see Figure 1b). In the 0% reinforcement rate block, no US was delivered. In the 50% reinforcement rate block, half of the CS+ and CS- were paired with the respective US. In the 100% reinforcement rate block, all CS+ and CS- were paired with the respective US. All participants had a short self-paced break after each block. The same three blocks with the same stimuli were presented before and after the sleep/wake interval. These six blocks thus covered four stages of fear conditioning: 0% block before the sleep/wake interval – habituation, 50% and 100% blocks before the sleep/wake interval – acquisition, 0% block after the sleep/wake interval – extinction, 50% and 100% blocks after the sleep/wake interval – re-acquisition.

Subjective arousal and valence ratings

Subjective arousal and valence ratings of the stimuli were assessed with 9-point self-assessment manikins to evaluate emotional responses towards the stimuli during the conditioning procedures (Bradley & Lang, 1994). Before the conditioning, for training, the US+ (loud noise) was presented three times, and participants were asked to rate the sound on arousal and valence scales. After each block of conditioning, each previously presented stimulus (e.g., after 0% reinforcement rate block only Standard, CS+, and CS- were presented) was played individually once again and participants rated them again for arousal and valence.

Vigilance

The Stanford sleepiness scale was used to assess the participants' subjective sleepiness (Hoddes et al., 1972). The participants filled out the questionnaire 4 times during each experimental day (before and after each conditioning session of 3 blocks).

For an objective assessment of vigilance, participants were asked to perform a psychomotor vigilance task (PVT) 2 times during each experimental day (before the first conditioning session and before the second conditioning session, i.e., 30 minutes after the end of the sleep/wake interval). In this vigilance test, the participants were asked to place left and right index fingers on two selected buttons of a computer keyboard. A red circle repetitively appeared on either

the left or on the right side of a dark screen every 2, 4, 6, 8, or 10 seconds. Participants were asked to press the corresponding left or right button as fast as possible. The task lasted 5 minutes. Median reaction times entered the analysis.

Sleep duration and quality before the experiment

As a part of the entry questionnaire, participants were asked to estimate their regular sleep duration. Participants' average reported sleep duration was 8±0.67 (mean±SD) hours. The SF-A/R (Schlaffragebogen, „sleep questionnaire"-A, revised) (Görtelmeyer, 2011) was used to evaluate the participants' sleep quality the night before each experimental day and also following the 2-hour nap on the experimental sleep day. Participants completed the SF-A/R regarding the previous night before the conditioning session. Sleep duration during the nights preceding the experiment was on average 7.5±0.75 hours. Sleep quality (scale range 1–5) on the nights preceding the experiment was within the normal range with 4.13±0.28 on average.

Electroencephalography and event-related potential analysis

A 32-channel EEG system with active electrodes (ActiCHamp amplifier and actiCAP slim electrodes, Brain Products) was used for the recording. The electrodes were placed according to the 10-20 system with Cz channel as the online reference and Fpz as the ground electrode. The level of impedance was maintained below 25 kOm. The sampling rate was 1000 Hz.

EEGLAB (Delorme & Makeig, 2004) was used for data preprocessing. Each recording was filtered by applying 0.1 Hz high-pass and 30 Hz low-pass filters (pop_eegfiltnew function in EEGLAB). Then, an Independent Component Analysis (ICA) was performed using the AMICA algorithm (Palmer et al., 2012). As a preliminary step for improving ICA decomposition, a high-pass 1 Hz filter was applied. The results of the ICA on the 1Hz filtered data were then imported to the data filtered with 0.1 high-pass filter. Components clearly related to eye movements and high amplitude muscle activity were removed. Additionally, components that were mapped onto one electrode and could be clearly distinguished from EEG signals were subtracted from the data. After this, data were re-referenced to average reference and epoched into [-200 800 ms] intervals with 0 representing CS and US onset, where the [-200 0] interval was used for baseline correction. Epochs still containing artifacts were visually identified and discarded. Finally, before entering the statistical analysis data were re-referenced to averaged mastoids.

We first aimed to test the classic auditory ERPs normally appearing in the auditory oddball paradigm (Kotchoubey & Pavlov, 2019). For this analysis, mean amplitudes of the N1, P2, P3a, P3b, and N3 were computed in time windows of 70-125, 140-180, 180-290, 290-360, and 360-500 ms post-stimulus, respectively. These time windows were chosen based on group averages collapsed across all experimental conditions and channels (flatten average (Bowman et al., 2020)). In the first step of the ERP analysis, we took the average of the CS+ and CS- waveforms and compared them with ERPs to Standards at three channels typically used in ERP analyses: Fz, Cz, and Pz.

In the second step, we directly compared the CS+ and CS- ERP waveforms in the same time windows. The initial analysis revealed a significant Stimulus effect in P3a, P3b, and N3 time windows. A visual inspection revealed, however, that the effects were not separate for distinct components, but rather that there was one slow negative potential shift starting at about 180 ms after conditioned stimulus onset overlapping all faster components. The difference between CS+ and CS- amplitude significantly correlated between P3a, P3b, and N3 components (P3a-P3b: $r = 0.7$, $p = 0.001$; P3a-N3: $r = 0.75$, $p < 0.001$; P3b-N3: $r = 0.87$, $p < 0.001$) but not with N1 and P2. This slow shift is reminiscent of the stimulus preceding negativity (SPN (Böcker et al., 1994, 2001)). Therefore, we focused on the average SPN amplitude between 180 and 500 ms post-stimulus in the main analyses exploring the effects of sleep/wake on fear extinction and re-acquisition.

Before further statistical analyses, we assessed the spatial distribution of the Stimulus effect (CS+ vs CS-) via cluster-based permutation tests as implemented in Fieldtrip toolbox (Oostenveld et al., 2011). We applied the tests with 5000 permutations and 0.05 alpha level throughout the epoch (0-800 ms) with at least 2 channels threshold for forming a cluster. The tests revealed a stable presence of the Stimulus effect at central channels (see Figure S1 in the supplementary materials) over about 180-650 ms time interval. Confirming our assumption concerning the nature of this ERP component, this distribution was more typical for the SPN than for any of the faster ERP components in an oddball paradigm (Böcker et al., 1994; Kotani et al., 2015). Thus, the region of interest (ROI) for SPN analyses included C3, C4, and Cz channels (note that exploratory analyses using other channels around vertex yielded similar results).

For estimation of the unconditioned response, we used the N1-P2 amplitude difference as a measure of primary sensory cortical response with an N1 time window of 590-650 ms and a P2 time window of 710-770 ms after CS onset (defined as 60 ms time window around the peaks in the flatten average), averaged over C3, C4, Cz channels. To compensate for a possible drift in the baseline due to the SPN, we used the difference score instead of the ERP amplitude in N1 and P2 separately.

Polysomnography

To assure that participants were asleep on the sleep day and awake on the wake day in the respective interval, in addition to EEG, chin electromyography (EMG) and electrooculography (EOG, positioned 1 cm lateral and below to the outer canthi of the left eye, and 1 cm lateral and above the outer canthi of the right eye) were assessed.

Before sleep scoring, data were referenced to average mastoids. Sleep scoring was performed visually on 30-s epochs according to standard criteria of the AASM (Iber et al., 2007). The channels F3, C3, and O1 were used for the analysis unless additional information was required to classify an epoch in which case other channels were used in addition.

Statistics

Most repeated-measures ANOVAs, unless specified otherwise, involved a factor Stimulus (2 levels: CS+ and CS-), Block (3 levels: 0%, 50%, 100% reinforcement rate), Intervention (2 levels: sleep and wake), and PrePost (2 levels: before and after the intervention). The Greenhouse-Geisser correction was applied in the cases of violation of sphericity. The complete statistical results of the analyses are reported in Supplementary materials.

All statistical analyses were performed with R version 4.1.0 (R Core Team, 2013).

Results

Subjective arousal and valence ratings

As expected, the valence ratings for the CS+ and CS- did not significantly differ after the habituation block (Figure 2; $t(17) = 0.529$, $p = 0.60$, $dz = 0.128$). Fear conditioning shifted ratings toward more negative valence for CS+ and slightly more positive valence for CS- ($p = 0.037$ for Block x Stimulus interaction and $p = 0.001$ for main effect of Stimulus; Table S1), with no difference between 50% and 100% reinforcement rate (Stimulus effect after 50%: $t(17) = 3.95$, $p = 0.001$, $dz = 0.958$ and after 100% reinforcement rate blocks: $t(17) = 3.37$, $p = 0.004$, $dz = 0.817$; Table S2). The arousal ratings were similar in dynamics but not significant (Tables S3 and S4). Neither valence nor arousal ratings were affected by time or intervening sleep (all $p > 0.20$ for main effects and interactions with the factors Pre/Post and Intervention).

An analysis of responses to the US revealed more negative valence and higher arousal in US+ than US- ratings ($p < 0.001$ for both main effects of Stimulus; $US\text{-}_{arousal}$: 3.54±1.63, $US+_{arousal}$: 6.31±1.67, $US\text{-}_{valence}$: 7.18±1.39, $US+_{valence}$: 2.40±1.60; Tables S5 and S6). Additionally, arousal ratings decreased over time for both US ($p = 0.017$ for main effect of PrePost; $before_{arousal}$: 5.15±0.99, $after_{arousal}$: 4.71±1.27), possibly indicating sensory habituation to both stimuli. This effect was not evident for valence ratings ($before_{valence}$: 4.80±1.06, $after_{valence}$: 4.79±1.09; $p = 0.899$). Both ratings of the USs were not affected by intervening sleep ($p > 0.05$ for all effects of Intervention).

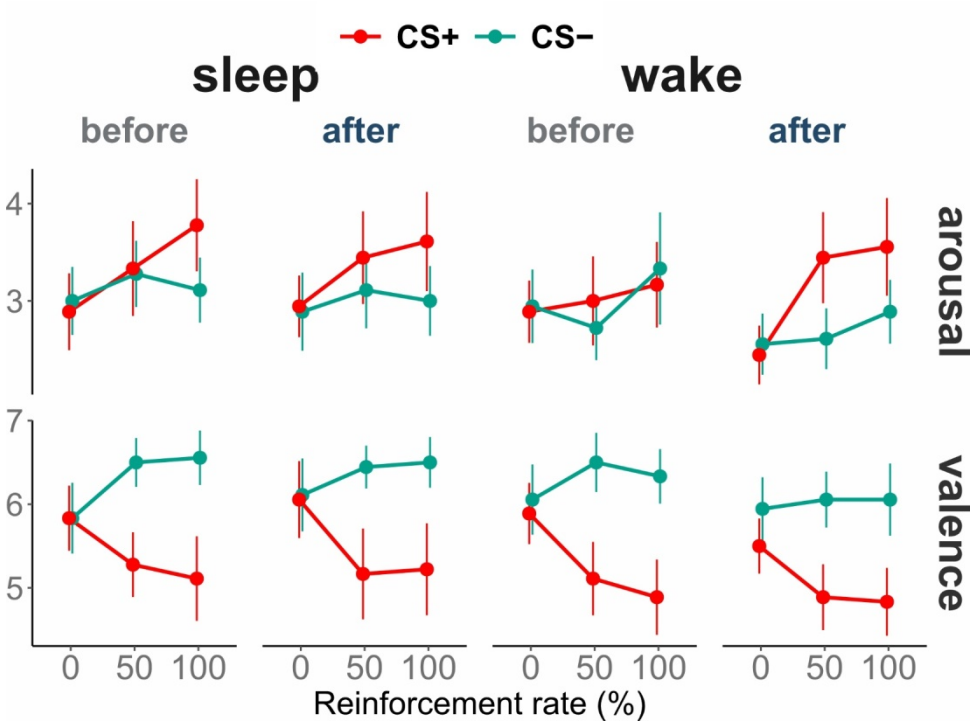

Figure 2 – Valence and arousal ratings to CS+ and CS-.

Sleep and time effects on event-related potentials

First, we ran a manipulation check to test for the expected oddball effect (i.e., CSs as rare deviant vs frequent standard stimuli). A strong oddball effect was present in the ERP components N1, P3a, and N3 (Figure 3a; Tables S7-S11: $p < 0.01$ for main effects of Stimulus (collapsed CSs vs. standards) with large effect sizes and central accentuation (Stimulus x Channel interaction). P3b was significantly larger to CSs only at Pz and the amplitude of P2 was slightly smaller to CSs, with the latter finding possibly reflecting a mismatch negativity to the CS deviants. Thus, the amplitude of all ERP components of interest was more pronounced (except P2) in response to rare stimuli serving as CSs than to the frequent standard stimuli (for a detailed description of the analyses see Supplementary materials).

Acquisition and re-acquisition

In a second step, we directly examined responses to the fear-conditioned stimulus (i.e. CS+ vs. CS-) during acquisition and re-acquisition (i.e., blocks 2 and 3 before the sleep/wake period and blocks 5 and 6 after the sleep/wake period). As can be seen in Figure 3b, the fear conditioning effect was manifested in a slow wave spreading over a time window of 180 to at least 500 ms ($p < 0.001$ for the main effect of Stimulus; Table S12), reflecting the stimulus-preceding negativity (SPN). There was a pronounced time effect on the SPN, with the difference between the CS+ and CS- becoming larger after the sleep/wake period, independently of whether the interval was filled with sleep or wakefulness ($p = 0.02$ for Stimulus x PrePost interaction; Figures 3c,d; Table S12). Generally, the amplitude of the SPN was larger after than before the intervention, larger in the wake than in the sleep condition, and larger with 100%

reinforcement than with 50% reinforcement ($p \leq 0.05$ for main effects of PrePost, Intervention, and Block, respectively; Table S12).

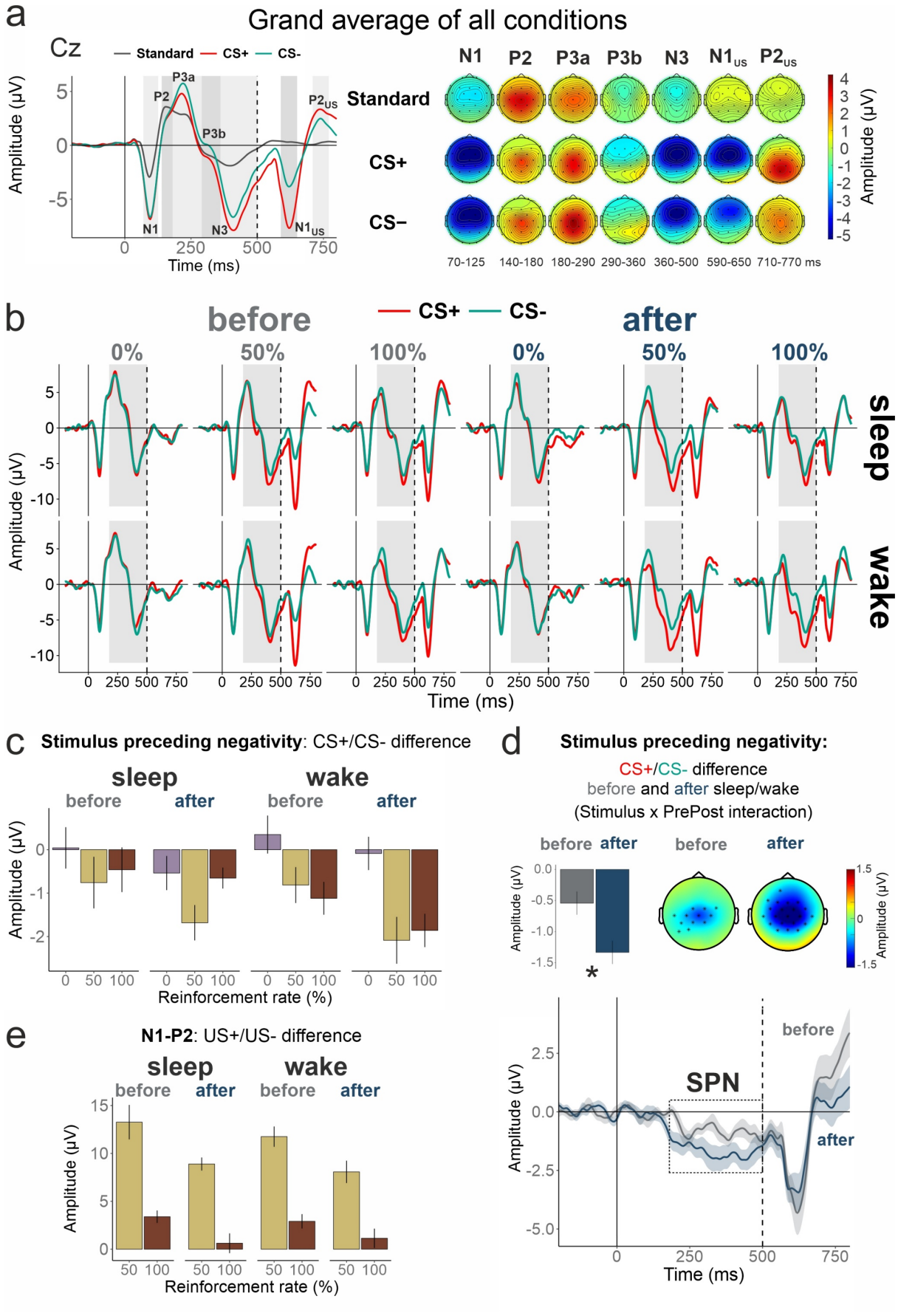

Figure 3 – (a) Overall responses to the rare CS+ and CS- compared to frequent standard stimuli. Left panel: ERP curves at Cz channel. The grey boxes indicate (from left to right): N1, P2, P3a, P3b, N3, $N1_{US}$, and $P2_{US}$ time windows. Right panel: topographical maps with averaged ERPs in the corresponding time windows. (b) ERP curves at Cz channel for CS+ and CS- separately for the sleep and wake condition as well as before and after the intervention and for the different reinforcement rates. The grey box indicates the stimulus preceding negativity (SPN) time window (180-500 ms). (c) Barplots of the CS+/CS- difference in the SPN time window in all conditions. (d) CS+/CS- difference before and after the intervention. Top panels: average SPN in Cz, C3, and C4 (bar plot), and topoplots with black dots indicating significant clusters from the cluster-based permutation tests over all channels averaged in the SPN time window. Bottom panel: CS+/CS- difference in time. The shading indicates the standard error of the mean. (e) Barplots of the US+/US- difference in P2-N1 amplitude. The error bars represent the standard errors of the mean.

Extinction learning

We then examined the effect of time and sleep on extinction learning during the first block after the sleep/wake period (block 4) compared to habituation before the sleep/wake interval (block 1). Unexpectedly, extinction learning was equally successful after sleep and wakefulness. Although the SPN to both CSs was overall more negative during extinction than during habituation ($p < 0.001$ for main effect Pre/Post), there was no difference between the CS+ and CS- nor between sleep and wakefulness or time points ($p > 0.30$ for all remaining main effects and interactions of Stimulus, Pre/Post and Intervention; Figure 3c, Table S13). To examine whether the lacking effects of sleep and time resulted from the excessive number of CS presentations (60 trials), we conducted an analysis of only the first 10 trials of extinction learning. However, the results remained essentially the same (Table S14).

Later effects in unreinforced trials

To explore whether there were any effects of sleep or time beyond the analyzed 180-500 ms interval (e.g., late positive potential (LPP)), we selected only unreinforced trials from the 50% reinforcement rate block (because in reinforced trials the US was presented at 500 ms) and compared the CS+ and CS- over the whole duration of the trial (0-800 ms). As shown in Figure S2, there were no late ERPs that differentiated CS+ and CS- (cluster-based permutation tests did not reveal any significant clusters beyond 500 ms).

Unconditioned responses

To test whether sleep or time might affect the processing of unconditioned stimuli, we compared the responses between US+ and US- in the acquisition and reacquisition blocks. Overall, the US+ elicited a larger amplitude of the N1-P2 difference as compared to US- (Figure 3e; Table S15). Both unconditioned responses in general and the US+/US- difference decreased over time from before to after the sleep/wake interval ($p < 0.001$ for main effect of PrePost, $p = 0.003$ for PrePost x Stimulus interaction), speaking for a habituation effect independent of sleep ($p > 0.20$ for all effects of Intervention). Furthermore, unconditioned responses in general as well as the US+/US- difference were stronger in 50% compared to 100% reinforcement blocks ($p < 0.001$ for main effect of Block and Stimulus x Block interaction; Figure 3e).

Vigilance and sleep

Subjective sleepiness measured by the Stanford Sleepiness Scale decreased after the 2-hour nap (before: 3.61±1.19, after: 2.83±0.92; $t(17) = 2.96$, $p = 0.009$, $dz = 0.718$), but remained stable after wakefulness (before: 3.17±1.25, after: 3.33±1.14; $t(17) = 0.55$, $p = 0.59$, $dz = 0.132$), which resulted in a significant Intervention x PrePost interaction ($F(1,17) = 5.36$, $p = 0.033$, $\eta^2 = .24$). However, post-hoc analyses revealed that subjective sleepiness did not differ significantly between sleep and wake days before the intervention ($t(17) = 1.29$, $p = 0.215$, $dz = 0.312$) as well as after the intervention ($t(17) = 1.84$, $p = 0.083$, $dz = 0.447$).

A different pattern was observed in the psychomotor vigilance test. The reaction times increased slightly after wakefulness (before: 355±14, after: 366±18 ms; $t(17) = 1.89$, $p = 0.075$, $dz = 0.46$) but remained stable after the sleep interval (before: 357±19, after: 352±15 ms; $t(17) = 0.756$, $p = 0.46$, $dz = 0.183$), leading to a significant Intervention x PrePost interaction ($F(1,17) = 5.65$, $p = 0.029$, $\eta^2 = .249$). Post-hoc tests revealed comparable vigilance performance between sleep and wake days before the intervention ($t(17) = 0.38$, $p = 0.71$, $dz = 0.093$), but faster reaction times on sleep days after the intervention ($t(17) = 2.83$, $p = 0.012$, $dz = 0.686$).

All participants successfully fell asleep during the sleep intervention while none of the participants showed any signs of sleep during the period of wakefulness (Table 1).

Table 1 – Results of polysomnography on the sleep day

Variable	Mean (SD)	minimum	maximum
N1, min	18.6 (13.9)	3.5	65.5
N2, min	43 (17.4)	6.5	65.5
N3, min	19.9 (16)	0	49
REM, min	9.9 (11.5)	0	33
N1, %	22.7 (19.9)	3.3	91
N2, %	46.6 (15.1)	9	66.7
N3, %	21.1 (18)	0	62.9
REM, %	9.4 (10.6)	0	29.3
Sleep latency, min	9.7 (7.2)	3	35
Total sleep time, min	91.6 (21.6)	25.5	112.5

N1 – stage 1 sleep, N2 – stage 2 sleep, N3 – slow wave sleep, REM – rapid eye movement sleep

Discussion

In our study, we investigated the effects of time and sleep on behavioral and neural indices of fear learning in humans. The data showed a strong modulation of ERPs during fear learning, with a larger stimulus preceding negativity (SPN) to the CS+ compared to the CS-, indicating successful fear conditioning. Paralleling the neural indices, subjective ratings indicated more negative valence for the previously neutral CS+ after having been paired with a loud noise. After extinction training, valence ratings returned to the baseline level, and similarly the difference between CS+ and CS- in the SPN vanished after very few extinction trials. Contrary to our expectations, both neural and behavioral conditioned responses were not affected by sleep,

neither for fear extinction nor for fear re-acquisition (i.e., relearning). Importantly though, after the retention interval, re-acquisition led to stronger neural conditioned responses compared to the original acquisition, evidenced by a larger SPN difference between the CS+ and CS- during re-acquisition.

The enhanced SPN to conditioned stimuli during re-acquisition can be interpreted as evidence for the consolidation of fear memories during the retention interval. Without any fear memory consolidation, we would have expected a completely new learning process after successful extinction that should be identical to the original fear memory acquisition. However, fear responses were stronger upon re-acquisition after the retention interval compared to the original acquisition before the interval, suggesting that consolidation processes strengthened (or protected) the fear memory during the retention interval, and thus facilitated subsequent re-learning. This interpretation is in line with previous evidence showing that successful memory consolidation facilitates new learning even when memory recall is not improved (Ashton et al., 2018; Mazza et al., 2016). The finding that fear memory consolidation was only evident in neural responses in the present study, but not in subjective ratings of valence and arousal suggests that neural responses are more sensitive to fear memory consolidation effects than behavioral measures. In fact, the present study is the first to provide evidence for consolidation of human fear memories over time in purely electrophysiological measures. Thus, future studies should include more sensitive neural measures such as ERP to capture subtle effects of consolidation that may be overlooked by less sensitive behavioral measures.

The finding that sleep did not affect fear memory consolidation was unexpected and contradicts previous evidence revealing consistent sleep benefits for memory consolidation (Diekelmann & Born, 2010; Schimke et al., 2021; Schmid et al., 2020). However, evidence of sleep benefits for emotional memory consolidation is more mixed (Schäfer et al., 2020), especially with regard to fear conditioning in human neuroscience. Menz and colleagues found an enhanced differential conditioned response after sleep compared to wakefulness, evident in skin conductance responses, US expectancy, and amygdala activation (Menz et al., 2013). Another study of the same group observed, however, no effect of sleep as compared to sleep deprivation (Menz et al., 2016). The opposite effect, i.e., a weaker differential response of the amygdala and the hippocampus after sleep compared to wakefulness, was observed in a recent study by Lerner and colleagues (Lerner et al., 2021).

Animal research has provided some insight into these mixed findings, suggesting that sleep plays a greater role in the consolidation of tasks that depend on the hippocampus, such as context conditioning, whereas tasks that mainly rely on the amygdala, such as cue conditioning, benefit to a lesser extent from sleep (Graves, 2003; Hagewoud et al., 2010; Phillips & LeDoux, 1992). Considering that the present paradigm is more akin to cue conditioning (i.e., a tone-cue preceding the US), the lack of a sleep effect might be explained by a relatively weak hippocampus dependence of this paradigm. Alternatively, the lacking effect of sleep on extinction learning might have been due to too many extinction trials in the present study (60 trials). However, additional analyzes of only the first 10 extinction trials likewise did not reveal any differences between the sleep and wake condition. Another possibility is that sleep effects may be evident only in later responses, i.e., after 500 ms. However, again additional analyses also showed no significant differences in time windows after 500 ms.

The finding that fear-conditioned neural evoked responses were reflected in a larger SPN probably indicates expectation and possible preparatory processes similar to flight or fight responses. An analogous increased SPN waveform has been shown to be predictive of emotionally charged events (Baas et al., 2002; Böcker et al., 2001; Dahl et al., 2020; Ferreira de Sá et al., 2019; Hellwig et al., 2008; Regan & Howard, 1995), while anticipatory attention to stimuli with no motivational value elicited reduced SPN (Brunia et al., 2011). This interpretation is in line with the observation that in the present study the SPN amplitude to the CS+ was generally larger in 100% reinforcement trials than in 50% reinforcement trials, whereas the responses to the US were weaker in 100% than 50% reinforcement trials. Possibly, the larger SPN in 100% reinforcement trials indicated stronger preparatory processes that, in turn, resulted in less pronounced unconditioned responses (MacNamara & Barley, 2018; Seidel et al., 2015).

Interestingly, our data did not reveal any effect of fear conditioning on early sensory ERP components such as N1 or P2. Instead, fear conditioning in our paradigm appeared to involve higher-order cognitive processing related to the anticipation of the unpleasant event. The absence of sensory or perceptual effects in conditioning contradicts some previous studies (Miskovic & Keil, 2012). Considering a pretty large number of averages in our paradigm (about 480 per condition per participant), the absence of these effects is unlikely to be caused by insufficient signal-to-noise ratio. Moreover, earlier studies argued that long-term (over days) and many trial paradigms are likely to affect early ERP components down to C1 in visual and P1 in auditory paradigms (Bublatzky & Schupp, 2012; Kluge et al., 2011; Stolarova et al., 2006). A possible factor contributing to this discrepancy may be an effect of latent inhibition, suggesting that unreinforced exposure of the to-be-conditioned stimulus slows down further associative learning with the same stimulus (Lubow, 1973). Our paradigm includes a relatively long habituation period preceding the acquisition block, which could have facilitated the engagement of higher order cognitive processes to overcome the effect of latent inhibition and to foster acquisition. In keeping with this explanation, other EEG studies that employed a habituation block immediately followed by the acquisition phase showed only late ERP effects (Bacigalupo & Luck, 2018; Danon-Kraun et al., 2021; Panitz et al., 2015; Stolz et al., 2019) as opposed to studies without the habituation phase (Bublatzky & Schupp, 2012; Pavlov & Kotchoubey, 2019; Sperl et al., 2021).

Overall, in the current study, we examined the neural mechanisms underlying the formation and consolidation of fear memories, with a particular focus on changes in fear responses over time and sleep. Applying sensitive electrophysiological measures, we provide evidence that fear memories are consolidated over time, with this effect being independent of sleep. These findings suggest that pathological fear responses as a result of exposure to strong fear-evoking events may increase over time, possibly contributing to the development of anxiety-related disorders. Sleep, however, does not seem to influence this process, neither enhancing nor reducing fear responses. This new understanding of the evolution of fear memories over time should be taken into account in the prevention and treatment of anxiety-related disorders such as PTSD and specific phobias.

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Author contributions statement

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Data availability statement

The data are publicly available on OSF (https://osf.io/xph69/).

Potential Conflicts of Interest

Nothing to report.

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