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Prior learning of relevant non-aversive information is a boundary condition for avoidance memory reconsolidation in the rat hippocampus

Abbreviated title: Conflicting memories and avoidance reconsolidation

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Abstract

Reactivated memories can be modified during reconsolidation, making this process a potential therapeutic target for post-traumatic stress disorder (PTSD), a mental illness characterized by the recurring avoidance of situations that evoke trauma-related fears. However, avoidance memory reconsolidation depends on a set of still loosely defined boundary conditions, limiting the translational value of basic research. In particular, the involvement of the hippocampus in fear-motivated avoidance memory reconsolidation remains controversial. Combining behavioral and electrophysiological analyses in male Wistar rats, we found that previous learning of relevant non-aversive information is essential to elicit the participation of the hippocampus in avoidance memory reconsolidation, which is associated with an increase in theta and gamma oscillations power and cross-frequency coupling in dorsal CA1 during reactivation of the avoidance response. Our results indicate that the hippocampus is involved in memory reconsolidation only when reactivation results in contradictory representations regarding the consequences of avoidance, and suggest that robust nesting of hippocampal theta-gamma rhythms at the time of retrieval is a specific reconsolidation marker.

Significance Statement

Post-traumatic stress disorder is characterized by maladaptive avoidance responses to stimuli or behaviors that represent or bear resemblance to some aspect of a traumatic experience. Disruption of reconsolidation, the process by which reactivated memories become susceptible to modifications, is a promising approach for treating PTSD patients. However, much of what is known about fear-motivated avoidance memory reconsolidation derives from studies based on fear conditioning instead of avoidance learning paradigms. Using a step-down inhibitory avoidance task in rats, we found that the hippocampus is involved in memory reconsolidation only when the animals

acquired the avoidance response in an environment they had previously learned as safe, and showed that increased theta-gamma oscillations coupling during reactivation is an electrophysiological signature of this process.

Introduction

Avoidance is a normal defensive behavior intended to avert uncomfortable or fearful situations. However, in patients with post-traumatic stress disorder (PTSD), avoidance of emotions, thoughts and stimuli that symbolize or resemble traumatic events is exacerbated and disproportionate. Reactivation may render memories transiently labile and, to persist, these memories must undergo a gene expression- and protein synthesis-dependent restabilization process referred to as reconsolidation, during which they can also be updated or enhanced (Misanin et al., 1968; Spear, 1973; Lewis, 1979; Przybyslawski and Sara, 1997; Nader et al., 2000, Haubrich and Nader, 2016). Consequently, it has been suggested that therapeutic interventions based on the interference of fear-motivated avoidance memory reconsolidation might help PTSD patients recontextualize intrusive recollections and cope with anxiety (Schwabe et al., 2014; Dunbar and Taylor, 2016). Nevertheless, perhaps because conditioned fear has long been associated with the reinforcement of fear-motivated avoidance responses (Mowrer and Lamoreaux, 1946; Miller, 1948), most studies on the relevance of reconsolidation for the treatment of stressor-related disorders have been carried out using fear conditioning learning paradigms (Johansen et al., 2011; Reichelt and Lee, 2013). However, there are important neuroanatomical and neurochemical differences between fear conditioning and fear-motivated avoidance memory processing (Wilensky et al., 2000; Tinsley et al., 2004; Alberini et al., 2005), and several reports have clearly dissociated fear-induced avoidance from the expression of conditioned fear (Riccio and Silvestri, 1973; Overmier and Brackbill, 1977; Mineka, 1979). Actually, there is a paucity of information about the behavioral conditions that constrain fear-induced

avoidance reconsolidation and the physiological properties that distinguish this process from other phenomena that depend on memory reactivation.

In particular, the role of the hippocampus, which is well documented in fear conditioning memory reconsolidation (de Oliveira Alvares et al., 2008; Besnard et al., 2013; Ishikawa et al., 2016), remains elusive for the case of avoidance, and some laboratories, including our own, have failed to find evidence that de novo hippocampal protein synthesis is necessary for restabilization of fear-induced avoidance memory after reactivation (Taubenfeld et al., 2001; Cammarota et al., 2004; Power et al., 2006; Arguello et al., 2013). One possible explanation for these negative results is that avoidance memory never undergoes reconsolidation, which is highly unlikely since it has been reported that systemic administration of protein synthesis blockers after fear-induced avoidance memory retrieval causes amnesia (Taubenfeld et al., 2001). Other possibility is that reactivation induces reconsolidation of avoidance memory but the hippocampus does not play any role in this process, which also seems implausible since the hippocampus is essential not only for consolidation, retrieval and extinction of the fear-induced avoidance response (Bernabeu et al., 1995; Cammarota et al., 2005; Bonini et al., 2006) but also for reconsolidation of avoidance extinction memory (Radiske et al., 2015). This last observation suggests a third hypothesis, which we investigated in this study, that the hippocampus is engaged in fear-motivated avoidance memory reconsolidation only when reactivation results in contradictory predictions regarding the possible outcomes of the avoidance response.

Modifications in hippocampal oscillatory activity are linked to memory processing (Lisman, 2005). In particular, increased theta-gamma interactions are associated with memory retrieval (Gruber et al., 2004; Montgomery and Buzsaki, 2007) and these oscillations serve to compute uncertainty signals (Garrido et al., 2015) and to distinguish between correct and incorrect responses (Sederberg et al., 2007), all of which have been related to some aspect of memory reconsolidation in different preparations (Fernández et al., 2016).

Therefore, we also posited that trace competition at the onset of reconsolidation enhances theta-gamma coupling in the hippocampus. To test these assumptions, we used the step-down inhibitory avoidance paradigm (SD-IA), a one-trial hippocampus-dependent learning task suited to study time-dependent changes associated with retrieval of learned avoidance in rats.

Materials and Methods

Subjects

We used 3-month-old naïve male Wistar rats weighting 300-350 g for the experiments. Animals were housed in groups of five and maintained on a 12:12 h light/dark cycle (lights on at 06:00 AM) at 23 °C with free access to food and water. We carried out the experiments during the light cycle. Animals were trained and tested only once. All procedures were in accordance with the USA National Institutes of Health Guidelines for Animal Care and were approved by the local institutional ethics committee (Comissão de Ética no Uso de Animais - CEUA). The experiments were conducted blind to the treatment condition of the animals.

Cannula and multielectrode arrays implants

We implanted animals with 22-gauge stainless steel guides aimed to the CA1 region of the dorsal hippocampus (stereotaxic coordinates, in mm: anteroposterior, −4.2; laterolateral, ±3.0; dorsoventral, −3.0). Six animals were chronically implanted with sixteen-channel electrode arrays in the left dorsal hippocampus (stereotaxic coordinates, in mm: anteroposterior, −3.6; laterolateral, +2.4; dorsoventral, −3.6 mm) and two epidural screws localized in the parietal bone as ground electrodes. Electrode arrays were made of 50 µm blunt-cut, PFA-coated, tungsten micro-wires (A-M Microsystems) positioned in a 2 by 8 configuration with spacing of 250 µm between adjacent electrodes. Implants were performed under ketamine (80 mg/kg) / xylazine

(10 mg/kg) anesthesia and immediately after surgery animals received a single subcutaneous dose of meloxicam (0.2 mg/kg) as analgesic. After surgery rats with electrode implants were housed individually. Behavioral procedures began 7-10 days after surgery.

Experimental Design and Statistical Analysis

Before training in SD-IA (see below), rats were submitted to one out of three different procedures, as follows. Control animals were handled for 5 min/day during 5 days (Control Group). Open Field Group animals (OF Group) were allowed to explore a 60x60x60 cm light grey open field arena for 5 min/day during 5 days. Training Box Group animals (TB Group) were put on the SD-IA training box platform and allowed to freely explore the apparatus for 5 min/day during 5 days. One day or twenty-eight days after the end of these procedures, rats were trained in the SD-IA task. The SD-IA apparatus was a 50x25x25 cm Plexiglas box with a 5 cm high, 8 cm wide, and 25 cm long platform on the left end of a series of bronze bars that made up the floor of the box. For training (a single session carried out between 8:00 A.M. and 11:00 A.M), animals were placed on the platform facing the left rear corner of the SD-IA apparatus. When they stepped down and placed their four paws on the grid, they received a 0.8 mA (Strong training) or a 0.4 mA (Weak training) scrambled footshock during 2 s and were immediately returned to their home cage. To reactivate the avoidance memory trace, 24 h after SD-IA training the animals were placed again on the training box platform for 40 s. During these 40 s, the rats explored the platform avoiding stepping down from it. Retention was assessed using independent groups of animals either 3 h, 1 day or 14 days after SD-IA memory reactivation. In order to do that, animals were placed on the SD-IA training box platform and the latency to step-down from it was measured. This session finished when the animal stepped down to the grid or after 500 s. No footshock was given. Because of the 500 s ceiling imposed on retention test session latency and the fact that there is no validated multifactorial ANOVA test for

non-parametric variables, data are expressed as median (interquartile ranges) and analyzed by two-tailed Mann–Whitney U test or Kruskal-Wallis test followed by Dunn's post hoc comparisons, when appropriate. Data from pre-exposure and training sessions (no ceiling imposed) are expressed as mean ± SEM and were analyzed using ANOVA followed by Bonferroni's multiple comparison test. Significance was set at $p < 0.05$. Data analyses were performed using the GraphPad Prism 6 software (RRID:SCR_002798).

Drugs and infusion procedures

All drug doses used in this work were based on previous studies and pilot experiments. Anisomycin (ANI; 160 µg/side; Rossato et al., 2007), α-amanitin (AMA; 45 ng/side Radiske et al., 2015), D(−)-2-Amino-5-phosphonopentanoic acid (AP5; 1 µg/side; Radiske et al., 2015) and isoproterenol (ISO; 5 mg/kg; Do-Monte et al., 2010) were purchased from Sigma-Aldrich. ANI, an antibiotic produced by *Streptomyces griseolus*, binds to the 60S subunit of eucaryotic ribosomes and reversibly inhibits the biosynthesis of proteins blocking peptidyl transferase activity and thereby preventing elongation (Grollman, 1967; Barbacid and Vazquez, 1974). The gene transcription blocker AMA is a cyclic peptide from *Amanita phalloides* that binds to the RNA polymerase II bridge helix interfering with the conformational change required to translocation and release of the active site (Bushnell et al., 2002). AP5 is a potent and selective NMDA receptor antagonist that interacts with the glutamate binding site on the NR2 subunit (Monaghan and Jane, 2009). ISO is an agonist of β-adrenergic receptors that induces adenylate cyclase activation and cAMP increase. Zif268 antisense (ASO; 5′-GGT AGT TGT CCA TGG TGG-3′; 2 nmol/side) and missense oligodeoxynucleotides (MSO; 5′-GTG TTC GGT AGG GTG TCA-3′; 2 nmol/side; Lee et al., 2004) were from GBToligos. ASO and MSO were phosphorothioated on the three terminal bases to avoid nuclease degradation. MSO had ASO base composition in a scrambled order and did not match any mammalian sequence in the GenBank

database. Drugs and oligos were dissolved upon arrival and stored at −20 °C until use. On the day of the experiment stock aliquots were thawed and diluted to working concentration in sterile saline (pH 7.2). At the time of intra-hippocampus drug delivery, infusion cannulas extending 1 mm beyond the guide cannulas were fitted into the guides and injections (1 µl/side at a rate of 0.5 µl/min) carried out using a 5 µl Hamilton syringe coupled to an infusion pump (Harvard Apparatus). Infusion cannulas were left in place for one additional minute to minimize backflow. Placement of the cannulas was verified postmortem: 2-4 h after the end of the behavioral experiments, 1 µl of 4% methylene-blue was infused as described above and the extension of the dye 30 min thereafter taken as indication of the previously injected vehicle/drug diffusion. Only data from animals with correct cannula implants (96%) were included in the statistical analyses.

In vivo electrophysiology

Neurophysiological signals were acquired continuously using the Cerebus Neural Signal Processor system (Blackrock Microsystems). Data were amplified, filtered at cut-off frequencies of 0.3 Hz and 150 Hz, sampled at 1000 Hz and analyzed offline in MATLAB (RRID:SCR_001622) using built-in and custom written routines (Signal Processing Toolbox). The CA1 pyramidal cell layer was identified by stereotaxic coordinates and standard electrophysiological parameters such as maximal theta power at the hippocampal fissure and phase-reversal of theta activity across the stratum radiatum (Brankack et al., 1993; Bragin et al. 1995). We used the Welch periodogram method (5 s Hamming windows, 75% overlap) for power spectra computing. Power ratio indicates power per unit frequency normalized by power during the baseline epoch (the first 40 s of stable recording in the recording cage). Baseline field potentials were acquired in the recording cage one hour before memory reactivation. Band power of theta, slow gamma and fast gamma were defined as the average power in the frequency range of 5-10 Hz, 35-55 Hz and 55-100 Hz,

respectively. For cross-frequency coupling analysis, slow and fast gamma amplitudes and the theta phases along the recording were computed from the Hilbert transform of the filtered versions of each frequency band. Theta phases were binned into 18 intervals of 20°. The mean amplitude of gamma bands was computed for each theta phase bin and normalized by the sum of amplitude values over all bins. The modulation strength between frequency bands was expressed by the modulation index (MI) which indicates the Kullback-Leiber distance between the uniform distribution and the probability function derived from mean amplitude per phase distribution (Tort et al., 2010). Comodulation maps were obtained by expressing the MI of several frequency band pairs (4 Hz bandwidths, 1 Hz steps for phase frequencies; 10 Hz bandwidths, 5 Hz steps for amplitude frequencies) in a bi-dimensional pseudo-color plot (Tort et al., 2010). Mean MI was obtained by averaging the corresponding MI values in the (5-10 Hz) x (35-55 Hz) or (5-10 Hz) x (55-100 Hz) regions of the comodulation maps. MIs were calculated from single electrodes using 40 s-long contiguous LFP recordings from the reactivation session. Events of slow and fast gamma amplitude were identified and the theta phase associated was determined. These events were defined as time intervals when gamma power surpassed by 2 s.d. their respective time-averaged power as in Colgin et al. (2009). To avoid the analysis of artefactual gamma events, we did not consider time intervals with power above 6 s.d. in the computations. Events separated by less than 100 ms were merged and considered as a single event. Theta phase at the time points corresponding to the maximum of each gamma event was extracted and the circular mean was computed, obtaining a single-phase value associated to the occurrence of high gamma amplitude. Digital video cameras fixed above the SD-IA apparatus and recording cages were used for tracking the animal's position. Video data were acquired at 30 frames/s and analyzed using the TopScan system (CleverSys). Data are expressed as mean ± SEM and were analyzed using unpaired Student's t-test or one-sample t test with theoretical mean = 1. Electrodes placement was verified postmortem. To do that, rats were deeply anesthetized and

perfused intracardially (first with saline, pH 7.2 then with 4% paraformaldehyde, pH 7.2). Brains were removed, left in 30% sucrose for 48 h and cut coronally (50 µm sections). Relevant sections were selected and stained with cresyl violet to confirm electrode location.

Results

Repeated non-reinforced pre-training exposure to the training apparatus elicits the participation of the hippocampus in avoidance memory reconsolidation.

To determine the effect of previous learning on fear-motivated avoidance memory reconsolidation, male Wistar rats (3-month-old; 300-350 g) were handled (Control Group) or allowed to freely explore either an open field arena (OF Group) or the SD-IA training box (TB Group) during 5 minutes once daily for 5 days. Twenty-four hours later, the animals were trained in SD-IA (0.8 mA/2 s footshock) and one day thereafter submitted to a 40 s-long non-reinforced memory reactivation session. Immediately after that, rats received bilateral injections of vehicle (VEH; 0.9% saline), the gene transcription blocker α-amanitin (AMA; 45 ng/side), or the protein synthesis inhibitor anisomycin (ANI; 160 µg/side) into the CA1 region of the dorsal hippocampus. Control and OF animals showed normal SD-IA memory retention during a test session carried out 24 h post-reactivation, regardless of treatment. TB animals that received VEH also showed normal retention, but those given AMA or ANI were amnesic (Figure 1B; Control Group: $H = 0.8501$, $p = 0.6537$; OF Group: $H = 0.1925$, $p = 0.9082$; TB Group: $H = 12.23$, $p = 0.0022$, VEH vs AMA $p < 0.05$, VEH vs ANI $p < 0.01$ in Dunn's multiple comparisons after Kruskal-Wallis test).

Post-reactivation intra-CA1 administration of AMA and ANI also caused amnesia to TB animals trained in SD-IA using a weak footshock (0.4 mA/2 s; Figure 1C; Control Group: $H = 0.2679$, $p = 0.8747$; TB Group: $H = 14.96$, $p = 0.0006$, VEH vs AMA $p < 0.05$, VEH vs ANI $p < 0.001$ in Dunn's multiple comparisons after Kruskal-Wallis test).

In agreement with the notion that prior learning of conflicting non-aversive information is a necessary condition for the involvement of the hippocampus in avoidance memory reconsolidation, the amnesia caused by AMA and ANI lasted for at least 14 days (Figure 1D; $H = 15.43$, $p = 0.0004$, VEH vs AMA $p < 0.001$, VEH vs ANI $p < 0.05$ in Dunn's multiple comparisons after Kruskal-Wallis test), was not observed when AMA and ANI were injected 6 h after (Figure 1E; $H = 2.376$, $p = 0.3049$) or in the absence of memory reactivation (Figure 1F; $H = 2.282$, $p = 0.3196$), when we tested the animals for retention 3 h instead of 24 h post-reactivation (Figure 1G; $H = 1.959$, $p = 0.3754$), or when we submitted the animals to a single training box pre-exposure session (Figure 1H; $H = 1.478$, $p = 0.4776$).

Repeated pre-exposure to the training box decreased step-down latency at training but did not affect SD-IA memory strength or persistence (Figure 2A; Left Panel: $F_{(2, 47)} = 26.46$, $p < 0.001$ pre-exposure effect; $t_{(47)} = 2.144$, $p > 0.05$ for Control Group vs OF Group; $t_{(47)} = 7.106$, $p < 0.001$ for Control Group vs TB Group; $t_{(47)} = 4.994$, $p < 0.001$ for OF Group vs TB Group in Bonferroni's multiple comparisons test after one-way ANOVA. Right Panel: Day 1: $H = 0.4478$, $p = 0.7994$; Day 14: $H = 0.2072$, $p = 0.9016$). Moreover, non-reinforced reactivation had no effect on the strength of the learned avoidance response regardless of the footshock intensity at training (Figures 2B and 2C; $U = 24.50$, $p > 0.9999$, No RA Group vs RA Group for Strong training and $U = 20.00$, $p > 0.5594$, No RA Group vs RA Group, for Weak training).

Expression of the transcription factor Zif268 is a selective hippocampal reconsolidation marker (Lee et al., 2004), and pharmacological activation of β-adrenergic receptor signaling enhances fear memory reconsolidation (Debiec et al., 2011; but see also Muravieva and Alberini, 2010). In TB animals, but not in Control animals, intra-CA1 infusion of Zif268 antisense oligodeoxynucleotides (2 nmol/side) 90 min before memory reactivation provoked amnesia 24 h later (Figure 3A; Control Group: $U = 49.50$, $p > 0.9999$, MSO vs ASO; TB Group: $U = 7.50$, $p = 0.0007$, MSO vs ASO in Mann Whitney test) whereas intra-peritoneal administration of the β-adrenergic receptor agonist

isoproterenol (5 mg/kg) immediately post-reactivation slowed down memory decay (Figure 3B; U = 15.00, p = 0.0073, VEH vs ISO in Mann Whitney test). Moreover, administration of AMA and ANI following SD-IA memory reactivation did not affect retention in animals that received the NMDAr antagonist AP5 (5 µg/side) in dorsal CA1 after every pre-exposure session (Figure 4A; Left panel: $F_{(4, 196)} = 5.472$, p = 0.0003 for treatment effect; $F_{(1, 49)} = 15.81$, p = 0.0002 for session effect; $F_{(4, 196)} = 5.248$, p = 0.0005 for interaction. Session 4-AP5: $t_{(245)} = 4.038$, p < 0.001 vs Session 4-VEH; Session 5-AP5: $t_{(245)} = 4.179$, p < 0.001 vs Session 5-VEH in Bonferroni's multiple-comparison test after two-way ANOVA; Right panel: VEH after repeated pre-exposure: H = 12.96, p = 0.0015, VEH vs AMA p < 0.01, VEH vs ANI p < 0.05; AP5 after repeated pre-exposure: H = 2.046, p = 0.3595 in Dunn's multiple comparisons after Kruskal-Wallis test), or when the time elapsed between the last pre-exposure session and the training session was increased from 1 day to 28 days. However, re-exposure to the SD-IA training box, but not to an open field arena, 27 days after the last pre-exposure session restored the amnesic effect of AMA and ANI (Figure 4C; Left Panel: Handled Group: H = 0.07045, p = 0.9654; Open field Group: H = 3.214, p = 0.2005; Re-exposed Group: H = 19.20, p < 0.0001, VEH vs AMA p < 0.001, VEH vs ANI p < 0.001 in Dunn's multiple comparisons after Kruskal-Wallis test).

Avoidance memory reconsolidation increases hippocampal theta-gamma coupling.

Memory reconsolidation has been extensively characterized at the pharmacological and molecular levels (Alberini, 2005; Tronson and Taylor, 2007; Haubrich and Nader, 2016). However, electrophysiological analyses of this process are missing, which has hitherto hindered the description of definite reconsolidation electrophysiological signatures.

In the hippocampus, local field potential (LFP) oscillations in the theta band (5-10 Hz) are associated with contingency detection (Nokia and Wikgren, 2010) while slow (35-

55 Hz) and fast gamma (55-100 Hz) oscillations are involved in the transfer of information from and to other brain areas (Fries, 2009). Slow gamma originates in CA3 and propagates to CA1 *stratum radiatum* via the Schaffer collaterals whereas fast gamma activity seems to be generated mainly in the medial entorhinal cortex and propagates to the *stratum lacunosum-moleculare*, although the true origin and nature of this oscillatory activity remain to be fully elucidated (Csicsvari et al., 2003; Colgin et al., 2009; Zemankovics et al., 2013; Lasztóczi and Klausberger, 2014, 2016). Slow and fast gamma oscillations can also be recorded from CA1 pyramidal layer (Butler et al., 2016) where their coupling to theta mirrors the integration of novel information with that retrieved from long term memory stores during learning (Fell and Axmacher 2011; Yaffe et al., 2014). To determine whether reactivation-induced hippocampal LFP activity differs between animals that just retrieved the avoidance response (Control Group) and animals that also reconsolidated that response (TB Group), we recorded LFPs in dorsal CA1 pyramidal cell layer and analyzed changes in the oscillatory pattern during SD-IA memory reactivation by measuring the relative power of theta and gamma bands. We found that the amplitude of slow gamma oscillations increased in both Control and TB groups during reactivation (Figure 5C; Control Group: $t_{(5)} = 2.605$, $p = 0.0480$; TB Group: $t_{(5)} = 5.182$, $p = 0.0035$ in one-sample t-test with theoretical mean = 1; Control Group vs TB Group: $t_{(10)} = 1.858$, $p = 0.0928$ in unpaired t-test), in agreement with reports suggesting that slow gamma is involved in memory retrieval (Colgin, 2015). TB animals, but not Control animals, also showed an increase in theta and fast gamma power (Figure 5C; TB Group: $t_{(5)} = 8.754$, $p = 0.0003$ for theta band; $t_{(5)} = 3.601$, $p = 0.0155$ for fast gamma band in one-sample t-test with theoretical mean = 1; Control Group vs TB Group: $t_{(10)} = 2.524$, $p = 0.0302$ for theta band; $t_{(10)} = 2.527$, $p = 0.0300$ for fast gamma band in unpaired t-test). Using the modulation index (MI; Canolty et al., 2006; Tort et al., 2010), we found that slow and fast gamma amplitudes were coupled to theta during SD-IA memory reactivation, and that this modulation was stronger in TB animals than in Control animals (Figure 5F; Control Group vs TB Group:

$t_{(10)}$ = 3.639, p = 0.0045 for theta-slow gamma; $t_{(10)}$ = 3.963, p = 0.0027 for theta-fast gamma in unpaired t-test). To investigate whether this difference in coupling strength indeed reflects an active memory process or was simply the result of improved phase identification due to increased theta power in TB animals (Canolty et al., 2006; Tort et al., 2008), we binned LFP responses recorded during the 40 s-long SD-IA reactivation session into 1 s-long intervals and equalized theta power between Control and TB animals (Figure 5G; Left panel: Control Group vs TB Group: $t_{(10)}$ = 0.1640, p = 0.8730 in unpaired t-test) to recalculate MI by just taking into account epochs with theta power values above or below the 50^{th} percentile, respectively. We found that, even under these stringent conditions, MI was higher in TB than in Control animals (Figure 5G; Right panel: Control Group vs TB Group: $t_{(10)}$ = 3.006, p = 0.0132 for theta-slow gamma; $t_{(10)}$ = 5.409, p = 0.0003 for theta-fast gamma in unpaired t-test).

Analysis of gamma normalized amplitude distribution over theta phases showed that in TB animals, but not in Control animals, maximal power of slow and fast gamma components occurred near the peak of the theta cycle during memory reactivation (Figure 5H; Left panel). We also determined the theta phase distribution of slow and fast gamma events, defined as periods when power of the selected gamma frequency sub-band exceeded 2 s.d. the mean power, and found that in TB animals slow and fast gamma events occurred at different phases of the theta cycle, with slow gamma episodes concentrated on the late ascending portion and fast gamma events on the early descending phase of the theta wave (Figure 5H; Right panel: 347.18° ± 5.33 for slow gamma events and 36.34° ± 13.03 for fast gamma events, mean phase ± angular deviation; F = 7.11, p = 0.048 in Hotelling paired sample test for equal angular means; non-uniform phase distribution p < 0.001 in Rayleigh test; 0° defined as the peak of the theta cycle).

The differential modulation of slow and fast gamma bands observed in TB animals during memory reactivation was independent on the number of gamma events (Figure

6I; slow gamma events: $t_{(10)} = 2.194$, $p = 0.0529$, Control Group vs TB Group; fast gamma events: $t_{(10)} = 1.470$, $p = 0.1724$, Control Group vs TB Group; unpaired t-test).

Discussion

Previous non-aversive learning is a boundary condition for avoidance memory reconsolidation

Reconsolidation is not a necessary consequence of memory reactivation but there are experimental conditions that constrain this process. Several of these boundary conditions have been already described although there have been conflicting reports about every one of them, which is not surprising given the amount of behavioral variables and physiological interactions that can affect memory reactivation and retrieval (Nader and Hardt, 2009). However, the finding that inhibition of hippocampal protein synthesis after fear avoidance reactivation does not result in persistent amnesia has been remarkably consistent over time (Taubenfeld et al., 2001; Cammarota et al., 2004; Power et al., 2006; Arguello et al., 2013), supporting the idea that the hippocampus is not involved in fear-motivated avoidance memory reconsolidation. Contradicting this view, our experiments demonstrate that the hippocampus does indeed participate in avoidance memory reconsolidation but only when the animals were repeatedly pre-exposed to the training environment before acquiring the avoidance memory trace. This assertion is based on results showing that intra-CA1 administration of AMA or ANI immediately after reactivation caused time-dependent amnesia for SD-IA memory in pre-exposed animals (TB Group) but not in control non-pre-exposed rats (Control Group) or in rats pre-exposed to an open field arena unrelated to the SD-IA training box (OF Group). Moreover, the amnesic effect of AMA and ANI did not occur when retention was assessed 3 h after reactivation, and was mimicked by blocking the expression of the reconsolidation marker Zif268 in the hippocampus. It is improbable that latent inhibition could account for our results, since

412 it has been repeatedly reported that, if something, pre-exposure to the training context
413 increases rather than decreases fear memory strength (Pisano et al., 2012) which in
414 turn should make memory resistant to reconsolidation (Suzuki et al., 2004; Wang et al.,
415 2009). In any case, pre-exposure to the SD-IA apparatus did not alter the strength or
416 the persistence of SD-IA memory, and the effect of AMA and ANI on reconsolidation
417 was independent on the strength of the avoidance response, which together with the
418 fact that repeated pre-exposure turned the trace susceptible instead of resistant to
419 hippocampal manipulations, allow us to discard also any possible influence of a pre-
420 exposure facilitation-like effect similar to that described for the formation of contextual
421 fear conditioning memory (Fanselow, 1990; Barrientos et al., 2002).
422 The hippocampus supports the associative schema that organizes previously acquired
423 knowledge and computes mismatch signals (Vinogradova, 2001; Lisman and Grace,
424 2005; Schiller et al., 2015). Hence, it has been proposed that the hippocampus is
425 specifically engaged in memory reconsolidation when reactivation occurs concomitantly
426 with novelty or prediction error detection (Morris et al., 2006; Rossato et al., 2007;
427 Fernández et al., 2016). However, in our experiments, neither Control nor TB animals
428 made any error or learned any new information during the reactivation session but they
429 doubtless had different expectations about the possible outcomes of this session. For
430 Control animals the only foreseeable consequence of stepping down from the safe
431 platform during reactivation was a footshock while for TB rats the consequences of this
432 action were not unambiguously predictable. Therefore, it is tempting to speculate that
433 what triggers the involvement of the hippocampus in fear-motivated avoidance memory
434 reconsolidation is not the discrepancy between facts and forecasts or the perception of
435 novelty, but the uncertainty about the aftereffects of avoidance brought about by the
436 comparison between competing contradictory representations.
437
438 *Oscillatory activity in the hippocampus and avoidance memory reconsolidation*

Hippocampal theta oscillations are linked to retrieval of choice-relevant information during decision-making (Womelsdorf et al., 2010) and coordinate reactivation of different inputs increasing the accuracy of comparisons (Vinogradova, 2001). Our electrophysiological recordings showed that CA1 theta power increased in TB animals, but not in Control animals, during memory reactivation, suggesting that hippocampal theta activity may reflect computing of conflicting information at the onset of reconsolidation. It has been suggested that slow gamma frequencies promote memory retrieval while fast gamma rhythms facilitate encoding and re-encoding of current contextual information (Colgin, 2016). In agreement with these reports, we found that both Control and TB animals showed increased slow gamma activity, while only TB animals presented changes in the fast gamma band during memory reactivation. In the amygdala, fast gamma power is associated with safety signals, and it is known that expression of aversive and safety states involves synchronized interaction of this structure with the hippocampus (Stujenske et al., 2014). Then, an alternative explanation for our findings is that the increased hippocampal fast gamma activity observed in TB animals mirrors reactivation of the non-aversive representation learned during repeated pre-exposure to the training apparatus.

Theta-gamma interactions are associated with synaptic plasticity, memory retrieval and communication between brain regions (Lisman, 2005; Canolty and Knight, 2010; Jutras and Buffalo, 2010; Lesting et al., 2011). We found that theta phase strongly modulates the amplitude of slow and fast gamma bands during memory reactivation in TB but not in Control animals, suggesting that the strength of this cross-frequency coupling in the hippocampus is an electrophysiological correlate of memory reconsolidation. Although speed-dependent variations in hippocampal LFP activity have been reported (Whishaw and Vanderwolf, 1973; Montgomery et al., 2009; Newman et al., 2013), it is unlikely that differences in motor activity could account for our results since both Control and TB animas stayed in the training box platform in a minimal movement state (mean velocity < 1cm/s) during the reactivation session.

Is previously acquired conflicting information a universal boundary condition for memory reconsolidation?

We cannot conclusively answer if the effect of previous conflicting learning is specific for SD-IA memory reconsolidation, but it is noteworthy that most, if not all, significant reports about memory reconsolidation published so far involved some sort of pre-exposure (or habituation) to the training apparatus. Indeed, such non-reinforced pre-exposure to the training environment and/or process is a standard procedure for both auditory and contextual fear conditioning as well as for novel object recognition training, conditioned taste aversion and almost every other preparation in which reconsolidation has been studied (Hall et al., 2001; Debiec et al., 2002; Rossato et al. 2007; Garcia-DeLaTorre et al., 2009), including learning paradigms in non-mammalian animal models such as conditioning in medaka fish (Eisenberg and Dudai, 2004), long-term sensitization of the siphon-withdrawal reflex in the marine snail *Aplysia californica* (Cai et al., 2012) and context-signal training in the crab *Chasmagnathus* (Pedreira et al., 2002). During these pre-exposure sessions, the animals can acquire information that clashes with that to be presented at the moment of training. Therefore, it is possible that the results we report here reveal a hitherto neglected universal boundary condition, although further research is certainly required to gauge the significance of this suggestion.

Conclusions and possible implications

Clinical interventions aimed to attenuate the persistent recollection of traumatic experiences can be based not only on the disruption of avoidance memory reconsolidation but also on the enhancement of avoidance memory extinction (Vervliet et al., 2013; Schwabe et al., 2014). Extinction is the process by which the probability of emission of a learned response declines upon repeated non-reinforced reactivation and

494 entails formation of an inhibitory memory that ends up competing with the original
495 trace. Reconsolidation and extinction are mutually exclusive processes (Merlo et al.,
496 2014) and it has been suggested that whether retrieval results in extinction learning or
497 memory reconsolidation depends on the boundary conditions prevailing during the
498 reactivation session. However, although extinction and reconsolidation are exclusive of
499 each other, the inhibitory memory trace induced by extinction learning is susceptible to
500 reconsolidation. For example, SD-IA extinction memory undergoes protein synthesis-
501 dependent reconsolidation in the hippocampus upon reactivation, and its manipulation
502 can either recover the avoidance response or enhance the extinction memory trace
503 (Radiske et al., 2015; Rosas-Vidal et al., 2015). These findings, together with the
504 results presented in this study, strongly suggest that the hippocampus is engaged in
505 memory reconsolidation when conflicting signals are detected during the reactivation
506 session, and that the mnemonic representation that actually controls the animal's
507 behavior in that session is the one that becomes vulnerable to pharmacological
508 interference, as suggested by the trace dominance theory (Eisenberg et al., 2003).
509 Within this framework, we propose that therapies based on the interference of memory
510 reconsolidation should be preferred to treat traumas and phobias associated with
511 familiar contexts, while interventions based on the facilitation of extinction should be
512 the prescription of choice when the traumatic events stem from unfamiliar
513 backgrounds. Lastly, our results also suggest that phase-amplitude coupling analyses
514 from EEG signals recorded during reconsolidation-based psychotherapies could be
515 useful to verify the actual occurrence of this process and predict the treatment's
516 efficacy.
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Figure Legends

Figure 1. *Repeated non-reinforced pre-training exposure to the training apparatus elicits the involvement of the hippocampus in avoidance memory reconsolidation.* **A. Left Panel:** Schematic representation of the experimental protocol. **Right Panel:** Schematic representation of bilateral cannula placement in dorsal CA1 (adapted from Paxinos and Watson, 2007) and representative microphotograph of Nissl-stained coronal section showing cannula/injection tracks. **B.** Rats were handled (Control Group) or allowed to freely explore either an open field arena (OF Group) or the step-down inhibitory avoidance (SD-IA) training box (TB Group) once daily for 5 min during 5 days. Twenty-four hours after the last session, the animals were trained in the SD-IA task (TR; 0.8 mA/2 s) and one day later submitted to a 40 s-long non-reinforced memory reactivation session (RA). Immediately after the RA session, animals received bilateral intra-CA1 infusions of vehicle (VEH; 0.9% saline), the mRNA synthesis blocker α-amanitin (AMA; 45 ng/side) or the protein synthesis inhibitor anisomycin (ANI; 160 µg/side). Memory retention was evaluated 1 day later (Test). AMA and ANI disrupted SD-IA retention in TB animals but not in Control or OF animals (Control Group: TR_{lat} = 22.85 ± 6.43, n = 13 for VEH; TR_{lat} = 15.15 ± 1.56, n = 13 for AMA; TR_{lat} = 17.70 ± 3.56, n = 10 for ANI. OF Group: TR_{lat} = 17.92 ± 3.58, n = 12 for VEH; TR_{lat} = 12.45 ± 2.22, n = 11 for AMA; TR_{lat} = 10.75 ± 1.14, n = 12 for ANI. TB Group: TR_{lat} = 4.9 ± 0.94, n = 10 for VEH; TR_{lat} = 5.88 ± 1.09, n = 9 for AMA; TR_{lat} = 5.70 ± 0.65, n = 10 for ANI). **C.** Control and TB animals were treated as in B, except that they were trained using a weak footshock (0.4 mA/2 s; Control Group: TR_{lat} = 14.29 ± 3.02, n = 7 for VEH; TR_{lat} = 11.86 ± 2.19, n = 7 for AMA; TR_{lat} = 9.14 ± 4.33, n = 7 for ANI. TB Group: TR_{lat} = 4.75 ± 1.54, n = 8 for VEH; TR_{lat} = 5.62 ± 1.59, n = 8 for AMA; TR_{lat} = 5.25 ± 2.99, n = 8 for ANI). **D.** TB animals were treated as in B, except that the retention test was carried out 14 days after RA (TR_{lat} = 5.08 ± 1.07, n = 12 for VEH; TR_{lat} = 8.16 ± 1.91, n = 12 for AMA; TR_{lat} = 6.36 ± 1.71, n = 11 for ANI). **E.** TB animals were treated as in B, except

that VEH, AMA and ANI were injected in dorsal CA1 6 h after RA (TR_{lat} = 6.25 ± 2.96, n = 8 for VEH; TR_{lat} = 5.12 ± 1.91, n = 8 for AMA; TR_{lat} = 5.12 ± 3.28, n = 8 for ANI). **F.** TB animals were treated as in B, except that VEH, AMA and ANI were injected into CA1 24 h after training in the absence of memory reactivation (TR_{lat} = 2.25 ± 0.25, n = 8 for VEH; TR_{lat} = 3.50 ± 1.06, n = 8 for AMA; TR_{lat} = 2.37 ± 0.98, n = 8 for ANI). **G.** TB animals were treated as in B, except that the retention test was carried out 3 h after RA (TR_{lat} = 5.87 ± 2.34, n = 8 for VEH; TR_{lat} = 3.75 ± 1.41, n = 8 for AMA; TR_{lat} = 2.28 ± 0.76 n = 7 for ANI). **H.** TB animals were treated as in B, except that they were submitted to a single SD-IA training box pre-exposure session (TR_{lat} = 7.41 ± 1.09, n = 12 for VEH; TR_{lat} = 7.00 ± 0.76, n = 10 for AMA; TR_{lat} = 8.4 ± 1.01, n = 10 for ANI). Data are expressed as median ± interquartile range for retention test step-down latency. TR_{lat}: mean training step-down latency in seconds ± SEM. Training step-down latencies did not differ between VEH and drug-treated groups. *p < 0.05, **p < 0.01, ***p < 0.001. INF: drug infusion.

Figure 2. *Neither repeated non-reinforced pre-exposure to the training box nor non-reinforced reactivation have effect on the learned avoidance response.* **A.** Rats were handled (Control Group) or allowed to freely explore either an open field arena (OF Group) or the step-down inhibitory avoidance (SD-IA) training box (TB Group) once daily for 5 min during 5 days. Twenty-four hours after the last session, the animals were trained in SD-IA (TR; 0.8 mA/2 s). Memory retention was evaluated 1 or 14 days later (Test). **Left Panel:** Step-down latency during the SD-IA training session for Control, OF and TB animals. **Right Panel:** Step-down latency during the SD-IA retention test session for Control, OF and TB animals (n = 17 for Control Group, n = 17 for OF Group, n = 16 for TB Group). **B.** Animals were trained in the SD-IA task using a 0.8 mA/2 s footshock and, one day later, they were handled (No RA Group) or submitted to a 40 s-long non-reinforced memory reactivation session (RA Group).

Memory retention was evaluated 24 h later (TR_{lat} = 16.43 ± 2.86, n = 7 for No-RA Group; TR_{lat} = 15.14 ± 3.61, n = 7 for RA Group). **C.** Animals were trained in the SD-IA task using a 0.4 mA/2 s footshock (Weak training) and one day later, they were handled (No RA Group) or submitted to a 40 s-long non-reinforced memory reactivation session (RA Group). Memory retention was evaluated 1 day later (TR_{lat} = 18.00 ± 6.09, n = 7 for No RA Group; TR_{lat} = 20.43 ± 6.68, n = 7 for RA Group). Data are expressed as median ± interquartile range for retention test step-down latency, or mean ± SEM for training step-down latency. TR_{lat}: mean training step-down latency in seconds ± SEM. ***p < 0.001.

Figure 3. A. *Avoidance memory reconsolidation requires Zif268 expression in dorsal CA1.* Rats were handled (Control Group) or allowed to freely explore the step-down inhibitory avoidance (SD-IA) training box (TB Group) once daily for 5 min during 5 days. Twenty-four hours after the last session, animals were trained in the SD-IA task (TR; 0.8 mA/2 s). One day later, they received bilateral intra-CA1 infusions of Zif268 antisense (ASO; 2 nmol/side) or missense (MSO; 2 nmol/side) oligodeoxynucleotides and, 90 min thereafter, were submitted to a 40 s-long non-reinforced memory reactivation session (RA). Memory retention was evaluated 1 day later (Control Group: TR_{lat} = 14.10 ± 3.32, n = 10 for MSO; TR_{lat} = 13.30 ± 2.51, n = 10 for ASO. TB Group: TR_{lat} = 6.80 ± 2.96, n = 10 for MSO; TR_{lat} = 5.87 ± 1.61, n = 8 for ASO). **B.** *Post-reactivation administration of isoproterenol delays avoidance memory decay in animals repeatedly pre-exposed to the training apparatus before SD-IA training.* TB animals were trained in the SD-IA task using a 0.4 mA/2 s footshock (Weak training) and one day later submitted to a 40 s-long non-reinforced memory reactivation session (RA). Immediately after the RA session, the animals received intraperitoneal isoproterenol (ISO; 5 mg/kg) or VEH and were tested for retention 28 days later (TR_{lat} = 4.88 ± 0.71, n = 9 for VEH; TR_{lat} = 4.27 ± 0.55, n = 11 for ISO). Data are expressed as median ±

interquartile range for retention test step-down latency. TR_{lat}: mean training step-down latency in seconds ± SEM. Training step-down latencies did not differ between VEH and drug-treated groups. **p < 0.01, ***p < 0.001.

Figure 4. *The effect of repeated non-reinforced pre-training exposure to the training apparatus on avoidance memory reconsolidation is time-dependent and requires NMDAr activation immediately after each pre-exposure session.* **A. Left panel:** *Bilateral intra-CA1 infusion of AP5 immediately after each pre-exposure session prevented the decrease in step-down latency caused by repeated non-reinforced exposure to the training environment.* Animals were allowed to explore the step-down inhibitory avoidance (SD-IA) training box once daily for 5 min during 5 days and immediately after each session received intra-CA1 infusions of vehicle (VEH; 0.9% saline) or AP5 (5 µg/side). **Right panel:** *Bilateral intra-CA1 infusion of AP5 immediately after each pre-exposure session prevented the amnesic effect of the post-reactivation administration of AMA and ANI.* Twenty-four hours after the last pre-exposure session, the animals showed in the left panel were trained in the SD-IA task (TR; 0.8 mA/2 s) and one day later submitted to a 40 s-long non-reinforced memory reactivation session (RA). Immediately after the RA session, rats received bilateral intra-CA1 infusions of VEH, the mRNA synthesis blocker α-amanitin (AMA; 45 ng/side) or the protein synthesis inhibitor anisomycin (ANI; 160 µg/side). Memory retention was evaluated 1 day later (TR_{lat} = 4.9 ± 0.97, n = 10 for VEH-VEH; TR_{lat} = 2.87 ± 0.29, n = 8 for VEH-AMA; TR_{lat} = 3.5 ± 0.50, n = 8 for VEH-ANI; TR_{lat} = 4.66 ± 0.76, n = 9 for AP5-VEH; TR_{lat} = 6.50 ± 0.84, n = 8 for AP5-AMA; TR_{lat} = 4.25 ± 0.92, n = 8 for AP5-ANI). **B.** Schematic representation of the experimental protocol. **C. Left Panel:** Animals were allowed to explore the SD-IA training box once daily for 5 min during 5 days (TB Group). Twenty-seven days after the last session, they were handled (Handled Group), allowed to explore an open field arena (Open field Group) or re-exposed to the SD-IA

training box (Re-exposed Group) for 5 min and, one day later, trained in the SD-IA task (0.8 mA/2 s). Twenty-four hours post-training, the animals were submitted to a RA session and immediately thereafter received bilateral intra-CA1 infusions of VEH, AMA or ANI. Retention was evaluated 1 day later. AMA and ANI impaired avoidance memory retention in animals re-exposed to the training box, but not in handled animals or in rats exposed to an open field arena (Handled Group: TR_{lat} = 4.75 ± 0.82, n = 12 for VEH; TR_{lat} = 5.25 ± 2.72, n = 8 for AMA; TR_{lat} = 4.00 ± 1.03, n = 8 for ANI. Open Field Group: TR_{lat} = 5.20 ± 0.74, n = 10 for VEH; TR_{lat} = 4.1 ± 1.02, n = 10 for AMA; TR_{lat} = 5.3 ± 1.7, n = 10 for ANI. Re-exposed Group: TR_{lat} = 3.30 ± 0.55, n = 10 for VEH; TR_{lat} = 4.33 ± 0.81, n = 9 for AMA; TR_{lat} = 6.70 ± 2.57, n = 10 for ANI). **Right Panel:** Animals were allowed to explore an open field arena once daily for 5 min during 5 days. Twenty-seven days after the last session, they were left to explore again the open field arena (Open field Group) or the SD-IA training box (SD-IA Group) for 5 min and, one day later, trained in the SD-IA task. Twenty-four hours post-training, the animals were submitted to a RA session and immediately thereafter received bilateral intra-CA1 infusions of VEH, AMA or ANI. Retention was evaluated 1 day later. AMA and ANI did not affect SD-IA memory retention (Open field Group: TR_{lat} = 15.18 ± 4.29, n = 11 for VEH; TR_{lat} = 20.4 ± 6.75, n = 10 for AMA; TR_{lat} = 17.7 ± 3.58, n = 10 for ANI. SD-IA box Group: TR_{lat} = 6.58 ± 0.80, n = 12 for VEH; TR_{lat} = 5.50 ± 0.60, n = 12 for AMA; TR_{lat} = 7.44 ± 2.23, n = 9 for ANI. Open field Group: H = 2.378, p = 0.3045; SD-IA box Group: H = 1.797, p = 0.4072). Data are expressed as median ± interquartile range for retention test step-down latency, or mean ± SEM for pre-exposure sessions step-down latency. TR_{lat}: mean training step-down latency in seconds ± SEM. Training step-down latencies did not differ between VEH and drug-treated groups. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 5. *Avoidance memory reactivation induces prominent hippocampal theta and gamma oscillatory activity as well as strong theta-gamma coupling only in animals repeatedly pre-exposed to the training apparatus before SD-IA training.* Rats were handled (Control Group) or allowed to explore the step-down inhibitory avoidance (SD-IA) training box (TB Group) once daily for 5 min during 5 days and, 24 h after the last session, they were trained in SD-IA (0.8 mA/2 s). One day later, animals were submitted to a 40 s-long non-reinforced memory reactivation session (RA) during which local field potential (LFP) signals from CA1 pyramidal layer were recorded. **A.** Representative power spectrum density plots from Control and TB animals during RA. **B.** Control and TB group mean power ratio (1-120 Hz) showing reactivation-induced alterations in hippocampal oscillatory activity; bold lines represent group mean and shaded areas represent SEM. **C.** Mean power ratio for theta (5-10 Hz), slow gamma (35-55 Hz) and fast gamma (55-100 Hz) frequency bands during RA. Avoidance memory reactivation increased slow gamma power in both Control and TB animals. Theta and fast gamma power was also increased in TB animals during RA. **D.** Example of filtered dorsal-CA1 LFP recordings of Control and TB animals during RA. Black lines: LFP filtered between 1-150 Hz; magenta lines: LFP filtered in the theta frequency range; green line: LFP filtered in the slow gamma frequency range; blue line: LFP filtered in the fast gamma frequency range. **E.** Representative phase-amplitude comodulograms for TB and Control animals during RA. **F.** Mean theta-slow gamma and theta-fast gamma modulation indexes (MI). TB animals showed stronger theta-gamma coupling than Control animals during RA. **G.** Mean theta-slow gamma and theta-fast gamma modulation indexes (MI) calculated using epochs with equalized theta power. **H. Left Panel:** Representative examples of mean slow gamma and mean fast gamma normalized amplitude distribution over theta phase (20° bins) during RA; two cycles are shown for clarity; theta phase trace is shown in black. **Right Panel:** Representative circular histograms showing the distribution of gamma events over theta phase for Control and TB animals during RA. **I.** The mean number of slow and fast gamma

events did not differ between Control and TB animals. **J. Upper Panels:** Schematic representation of multielectrode array placement in dorsal CA1 (adapted from Paxinos and Watson, 2007) and representative microphotograph of Nissl-stained coronal section showing electrodes tracks. **Lower Panels:** LFPs and theta phase difference between electrodes placed in dorsal CA1. Light blue: Control Group; red: TB Group; bars represent mean ± SEM; floating bars show minimum, maximum and mean values; γ_S: slow gamma, γ_F: fast gamma; 0° was defined as the peak of the theta cycle. *p < 0.05, **p < 0.01, ***p < 0.001.