Medial anterior prefrontal cortex stimulation down-regulates implicit reactions to threats and prevents the return of fear

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Information about the manuscript

Medial anterior prefrontal cortex stimulation down-regulates implicit reactions to threats and prevents the return of fear

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Title: Medial anterior prefrontal cortex stimulation down-regulates implicit reactions to threats and prevents the return of fear

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Abstract

Down-regulating emotional overreactions toward threats is fundamental for developing treatments for anxiety and post-traumatic disorders. The prefrontal cortex (PFC) is critical for top-down modulatory processes, and despite previous studies adopting repetitive Transcranial Magnetic Stimulation (rTMS) over this region provided encouraging results in enhancing extinction, no studies have hitherto explored the effects of stimulating the medial anterior PFC (aPFC, encompassing the Brodmann area 10) on threat memory and generalization. Here we showed that rTMS over the aPFC applied before threat memory retrieval immediately decreases implicit reactions to learned and novel stimuli in humans. These effects enduringly persisted one week later in the absence of rTMS. No effects were detected on explicit recognition. Critically, rTMS over the aPFC resulted in a more pronounced reduction of defensive responses compared to rTMS targeting the dorsolateral PFC. These findings reveal a previously unexplored prefrontal region, the modulation of which can efficiently and durably inhibit implicit reactions to learned threats. This represents a significant advancement towards the long-term deactivation of exaggerated responses to threats.

Introduction

Emotional memories related to past threat experiences allow humans to predict future dangers and trigger adaptive defensive reactions when encountering learned threat-signaling cues[1]. However, extremely dangerous situations may lead to psychological disorders[2]. Furthermore, the ability to generalize defensive reactions to new stimuli enables organisms to anticipate potential threats and respond to them based on similar perilous experiences lived in the past. On the other hand, evaluation mechanisms excessively biased toward threat generalization (i.e. overgeneralization) may underlie anxiety disorders and trauma[3]. At the base of these processes, in a previous work[4] we observed that autonomic-implicit and cognitive-explicit tunings may diverge when humans are exposed to the same new stimuli, where cognitive generalization may enable a flexible evaluation of incoming cues to develop adaptive predictions of potential dangers. The crosswise presence of overgeneralization in anxiety diseases, and the dissociation between autonomic and cognitive defensive response patterns, highlight the importance of including both implicit and explicit generalization tasks to characterize fear-related processes in humans.

Attempting to down-regulate the emotional overreactions toward threat-predictive and new stimuli is one of the main routes for developing effective treatments for anxiety and post-traumatic disorders. Common approaches such as pharmacological treatments and cognitive-behavioral therapy (CBT) have demonstrated partial efficacy[5], and recent evidence suggests that the functional outcome of behavioral methods may depend on the extent to which the prefrontal cortex is recruited during these processes[6]. Hence, new intervention strategies influencing the prefrontal dynamics would represent an important advance in the field[7].

Previous studies adopted transcranial direct current stimulation (tDCS) or transcranial electrical stimulation (tES) to disrupt the consolidation of these memories[8–10], potentiate extinction processes[11,12], and narrow threat generalization patterns[13], leading to contradictory results. According to one work[8], cathodal stimulation over the dorsolateral prefrontal cortex (dlPFC) disrupted threat memory consolidation, with no enhancing effect of anodal stimulation. In contrast,

other studies found an increase in implicit responses with anodal stimulation[9] and no effect of cathodal stimulation[10] over the same site. Moreover, one study employing anodal stimulation over the dlPFC[12] revealed an improvement in extinction learning but no delayed effects on the recall of the extinction memory. A further investigation[11] reported that low-frequency alternating-current (AC) stimulation of the medial prefrontal cortex (mPFC) augmented the defensive responses, whereas direct-current (DC) stimulation widened threat generalization profiles.

An alternative neurostimulation approach is repetitive transcranial magnetic stimulation (rTMS), which ensures greater focality[14,15]. Some rTMS studies targeted the mPFC[16] and the posterior PFC[17] to obtain a successful enhancement of extinction learning, while others[18,19] targeted the dlPFC to disrupt threat-memory reconsolidation. Indeed, most rTMS-based research targeting the PFC has pursued an improvement of fear extinction, which may be followed by a return of fear with a change of context (i.e. renewal)[20] where prevention of relapse over time is the main challenge for therapies dedicated to post-traumatic and anxiety disorders. No previous studies reported significant effects in down-modulating the defensive responses triggered by a learned threatening stimulus without adopting fear extinction.

So far, human brain stimulation studies have been mainly focused on the dorsolateral region of the PFC[7], partly because other prefrontal areas involved in the top-down regulation of subcortical threat-detection systems –such as the ventromedial PFC (vmPFC), are too deep to be reached with TMS[17]. However, within the PFC, a brain structure that is emerging to be engaged in downstream emotional regulation is the anterior prefrontal cortex (aPFC), also known as the frontopolar cortex or rostral frontal cortex. The aPFC encompasses the most anterior portion of the prefrontal cortex (Brodmann area 10, BA 10)[21] and extends over a wider cortical space in humans than in other species[22]. Even if it has not been included in fear network models so far, many studies[23–25] highlighted its role in emotional down-regulation. Anatomical projections have been found between the lateral[25,26] and the medial aPFC[27] and the amygdala, and functional connectivity has been detected between the aPFC and the vmPFC during fear down-regulation[28]. Notably, hypoactivation,

reduced connectivity, and altered thickness of aPFC were reported in PTSD patients[29–32], whereas a longitudinal study[33] showed that strong activation of the aPFC resulted in a higher resilience against PTSD onset. Accordingly, enhanced aPFC activity and potentiated aPFC-vmPFC connectivity were detected after an effective therapy in PTSD patients[34]. Crucially, the aPFC is a surface area easily accessible with rTMS. However, to our knowledge, no study has been so far conducted to explore the effects of aPFC stimulation on the expression of a threat memory without extinction learning in humans.

In our current study, we posited that applying rTMS to the aPFC could influence implicit defensive responses to a learned threat-predictive stimulus and/or the conscious recognition of it. Subsequently, we explored additional hypotheses. The second hypothesis centered on the potential extension of rTMS dampening effects to new stimuli, thereby reducing threat generalization. The third hypothesis focused on the enduring persistence of rTMS effects on defensive responses over time. The final hypothesis proposed that the dampening effects achieved by stimulating the aPFC might surpass those observed when targeting the dorsolateral PFC.

Results

aPFC-focused rTMS effects on implicit defensive reactions toward threat-predictive and new cues

To explore the effects of an aPFC-centered rTMS on the implicit responses to a learned threat, we designed a three-session experiment starting with a threat learning session followed by an implicit retention test and a follow-up implicit re-test (Figure 1).

During the learning session, participants learned to associate an auditory cue (conditioned stimulus, CS, 800Hz) with a mild electric stimulation (unconditioned stimulus, US, individually calibrated intensity) in a given environment (context A). We adopted a single-cue learning paradigm because it more ecologically reflects real-life traumatic experiences[35–39]. To validate the between-groups homogeneity in the painful stimuli perception, we compared the post-conditioning US ratings, and we observed no significant differences between groups (Student's unpaired *t* test, $t_{(58)} = 0.799$, $P = 0.428$, $\eta_p^2 = 0.011$) (Table 1). We also did not observe significant differences between groups in SCRs to the CS during the preconditioning phase ($t_{(58)} = 0.418$, $P = 0.677$, $\eta_p^2 = 0.003$), to the CS during the conditioning phase (2 × 15 mixed ANOVA; main effect of group: $F_{(1,52)} = 2.367$, $P = 0.130$, $\eta_p^2 = 0.044$; main effect of trial: $F_{(8.762,455.600)} = 13.366$, $P < 0.001$, $\eta_p^2 = 0.204$; group × trial interaction: $F_{(8.762,455.600)} = 1.619$, $P = 0.109$, $\eta_p^2 = 0.030$; Student's unpaired *t* test on the averaged response, $t_{(58)} = 1.290$, $P = 0.202$, $\eta_p^2 = 0.028$), nor to the US during the conditioning phase ($t_{(58)} = 1.011$, $P = 0.316$, $\eta_p^2 = 0.017$) (Figure 2-figure supplement 1).

One week later, we tested the implicit memory of the learned association in control sham-stimulated subjects and in those who received rTMS over the aPFC shortly before the memory test. To locate this brain region, which corresponds to the BA 10[40], we positioned the coil over the frontopolar midline electrode (Fpz) adopting the international 10–20 electroencephalogram (EEG) coordinate system[41] since previous rTMS studies[16,42,43] ensured this placement reached the aPFC. An offline 10-min session of 1Hz-rTMS targeting this neural site (aPFC, $n = 30$) was applied

immediately before memory retrieval (Figure 2A). Control subjects underwent a 10-min sham stimulation procedure over the same cortical area (sham, n = 30).

Memory retention was tested in a different environment from that where the learning had occurred (context B) to avoid any contextual influence on retrieval[4,44–47]. Indeed, the context shift for this session mirrors a real-life treatment setting –which unlikely takes place in the threatening location. To test implicit threat memory, we performed an implicit recognition task in which subjects were exposed to the CS while being recorded in their evoked autonomic reactions (i.e., electrodermal skin conductance responses, SCRs). No US shocks were delivered during this phase. Besides the CS, participants were presented with two novel but perceptually similar tones (NS_1, 1000Hz; NS_2, 600Hz) to study threat generalization. Auditory frequencies of NSs were selected to obtain a slowly decaying gradient of defensive tunings[4,48,49]. To test the effects of rTMS on memory retention, we compared the between-group differences as well as the within-group differences from the acquisition phase to the testing phase through a 2 × 2 mixed ANOVA. This analysis yielded a not significant main effect of group ($F_{(1,58)}$ = 2.015, P = 0.161, η_p^2 = 0.034), a not significant main effect of phase ($F_{(1,58)}$ = 0.053, P = 0.818, η_p^2 = 0.001) and a significant group × phase interaction ($F_{(1,58)}$ = 13.445, P = 0.001, η_p^2 = 0.188). Simple main effects analysis revealed no significantly different mean CS-evoked SCRs between groups during the conditioning phase (P = 0.506; Bonferroni corrected). On the contrary, during the test phase subjects that received rTMS over the aPFC exhibited weakened CS-related SCRs than those observed in the sham group (P = 0.006; Bonferroni corrected). Moreover, the aPFC group showed reduced autonomic responses to the CS from conditioning to test (P = 0.008; Bonferroni corrected) whereas the sham group displayed increased mean SCRs to the CS from conditioning to test (P = 0.018; Bonferroni corrected) (Figure 2B,C). This data indicates that the rTMS procedure affected SCRs triggered by memory retrieval performed shortly after rTMS. To the best of our knowledge, this is the first evidence that brain stimulation may promptly attenuate implicit defensive reactions during memory retrieval.

In the test session, we also analyzed threat generalization to the NSs through a 2 × 3 mixed ANOVA, which showed a significant main effect of group ($F_{(1,58)} = 5.310$, $P = 0.025$, $\eta_p^2 = 0.084$), a not significant main effect of tone ($F_{(2,116)} = 0.690$, $P = 0.504$, $\eta_p^2 = 0.012$) and a not significant group × tone interaction ($F_{(2,116)} = 1.301$, $P = 0.276$, $\eta_p^2 = 0.022$), revealing that the aPFC group displayed overall attenuated responses to tones relative to the sham condition (Figure 2D,E).

We next sought to disambiguate whether the rTMS effects were due to a general down-regulation of electrodermal responsivity, or whether they specifically targeted the threat memory. To this end, subjects were presented with an unconditioned threatening stimulus consisting of a female scream sample (unconditioned stimulus 2, US_2) while being recorded in their SCRs. No significant differences emerged between conditions ($t_{(58)} = 0.334$, $P = 0.739$, $\eta_p^2 = 0.002$), indicating that the rTMS did not cause an overall inhibition of electrodermal reactivity (Figure 2F).

To test whether and to what extent rTMS-related outcomes endured beyond the after-effect window and persisted over a long-term period, we planned a follow-up session. One week after the threat memory retrieval test, all participants returned to the conditioning room (context A) and underwent a re-testing phase, identical to the testing one except for the absence of rTMS administration. This phase also allowed us to test a possible renewal effect[20] since subjects were re-exposed to the original threatening environment.

Concerning the implicit responses to the CS, a 2 × 2 mixed ANOVA showed a not significant main effect of group ($F_{(1,58)} = 1.952$, $P = 0.168$, $\eta_p^2 = 0.033$), a significant main effect of phase ($F_{(1,58)} = 7.690$, $P = 0.007$, $\eta_p^2 = 0.117$) and a significant group × phase interaction ($F_{(1,58)} = 9.966$, $P = 0.003$, $\eta_p^2 = 0.147$). Simple main effects analysis revealed that participants of the aPFC group persisted in displaying weaker SCRs than those observed in the sham group ($P = 0.006$; Bonferroni corrected). Moreover, the aPFC group persisted in showing a decrease of defensive reactions to the CS from conditioning to follow-up ($P < 0.001$; Bonferroni corrected), while the sham group did not display significantly different SCRs in the two phases ($P = 0.787$; Bonferroni corrected) (Figure 2G,H).

These findings support an enduring effect of the aPFC-rTMS in attenuating the long-term implicit defensive responses to the learned threat-predictive cue, even with the re-exposition to the environment where threat learning had occurred. We next analyzed the autonomous response patterns to the female scream sample (unconditioned stimulus 2, US_2) and again we found that reactions did not differ between groups ($t_{(58)} = 0.057$, $P = 0.955$, $\eta_p^2 < 0.001$) (Figure 2I). Thus, the persistent effect was expressed notwithstanding an unaffected electrodermal overall reactivity.

An important aspect to consider is that rTMS application over the forehead can be subjectively perceived as unpleasant. We, therefore, investigated whether an rTMS-related discomfort before memory retrieval might have provoked habituation to unpleasant stimulations, leading to a reduction in SCR levels during CS presentations. We repeated the entire experiment in one further group (ctrl discomfort, $n = 10$) by replacing the rTMS procedure with a 10-min discomfort-inducing procedure over the same site of the forehead to mimic the rTMS-evoked unpleasant sensations in the absence of neural stimulation effects. This group showed no significantly different CS-evoked SCR levels to those of the sham group during the test session as well as during the follow-up session (Figure 2-figure supplement 2). Thus, the discomfort experienced during the rTMS procedure did not contribute to the reduction of electrodermal responses observed in the aPFC-stimulated group.

aPFC-focused rTMS effects on the explicit memory recognition and perceptual discrimination

We then investigated the effect of rTMS over the aPFC on the retention of explicit-declarative threat memories. A further group of subjects that received the identical 1Hz-rTMS procedure over the aPFC (aPFC-E, $n = 21$) and a further control group (sham-E, $n = 21$) underwent an explicit two-alternative forced-choice (2AFC) recognition task, in which they were presented with a random sequence of tone pairs, each composed of the CS and one of the two NSs. Subjects were asked to consciously identify which stimulus of each pair was the one previously paired with the US (i.e., the CS), and to provide a subjective confidence level for each choice using a scale ranging from 0

(completely unsure) to 10 (completely sure)[4,50]. Both groups reported not significantly different post-conditioning US ratings ($t_{(40)} = 0.339$, $P = 0.737$, $\eta_p^2 = 0.003$) and successfully identified the CS amongst the NSs with an accuracy level above the 50% chance level (aPFC-E: $t_{(20)} = 9.226$, $P < 0.001$, $\eta_p^2 = 0.810$; sham-E: $t_{(20)} = 14.240$, $P < 0.001$, $\eta_p^2 = 0.910$). A between-groups comparison ($t_{(40)} = 1.114$, $P = 0.272$, $\eta_p^2 = 0.030$) showed no differences in the explicit recognition accuracy (Figure 3A). The two groups were not differently confident when making their choices ($t_{(40)} = 0.842$, $P = 0.405$, $\eta_p^2 = 0.017$) (Figure 3B), thereby supporting the lack of rTMS-related effects.

Next, since a previous study[13] targeting the vmPFC modulated perceptual discrimination processes, we implemented a 2AFC perceptual task in which we investigated the ability of participants to sensory discriminate between the CS and the two NSs by collecting binary 'same or different' judgments as well as confidence ratings. The perceptual discrimination test yielded no significant between-groups differences in accuracy ($t_{(40)} = 1.362$, $P = 0.181$, $\eta_p^2 = 0.044$) as well as confidence levels ($t_{(40)} = 0.917$, $P = 0.365$, $\eta_p^2 = 0.021$). Indeed, both groups discriminated the CS from the NSs with high precision (aPFC-E: 0.980 ± 0.015 SEM; sham-E: 1.000 ± 0.000 SEM) and with no different confidence levels (aPFC-E: 9.409 ± 0.153 SEM; sham-E: 9.586 ± 0.117 SEM), thereby showing no rTMS effects on sensory abilities.

These data suggest that the pre-retrieval rTMS procedure over the aPFC did not affect the explicit recognition nor the perceptual discrimination of a learned threat.

During the follow-up session, explicit recognition patterns demonstrated an over-chance accuracy level for each group (aPFC-E: $t_{(20)} = 13.780$, $P < 0.001$, $\eta_p^2 = 0.905$; sham-E: $t_{(20)} = 7.162$, $P < 0.001$, $\eta_p^2 = 0.720$). Again, here there were no between-group differences ($t_{(40)} = 1.024$, $P = 0.312$, $\eta_p^2 = 0.026$) since both groups achieved a high recognition accuracy (Figure 3C). Groups did also not report different confidence levels ($t_{(40)} = 0.084$, $P = 0.934$, $\eta_p^2 < 0.001$) (Figure 3D).

As in the case of the previous session, we did not observe significant between-group differences in the perceptual discrimination ($t_{(40)} = 1.000$, $P = 0.323$, $\eta_p^2 = 0.024$) and the respective confidence ratings ($t_{(40)} = 0.149$, $P = 0.882$, $\eta_p^2 < 0.001$). Indeed, the discrimination accuracy

(aPFC-E: 1.000 ± 0.000 SEM; sham-E: 0.993 ± 0.007 SEM) and the self-assessed confidence (aPFC-E: 9.598 ± 0.147 SEM; sham-E: 9.633 ± 0.182 SEM) were high in each condition.

Topographical selectivity of rTMS effects on implicit defensive responses to threat-predictive and new cues

To ascertain the topographical selectivity, in one further condition (OC, n = 30) we applied the rTMS over the left occipital cortex as an active control site (Figure 4A) and we contrasted its implicit reactions with those of the group stimulated over the aPFC.

No differences emerged between the two conditions in terms of post-conditioning US ratings ($t_{(58)}$ = 0.000, P = 1.000, η_p^2 = 0.000) (Table 1), SCR responses to the CS during the preconditioning phase ($t_{(58)}$ = 1.037, P = 0.304, η_p^2 = 0.018), to the CS during the conditioning phase (2 × 15 mixed ANOVA; main effect of group: $F_{(1,54)}$ = 0.124, P = 0.726, η_p^2 = 0.002; main effect of trial: $F_{(9.368,505.856)}$ = 13.341, P < 0.001, η_p^2 = 0.198; group × trial interaction: $F_{(9.368,505.856)}$ = 0.994, P = 0.445, η_p^2 = 0.018; Student's unpaired t test on the averaged response, $t_{(58)}$ = 0.162, P = 0.872, η_p^2 < 0.001), and to the US during the conditioning phase ($t_{(58)}$ = 1.210, P = 0.231, η_p^2 = 0.025) (Figure 4-figure supplement 1).

Next, we analyzed implicit reactions toward the CS in both conditioning and test sessions. A 2 × 2 mixed ANOVA revealed a not significant group main effect ($F_{(1,58)}$ = 2.952, P = 0.091, η_p^2 = 0.048), a not significant phase main effect ($F_{(1,58)}$ = 2.027, P = 0.160, η_p^2 = 0.034), and a significant group × phase interaction ($F_{(1,58)}$ = 4.705, P = 0.034, η_p^2 = 0.075). CS-related SCRs did not differ between groups during conditioning (P = 0.798; Bonferroni corrected) but, during the test, the aPFC group exhibited weaker defensive responses than the OC group (P = 0.019; Bonferroni corrected). Unlike the aPFC group, whose implicit reactions to the CS diminished from conditioning to test (P = 0.014; Bonferroni corrected), the OC group's responses did not differ in the two phases (P = 0.600; Bonferroni corrected) (Figure 4B,C).

No significant between-group differences were observed in implicit responses to new tones (2 × 3 mixed ANOVA; main effect of group: $F_{(1,58)} = 2.775$, $P = 0.101$, $\eta_p^2 = 0.046$; main effect of tone: $F_{(2,116)} = 5.857$, $P = 0.004$, $\eta_p^2 = 0.092$; group × tone interaction: $F_{(2,116)} = 3.739$, $P = 0.027$, $\eta_p^2 = 0.061$). While the CS triggered weaker reactions in the aPFC group ($P = 0.019$; Bonferroni corrected), both the NS_1 ($P = 0.203$; Bonferroni corrected) and the NS_2 ($P = 0.323$; Bonferroni corrected) elicited not significantly different responses in the two conditions. These findings underscored the selectivity of divergent rTMS effects in the aPFC and OC groups specifically for the CS. Fear tuning analysis of the aPFC group's implicit reactions unveiled no differences in SCR amplitudes elicited by the CS and the NS_1 ($P = 0.378$; Bonferroni corrected), by the CS and the NS_2 ($P = 1.000$; Bonferroni corrected), and by the NSs ($P = 0.552$; Bonferroni corrected). In the case of the OC group, implicit reactions were not different for the CS and the NS_1 ($P = 0.876$; Bonferroni corrected) but the NS_2 evoked lower SCRs than the CS ($P < 0.001$; Bonferroni corrected) and the NS_1 ($P = 0.041$; Bonferroni corrected) (Figure 4D,E). Furthermore, no significant group differences were detected in SCRs elicited by US_2 during the test session ($t_{(58)} = 0.175$, $P = 0.862$, $\eta_p^2 < 0.001$) (Figure 4F).

The distinctive pattern toward the learned threatening cue persisted during the follow-up session (2 × 2 mixed ANOVA; main effect of group: $F_{(1,58)} = 2.141$, $P = 0.149$, $\eta_p^2 = 0.036$; main effect of phase: $F_{(1,58)} = 26.023$, $P < 0.001$, $\eta_p^2 = 0.310$; group × phase interaction: $F_{(1,58)} = 3.167$, $P = 0.080$, $\eta_p^2 = 0.052$). The aPFC group continued to react more dimly to the CS compared to the OC group ($P = 0.026$; Bonferroni corrected). Both the aPFC ($P < 0.001$; Bonferroni corrected) and the OC ($P = 0.022$; Bonferroni corrected) groups showed decreased responses relative to conditioning (Figure 4G,H). Conversely, no significant differences were observed in SCRs evoked by US_2 during the follow-up session ($t_{(58)} = 0.574$, $P = 0.568$, $\eta_p^2 = 0.006$) (Figure 4I).

Comparison between the effects of rTMS administered over the anterior versus the dorsolateral prefrontal cortex

Next, we asked whether the findings we obtained by targeting the aPFC were finely specific for this site or, alternatively, they overlapped with those observed by targeting other prefrontal sub-regions. For this purpose, in one further group (dlPFC, n = 30) we applied the same rTMS procedure over the left dorsolateral PFC (Figure 5A) and we then compared the implicit patterns of this group with those displayed by the aPFC condition. We selected the left dlPFC since previous studies[e.g. 17] targeted the left hemisphere for testing the rTMS effects on the PFC, and some evidence[see 7] suggested that inhibitory tDCS and rTMS over the left dlPFC may disrupt threat memory consolidation.

We found no significant differences between the two conditions in the post-conditioning US ratings ($t_{(58)}$ = 0.908, P = 0.368, η_p^2 = 0.014) (Table 1), in SCRs to the CS during the preconditioning phase ($t_{(58)}$ = 0.967, P = 0.337, η_p^2 = 0.016), to the CS during the conditioning phase (2 × 15 mixed ANOVA; main effect of group: $F_{(1,51)}$ = 0.026, P = 0.873, η_p^2 = 0.001; main effect of trial: $F_{(8.026,409.333)}$ = 12.135, P < 0.001, η_p^2 = 0.192; group × trial interaction: $F_{(8.026,409.333)}$ = 1.042, P = 0.403, η_p^2 = 0.020; Student's unpaired t test on the averaged response, $t_{(58)}$ = 0.378, P = 0.707, η_p^2 = 0.002), and to the US during the conditioning phase ($t_{(58)}$ = 1.752, P = 0.085, η_p^2 = 0.050) (Figure 5-figure supplement 1).

Then we compared the implicit reactions toward the CS during conditioning and test sessions. A 2 × 2 mixed ANOVA indicated a not significant main effect of group ($F_{(1,58)}$ = 1.874, P = 0.176, η_p^2 = 0.031), a not significant main effect of phase ($F_{(1,58)}$ = 0.122, P = 0.729, η_p^2 = 0.002), and a significant group × phase interaction ($F_{(1,58)}$ = 10.810, P = 0.002, η_p^2 = 0.157). CS-evoked SCRs did not differ between the two groups during conditioning (P = 0.647; Bonferroni corrected) while during the test we found weaker defensive responses in the aPFC group relative to the dlPFC group (P = 0.009; Bonferroni corrected). At odds with the aPFC group whose implicit reactions to the CS were diminished from conditioning to test (P = 0.013; Bonferroni corrected), the dlPFC group increasingly responded during the test relative to conditioning (P = 0.042; Bonferroni

corrected) (Figure 5B,C). This incremental trend is in line with a previous study that delivered a 1Hz-rTMS protocol over the left dlPFC[18].

We found no between-groups differences in the implicit responses to the new tones (2 × 3 mixed ANOVA; main effect of group: $F_{(1,58)} = 3.967$, $P = 0.051$, $\eta_p^2 = 0.064$; main effect of tone: $F_{(2,116)} = 2.819$, $P = 0.064$, $\eta_p^2 = 0.046$; group × tone interaction: $F_{(2,116)} = 3.286$, $P = 0.041$, $\eta_p^2 = 0.054$) since to both the NS_1 ($P = 0.188$; Bonferroni corrected) and the NS_2 ($P = 0.110$; Bonferroni corrected) were not significantly different. These data showed that the divergent rTMS effects in the aPFC and the dlPFC groups were selective for the CS. Fear tuning analysis of the dlPFC group's implicit reactions revealed no different SCR amplitudes elicited by the CS and the NS_1 ($P = 0.158$; Bonferroni corrected) and by the NSs ($P = 0.721$; Bonferroni corrected), but the NS_2 evoked lower SCRs than the CS ($P = 0.014$; Bonferroni corrected) (Figure 5D,E). We also detected no significant differences between groups in the SCRs elicited by the US_2 during the test session ($t_{(58)} = 1.762$, $P = 0.083$, $\eta_p^2 = 0.051$) (Figure 5F).

The different pattern toward the learned threatening cue was replicated during the follow-up session (2 × 2 mixed ANOVA; main effect of group: $F_{(1,58)} = 3.751$, $P = 0.058$, $\eta_p^2 = 0.061$; main effect of phase: $F_{(1,58)} = 3.114$, $P = 0.083$, $\eta_p^2 = 0.051$; group × phase interaction: $F_{(1,58)} = 15.248$, $P < 0.001$, $\eta_p^2 = 0.208$) since the aPFC group persisted in more dimly reacting to the CS with respect to the dlPFC group ($P = 0.001$; Bonferroni corrected), and the aPFC group endured in displaying attenuated responses relative to conditioning ($P < 0.001$; Bonferroni corrected) while the dlPFC group did not ($P = 0.136$; Bonferroni corrected) (Figure 5G,H). No significant differences were instead observed in SCRs evoked by the US_2 during the follow-up session ($t_{(58)} = 1.927$, $P = 0.059$, $\eta_p^2 = 0.060$) (Figure 5I).

These findings demonstrated that rTMS over the left dorsolateral PFC did not diminish implicit defensive reactions in the absence of an extinction paradigm, as in other previous studies[16–18]. Meanwhile, rTMS targeting the aPFC proved to be effective in achieving this outcome.

Discussion

In this study, we found that implicit reactions to both learned and novel stimuli were significantly down-regulated following a 1Hz-rTMS procedure over the aPFC.

So far, most rTMS studies targeting the prefrontal cortex have been conducted to enhance fear extinction processes. A study[16] administering one session of 10Hz-rTMS over the mPFC observed enhancement of extinction learning. These behavioral results were mirrored by the functional near-infrared spectroscopy (fNIRS) findings, which revealed increased mPFC activity in the stimulated group relative to the sham group[16]. Subsequently, Raij *et al.* (2018) delivered brief 20Hz-rTMS trains over the left posterior PFC –a region that showed robust functional connectivity with the vmPFC– during extinction learning and found a reduction of defensive responses during extinction recall.

Our study differs from the previous ones because we tested rTMS effects over the medial anterior prefrontal cortex (medial BA 10), and we did not include extinction training before retrieval. We observed a significant decrease in defensive reactions shortly after rTMS, and this effect was maintained until the follow-up session. Thus, we identified a previously unexplored prefrontal region, the modulation of which can efficiently and durably inhibit implicit reactions to learned threats. These dampening effects may be due to the fact that rTMS over the aPFC have directly modulated the defensive responses activated by the implicit threat memory trace. Alternatively, the rTMS procedure over the aPFC may have inhibited the recall of the CS-US association, preventing the defensive responses from being activated by the CS. This possibility would be in line with a large body of literature on humans[see 51] which demonstrates the importance of the medial PFC for value-based processing.

Autonomic reactions to the new tones in the aPFC group relative to the sham control group did not support the conclusion that rTMS targeted threat generalization, leaving open the question of the specificity of rTMS effects. However, the lack of between-group differences in the autonomic responses to the US_2 seems to suggest that the observed effect may be memory-related and not due

to a general dampening of autonomic reactivity. Interestingly, defensive responses toward the NSs were decreased following the stimulation of the left occipital cortex (OC group, BA18/19). This effect might be explained by the fact that anatomical and functional reciprocal projections between the medial BA10 and visual association cortices (including BA17/18/19) have been traced via the fronto-occipital fasciculus (FOF) of the human brain[52–54 but see 27].

Regarding the persistence of inhibitory effects during the follow-up session, different factors may have contributed to this result. Firstly, the inhibition of SCR responses induced by rTMS during the mnemonic retention test could have persistently reduced such conditioned responses even at a distance from the treatment. Moreover, the inhibition of these responses during the test might have boosted the extinction of these responses, contributing to keeping them low over time. On this possibility, it should be pointed out that one core knowledge about extinction is that under certain circumstances –such as a simple passage of time (i.e., spontaneous recovery) or a change in surrounding context (i.e., renewal)– extinguished reactions triggered by the CS may reoccur, giving rise to the phenomenon known as return of fear[20,55,56]. To test potential renewal phenomena, which have not been investigated in the aforementioned studies[16,17], we opted for a context-shift amongst the learning (context A), the test (context B), and the follow-up phase (context A), and we found down-regulated defensive reactions in both the test and the follow-up phases. These data demonstrated that the aPFC-rTMS protocol long-term reduced threat memory expression in a different context as well as in the context in which the threatening experience had occurred, thus preventing the return of fear. Finally, we cannot exclude that the rTMS applied immediately before the mnemonic retention test interfered with the reconsolidation process that is known to occur after this test[19], resulting in a persistent impairment in the retention of this mnemonic trace.

To potentiate the neural activity of the PFC, both the aforementioned studies[16,17] adopted high-frequency rTMS protocols –which are conventionally considered excitatory of proximal brain activity[57]. In our study, we adopted a low-frequency rTMS protocol –which is conventionally considered inhibitory[57]. Recent evidence, however, challenged this common frequency-dependent

rule[58]. Resting-state functional magnetic resonance imaging (fMRI) studies demonstrated that 1Hz-rTMS protocols may also induce downstream distal effects and enhance functional connectivity amongst the brain regions located underneath the coil and remote brain areas of the stimulated neural network[59]. Additionally, some studies[60,61] reported that 1Hz-rTMS procedures delivered over the PFC may paradoxically increase regional cerebral blood flow (rCBF).

The dorsolateral PFC is another prefrontal region that is assumed to be critically involved in threat learning[62,63] and the down-regulation of the cortico-meso-limbic network[64]. One investigation[18] probed the effects of a 1Hz-rTMS over the dlPFC after memory reactivation to disrupt threat-memory reconsolidation. Stimulated groups failed to discriminate between threatening and safe stimuli, with an increase in autonomic responses to these last ones. A more recent study[19] adopted the continuous theta-burst stimulation (cTBS) over the right dlPFC during the reconsolidation window and successfully decreased the defensive responses for threat memories. In our study, we found an immediate and long-term reduction of defensive responses to the CS only in subjects that were stimulated over the aPFC, while reactions to the NSs were decreased in both conditions. This evidence suggests that targeting the aPFC might represent a more promising approach for therapeutic applications. The lack of any down-regulation of CS-evoked reactions that we found in the dlPFC group, at odds with previous studies targeting the same cortical area[18,19], might be due either to the fact that we did not adopt an extinction paradigm, or to the different brain stimulation approach (rTMS vs cTBS).

The neural mechanisms by which rTMS over the aPFC decreases threat-conditioned responses can be manifold. Fear memories are formed and retrieved by an intricate neural network encompassing the amygdala[65], the cerebellum[66–68], and sensory cortices[46,69–77]. Indeed, previous evidence showed both structural connections between the aPFC and the amygdala[25–27] and a connectivity pathway of downstream modulation from the aPFC to the vmPFC[34]. This projection is activated during fear regulation[28], possibly supporting the vmPFC in top-down modulating the amygdala[78]. Through the direct or indirect connections of the aPFC with these areas, it might be that

the effects of focal manipulations of aPFC activity reflect complex and dynamic changes in the overall neural network state and/or influence the activity of some of these areas.

Although previous studies enlightened the role of the medial BA10 and BA10–posterior hippocampus functional connectivity in episodic memory retrieval[see 79], we did not detect any rTMS-driven effect on explicit recognition memory. The observed divergence between autonomous and declarative patterns might have been due to a selective rTMS action upon the neural system supporting implicit threat processing, which has been widely dissociated from the neural system underlying explicit memory processes[80–82]. Critically, an rTMS procedure that shapes implicit overreactions to learned threats without affecting conscious knowledge of danger might represent a strategic advantage for therapeutic applications.

Since prevention of relapse is the main challenge for therapies dedicated to post-traumatic and anxiety disorders, our findings may represent an advance in this direction by providing a potential strategy to deactivate emotional overreactions and, most of all, to prevent the return of fear. Future research perspectives might consist of exploring this rTMS application over the aPFC in clinical populations displaying high levels of anxiety or suffering from anxiety disorders and PTSDs.

Materials and Methods

Participants

All participants (n = 183) were healthy volunteers (mean age: 23.86 ± 2.90, 74 males and 109 females) with no history of psychiatric disorders, neurological illnesses, cardiovascular diseases, illegal drug use, musical training, or any other exclusion criteria for rTMS administration[83]. During the pre-experimental screening phase, each volunteer was also administered the *State-Trait Anxiety Inventory Form Y*[84,85], and those who showed a score >80 in the sum of the two subscales (State + Trait anxiety) were not included in the sample (see Table 1 for all groups' mean State-Trait Anxiety Inventory scores). Participants were then randomly assigned to each experimental condition, based on sex and age (see Table 1 for all groups' mean age and sex distribution). We discarded eleven participants because of a complete absence of skin conductance responses (SCRs) during the test session, leaving a total of 172 participants. Each participant provided written informed consent after receiving a complete description of the experimental procedures. All experimental procedures were performed in accordance with the ethical standards of the Declaration of Helsinki and were approved by the Bioethics Committee of the University of Turin (protocols N. 19961 and N. 161427).

Auditory stimuli

Auditory stimuli were pure sine wave tones with oscillation frequencies of 800Hz (CS), 1000Hz (NS_1), and 600Hz (NS_2), lasting 6s with onset/offset ramps of 5ms. Tones were digitally generated using Audacity 2.1.2 software (Audacity® freeware). The unconditioned threatening stimulus (US_2) consisted of a woman scream sample lasting 4s. All auditory stimuli were binaurally delivered through headphone speakers (Direct Sound EX29) at 50 dB intensity. All experimental scenarios were controlled by Presentation® 21.1 software (NeuroBehavioral Systems, Berkeley, CA).

444

445 **Preconditioning**

446 This phase consisted of the presentation of 4 trials of the CS (800Hz) with an inter-trial-interval
447 (ITI) randomly ranging between 21s and 27s. SCRs were recorded during this phase to provide a
448 baseline response pattern to the 800Hz tone for each participant. At the end of this phase,
449 participants were asked to confirm whether the tones were easily audible but not too loud or
450 annoying.

451

452 **Unconditioned stimulus calibration procedure**

453 Before starting with the calibration procedure, systolic and diastolic blood pressure was measured to
454 prevent possible hypo-arousal reactions caused by basal hypotension. The unconditioned stimulus
455 (US) consisted of a mild electrical shock (train pulse at 50Hz lasting 200ms, with a single pulse
456 duration of 1000μs) generated with a direct current stimulator (DS7A Constant Current Stimulator,
457 Digitimer). Impulses were delivered through a bar stimulating electrode connected by a Velcro strap
458 on the upper surface of the dominant hand's index finger. The electrical stimulation intensity was
459 individually calibrated through a staircase procedure[4,50,86], starting with a low current near the
460 perceptible tactile threshold (~0.5 mA). Participants were asked to rate the painfulness of each train
461 pulse on a scale ranging from 0 (not painful at all), 1 (pain threshold) to 10 (highly painful if
462 protracted in time). At the end of the procedure, the US amplitude was then set at the current level
463 (mA) corresponding to the mean rating of '7' on the subjective analog scale.

464

465 **Conditioning**

466 After a 1-min resting period, participants underwent a single-cue auditory threat conditioning,
467 which consisted of the presentation of 15 trials of the conditioned stimulus (CS, 800Hz), with an
468 ITI randomly ranging between 21s and 27s. The CS co-terminated with the US 12 times (80%
469 reinforcement rate). Subjects were not informed about any possible CS-US contingency. To validate

the threat learning experience, immediately following this phase subjects rated the painfulness of the US using the same analog scale as in the preconditioning calibration procedure (see Table 1 for all groups' US current intensity and US analog ratings).

Transcranial Magnetic Stimulation

Transcranial Magnetic Stimulation was performed with a Magstim Rapid^2 Stimulator (Magstim Co., Whitland, Dyfed, UK). A 70-mm figure-of-eight coil was positioned over the subject's M1 cortical area at the optimum scalp position to elicit a contraction of the contralateral abductor *pollicis brevis* muscle (APB). Resting motor threshold (rMT) was defined as the minimum stimulation intensity that induced a visible finger movement in at least 5 out of 10 single pulses over the right-hand area of the left primary motor cortex[16,87]. After having determined each individual's rMT, we applied a single train of 1Hz-rTMS[88,89] for a total duration of 10 min (600 pulses) to the target area. The rTMS intensity was set at 80% of the rMT for subjects whose rMT was ≤ 50% of the machine's maximum deliverable power (e.g., the intensity corresponded to 40% of the maximum power when the rMT was equal to 50% of the same parameter). For subjects with an rMT > 50%, the stimulation intensity was always set to a ceiling corresponding to 40% of the machine's maximum deliverable power (see Table 1 for each group's mean rMT and mean stimulation intensity). During the rTMS procedure participants were seated in a comfortable recliner that we adjusted to allow their upper body to be in a sloped position, thus ensuring an optimal positioning of the coil.

To target the medial anterior portion of the prefrontal cortex (BA 10; aPFC and aPFC-E groups), the coil was centered over Fpz (10% of nasion-inion distance) according to the international 10–20 electroencephalogram (EEG) system[41] (Figure 1). This placement should –with an rTMS reach of 1.5 to 2 cm beneath the scalp[90,91]– ensure the targeting of the medial aPFC as in previous studies[16,42,43] and avoid the targeting of the dorsomedial prefrontal cortex (dmPFC), which would have been localizable with a scalp-based heuristic approach of 25.84% nasion-inion distance[92]. In

the case of left occipital cortex stimulation (OC group), the coil was positioned over O1 using the 10–20 EEG system (BA 18/19), which functionally corresponds to associative visual cortices V3, V4, and V5[93,94] (Figure 1). For the stimulation of the left dorsolateral prefrontal cortex (dlPFC group), the coil was placed over F3 using the 10–20 EEG system (BA 8/9)[18,95] (Figure 1). For sham stimulation (sham and sham-E groups), the coil was centered over Fpz and positioned perpendicular to the scalp surface, so that no effective stimulation reached the brain during the procedure but allowed subjects to feel a comparable coil-scalp contact and hear the same noise as in real stimulation (Figure 1).

All participants were blinded to their experimental condition (i.e., active or sham), and were not informed about the potential cognitive or emotional effects of the stimulation.

Discomfort-inducing procedure

The discomfort-inducing procedure mirrored the rTMS protocol and consisted of the delivery of mild electrical shocks (single 1-Hz train of 600 pulses lasting 10min, with a single pulse duration of 500μs to mimic the duration of a single TMS pulse) generated with a direct current stimulator (DS7A Constant Current Stimulator, Digitimer). Impulses were delivered through two cup-stimulating electrodes attached to the surface of the subject's forehead in correspondence with Fpz according to the 10-20 EEG system. As in the case of the US calibration, the electrical stimulation intensity was individually calculated through a staircase procedure[86], starting with a low current near the perceptible tactile threshold (~0.5 mA). Participants were asked to evaluate the perceived discomfort of each pulse on a scale from 0 (no discomfort) to 10 (high discomfort). At the end of the procedure, the shock amplitude was set at the current level (mA) corresponding to the mean rating of '4' on the subjective analog scale. To quantify the habituation to the uncomfortable stimulations, at the end of every minute of the 10-min procedure (i.e. every 60 pulses), subjects were requested to rate the level of the present discomfort on the same scale adopted during the calibration procedure.

Implicit recognition test

After a 1-min resting period, participants underwent this task, which consisted of the presentation of 12 auditory stimuli in a completely random sequence: 4 × CS, 4 × NS_1, 4 × NS_2, with an ITI whose duration randomly ranged between 21s and 27s. SCRs were recorded throughout this phase, and the stimulating electrode was kept attached to create the expectation of receiving the US[44]. Differently from other paradigms[48,96–98], here no shocks were delivered to avoid any reacquisition effect[4,50].

Implicit unconditioned threatening test

This task was designed to elicit an unconditioned electrodermal response and consisted of the presentation of 4 trials of a woman scream sample lasting 4s, with an ITI randomly ranging between 21s and 27s. SCRs were recorded throughout this phase, and the stimulating electrode was kept attached.

Two-alternative forced-choice (2AFC) explicit recognition test

This procedure involves the presentation of two stimuli on each trial and the subject chooses the one that was previously encoded (i.e. the first or the second one). As in our previous works[4,50], a 2AFC design was preferred over a new-old paradigm, which involves one single stimulus on each trial, and the subject judges whether the stimulus has been previously encoded (old), or whether it is new. Our choice was motivated by the evidence that a 2AFC task improves recognition performance and discourages response biases such as the familiarity-based decision bias, namely the heuristic to endorse novel cues as 'old' when their familiarity is high[99].

The task consisted of the presentation of 16 tone-pairs, each composed of the CS (800Hz) and one of the two NSs (NS_1, 1000Hz or NS_2, 600Hz) in a completely random sequence: 4 × CS vs NS_1, 4 × NS_1 vs CS, 4 × CS vs NS_2, 4 × NS_2 vs CS. On each trial, the two stimuli were presented with an intra-trial-interval of 1000ms. After each pair offset, an ITI randomly ranging between 21s and 27s

548 occurred. Participants were explained that in each couple of sounds, there was a tone that they had
549 heard on the first session (one week before or, in the case of the follow-up session, two weeks
550 before) and a new tone. Participants were then instructed to recognize and verbally report which one
551 (the first or the second) was the tone heard in the first session, paired with the US-shock (CS).
552 Participants were further asked to verbally provide a confidence rating about each response, on a
553 scale from 0 (completely unsure) to 10 (completely sure). No feedback was supplied. As in the
554 implicit task, the stimulating electrode was kept attached, but no shock was delivered.
555
556 **Two-alternative forced-choice (2AFC) perceptual discrimination test**
557 The task consisted of the presentation of 7 pairs of auditory stimuli (i.e. CS vs NS_1, NS_1 vs CS, CS
558 vs NS_2, NS_2 vs CS, CS vs CS, NS_1 vs NS_1, NS_2 vs NS_2) with a 1000-ms intra-pair-interval in a
559 completely random sequence (ITI randomly ranging between 21s and 27s). For each pair, subjects
560 were asked to report whether the two tones were "the same tone or different tones", and to provide a
561 confidence rating on an analog scale from 0 (completely unsure) to 10 (completely sure). No
562 feedback was supplied, and the stimulating electrode was kept attached.
563
564 **Psychophysiological recording and analysis**
565 Event-related skin conductance responses (SCRs) were used as an implicit index of defensive
566 responses. To record the autonomic signal, two Ag-AgCl non-polarizable electrodes filled with
567 isotonic paste were attached to the index and middle fingers of the non-dominant hand by Velcro
568 straps. The transducers were connected to the GSR100C module of the BIOPAC MP-150 system
569 (BIOPAC Systems, Goleta, CA) and signals were recorded at a channel sampling rate of 1000 Hz.
570 SCR waveforms were analyzed offline using AcqKnowledge 4.1 software (BIOPAC Systems,
571 Goleta, CA), and were performed blindly to the subject's experimental condition and the
572 randomized sequence of stimuli. Each SCR was evaluated as event-related if the trough-to-peak
573 deflection occurred 1–6 s (for the CS and the NSs) or 1–4 s (for the US_2) after the stimulus onset,

574 the duration was comprised between 0.5 and 5.0 s, and the amplitude was greater than 0.02 micro
575 siemens (μS). Responses that did not fit these criteria were scored zero. To account for inter-
576 individual variability, these raw values were then scaled according to each participant's average
577 unconditioned response by dividing each response by the mean unconditioned stimulus (US)
578 response during the conditioning phase[100,101]. Scaled SCR data were square-root transformed to
579 normalize the distributions[102].
580
581 **Statistical analyses**
582 We computed the appropriate sample size based on a power analysis performed through G*Power
583 3.1.9.2. For the main statistics, i.e. mixed ANOVA (within-between interaction) with two groups
584 and two measurements, with the following input parameters: α equal to 0.05, power (1-β) equal to
585 0.95, and a hypothesized effect size (f) equal to 0.25, the estimated sample size resulted in n = 30
586 per experimental group.
587 Since most variables passed the D'Agostino-Pearson omnibus normality test, parametric statistics
588 were adopted in each experiment.
589 To test the between-group differences in post-conditioning US ratings, preconditioning mean SCRs
590 levels, mean SCRs to the CS and the US during conditioning, and mean SCRs to the US_2 during the
591 test and the follow-up sessions, we performed Student's unpaired t tests. Potential differences in
592 CS-related SCRs over the 15 trials of the conditioning phase were tested through 2 × 15 mixed
593 ANOVAs with Group (aPFC *vs* sham, aPFC *vs* OC, aPFC *vs* dlPFC) as between-subject variable
594 and Trial (1–15) as within-subject variable.
595 To test the potential between-group differences in the implicit reactions to the CS during the
596 conditioning session, the test session, and the follow-up session, as well as the within-group
597 differences from conditioning to test/follow-up phases, we computed 2 × 2 mixed ANOVAs with
598 Group (aPFC *vs* sham, aPFC *vs* OC, aPFC *vs* dlPFC, sham *vs* ctrl discomfort) as between-subject
599 variable and Phase (conditioning *vs* test, conditioning *vs* follow-up) as within-subject variable.

Bonferroni adjustment was applied for simple main effects analyses. To compare between-group and within-group responses to the CS and the NSs during the test session, we performed 2 × 3 mixed ANOVAs with Group (aPFC *vs* sham, aPFC *vs* OC, aPFC *vs* dlPFC) as between-subject variable and Tone (NS_1, CS, and NS_2) as within-subject variable. Bonferroni adjustment was applied for simple main effects analyses.

To test the between-group differences in the explicit recognition and respective confidence ratings, as well as in the perceptual discrimination and respective confidence ratings during the test and the follow-up sessions (aPFC-E *vs* sham-E), we performed Student's unpaired *t* tests. To test whether explicit recognition levels were significantly higher than the 50% chance level for each condition during the test and the follow-up sessions, we calculated Student's one sample *t* tests against 0.50.

For each ANOVA we assessed the Sphericity assumption through Mauchly's Test. Where it was violated, we applied the Greenhouse-Geisser correction accordingly.

The null hypothesis was rejected at $P < 0.05$ significance level. All statistical analyses were performed using SPSS Statistics 22 (IBM) and Prism 9 (GraphPad).

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Competing interests

The authors declare no financial interests or potential conflicts of interest.

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Table 1. Experimental groups' descriptive, experimental, and clinical data. The table reports, for each experimental condition: sample size (*N*), sex distribution (F = Female, M = Male), mean age, State-Trait Anxiety Inventory Form Y (STAI-Y) State subscale score during session 1 (S1), session 2 (S2), and session 3 (S3), and Trait subscale score, US current intensity (mA), post-conditioning US rating, rTMS resting motor threshold (rMT), rTMS power, and discomfort stimulation (DS) current intensity (mA). All data are mean ± standard deviation.

Group	*N*	Sex	Age	STAI-Y State (S1)	STAI-Y State (S2)	STAI-Y State (S3)	STAI-Y Trait	US (mA)	US rating	rTMS rMT	rTMS power	DS (mA)
aPFC	30	18F 12M	24.45 ± 3.78	30.97 ± 4.07	32.47 ± 7.16	30.60 ± 6.04	39.27 ± 6.18	4.92 ± 2.06	5.28 ± 0.90	58.20 ± 6.40	39.73 ± 1.11	-
sham	30	18F 12M	23.35 ± 2.35	33.23 ± 5.86	32.70 ± 7.74	31.87 ± 6.51	38.77 ± 4.02	4.88 ± 2.45	5.47 ± 0.88	-	-	-
OC	30	18F 12M	24.14 ± 2.62	32.33 ± 5.51	31.53 ± 7.57	30.60 ± 6.75	39.03 ± 5.12	4.99 ± 3.17	5.28 ± 1.06	60.90 ± 6.67	39.70 ± 1.47	-
dlPFC	30	18F 12M	23.91 ± 3.15	31.70 ± 5.40	30.83 ± 7.04	30.13 ± 5.88	39.17 ± 5.85	5.16 ± 2.43	5.57 ± 1.45	58.77 ± 5.89	39.90 ± 0.40	-
aPFC-E	21	13F 8M	24.39 ± 2.43	31.71 ± 4.89	30.90 ± 5.66	30.48 ± 4.96	38.29 ± 6.21	5.13 ± 1.86	5.43 ± 0.94	58.67 ± 7.16	39.52 ± 1.54	-
sham-E	21	13F 8M	23.83 ± 2.73	33.10 ± 5.59	31.48 ± 5.54	30.38 ± 7.73	38.29 ± 5.22	5.27 ± 3.19	5.31 ± 1.31	-	-	-
ctrl discomfort	10	5F 5M	22.34 ± 3.67	34.40 ± 4.20	36.50 ± 6.47	34.20 ± 5.98	39.70 ± 4.03	6.97 ± 4.14	5.65 ± 1.11	-	-	6.65 ± 2.25

Figure legends

Figure 1. Schematic diagram depicting the experimental outline and rTMS conditions. In the first session (day 1, context A), participants underwent a single-cue threat conditioning in which a tone (CS) was paired with a mild electrical shock (US). In the second session (day 8, context B), a 1Hz-rTMS procedure was actively applied over the medial anterior prefrontal cortex (aPFC, *n* = 30; aPFC-E, *n* = 21), sham-applied over the same site (sham, *n* = 30; sham-E, *n* = 21), actively applied over the left occipital cortex (OC, *n* = 30) and over the left dorsolateral prefrontal cortex (dlPFC, *n* = 30). In the implicit conditions (aPFC, sham, OC, dlPFC), subjects underwent an implicit test during which they were presented with the CS and two new stimuli (NS_1 and NS_2) and then an unconditioned threat test while being recorded in their SCRs. In the explicit conditions (aPFC-E, sham-E), participants underwent an explicit 2AFC recognition task during which they were presented with tone pairs each composed of the CS and one of the two NSs, and they were asked to recognize the CS providing a confidence level for each choice. Last, participants underwent a 2AFC perceptual discrimination test, in which they had to judge whether the two tones in each pair (CS and/or NSs) were “the same tone” or “different tones”. The third session (day 15, context A) was identical to the second one except for the absence of the rTMS.

Figure 2. Effects of rTMS over the aPFC on immediate and remote implicit threat memory, threat generalization to new stimuli, and overall electrodermal responsivity. (**A**) Simulation of rTMS effects on the neural tissue of the medial aPFC (medial BA 10), performed with SimNIBS 4.0 software. The magnitude of the electric field is expressed in V/m. (**B**, **C**) Dot plot and line chart representing the mean SCRs elicited by the CS during the conditioning session and test session in the two different conditions. Groups' reactions were not different during the conditioning phase, whereas during the test phase the group stimulated over the aPFC showed attenuated implicit reactions relative to the sham condition. The aPFC group displayed reduced autonomic reactions to the CS from conditioning to test, while the sham group showed an increase in defensive responses. (**D**, **E**) Implicit reactions to all the tones (NS_1, CS, and NS_2) during the test session were decreased in the aPFC group relative to the sham group. Although we found a significant main effect of Group and no Group × Tone interaction effect, we reported the statistical significance marks of simple main effects. (**F**) Implicit reactions to the US_2 during the test session were not different between conditions, showing no rTMS effects on the overall electrodermal responsivity. (**G**, **H**) In the follow-up session, the aPFC group enduringly demonstrated reduced implicit reactions to the CS relative to the sham group and to the conditioning phase. (**I**) Implicit reactions to the US_2 during the follow-up session were not different between groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. All data are mean and SEM. 2×2 mixed ANOVA followed by Bonferroni-adjusted *post hoc* comparisons (B, C, G, H); 2×3 mixed ANOVA followed by Bonferroni-adjusted *post hoc* comparisons (D, E); Student's unpaired *t* test (F, I).

Figure 3. Effects of rTMS over the aPFC on immediate and remote explicit threat memory. (**A**) During the test session, explicit recognition patterns were not different between the group stimulated over the aPFC and the sham group. (**B**) During the test session, confidence ratings did not differ between the two conditions. (**C**) During the follow-up session, aPFC-E and sham-E groups identified the CS between the NSs in a not different manner. (**D**) During the follow-up session, aPFC-E and sham-E groups were not differently confident about their explicit choices. All data are mean and SEM. Student's unpaired *t* test (A, B, C, D).

Figure 4. Selective effects of rTMS over the aPFC and the left OC on the defensive responses to threat-predictive cues. (**A**) Simulation of rTMS effects on the neural tissue of the left OC (BA 18/19), performed with SimNIBS 4.0 software. The magnitude of the electric field is expressed in V/m. (**B**, **C**) Dot plot and line chart representing the mean SCRs elicited by the CS during the conditioning session and test session in the OC group, compared with the same aPFC group of Fig. 2. The two groups did not differently respond during the conditioning phase, but during the test phase the group stimulated over the aPFC showed weaker reactions than the OC group. While the defensive reactions of the aPFC group decreased from conditioning to test, those of the OC group remained not differently high. (**D**, **E**) Implicit reactions to NSs during the test session did not differ between groups. In the OC group, the responses elicited by the NS_2 were lower than those evoked by the CS and the NS_1. (**F**) Implicit reactions to the US_2 during the test session were not different between groups. (**G**, **H**) In the follow-up session, the aPFC group persisted in showing reduced implicit reactions to the CS relative to the OC group. Defensive reactions of both groups decreased from the conditioning phase. Although we found a significant main effect of Phase and no Group × Phase interaction effect, we reported the statistical significance marks of simple main effects. (**I**) Implicit reactions to the US_2 during the follow-up session were not different between groups. * $P < 0.05$, *** $P < 0.001$. All data are mean and SEM. 2×2 mixed ANOVA followed by Bonferroni-adjusted *post hoc* comparisons (B, C, G, H); 2×3 mixed ANOVA followed by Bonferroni-adjusted *post hoc* comparisons (D, E); Student's unpaired *t* test (F, I).

Figure 5. Different effects of rTMS over the aPFC and the left dlPFC on immediate and remote implicit threat memory. (**A**) Simulation of rTMS effects on the neural tissue of the left dlPFC (BA 8/9), performed with SimNIBS 4.0 software. The magnitude of the electric field is expressed in V/m. (**B**, **C**) Dot plot and line chart representing the mean SCRs elicited by the CS during the conditioning session and test session in the dlPFC group, compared with the same aPFC group of Fig. 2. The two conditions did not differently react during the conditioning phase, whereas during the test phase the group stimulated over the aPFC displayed lower reactions than the dlPFC group. Implicit reactions of the aPFC group decreased from conditioning to test, while those of the dlPFC group increased. (**D**, **E**) Implicit reactions to NSs during the test session did not differ between groups. In the dlPFC group, the responses elicited by the NS_2 were lower than those evoked by the CS. (**F**) The two groups did not differently react to the US_2 during the test session. (**G**, **H**) In the follow-up session, the aPFC group persisted in more dimly reacting to the CS relative to the dlPFC group and to the conditioning phase. (**I**) Implicit reactions to the US_2 during the follow-up session were not different between groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. All data are mean and SEM. 2×2 mixed ANOVA followed by Bonferroni-adjusted *post hoc* comparisons (B, C, G, H); 2×3 mixed ANOVA followed by Bonferroni-adjusted *post hoc* comparisons (D, E); Student's unpaired *t* test (F, I).

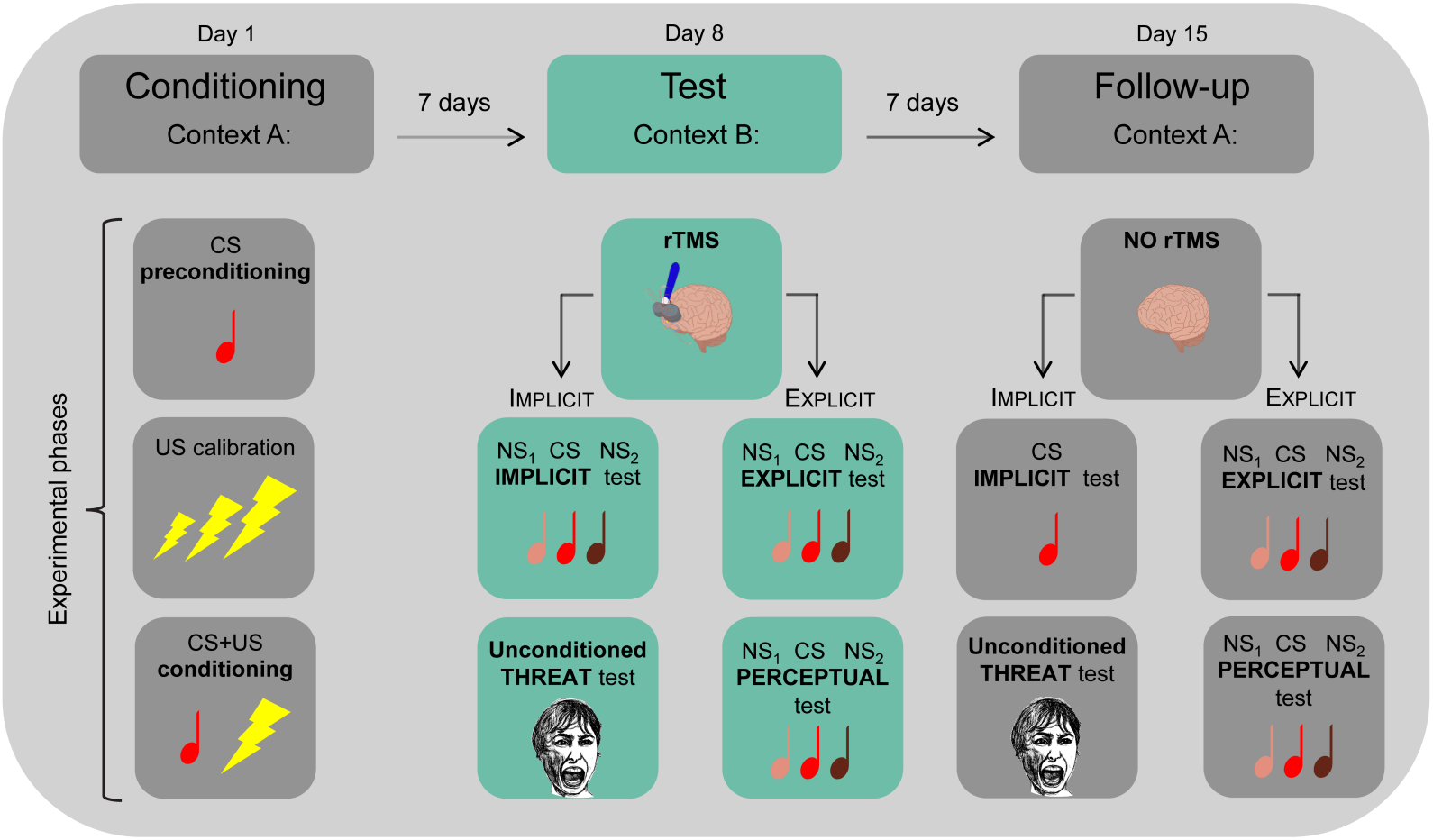
Figure 2-figure supplement 1. Implicit reactions during preconditioning (CS) and conditioning (CS, US) in the aPFC and sham groups. (**A**) Dot plot representing the mean SCRs elicited by the CS during the preconditioning phase in the aPFC group (*n* = 30) compared with the sham group (*n* = 30). Implicit reactions were not significantly different. (**B**) Mean SCRs elicited by the US during the conditioning phase in the aPFC group compared with the sham group. Responses were not significantly different. (**C**) Mean SCRs evoked by the CS over the 15 trials of the conditioning phase, and averaged SCRs (trials 1-15) in the aPFC and sham groups. Autonomic reactions were not significantly different. All data are mean and SEM. Student's unpaired *t* test (A, B, C); 2×15 mixed ANOVA (C).

Figure 2-figure supplement 2. Effects of a discomfort-inducing procedure on immediate and remote implicit threat memory. (**A**) Mean discomfort ratings provided by the subjects of the ctrl discomfort group (n = 10) during the 10-min discomfort-inducing procedure. (**B**) Dot plot representing the mean SCRs elicited by the CS during the conditioning phase and the test phase in the ctrl discomfort group and the sham group (n = 30). The groups' reactions were not significantly different during the conditioning phase as well as during the test phase (2 × 2 mixed ANOVA; main effect of group: $F_{(1,38)} = 0.712$, $P = 0.404$; main effect of phase: $F_{(1,38)} = 1.713$, $P = 0.198$; group × phase interaction: $F_{(1,38)} = 0.956$, $P = 0.335$). (**C**) In the follow-up session, SCRs to the CS did not differ between groups (2 × 2 mixed ANOVA; main effect of group: $F_{(1,38)} = 1.335$, $P = 0.255$; main effect of phase: $F_{(1,38)} = 0.042$, $P = 0.838$; group × phase interaction: $F_{(1,38)} = 0.175$, $P = 0.678$). All data are mean and SEM.

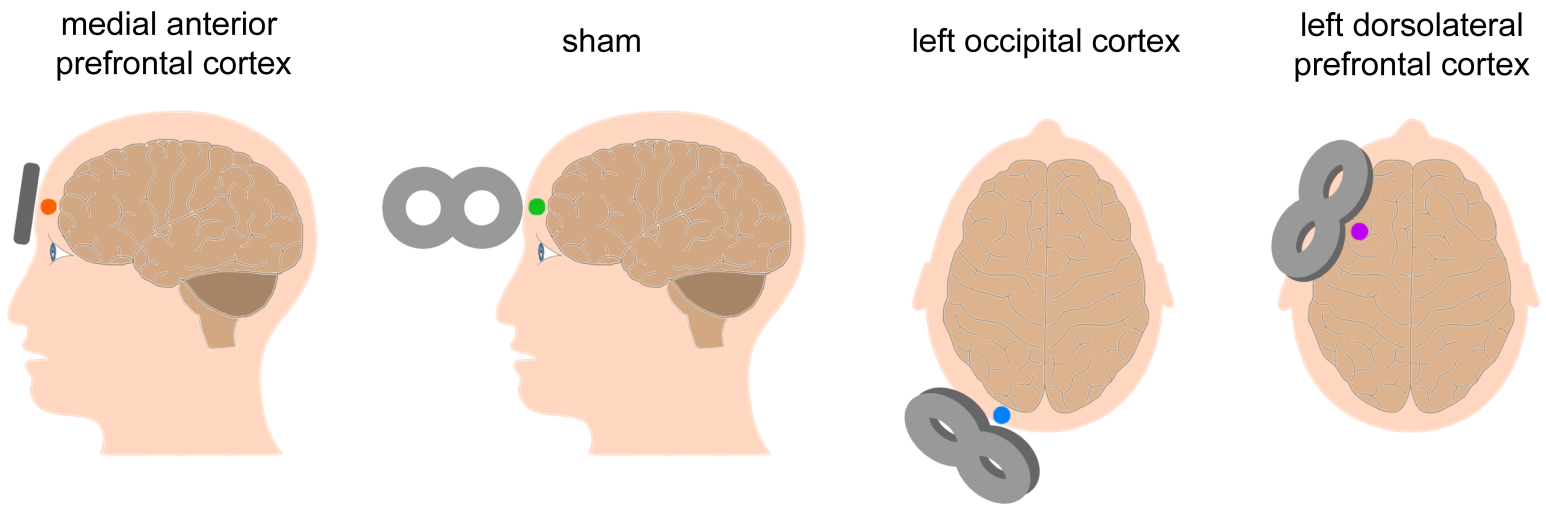
977 **Figure 4-figure supplement 1. Implicit reactions during preconditioning (CS) and**
978 **conditioning (CS, US) in the aPFC and OC groups.** (**A**) Dot plot representing the mean SCRs
979 elicited by the CS during the preconditioning phase in the aPFC group (*n* = 30) compared with the
980 OC group (*n* = 30). Implicit reactions were not significantly different. (**B**) Mean SCRs elicited by
981 the US during the conditioning phase in the aPFC group compared with the OC group. Responses
982 were not significantly different. (**C**) Mean SCRs evoked by the CS over the 15 trials of the
983 conditioning phase, and averaged SCRs (trials 1-15) in the aPFC and OC groups. Autonomic
984 reactions were not significantly different. All data are mean and SEM. Student's unpaired *t* test (A,
985 B, C); 2×15 mixed ANOVA (C).

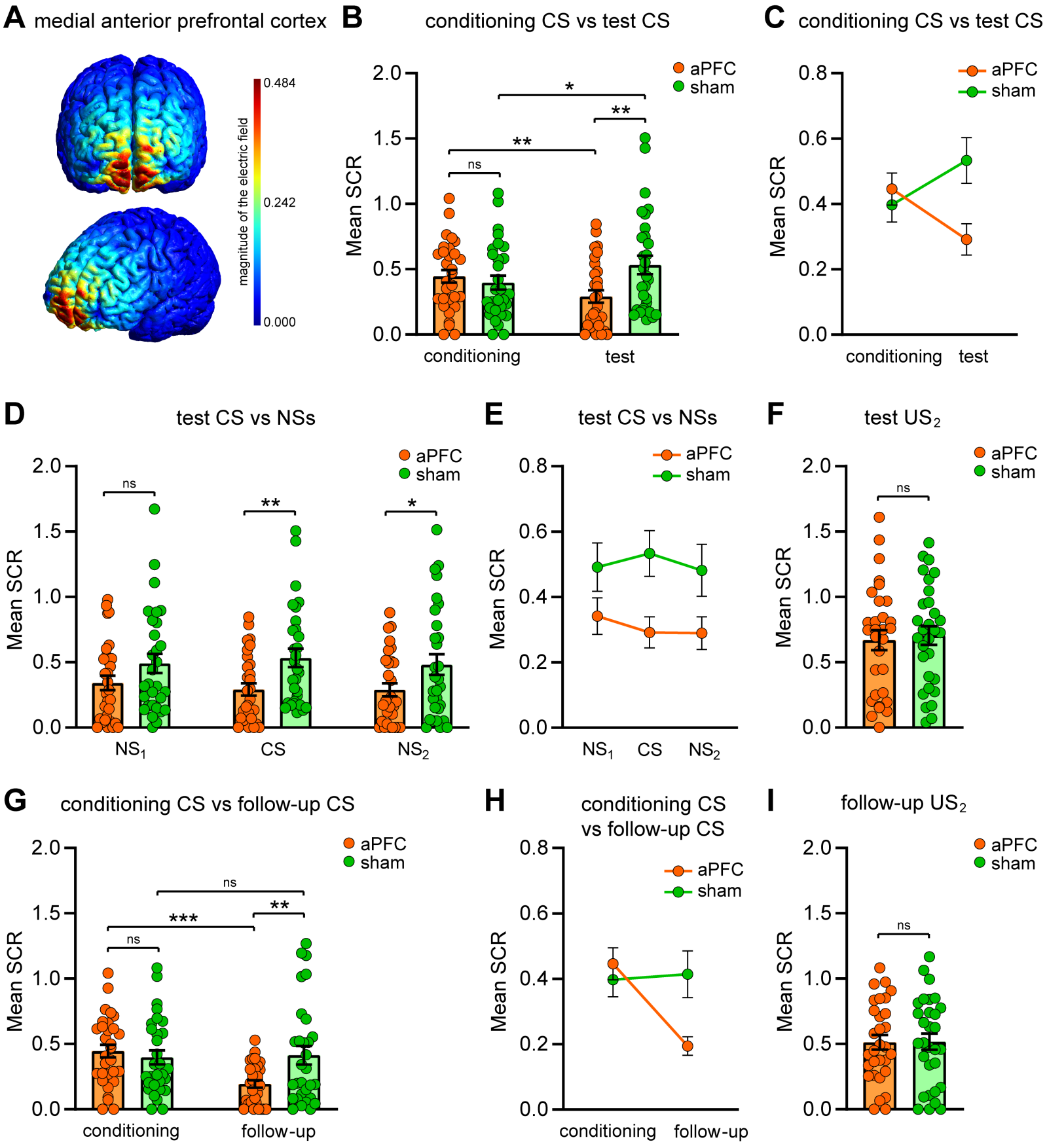
Figure 5-figure supplement 1. Implicit reactions during preconditioning (CS) and conditioning (CS, US) in the aPFC and dlPFC groups. (**A**) Dot plot representing the mean SCRs elicited by the CS during the preconditioning phase in the aPFC group (n = 30) compared with the dlPFC group (n = 30). Implicit reactions were not significantly different. (**B**) Mean SCRs elicited by the US during the conditioning phase in the aPFC group compared with the dlPFC group. Responses were not significantly different. (**C**) Mean SCRs evoked by the CS over the 15 trials of the conditioning phase, and averaged SCRs (trials 1-15) in the aPFC and dlPFC groups. Autonomic reactions were not significantly different. All data are mean and SEM. Student's unpaired t test (A, B, C); 2×15 mixed ANOVA (C).

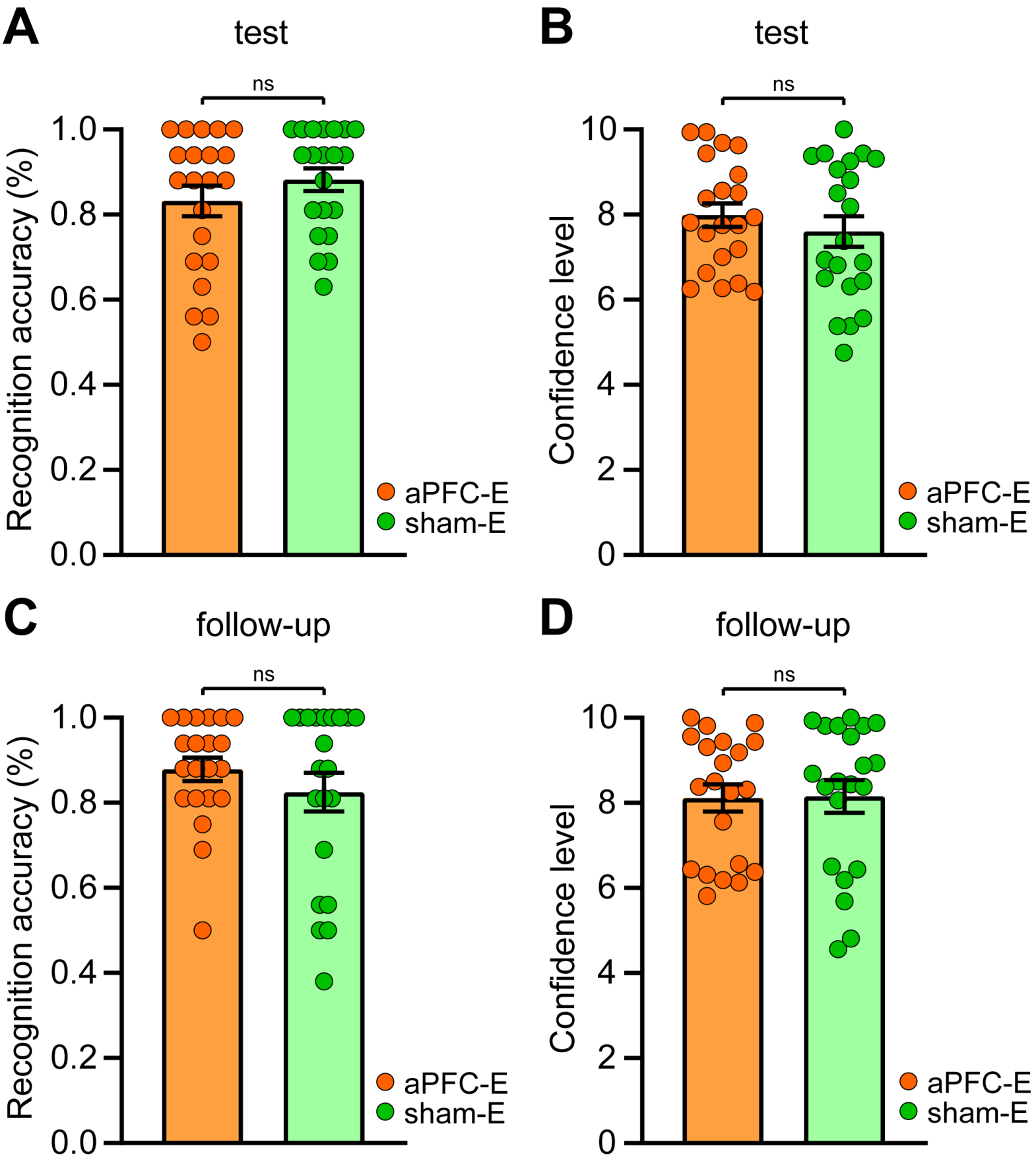
Experimental outline

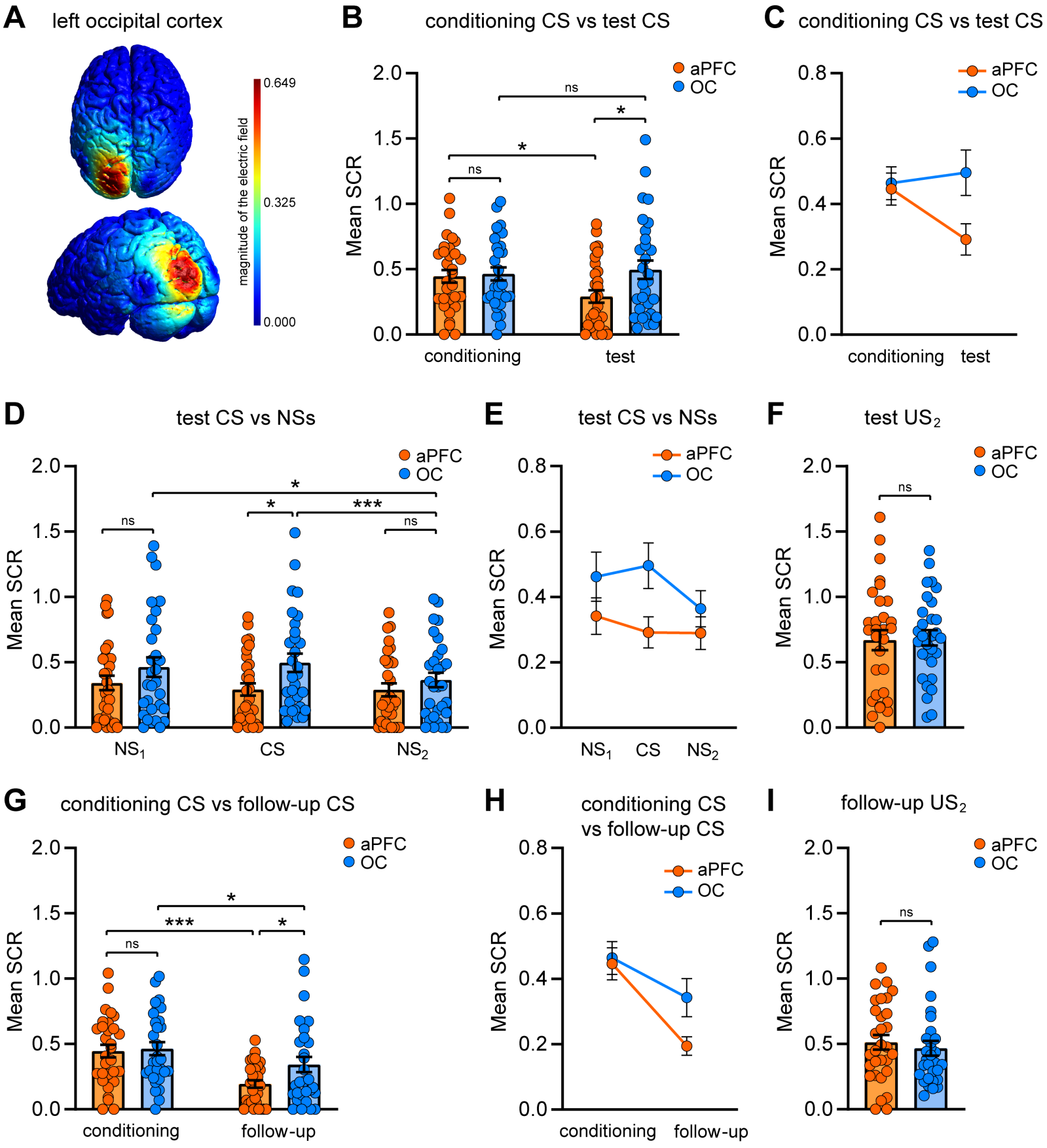


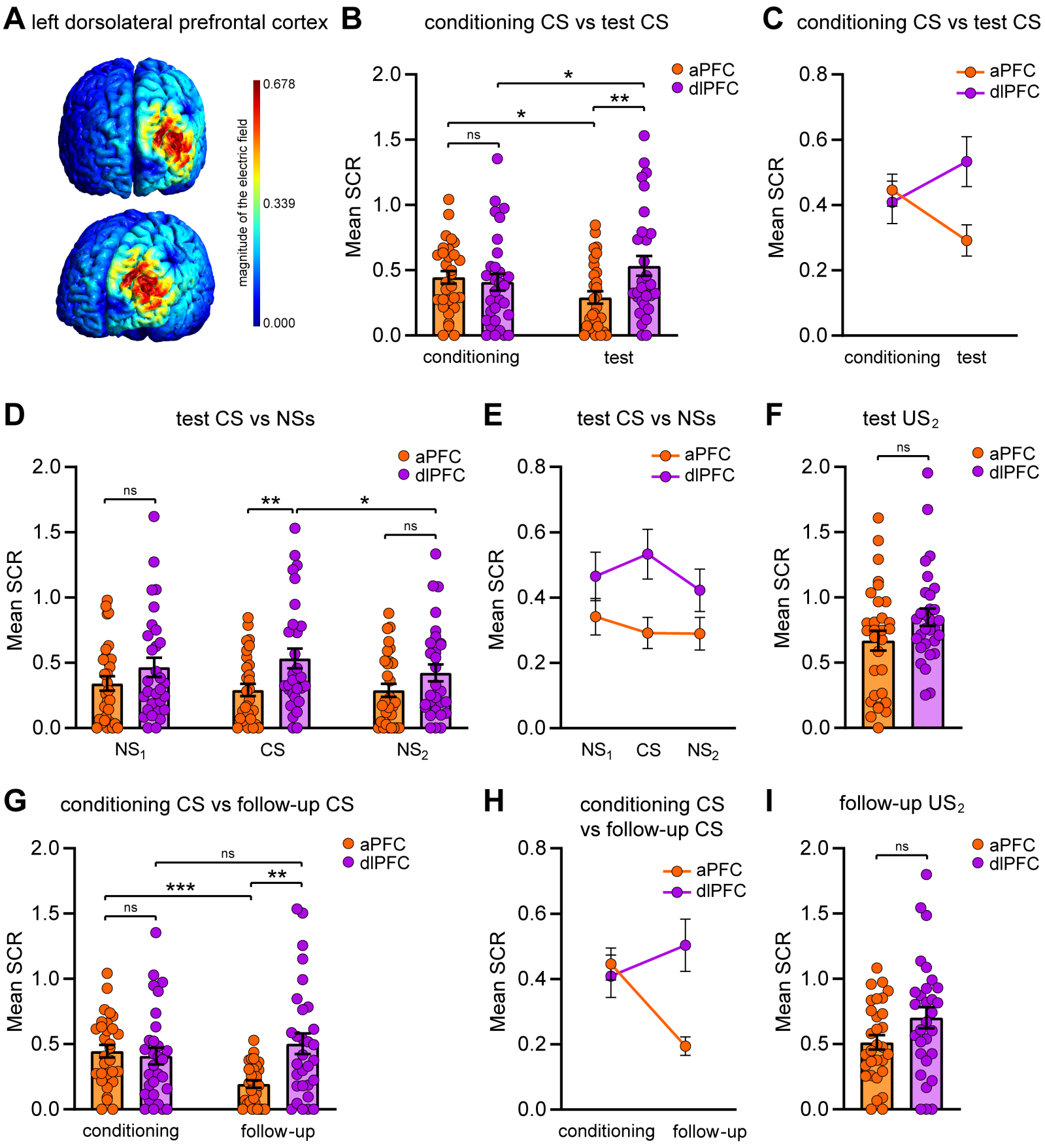
rTMS conditions

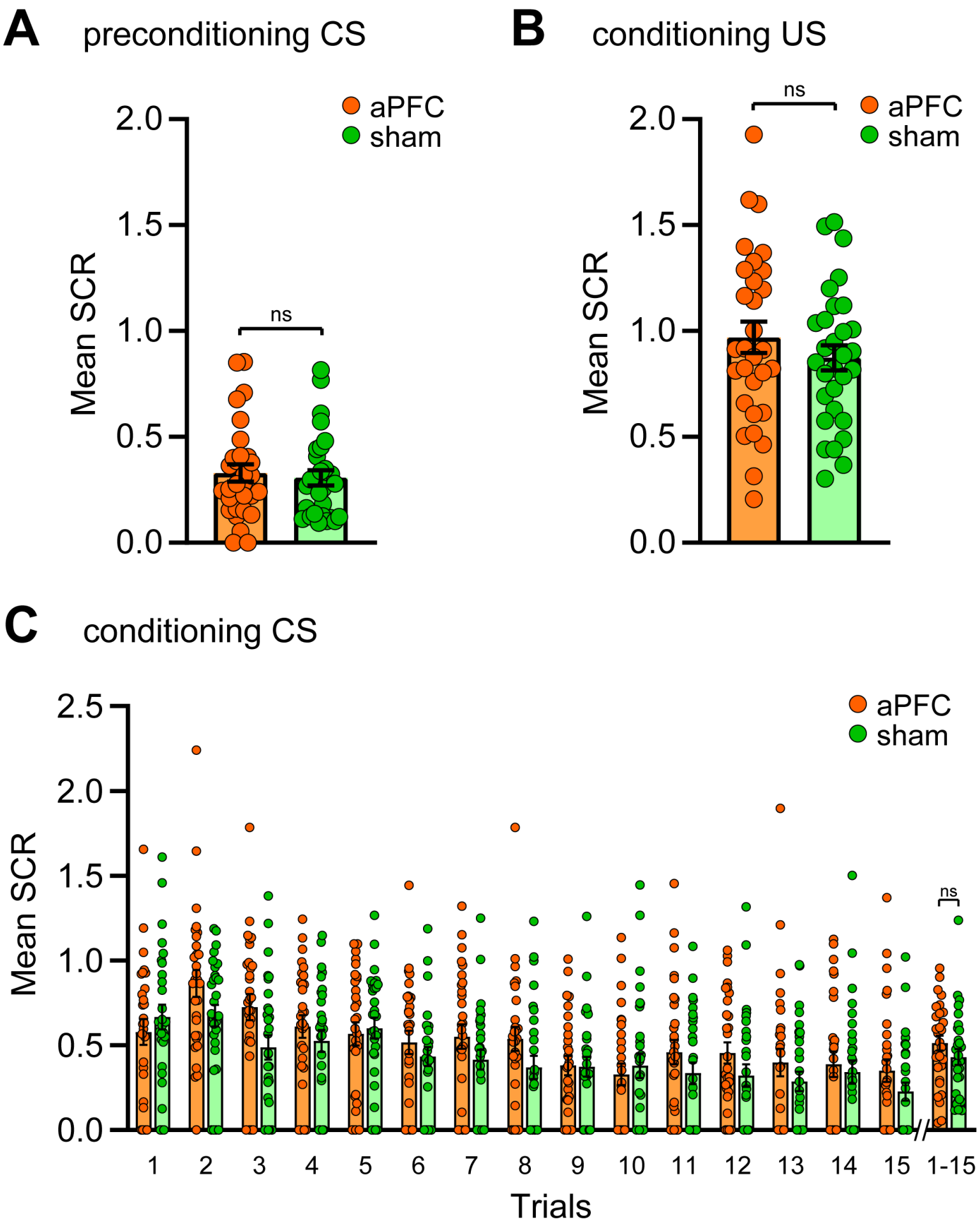


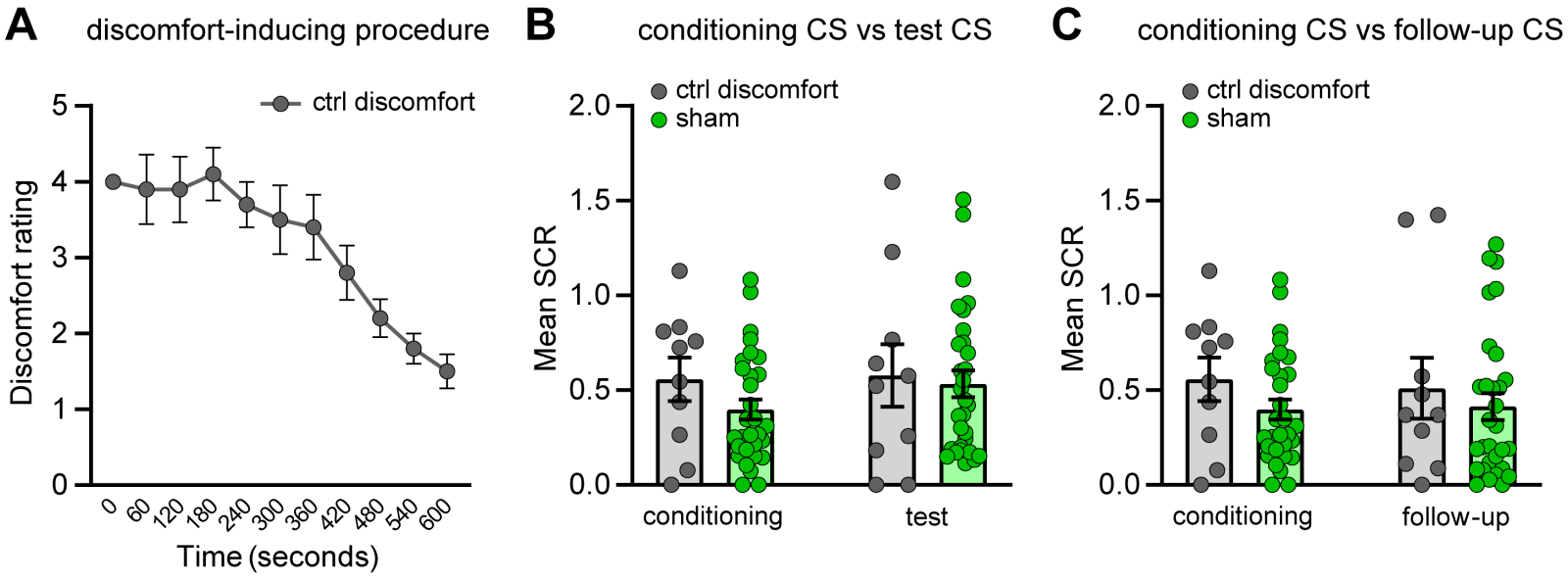

A medial anterior prefrontal cortex
magnitude of the electric field
0.484
0.242
0.000
B conditioning CS vs test CS
aPFC
sham
Mean SCR
2.0
1.5
1.0
0.5
0.0
ns
**
**
*
conditioning
test
C conditioning CS vs test CS
aPFC
sham
Mean SCR
0.8
0.6
0.4
0.2
0.0
conditioning
test
D test CS vs NSs
aPFC
sham
Mean SCR
2.0
1.5
1.0
0.5
0.0
ns
**
*
NS1
CS
NS2
E test CS vs NSs
aPFC
sham
Mean SCR
0.8
0.6
0.4
0.2
0.0
NS1
CS
NS2
F test US2
aPFC
sham
Mean SCR
2.0
1.5
1.0
0.5
0.0
ns
G conditioning CS vs follow-up CS
aPFC
sham
Mean SCR
2.0
1.5
1.0
0.5
0.0
ns
***
ns
**
conditioning
follow-up
H conditioning CS vs follow-up CS
aPFC
sham
Mean SCR
0.8
0.6
0.4
0.2
0.0
conditioning
follow-up
I follow-up US2
aPFC
sham
Mean SCR
2.0
1.5
1.0
0.5
0.0
ns

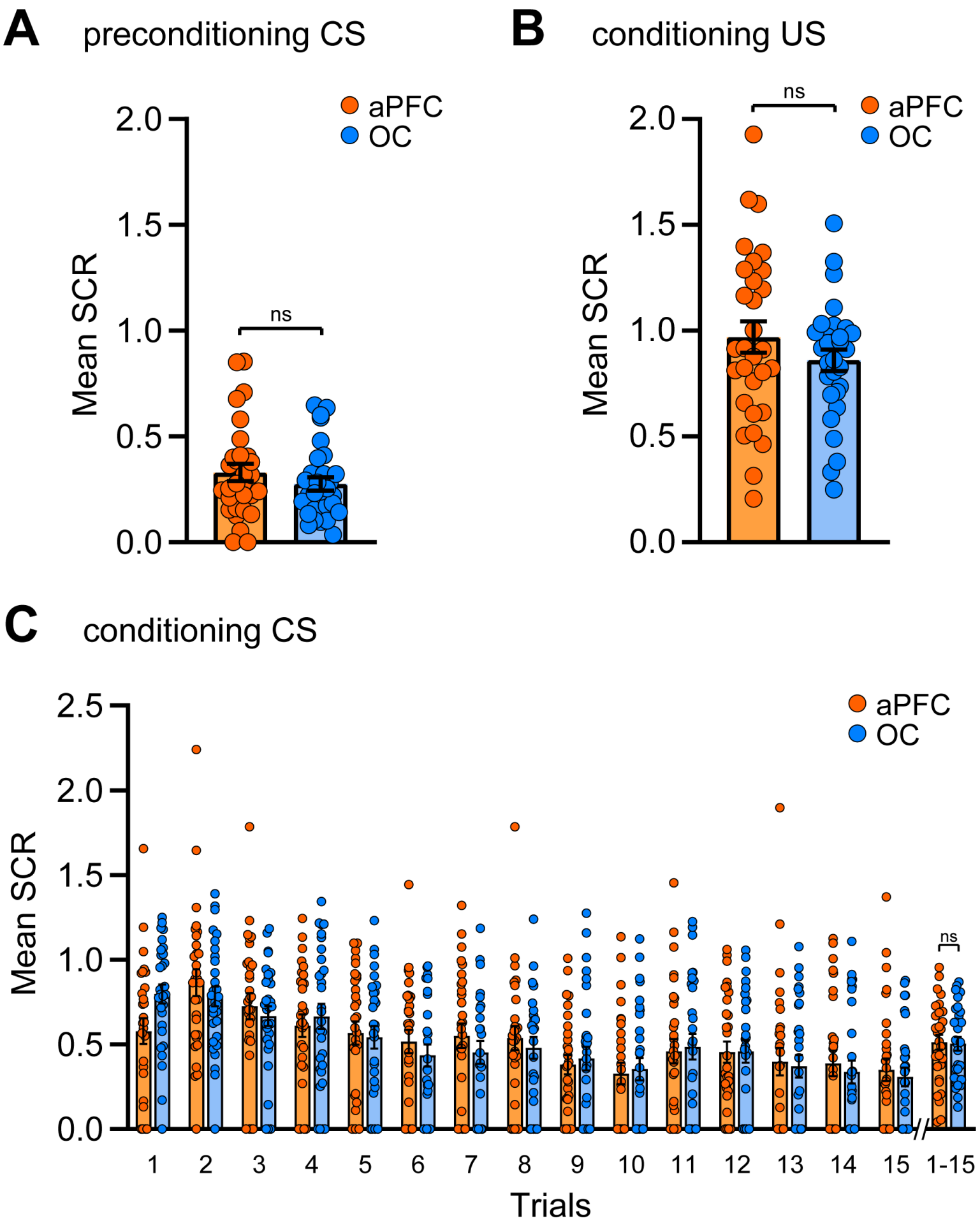

A
test
ns
Recognition accuracy (%)
1.0
0.8
0.6
0.4
0.2
0.0
aPFC-E
sham-E
B
test
ns
Confidence level
10
8
6
4
2
0
aPFC-E
sham-E
C
follow-up
ns
Recognition accuracy (%)
1.0
0.8
0.6
0.4
0.2
0.0
aPFC-E
sham-E
D
follow-up
ns
Confidence level
10
8
6
4
2
0
aPFC-E
sham-E

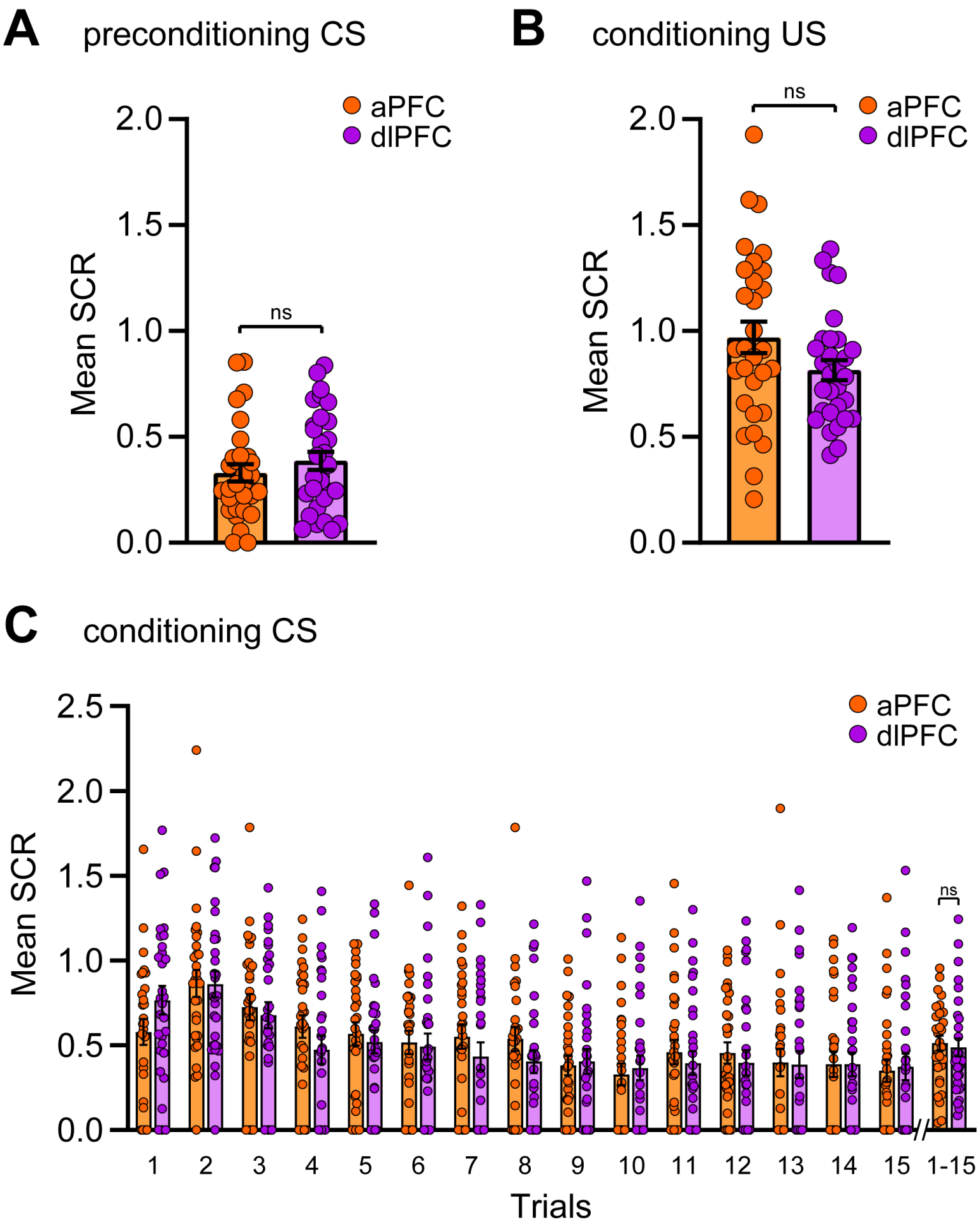

A
left occipital cortex
magnitude of the electric field
0.649
0.325
0.000
B
conditioning CS vs test CS
aPFC
OC
Mean SCR
ns
*
*
ns
conditioning
test
C
conditioning CS vs test CS
aPFC
OC
Mean SCR
conditioning
test
D
test CS vs NSs
aPFC
OC
Mean SCR
*
*
***
ns
ns
NS1
CS
NS2
E
test CS vs NSs
aPFC
OC
Mean SCR
NS1
CS
NS2
F
test US2
aPFC
OC
Mean SCR
ns
G
conditioning CS vs follow-up CS
aPFC
OC
Mean SCR
*
***
*
ns
conditioning
follow-up
H
conditioning CS
vs follow-up CS
aPFC
OC
Mean SCR
conditioning
follow-up
I
follow-up US2
aPFC
OC
Mean SCR
ns


A left dorsolateral prefrontal cortex
magnitude of the electric field
0.678
0.339
0.000
B conditioning CS vs test CS
aPFC
dlPFC
Mean SCR
conditioning
test
C conditioning CS vs test CS
D test CS vs NSs
NS1
CS
NS2
E test CS vs NSs
F test US2
G conditioning CS vs follow-up CS
follow-up
H conditioning CS vs follow-up CS
I follow-up US2
ns


A
preconditioning CS
aPFC
sham
Mean SCR
2.0
1.5
1.0
0.5
0.0
ns
B
conditioning US
aPFC
sham
Mean SCR
2.0
1.5
1.0
0.5
0.0
ns
C
conditioning CS
aPFC
sham
Mean SCR
2.5
2.0
1.5
1.0
0.5
0.0
ns
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
1-15
Trials


A
discomfort-inducing procedure
ctrl discomfort
Discomfort rating
0
1
2
3
4
5
0
60
120
180
240
300
360
420
480
540
600
Time (seconds)
B
conditioning CS vs test CS
ctrl discomfort
sham
Mean SCR
0.0
0.5
1.0
1.5
2.0
conditioning
test
C
conditioning CS vs follow-up CS
ctrl discomfort
sham
Mean SCR
0.0
0.5
1.0
1.5
2.0
conditioning
follow-up


A
preconditioning CS
aPFC
OC
Mean SCR
2.0
1.5
1.0
0.5
0.0
ns
B
conditioning US
aPFC
OC
Mean SCR
2.0
1.5
1.0
0.5
0.0
ns
C
conditioning CS
aPFC
OC
Mean SCR
2.5
2.0
1.5
1.0
0.5
0.0
ns
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
1-15
Trials


A
preconditioning CS
aPFC
dlPFC
Mean SCR
2.0
1.5
1.0
0.5
0.0
ns
B
conditioning US
aPFC
dlPFC
Mean SCR
2.0
1.5
1.0
0.5
0.0
ns
C
conditioning CS
aPFC
dlPFC
Mean SCR
2.5
2.0
1.5
1.0
0.5
0.0
ns
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
1-15
Trials