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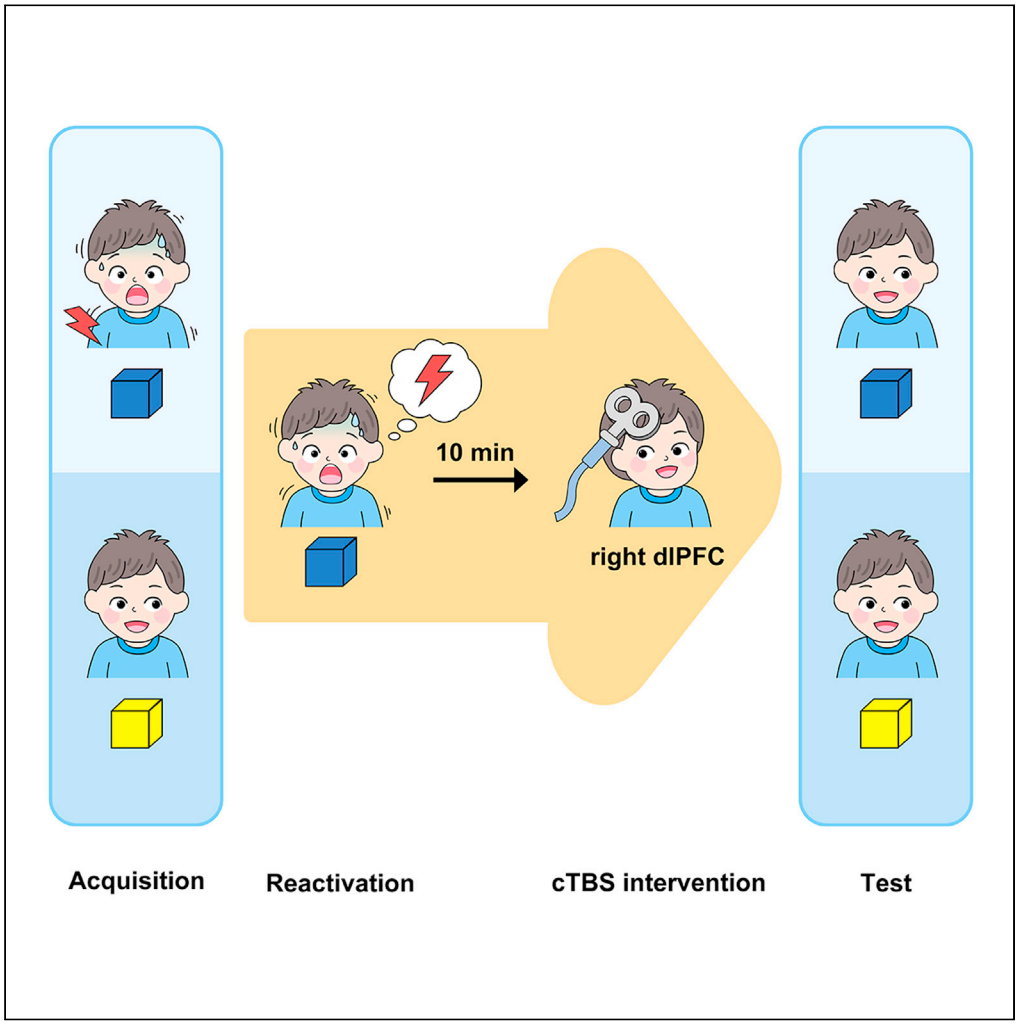

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Highlights

The fear response was decreased by cTBS over the right dlPFC after memory reactivation

cTBS prevented the return of fear memory only during the reconsolidation window

The impact of cTBS on fear memory reconsolidation was delay-dependent

Remote fear memory expression was blocked effectively by cTBS after memory reactivation

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Continuous theta-burst stimulation over the right dorsolateral prefrontal cortex disrupts fear memory reconsolidation in humans

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SUMMARY

Continuous theta-burst stimulation (cTBS), a non-invasive brain stimulation technique, can induce long-lasting changes in synaptic plasticity, vital for memory reconsolidation. For this study, a total of 170 participants completed four experiments by a randomized controlled design. Succeeding fear conditioning, the subjects received cTBS over the right dorsolateral prefrontal cortex (dlPFC) or vertex (control) with or without exposure to the conditioned stimulus to reactivate the original fear memory, and then underwent fear response tests. Compared with cTBS over the vertex and without memory reactivation, only cTBS over the right dlPFC after reactivation decreased the fear response for both recent and remote fear memories. This procedure was effective only during the reconsolidation window. The disruptive effect of cTBS over the right dlPFC on fear memory reconsolidation was delay-dependent. These findings demonstrate that cTBS time-dependently and delay-dependently prevents the return of fear and may have clinical potential for treating fear-related disorders.

INTRODUCTION

The pathological fear memory is considered an aversive emotional memory and can be associated with various mental disorders, such as anxiety disorders and post-traumatic stress disorder (PTSD) (Kindt, 2014; Mineka and Zinbarg, 2006). It can be so strong and persistent that patients seek help long after the negative events are experienced, a time when the fear memories are fully consolidated (Kindt, 2018). Pavlovian fear conditioning involves establishing an association between a neutral conditioned stimulus (CS; e.g., tone or picture) and adverse unconditioned stimulus (US; e.g., electric shock). This form of conditioning is widely used as an experimental analog for pathological fear memory across various animal species and humans (Pavlov, 1927). Labile short-term memory can become a stable long-term memory after establishing CS–US associations, which results from the synthesis of new proteins in the consolidation process (Davis and Squire, 1984; Dudai et al., 2015; Goelet et al., 1986; McGaugh, 2000).

Memories can be modulated via a reconsolidation process after being triggered by CS or US exposure (Misanin et al., 1968; Nader et al., 2000). The reactivated memory is unstable and can be modifiable during the reconsolidation window, which lasts for approximately 6 h after reactivation (Duvarci and Nader, 2004; Phelps and Hofmann, 2019; Walker et al., 2003). During reconsolidation, the original memory is labile and susceptible to modification (Gershman et al., 2017). Pharmacological or non-pharmacological manipulations can modify the reconsolidation, and change the memory expression (Fernández et al., 2016; Kindt and Soeter, 2018; Lee et al., 2017; Monfils et al., 2009; Schiller et al., 2010; Tronson et al., 2006). In animal studies, pharmacological agents have been shown to interfere with molecular cascades and neural plasticity to disrupt the reconsolidation process of fear and addiction memories (Alberini and Ledoux, 2013; Luo et al., 2015; Nader et al., 2000; Xue et al., 2012; Yuan et al., 2020). A clinical study found that after reactivation of the target memory, some interventions can interfere with traumatic memory and even PTSD (Kredlow and Otto, 2015). A previous study showed that the reactivation–extinction intervention might be a promising method for disrupting the pathological fear memory in humans (Schiller et al., 2010). However, other studies showed that this paradigm could not prevent the return of fear memory (Chalkia et al., 2020a, 2020b; Elsey et al., 2018). Moreover, not every reconsolidation intervention can be successfully translated into the clinical treatment of mental disorders, depending on the mode of such interventions and possible

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side effects (Phelps and Hofmann, 2019; Schiller and Phelps, 2011). These challenges prompted us to develop new interference approaches to modulate memory reconsolidation.

Theta-burst stimulation (TBS) is a non-invasive neuromodulation technique that alters the efficiency of synaptic transmission and consequently alters learning and memory processes (Huang et al., 2005, 2011; Lee and Hynds, 2013; Marin et al., 2018; Suppa et al., 2016). Continuous TBS (cTBS) is an advanced form of repetitive transcranial magnetic stimulation (rTMS), involving a burst of three stimuli (50 Hz) that are repeated at 5-Hz intervals. This form of stimulation produces more powerful and long-lasting effects than regular rTMS (Huang et al., 2005; Suppa et al., 2016). Moreover, cTBS takes less time to administer than rTMS, thus becoming more tolerable for patients (Suppa et al., 2016). Long-lasting cTBS is believed to partially block N-methyl-D-aspartate receptors (NMDARs) (Huang et al., 2005, 2011; Suppa et al., 2016), which is critical for the reconsolidation of fear memory (Ben Mamou et al., 2006; Lee and Hynds, 2013).

The dorsolateral prefrontal cortex (dlPFC) is a major cortical area that is targeted by cTBS. This region plays a critical role in modulating memory, especially the reconsolidation process (Benoit and Anderson, 2012; Li et al., 2019; Ragland et al., 2004). Activity of the dlPFC increased after memory retrieval (Ragland et al., 2004), and the suppression of dlPFC activity was related to the inhibition of unwanted memory (Anderson et al., 2004). Stimulation of the dlPFC by cTBS can affect the cortical function and potentiate prefrontal connections (Dayan et al., 2013; Gratton et al., 2013), thus improving unconscious perceptual memory (Lee et al., 2013) or impairing episodic memory (Marin et al., 2018). However, still unknown are the ways in which cTBS over the dlPFC influences fear memory reconsolidation. A recent study showed that the expression of fear memory was disrupted by low-frequency rTMS over the dlPFC after reactivation (Borgomaneri et al., 2020). Thus, the present study investigated whether cTBS over the dlPFC influences the reconsolidation of fear memory using a 3-day design with the fear acquisition on day 1, cTBS intervention after reactivation on day 2, and the recall test, extinction, and reinstatement test on day 3.

RESULTS

cTBS over the right dlPFC disrupts the reconsolidation of recent fear memory after CS exposure

In Experiment 1, we first investigated whether cTBS over the right dlPFC after memory reactivation disrupts recent fear memory reconsolidation. Seventy-two participants who acquired fear on day 1 were randomized into four groups: R dlPFC (cTBS over the right dlPFC 10 min after reactivation, n = 18), R vertex (cTBS over the vertex 10 min after reactivation, n = 18), NR dlPFC (cTBS over the right dlPFC without reactivation, n = 18), and NR vertex (cTBS over the vertex without reactivation, n = 18). The recall test, extinction, and reinstatement test occurred 24 h after cTBS (Figure 1A). No significant differences in age, gender, education, BMI, SDS score, SAS score, MoCA score, DST score (forward and backward), shock intensity, or AMT were found among the four groups (all $p > 0.05$; Table S1).

The differential skin conductance response (SCR) during fear acquisition was assessed using repeated-measures ANOVA, with group (R dlPFC, R vertex, NR dlPFC, and NR vertex) as the between-subjects factor and time (first and last phases of fear acquisition) as the within-subjects factor. This analysis showed a main effect of time ($F_{1,68}$ = 97.147, $p < 0.001$) but no effect of group ($F_{3,68} = 1.434$, $p = 0.241$) and no time × group interaction ($F_{3,68} = 0.973$, $p = 0.411$; Figure 1B). In addition, the SCR of CS+ was significantly higher than the SCR of CS− in the last phase of fear acquisition in the four groups (Figure S1). These results suggest that these four groups acquire comparable fear.

The effects of cTBS over the right dlPFC in the fear recall test were assessed using three-way ANOVA, with stimulus (dlPFC and vertex), reactivation (reactivation and no reactivation), and time (last phase of fear acquisition and recall test) as factors. The results of differential SCR indicated main effects of stimulus ($F_{1,68}$ = 5.495, $p = 0.022$) and time ($F_{1,68}$ = 6.397, $p = 0.014$), no main effect of reactivation ($F_{1,68}$ = 3.480, $p = 0.066$), and a significant stimulus × reactivation × time interaction ($F_{1,68}$ = 8.715, $p = 0.004$). The *post hoc* analysis showed that the mean differential SCR decreased in the R dlPFC group ($p < 0.001$) but not in the other three groups in the recall test (all $p > 0.05$; Figure 1B). These results show that fear response is reduced in the recall test in the R dlPFC group. In addition, no significant difference in the mean differential SCR in the last phase of extinction was found among four groups ($p = 0.172$; Figure 1B).

In the reinstatement test, the three-way ANOVA, with stimulus (dlPFC and vertex), reactivation (reactivation and no reactivation), and time (last phase of extinction and first phase of reinstatement test) as factors,

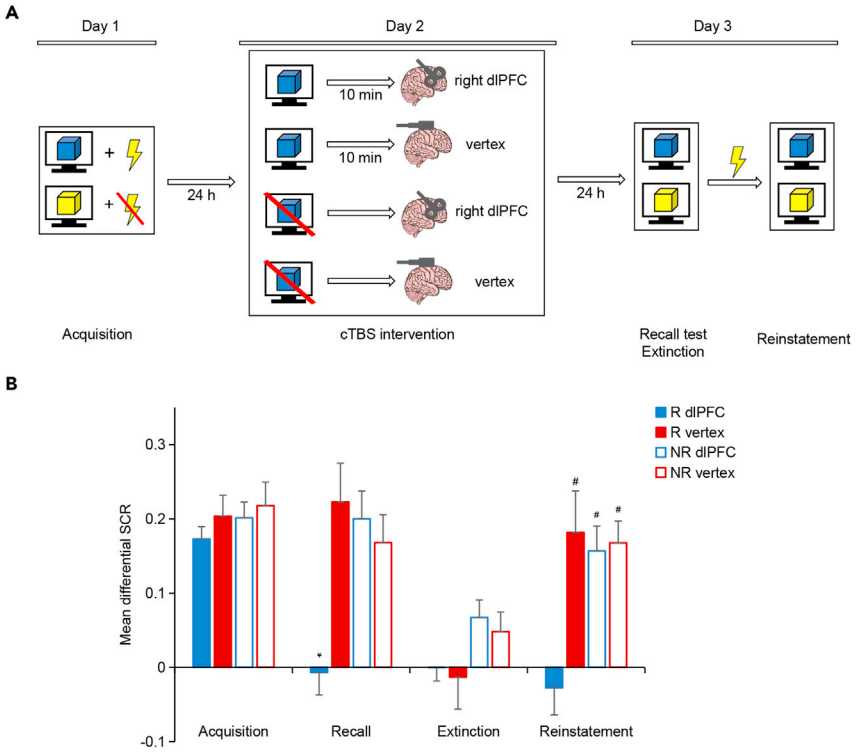

Figure 1. cTBS over the right dlPFC disrupts the reconsolidation of recent fear memory

(A) Experimental design and timeline of Experiment 1.

(B) Mean differential skin conductance response (SCR; CS+ minus CS–) during fear acquisition, the recall test, extinction, and the reinstatement test for each group. R dlPFC, cTBS over the right dlPFC after reactivation; R vertex, cTBS over the vertex after reactivation; NR dlPFC, cTBS over the right dlPFC without reactivation; NR vertex, cTBS over the vertex without reactivation. *p< 0.05, comparisons between last phase of acquisition and recall test (all within-group); #p< 0.05, comparisons between last phase of extinction and first phase of reinstatement (all within-group). The data are expressed as mean ± SEM.

revealed main effects of time ($F_{1,68}$ = 17.991, p< 0.001) and reactivation ($F_{1,68}$ = 7.613, p = 0.007), no main effect of stimulus ($F_{1,68}$ = 2.997, p = 0.088), and a significant stimulus × reactivation × time interaction ($F_{1,68}$ = 4.714, p = 0.033). The *post hoc* analysis showed that significant reinstatement of fear responses occurred in the R vertex, NR dlPFC, and NR vertex groups (all p< 0.05) but not in the R dlPFC group (p> 0.05; Figure 1B). Moreover, in the recall test and the first phase of reinstatement test, the SCR of CS+ was significantly higher than the SCR of CS− in the R vertex, NR dlPFC, and NR vertex groups, and no difference between the SCR of CS+ and the SCR of CS− was observed in the R dlPFC group (Figure S1). These results indicate that cTBS over the right dlPFC after CS exposure decrease recent fear memory expression and inhibit the return of fear.

Disruptive effect of cTBS on fear memory reconsolidation requires a specific time-window

In Experiment 2, we explored whether the effect of cTBS over the right dlPFC on fear memory reconsolidation requires a specific time-window. Thirty-six participants who acquired fear on day 1 were randomized into two groups: 10 min (cTBS over the right dlPFC 10 min after reactivation, *n* = 18) and 6 h (cTBS over the right dlPFC 6 h after reactivation, *n* = 18). The recall test, extinction, and reinstatement test occurred 24 h after cTBS (Figure 2A). No differences in demographic data, SDS, SAS, MoCA, DST scores (forward and backward), shock intensity, or AMT were found between the 10 min and 6 h group (all p> 0.05; Table S3).

During fear acquisition, the repeated-measures ANOVA, with group (10 min and 6 h) as the between-subjects factor and time (first and last phases of fear acquisition) as the within-subjects factor, showed a main effect of time ($F_{1,34}$ = 28.328, p< 0.001), no main effect of group ($F_{1,34}$ = 0.067, p = 0.798), and no group × time interaction ($F_{1,34}$ = 0.137, p = 0.714; Figure 2B). Furthermore, there were significantly higher SCRs to

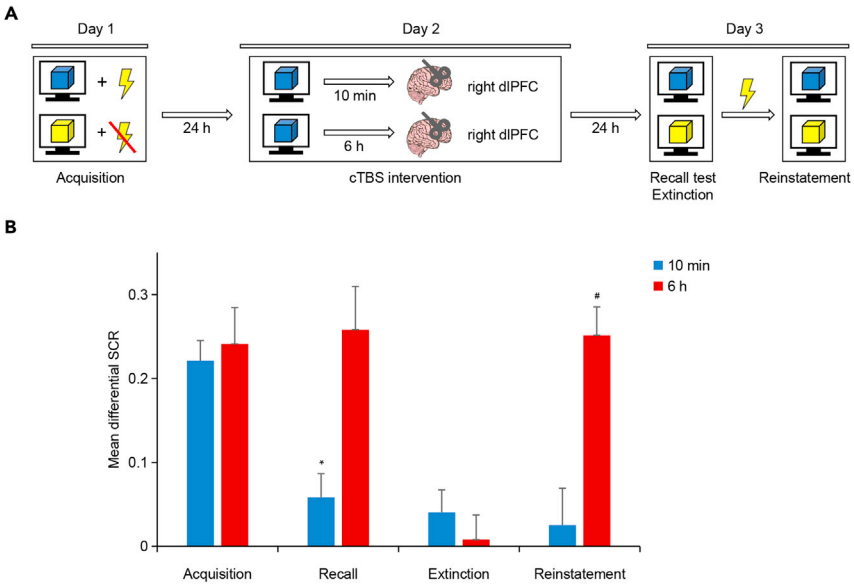

Figure 2. cTBS over the right dlPFC disrupts fear memory within a specific time-window

(A) Experimental design and timeline of Experiment 2.

(B) Mean differential skin conductance response (SCR; CS+ minus CS–) during fear acquisition, the recall test, extinction, and the reinstatement test for each group. 10 min, cTBS over the right dlPFC 10 min after reactivation; 6 h, cTBS over the right dlPFC 6 h after reactivation. *p< 0.05, comparisons between last phase of acquisition and recall test (all within-group); #p< 0.05, comparisons between last phase of extinction and first phase of reinstatement (all within-group). The data are expressed as mean ± SEM.

the CS+ than to the CS− in the last phase of fear acquisition in the two groups (Figure S2). These results indicate successful fear acquisition in both 10 min and 6 h groups.

The repeated-measures ANOVA of the differential SCR, with group (10 min and 6 h) as the between-subjects factor and time (last phase of fear acquisition and recall test) as the within-subjects factor, revealed no main effect of time ($F_{1,34}$ = 3.625, p = 0.065), but a significant main effect of group ($F_{1,34}$ = 8.088, p = 0.007) and a significant group × time interaction ($F_{1,34}$ = 5.502, p = 0.025) in the recall test. The *post hoc* analysis showed that the mean differential SCR decreased in the 10 min group (p = 0.005) but not in the 6 h group in the recall test (p = 0.757; Figure 2B). These results show that fear response is reduced in the recall test when cTBS is administered during the reconsolidation window. Mean differential SCR in the last phase of extinction were similar between the 10 min group and the 6 h group (p = 0.420; Figure 2B).

Reinstatement was assessed using repeated-measures ANOVA, with group (10 min and 6 h) as the between-subjects factor and time (last phase of extinction and first phase of the reinstatement test) as the within-subjects factor. We found significant main effects of group ($F_{1,34}$ = 8.358, p = 0.007) and time ($F_{1,34}$ = 10.818, p = 0.002) and a significant group × time interaction ($F_{1,34}$ = 13.864, p = 0.001). The *post hoc* analysis showed reinstatement of the conditioned fear response occurred in the 6 h group (p< 0.001) but not in the 10 min group (p = 0.761; Figure 2B). Moreover, in the recall test and the first phase of reinstatement test, the SCR of CS+ was significantly higher than the SCR of CS− in the 6 h group, and no difference between the SCR of CS+ and the SCR of CS− was found in the 10 min group (Figure S2). These results show that the modulation of fear memory expression by cTBS over the right dlPFC after CS exposure requires a specific time-window.

Disruptive effect of cTBS on reconsolidation is delay-dependent

We investigated whether the effect of cTBS over the right dlPFC on fear memory is time-dependent by measuring the SCR in recall tests 12 and 24 h after cTBS. Thirty-eight participants acquired fear on day 1 and received cTBS over the right dlPFC 10 min after reactivation on day 2. The first recall test was conducted exactly 12 h after cTBS, and the second recall test, extinction, and the reinstatement test occurred 24 h after cTBS. Considering the impact of sleep on the reconsolidation process, participants were randomized

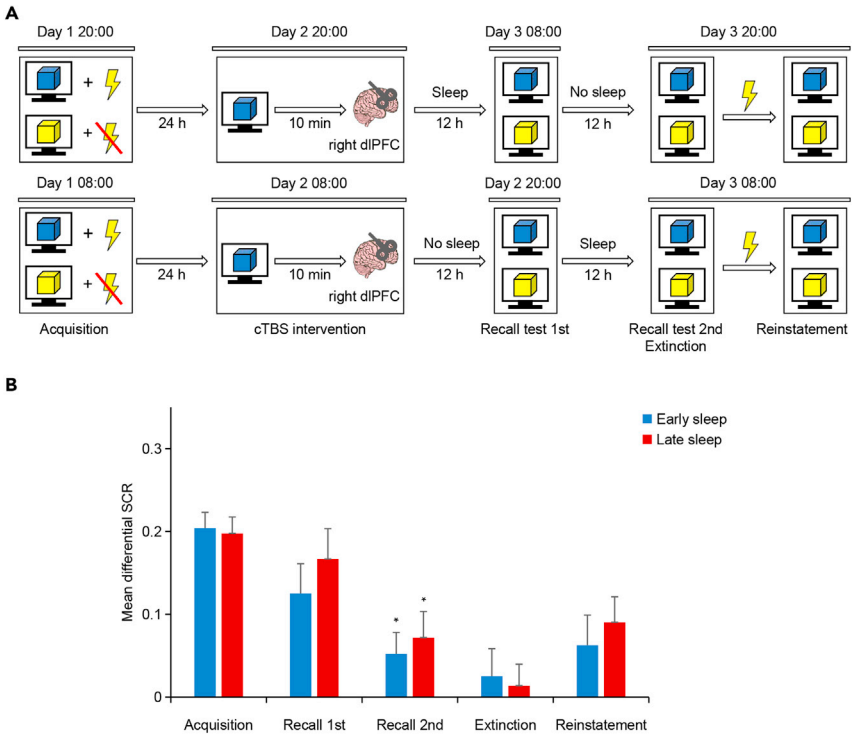

Figure 3. Disruptive effect of cTBS on reconsolidation is delay-dependent

(A) Experimental design and timeline of time-dependent effect of cTBS in Experiment 3.

(B) Mean differential skin conductance response (SCR; CS+ minus CS–) during fear acquisition, the recall test (first and second), extinction, and the reinstatement test for each group. Early sleep, nocturnal sleep before the first recall test; Late sleep, nocturnal sleep after the first recall test. *p< 0.05, comparisons between the first recall test and second recall test (all within-group). The data are expressed as mean ± SEM.

into two groups: early sleep (nocturnal sleep before the first recall test, n = 19) and late sleep (nocturnal sleep after the first recall test, n = 19; Figure 3A). The demographic and sleep data of 38 participants are presented in Table S5.

The repeated-measures ANOVA, with group (early sleep and late sleep) as the between-subjects factor and time (first and last phases of fear acquisition) as the within-subjects factor, showed a significant increase in the mean differential SCR in both groups, with a significant main effect of time ($F_{1,36}$ = 40.490, p< 0.001), no main effect of group ($F_{1,36}$ = 0.241, p = 0.626), and no group × time interaction ($F_{1,36}$ = 0.620, p = 0.436; Figure 3B). In addition, the SCR of CS+ was significantly higher than the SCR of CS− in the last phase of fear acquisition in both two groups (Figure S3). These results suggest that these two groups acquire comparable fear.

The repeated-measures ANOVA, with group (early sleep and late sleep) as the between-subjects factor and time (last phase of fear acquisition and first recall test) as the within-subjects factor, showed that both groups still had a high mean differential SCR in the first recall test that was assessed 12 h after cTBS, with no main effect of group ($F_{1,36}$ = 0.355, p = 0.555) or time ($F_{1,36}$ = 3.644, p = 0.064) and no group × time interaction ($F_{1,36}$ = 0.698, p = 0.409; Figure 3B). The SCR of CS+ was significantly higher than the SCR of CS− in the first recall test in the two groups (Figure S3). In the second recall test which was assessed 24 h after cTBS, fear responses were assessed by repeated-measures ANOVA, with group (early sleep and late sleep) as the between-subjects factor and time (first and second recall tests) as the within-subjects factor. The results of differential SCR showed a significant main effect of time ($F_{1,36}$ = 6.941, p = 0.012), no main effect of group ($F_{1,36}$ = 0.829, p = 0.369), and no group × time interaction ($F_{1,36}$ = 0.117, p = 0.735; Figure 3B). These results suggest that the fear responses in the two groups do not decrease until 24 h after cTBS. Moreover, mean differential SCR in the last phase of extinction were similar in the two groups (p = 0.784; Figure 3B).

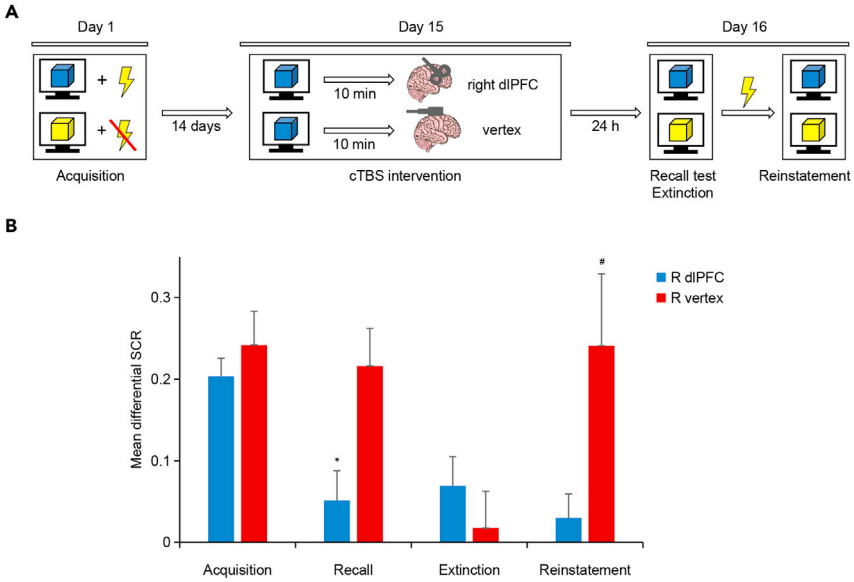

Figure 4. cTBS over the right dlPFC disrupts the reconsolidation of remote fear memory

(A) Experimental design and timeline of Experiment 4.

(B) Mean differential skin conductance response (SCR; CS+ minus CS–) during fear acquisition, the recall test, extinction, and the reinstatement test for each group. R dlPFC, cTBS over the right dlPFC after reactivation; R vertex, cTBS over the vertex after reactivation. *p< 0.05, comparisons between last phase of acquisition and recall test (all within-group); #p< 0.05, comparisons between last phase of extinction and first phase of reinstatement (all within-group). The data are expressed as mean ± SEM.

During the reinstatement test, the repeated-measures ANOVA, with group (early and late sleep) as the between-subjects factor and time (last phase of extinction and first phase of the reinstatement test) as the within-subjects factor, showed no main effect of group ($F_{1,36}$ = 0.051, p = 0.822) or time ($F_{1,36}$ = 4.119, p = 0.050) and no group × time interaction ($F_{1,36}$ = 0.488, p = 0.489; Figure 3B). In the second recall test and the first phase of the reinstatement test, no difference between the SCR of CS+ and the SCR of CS− was found in the early sleep group, but the SCR of CS+ was still higher than the SCR of CS− in the late sleep group (Figure S3). However, no difference between the early sleep group and the late sleep group was found in the SCRs (CS+, CS−, and differential SCR; Table S6 and Figure 3B). These findings indicate that a decrease in the fear response occurs after 24 h and not 12 h after cTBS, and one night of sleep does not accelerate this process.

cTBS over the right dlPFC on reconsolidation disrupts reconsolidation of remote fear memory

In Experiment 4, we investigated whether cTBS over the right dlPFC during reconsolidation has disruptive effects on remote fear memory. Twenty-four participants who acquired fear on day 1 were randomized into two groups: R dlPFC (cTBS over the right dlPFC 10 min after reactivation, n = 13) and R vertex (cTBS over the vertex 10 min after reactivation, n = 11). The cTBS over the right dlPFC or vertex after memory reactivation was performed on day 15, and the recall test, extinction, and reinstatement test occurred 24 h after cTBS (Figure 4A). No differences in age, gender, education, BMI, SDS score, SAS score, MoCA score, DST score (forward and backward), shock intensity, or AMT were found between the R dlPFC and R vertex group (all p> 0.05; Table S7).

The repeated-measures ANOVA, with group (R dlPFC and R vertex) as the between-subjects factor and time (first and last phases of fear acquisition) as the within-subjects factor, showed that all of the participants achieved the successful acquisition of fear, with a significant main effect of time ($F_{1,22}$ = 37.590, p< 0.001), no main effect of group ($F_{1,22}$ = 2.423, p = 0.134), and no group × time interaction ($F_{1,22}$ = 0.559, p = 0.463; Figure 4B). In the last phase of fear acquisition, the SCR of CS+ was significantly higher than the SCR of CS− in the two groups (Figure S4). These results suggest that these two groups acquire comparable fear.

The recall test was assessed using repeated-measures ANOVA, with group (R dlPFC and R vertex) as the between-subjects factor and time (last phase of fear acquisition and recall test) as the within-subjects factor. The results showed significant main effects of group ($F_{1,22}$ = 5.377, p = 0.030) and time ($F_{1,22}$ = 10.224, p = 0.004) and a significant group × time interaction ($F_{1,22}$ = 5.158, p = 0.033). The *post hoc* analysis showed that only participants in the R dlPFC group exhibited a significant decrease in the differential fear response (p = 0.001; Figure 4B). These results show that fear response is decreased in the recall test even though cTBS over the right dlPFC is performed on day 15. The mean differential SCR in the last phase of extinction was similar in both groups (p = 0.374; Figure 4B).

For the reinstatement test, the repeated-measures ANOVA, with group (R dlPFC and R vertex) as the between-subjects factor and time (last phase of extinction and first phase of reinstatement test) as the within-subjects factor, showed no main effects of group ($F_{1,22}$ = 2.697, p = 0.115) and time ($F_{1,22}$ = 2.794, p = 0.109), but a significant group × time interaction ($F_{1,22}$ = 5.678, p = 0.026). The *post hoc* analysis showed reinstatement of the conditioned fear occurred in the R vertex group (p = 0.012) but not in the R dlPFC group (p = 0.605; Figure 4B). In the recall test and the first phase of reinstatement test, the SCR of CS+ was significantly higher than the SCR of CS− in the R vertex group, and no difference between the SCR of CS+ and the SCR of CS− was observed in the R dlPFC group (Figure S4). These results indicate that cTBS over the right dlPFC after memory reactivation effectively decreases the fear response of remote fear memory.

DISCUSSION

The present findings authenticate that cTBS over the right dlPFC after CS exposure effectively decreases the fear response and prevents the return of fear. The fear response decreased when cTBS was delivered only during the reconsolidation window. The delivery of cTBS over the right dlPFC was effective for both recent and remote fear memories. Moreover, the impact of cTBS on reconsolidation was delay-dependent. These findings indicate that cTBS has a disruptive effect on fear memory reconsolidation, which may have implications for the clinical treatment of fear memory-related disorders.

Fear responses did not return after cTBS over the right dlPFC during reconsolidation, suggesting that the reconsolidation of fear memory was efficiently disrupted by cTBS. Consistent with the reconsolidation hypothesis, we further confirmed that this effect was only observed when cTBS was delivered 10 min after reactivation rather than no reactivation and 6 h after reactivation (Lee et al., 2017; Phelps and Hofmann, 2019). Moreover, in the present study, cTBS over the right dlPFC disrupted fear memory reconsolidation and eventually inhibited the expression of remote fear memory. Therefore, cTBS over the right dlPFC within the reconsolidation window appears to have clinical potential for treating fear-related disorders. Further studies are necessary to investigate the effect of cTBS combined with memory reactivation procedures on pathological emotional memory in psychiatric disorders.

The right dlPFC plays a critical role in both the reactivation process (de Chastelaine et al., 2016; Ragland et al., 2004; van Buuren et al., 2014) and the reconsolidation process following reactivation (Borgomaneri et al., 2020; Bridge et al., 2017; Marin et al., 2018). In the present study, fear memory expression decreased following cTBS over the right dlPFC after CS exposure. These findings are consistent with previous studies that showed that memory performance at recall was impaired after rTMS over the dlPFC during or after retrieval (Borgomaneri et al., 2020; Gagnon et al., 2010; Marin et al., 2018; Rossi et al., 2001). Additionally, interactions between the right dlPFC and other brain regions are involved in memory retrieval and reconsolidation. Compared with standard extinction, the reconsolidation process is related to lower functional connectivity of the dlPFC-anterior cingulate cortex and inferior temporal cortex-dlPFC (Li et al., 2019). Furthermore, inhibition of the dlPFC-hippocampus process disengages memory reactivation (Benoit and Anderson, 2012). However, the present study did not reveal the detailed role of the dlPFC in the effect of cTBS on the reconsolidation of fear memory. Future studies are needed to elucidate potential mechanisms of involvement of the dlPFC in reconsolidation.

The cTBS paradigm is a safe and non-invasive brain stimulation technique that can instantaneously and effectively modulate the function of cortical synapses. cTBS induces long-term drepression (LTD) at presynaptic neurons through the mild activation of NMDARs, which enhances lasting and moderate increases in Ca^{2+} and accelerates the dephosphorylation and regulation of AMPARs (Huang et al., 2011). Our findings are consistent with recent reports that low-frequency rTMS over the dlPFC after memory reactivation reduced physiological fear responses and prevented the return of fear after reinstatement (Borgomaneri

et al., 2020). The LTD-like effect of cortical plasticity that is induced by cTBS is similar to low-frequency rTMS, but the former uses shorter duration and lower intensity stimulus pulses and is more tolerable to subjects (Suppa et al., 2016). cTBS produces a longer-term effect, which persists for approximately 60 min, of suppressing synaptic transmission and reducing cortical excitability compared with rTMS (Suppa et al., 2016). cTBS over the right dlPFC has also been shown to impair memory selectively after reactivation (Marin et al., 2018), but the recognition accuracy of memory improved when cTBS was confined to the left dlPFC (Lee et al., 2013). Nevertheless, the mechanisms by which cTBS alters the function of the right and left dlPFC remain unclear. Further studies are required to explore the potential mechanisms of the action of cTBS on the reconsolidation of fear memory using electroencephalography and magnetic resonance imaging.

The present results provide additional evidence of a delay-dependent role for cTBS in the reconsolidation of fear expression. A previous study showed that rTMS over the dlPFC had no effect on fear memory expression when tested immediately after stimulation but significantly impaired fear memory expression 24 h later (Borgomaneri et al., 2020). Moreover, the fear response was still intact 4 h after an infusion of anisomycin, a protein synthesis inhibitor, after memory reactivation but decreased 24 h later in an animal study (Nader et al., 2000). Many previous studies tested the fear response 24 h after intervention, but little is known about other intervals within 24 h (Chuang et al., 2012; Nader et al., 2000; Schiller et al., 2010). Therefore, we tested the fear response 12 h after the reconsolidation intervention and found that cTBS during reconsolidation exerted a significant effect on fear memory expression only 24 h after cTBS and not at 12 h. These findings imply that reconsolidation requires sufficient time for the modulation of protein synthesis and synaptic plasticity (Bonin and De Koninck, 2015; Lee et al., 2017; Nader, 2003; Tronson et al., 2006). Moreover, sleep is essential for reconsolidation to modify memory (Diekelmann and Forcato, 2015; Lane et al., 2015; Stickgold and Walker, 2005; Walker et al., 2003). We further measured the fear response after nocturnal sleep 12 h after the cTBS intervention and found that the delay-dependent effect was unaffected by sleep. The effect of sleep on reconsolidation is different from a previous study, and one possible reason may be the diverse molecular and neural mechanisms of cTBS and propranolol (Kindt and Soeter, 2018). These results further demonstrate that the disruption of reconsolidation requires adequate time to modify memory, and sleep cannot always accelerate the reconsolidation update process.

In conclusion, the present results showed that disrupting reconsolidation with cTBS over the right dlPFC within the reconsolidation window blocked recent and remote fear memory expression. Moreover, cTBS prevents the return of fear memory with delay-dependent. These findings indicate a potential role for cTBS in modulating fear memories. Future studies should investigate the application of cTBS combined with memory reactivation procedures in the clinical setting and the neural mechanisms to better understand memory.

Limitations of the study

The present study has a few limitations. First, we did not assess the long-term resilience against fear recovery, and a longer follow-up period is required to determine the sustainability of treatment efficacy and evaluate the effect of fear incubation after the proposed reconsolidation interventions in the future. Second, the fear response was assessed only by the manually analyzed SCR, and we did not compare the SCR during the pre-CS period with the SCR during the CS period, which may induce a methodological bias (Kuhn et al., 2021; Lonsdorf et al., 2019). Additional behavioral and physiological measures and more accurately automatic analysis by the software are needed to confirm the effects of cTBS on fear memory expression. Third, we only investigated the effect of cTBS over the right dlPFC on reconsolidation. Future studies should investigate whether cTBS over the left dlPFC is also able to disrupt fear memory reconsolidation. Fourth, the mechanisms of action of cTBS on fear memory reconsolidation should be investigated in electroencephalography and magnetic resonance imaging studies. Fifth, we only tested the fear response 12 and 24 h after cTBS. The precise times at which the proposed cTBS intervention is effective remain unclear. Sixth, the participants in this study were healthy subjects. Future studies should also investigate the effect of cTBS combined with memory reactivation procedures in clinical populations, such as patients with PTSD and anxiety disorder. Seventh, one-third of participants were excluded from the experiments on days 2 and 3 when their differential SCR was less than 0.1 in the last phase of fear acquisition on day 1. This exclusion criterion may contribute to a sample bias because it is likely to exclude many factual learners (Lonsdorf et al., 2019; Marin et al., 2020). Indeed, we did not test the responses to the US at baseline (Lonsdorf et al., 2019). A more practical exclusion criterion needs to be used in future studies when investigating the effect of cTBS on fear memory reconsolidation.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.103614.

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AUTHOR CONTRIBUTIONS

Su S and Deng J designed and performed the experiments, interpreted the results, and wrote the initial draft of the manuscript. Su S, Deng J, Yuan K, Gong Y, Zhang Y, Li H, and Cao K contributed to recruiting participants and data collection. Su S, Deng J, Huang X, and Bao Y conducted the statistical analysis. Lin X, Wu P, Xue Y, and Shi J commented on and revised the manuscript. Lu L and Shi L commented on the study, revised the manuscript, and wrote the final version. All of the authors contributed to the final draft of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
Raw datasets	This paper	https://data.mendeley.com/datasets/p4k7jcnff8/1
Software and algorithms		
AcqKnowledge (version 5.0)	BIOPAC Systems, Inc.	https://www.biopac.com/
E-Prime (version 2.0)	Psychology Software Tools	https://pstnet.com/products/e-prime/
SPSS (version 20.0)	IBM SPSS Statistics	https://www.ibm.com/analytics/spss-statistics-software
AcqKnowledge (version 5.0)	BIOPAC Systems, Inc.	https://www.biopac.com/
Other		
Biopac MP160	BIOPAC Systems, Inc.	https://www.biopac.com/
Biopac EDA100C	BIOPAC Systems, Inc.	https://www.biopac.com/
Biopac TSD203 electrodes	BIOPAC Systems, Inc.	https://www.biopac.com/
STM 200 constant-current stimulator	BIOPAC Systems, Inc.	https://www.biopac.com/
Magstim TMS Super Rapid2	Magstim Company Limited	https://www.magstim.com/product/rapid-family/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Lin Lu (linlu@bjmu.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The datasets generated during this study are publicly available on the Mendeley Data https://data.mendeley.com/datasets/p4k7jcnff8/1.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Participants

For this study, 170 healthy volunteers were enrolled through campus flyers and online advertisements. The inclusion criteria included: (*i*) between 18 and 35 years of age and (*ii*) generally in good health as determined by a physician. The exclusion criteria included: (*i*) current or past history of medical or psychiatric illness, diagnosed by the Structured Clinical Interview for *Diagnostic and Statistical Manual of Mental Disorders*, 4th edition, Axis I Disorders, (*ii*) the use of any medications, (*iii*) any contraindication to TMS, (*iv*) self-reported pregnancy or menstrual period, (*v*) lifetime history of head injury, (*vi*) current or past neurological disorder (e.g., seizure disorder, brain tumors, stroke, and cerebral aneurysm), and (*vii*) past or current participation in other electric shock-related fear memory experiments. Additionally, all of the participants were given further details about the experimental protocol, and they signed an informed consent form that was approved by the Institutional Review Board of Peking University Sixth Hospital. The

participants were paid for their participation. To control for potential confounders, all the participants completed questionnaires to collect basic demographic information, including gender, age, education, and body mass index (BMI). Baseline mental health conditions were assessed with the Self-Rating Depression Scale (SDS) (Zung, 1965), Self-Rating Anxiety Scale (SAS) (Zung, 1971), Montreal Cognitive Assessment (MoCA) (Nasreddine et al., 2005), digit span test (DST) (Blankenship, 1938), and Pittsburgh Sleep Quality Index (PSQI) (Buysse et al., 1989).

METHOD DETAILS

Fear acquisition and reactivation

We used a modified fear acquisition procedure based on our previous studies (Deng et al., 2020; Liu et al., 2014; Yue et al., 2018). The participants were asked to specify the relationship between different CSs (colored square pictures on the computer screen) and a US (mild electric shock to the wrist). Before fear conditioning, the US intensity began at a mild level of shock (15 V) and then gradually increased until reaching the maximum that the participants felt uncomfortable but not painful (the highest intensity was 75 V). All the shocks were presented for 200 ms at a current of 50 pulses per second. The CSs were presented for 4 s with a variable interval of 8–12 s. The CS+ was paired with an electric shock on a partial reinforcement schedule (50% reinforced). The CS− was not paired with an electric shock. The CS+ and CS− were counterbalanced and randomized between participants. The fear acquisition consisted of 12 nonreinforced presentations of each CS and 12 additional reinforced presentations of the CS+. Since unconditioned shock stimulation might influence the reinforced CS+, only the skin conductance response (SCR) of the non-reinforced CS+ was used to assess the acquired fear response. The criterion for the establishment of fear conditioning was a mean differential SCR to the CS (CS+ minus CS−) $\geq$ 0.1 (Schiller et al., 2010). Finally, 170 participants completed the study. For reactivation, one non-reinforced CS+ presentation was given to reactivate the fear memory (Sevenster et al., 2013).

Recall test, extinction, and reinstatement test

The participants were presented with non-reinforced presentations of the CS. The recall test consisted of three trials of the CS+ and CS−, followed by extinction that consisted of 12 CS+ and 12 CS− presentations. One minute after extinction, the participants received four unsignaled US presentations, followed by the reinstatement test, during which 15 CS+ and 15 CS− were presented. During the extinction and tests, the CSs were presented for 4 s with an interstimulus interval of 8–12 s, during which the participants looked at a fixation point on the computer screen.

cTBS intervention

Non-invasive cTBS was delivered using the Magstim Rapid 2 system (Magstim, Whitland, Wales, UK). Before stimulation, resting motor thresholds were applied over the primary motor cortex to determine the lowest stimulation intensity that was at 80% of the active motor threshold (AMT). The neurophysiological effect of the 30 Hz TBS protocol coincided with the original TBS protocol (Deng et al., 2021; Hong et al., 2015; Huang et al., 2005; Wu et al., 2012). The cTBS protocol in the present study was 1,800 pulses and triplet bursts with a pulse frequency of 30 Hz and a burst frequency of 5 Hz. Given the evidence that the right dlPFC is involved in reactivation and reconsolidation in memory tasks (Benoit and Anderson, 2012; Gagnon et al., 2010; Rossi et al., 2001), cTBS was confined to the right hemisphere only in this study. Previous studies showed that stimulation over the vertex was one of the most common control conditions because the stimulation over the dlPFC and vertex induced similar noise, twitches, and even some cortical activity to dlPFC (Deng et al., 2021; Parkin et al., 2015; Sandrini et al., 2011). Thus, the coil handle was directed posteriorly, and cTBS was applied over the right dlPFC and vertex, which were determined by standard F4 and Cz locations based on international electroencephalogram 10–20 system measurements, an orientation that was in accordance with the longitudinal fissure (Censor et al., 2010).

Experimental design

In Experiment 1, we investigated the effect of cTBS over the right dlPFC after CS exposure on recent fear memory. Seventy-two participants were included and randomized into four groups using random numbers: R dlPFC (cTBS over the right dlPFC 10 min after reactivation, n = 18), R vertex (cTBS over the vertex 10 min after reactivation, n = 18), NR dlPFC (cTBS over the right dlPFC without reactivation, n = 18), and NR vertex (cTBS over the vertex without reactivation, n = 18). All participants underwent fear acquisition training on

day 1. On day 2, participants received cTBS over the right dlPFC or the vertex. The recall test, extinction, and reinstatement test occurred 24 h after cTBS stimulation (Figure 1A).

In Experiment 2, we explored whether the effect of cTBS on fear memory reconsolidation depends on a specific time-window. Thirty-six participants were included and randomized into two groups using random numbers: 10 min (cTBS over the right dlPFC 10 min after reactivation, $n = 18$) and 6 h (cTBS over the right dlPFC 6 h after reactivation, $n = 18$). Fear acquisition training was conducted on day 1. Participants received cTBS after 10 min or 6 h on day 2. The recall test, extinction, and reinstatement test occurred 24 h later (Figure 2A).

In Experiment 3, we investigated the time-dependent effect of cTBS over the right dlPFC during the reconsolidation process on fear expression. Fear conditioning was conducted with 38 participants on day 1. On day 2, they all received cTBS over the right dlPFC 10 min after reactivation. The first recall test was conducted exactly 12 h after cTBS, and the second recall test, extinction, and the reinstatement test occurred 24 h after cTBS. Considering the impact of sleep on the reconsolidation process, thirty-eight participants were included and randomized into two groups using random numbers: early sleep (nocturnal sleep before the first recall test, $n = 19$) and late sleep (nocturnal sleep after the first recall test, $n = 19$; Figure 3A). Moreover, the participants wore an Actiwatch (Phillips Actiwatch Spectrum Pro, Murrysville, PA, USA) and completed a sleep diary to evaluate their sleep duration and sleep-wake cycles during the first 24 h after cTBS.

In Experiment 4, we investigated the role of cTBS over the right dlPFC in remote fear memory. Twenty-four participants were included and randomized into two groups using random numbers: R dlPFC (cTBS over the right dlPFC 10 min after reactivation, $n = 13$) and R vertex (cTBS over the vertex 10 min after reactivation, $n = 11$). Fear acquisition training was conducted on day 1. The cTBS over the right dlPFC or vertex was performed 14 days later. The recall test, extinction, and reinstatement test occurred 24 h after cTBS (Figure 4A).

Psychophysiological stimulation and assessment

An STM 200 constant-current stimulator (BIOPAC Systems, Goleta, CA, USA) was used to deliver the electric shocks. We attached the stimulating electrode to the right inner wrist of the participants. Stimulus presentation was controlled by a computer using E-Prime software (Psychology Software Tools, Sharpsburg, PA, USA). The SCR was measured using two electrodes (BIOPAC TSD203 electrodes) attached to the second and third fingers of the left hand via a BIOPAC MP160 and EDA100C. Moreover, the SCR was manually analyzed using Acknowledge software (BIOPAC Systems, Goleta, CA, USA), and the CS trial was revised when the limb motion or forced breathing occurred (Boucsein et al., 2012; Lonsdorf et al., 2017). We recorded the greatest trough-to-peak change in the SCR in an 8-s time window after an interval of 2-s post each CS onset (Kuhn et al., 2021; Lonsdorf et al., 2017). These raw SCR values were square-root transformed to normalize the distribution (Kuhn et al., 2021).

QUANTIFICATION AND STATISTICAL ANALYSIS

Subjective data and SCR analysis

Demographic data, shock intensity, and the AMT are presented as the mean ± standard error of the mean (SEM). Differences in age, education levels, BMI, SDS scores, SAS scores, MoCA scores, DST scores, shock intensity, and AMT between groups were analyzed using one-way analysis of variance (ANOVA) in Experiment 1 and independent-sample *t*-tests in Experiments 2–4. Differences in gender frequencies between groups were analyzed using the χ^2 test in all of the experiments. The dependent variables of all tests were the difference of the mean SCRs to CS+ and CS− for the different experimental phases. A previous study showed that the first trial was susceptible to trial sequence effects and might cause noise and bias of CS+/CS− discrimination (Lonsdorf et al., 2019), so the first trial (CS−) for recall test and reinstatement test was excluded in this study. Every experimental phase was consisted of three trials of CS+ and three trials of CS− except the recall test and the first phase of reinstatement test, which comprised three trials of CS+ and two trials of CS−. Therefore, there were four phases in the fear acquisition, one phase in the recall test, four phases in the extinction, and five phases in the reinstatement test. The SCR results are expressed as the mean ± SEM. Mean differential SCRs (CS+ minus CS− in corresponding trials) for fear acquisition (first and last phases), the recall test, extinction (last phase), and the reinstatement test (first phases) were analyzed using repeated-measures ANOVA with appropriate between- and within-subjects factors, followed by the Bonferroni *post hoc* t-tests. Moreover, the differences of SCR between CS+ and - in each group during each phase were analyzed using paired sample t-tests in four experiments (Figures S1–S4).

The differences of SCRs (CS+, CS-, and CS+ minus CS-) among groups during different experimental phases were analyzed using one-way ANOVA in Experiment 1 and independent-sample t-tests in Experiments 2–4 (Tables S2, S4, S6, and S8). The level of significance for the statistical comparisons was a two-tailed $p < 0.05$. The statistical analyses were performed using SPSS 20.0 software (SPSS, Chicago, IL, USA).