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Abbreviated title: **Memory reconsolidation and PKMζ**

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Abstract

Object recognition memory (ORM) confers the ability to discriminate the familiarity of previously encountered items. Reconsolidation is the process by which reactivated memories become labile and susceptible to modifications. The hippocampus is specifically engaged in reconsolidation to integrate new information into the original ORM through a mechanism involving activation of brain-derived neurotrophic factor (BDNF) signaling and induction of long-term potentiation (LTP). It is known that BDNF can control LTP maintenance through protein kinase Mζ (PKMζ), an atypical protein kinase C isoform that is thought to sustain memory storage by modulating glutamatergic neurotransmission. However, the potential involvement of PKMζ in ORM reconsolidation has never been studied. Using a novel object recognition memory task combined with pharmacological, biochemical and electrophysiological tools, we found that, in adult male Wistar rats, hippocampal PKMζ is essential to update ORM through reconsolidation, but not to maintain the inactive recognition memory trace stored over time. Our results also indicate that hippocampal PKMζ acts downstream BDNF and controls AMPAR synaptic insertion to elicit reconsolidation, and suggest that blocking PKMζ activity during this process deletes active ORM.

Significance Statement

Object recognition memory (ORM) is essential to remember facts and events. Reconsolidation integrates new information into ORM through changes in hippocampal plasticity and BDNF signaling. In turn, BDNF enhances synaptic efficacy through protein kinase Mζ (PKMζ), which might preserve memory. Here, we present evidence that hippocampal PKMζ acts downstream BDNF to regulate AMPAR recycling during ORM reconsolidation, and show that this kinase is essential to update the reactivated recognition memory trace but not to consolidate or maintain an inactive ORM. We also demonstrate that the amnesia provoked by disrupting ORM reconsolidation through PKMζ inhibition is due to memory erasure and not to retrieval failure.

Introduction

To persist, long-term memories (LTM) must overcome the turnover of their molecular substrates. There are several hypotheses about the mechanisms responsible for LTM maintenance including, to name but a few examples, suggestions that LTM is stored as modifications in DNA methylation/sequence (Vanyushin et al., 1974; Crick, 1984), prion-like changes in synaptic growth (Bailey et al., 2004) or proteins able to auto-perpetuate their active state (Fukunaga and Miyamoto, 2000; Lisman and McIntyre, 2001). More recently, Sacktor and coworkers proposed that PKMζ, an atypical PKC isoform expressed in neural tissue and enriched in the hippocampus, is sufficient to maintain LTM (Sacktor, 2008). In vertebrates, PKMζ is transcribed from an internal promoter within the PKCζ gene to produce mRNAs lacking PKCζ auto-inhibitory domain. Therefore, although PKMζ is persistently active, under basal conditions translation of its mRNA is repressed (Bal et al., 2016). This block on PKMζ synthesis can be released through neurotrophin signaling (Adasme et al., 2011), allowing PKMζ to perpetuate LTM by promoting AMPAR expression at post-synaptic sites (Migues et al., 2010). As with other hypothesis about memory persistence, the notion that PKMζ sustains LTM is controversial (Lisman, 2012), has been validated (Cai et al., 2011; Wang et al., 2016; Hu et al., 2017) and refuted repeatedly (Wu-Zhang et al., 2012; Lee et al., 2013; Volk et al., 2013), and fails to accommodate comfortably the fact that consolidated LTMs are not immutable but can incorporate information at the time of reactivation through a protein synthesis-dependent reconsolidation process (Nader and Hardt, 2009). In this regard, it has been reported that PKMζ inhibition can cause amnesia when memory is reactivated within a certain period of time after PKMζ antagonists administration (Parsons and Davis, 2011) suggesting that, besides inactive memory maintenance, this kinase may also mediate active memory reconsolidation (Kwapis and Helmstetter, 2014). However, the few reports published so far about the putative involvement of PKMζ in reconsolidation have been inconclusive (Crespo et al., 2012; Levitan et al., 2016), mainly because they utilized behavioral tasks that cannot distinguish clearly

between reconsolidation and other phenomena induced by memory reactivation, such as extinction.

ORM is a core component of declarative memory. Its failure is one of the earliest signs of Alzheimer's disease, which is also associated with impaired hippocampal BDNF signaling (O'Bryant et al., 2009). ORM consolidation and retrieval require the functional participation of the hippocampus (DeVito and Eichenbaum, 2010; Cohen and Stackman, 2015). However, ORM reconsolidation only recruits this brain region when memory reactivation occurs concomitantly with novelty detection (Rossato et al., 2007; Winters et al., 2011). When induced by presentation of a novel object, reconsolidation mediates integration of new information into the reactivated ORM trace and is controlled by BDNF (Radiske et al., 2017b) and accompanied by a brief early post-reactivation depotentiation period followed by a late stage of synaptic efficacy enhancement (Clarke et al., 2010). Because PKMζ controls the facilitatory effect of BDNF on hippocampal LTP (Mei et al., 2011), and both PKMζ and BDNF regulate AMPAR surface expression during synaptic potentiation (Caldeira et al., 2007; Yao et al., 2008; Jourdi and Kabbaj, 2013), which seems necessary for reconsolidation (Rao-Ruiz, et al., 2011; Bhattacharya et al., 2017), we hypothesized that PKMζ modulates ORM reconsolidation by mediating the interplay between BDNF and AMPAR recycling, and hence, that its inhibition after retrieval in the presence of a novel object impairs ORM reconsolidation and causes amnesia by deleting the reactivated recognition memory trace.

Here, we confirm that inactive ORM does not require hippocampal PKMζ activity in order to persist (Hardt et al., 2010) and demonstrate that it becomes vulnerable to PKMζ inhibition following reactivation in the presence of a novel object, suggesting that PKMζ is necessary to update ORM through reconsolidation but not to consolidate or maintain the dormant recognition trace. We also present evidence that hippocampal PKMζ acts downstream BDNF to control AMPAR recycling during reconsolidation and that blocking this process with PKMζ inhibitors erases ORM.

Materials and Methods

Subjects

All experiments were performed in accordance with the USA National Institutes of Health Guidelines for Animal Care and the local institutional ethics committee recommendations (Comissão de Ética no Uso de Animais - CEUA, UFRN). A total of 1174 adult male Wistar rats (3 moth old; 300-350 g) were utilized; 150 were used to revalidate the ORM task and 1024 to test our hypotheses. Animals were housed in groups of 5 per cage with free access to food and water and kept in the institutional vivarium on a 12 h light on/off schedule (lights on at 06:00 AM) at 23°C. All behavioral experiments were performed during the light phase of the cycle. Researchers were blind as to the animal's treatment condition.

Stereotaxic surgery for cannula and multielectrode array implants

Rats were anesthetized with ketamine (80 mg/kg)/xylazine (10 mg/kg) and bilaterally implanted with 22-gauge stainless steel guides aimed to the CA1 region of the dorsal hippocampus (AP −4.2; LL, ±3.0; DV, −3.0) and/or the entorhinal cortex (AP −6.8; LL ±5.0; DV −8.1). Some animals were also implanted with electrode arrays aimed to the dorsal hippocampus (AP −3.6; LL +2.4; DV −3.6 mm). Coordinates were taken from Paxinos and Watson (2007). Arrays were made of 16 tungsten electrodes (50 µm, PFA coated; from A-M Microsystems) organized in two rows spaced by 250 µm. Ground screw electrodes were localized in the parietal bone. Meloxicam (0.2 mg/kg) was administered by subcutaneous injection at the end of all surgical procedures as analgesic. Animals implanted with electrode arrays were housed individually. Rats were allowed a recovery period of at least 7 days to regain pre-surgery weight before behavioral procedures. During this period, the animals were handled daily for 1-2 min.

Experimental Design and Statistical Analysis

Novel object recognition task (NOR task)

Object recognition memory was assessed using a novel object recognition task (NOR task) based on the spontaneous exploratory behavior of rats (Ennaceur and Delacour, 1988). If rats are put in the presence of a familiar and a novel object in an open field arena, they will preferentially explore the novel one. The NOR task was conducted in a grey plywood open field arena (60 x 60 x 60 cm) placed in a dim-light illuminated room acclimatized at 23-24°C. Rats were handled and allowed to freely explore the training arena in the absence of stimuli objects for 20 min/day during 4 days (Habituation sessions). One day after the last habituation session, animals were exposed to two novel stimuli objects (Objects A and B) for 5 min in the training arena (Training session; TR). Memory reactivation was conducted by re-exposing the animals to one of the objects presented during training (Object A) together with a novel object (Object C) for 5 min in the arena (Reactivation session; RA). RA sessions were carried out one or seven days after TR. ORM retention was evaluated 3 h, 1 day or 7 days after RA by exposing the animals to a novel object D alongside familiar object A, B or C for 5 min (Test session). One hour before the experimental sessions, animals were transported from the vivarium to the experimental anteroom. From there, each rat was individually brought to the experiment room in a transport cage. At the end of each session, animals were return to the experimental anteroom and stayed there for an additional hour before being transported back to the vivarium. Stimuli objects were made of metal, glass, or glazed ceramic and had no significant innate preference for rats (Table 1). The open field arena and the stimuli objects were cleaned with ethanol (50%) before each trial to ensure the absence of olfactory cues. Object exploration was defined as sniffing and touching the stimuli objects with the muzzle and/or forepaws. Sitting on or turning around the objects was not considered as exploratory behavior. Digital video cameras fixed above the open field arenas were used for tracking the animal's position and behavior. Video data were acquired at 30 frames/s and analyzed using the ObjectScan system (CleverSys). We validated the performance of the ObjectScan system by comparing it to human scores before performing the experiments reported here. Discrimination index (DI) was calculated as: (Time exploring novel object – Time exploring familiar object) / Total object exploration time), considering

data from the 5-min session (Akirav and Maroun, 2006; Maroun and Akirav, 2008). Naive rats discriminate between novel and familiar objects throughout the entire retention test session (Table 2). DI can vary between −1 and +1; positive DI score indicates preference for the novel object while DI score close to zero indicates that animals explore both objects to the same extent, suggesting absence of discrimination.

Morris water maze task (MWM task)

The MWM was a black circular pool (200-cm in diameter) conceptually divided in four equal imaginary quadrant from which rodents learned to escape onto a hidden platform whose top surface was 1.5 cm beneath the water surface. Water temperature was regulated at 24-25°C. The maze was located in a well-lit white room with several posters and other distal visual stimuli hanging on the walls to provide spatial cues. A curtain separated the maze from the area where the computer was set up and the animals temporarily housed during the behavioral sessions. Training was carried out during five consecutive days as previously described (Rossato et al., 2007). On each day, rats received eight consecutive training trials during which the hidden platform was kept in a constant location. A different starting location was used on each trial, which consisted of a swim followed by a 30 s platform sit. The experimenter guided any rat that did not find the platform within 60 s to it. The intertrial interval was 30 s. Memory retention was evaluated in a 60 s probe trial carried out in the absence of the escape platform 24 h after the last training session. Video data were acquired at 30 frames/s and analyzed on-line using the TopScan system (CleverSys).

Step-down inhibitory avoidance task (IA task)

The training apparatus was a 50 x 25 x 25 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, animals were placed on the platform facing the left rear corner of the training box and when stepped down and placed their four paws on the grid received a 2 s, 0.4 mA scrambled footshock. Memory retention was evaluated in a non-reinforced test

session carried out 24 h after training. Test session finished when the animal stepped down to the grid or after 300 s.

Drugs and infusion procedures

All drug doses used in this work were based on previous studies and pilot experiments. Anisomycin (ANI; 160 µg/µl), autocamptide-2 related inhibitor peptide (AIP; 5 nmol/µl), dynasore hydrate (DYN; 120 pmol/µl), lidocaine (LID; 4%), muscimol (MUS; 0.1 µg/µl) and recombinant human BDNF (BDNF; 0.25 µg/µl) were purchased from Sigma-Aldrich. Pep2m (PEP; 5 pmol//µl), myristoylated zeta-inhibitory peptide (ZIP; 1 nmol/µl) and myristoylated scrambled ZIP (Scr-ZIP; 1 nmol/µl) were purchased from Tocris. PKMζ antisense (ASO; 5′-CTCTTG GGA AGGCATGA-3′; 2 nmol/µl) and PKMζ scrambled antisense oligonucleotides (sASO; 5′-AACAATGGGTCGTCTCG-3′; 2 nmol/µl) were from GBT-Oligos. ASO and sASO were phosphorothioated on the three terminal bases to avoid nuclease degradation. sASO had ASO base composition in a scrambled order and did not match any mammalian sequence in the GenBank database. ICAP (0.01-1 nmol/µl) was a generous gift from Dr. Robert V. Farese, University of South Florida). Drugs and oligonucleotides 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; VEH). At the time of drug delivery, infusion cannulas were fitted into the guides and injections (1 µl/side at a rate of 0.5 µl/min) carried out using a 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. All infusions took place in a room separated from the experimental room and anteroom.

Immunoblotting

Animals were killed by decapitation without anesthesia and immediately thereafter the CA1 region of the dorsal hippocampus was dissected out and homogenized in ice-chilled homogenization buffer (10 mM Tris-HCl, pH 7.4, containing 0.32 M sucrose, 1 mM EDTA, 1

mM EGTA, 1 mM PMSF, 10 µg/ml aprotinin, 15 µg/ml leupeptin, 10 µg/ml bacitracin, 10 µg/ml pepstatin, 50 mM NaF, and 1 mM sodium orthovanadate). Dorsal CA1 microdissection was carried out on an ice-chilled surface with the help of a stereo zoom microscope. Subcellular fractions were prepared at 4° C. To do that, hippocampal homogenates were centrifuged at 1,000xg for 10 min and the supernatant transferred to a new tube for a second centrifugation round at 10,000xg during 15 min to obtain a crude synaptosomal fraction (CSF) enriched in synaptic plasma membranes. The CSF pellet was washed twice and finally resuspended in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 µg/ml aprotinin, 15 µg/ml leupeptin, 10 µg/ml bacitracin, 10 µg/ml pepstatin, 50 mM NaF, and 1 mM sodium orthovanadate. Protein concentration was determined using the BCA protein assay kit (Pierce). SDS/PAGE was performed under reducing conditions and proteins were electro-transferred onto PVDF membranes (Immobilon-P, Merck Millipore). Blots were blocked for 2 h in TTBS (100 mM Tris-HCl, 0.9% NaCl, 0.1% Tween 20, pH 7.4) and incubated overnight at 4 °C with anti-pThr286CaMKII (1:5,000 dilution; rabbit polyclonal; Cell Signaling), anti-pThr305CaMKII (1:5,000 dilution; rabbit polyclonal; Sigma), anti-αCaMKII (1:5,000 dilution; goat polyclonal; Santa Cruz), anti-PKMζ (1:80,000 dilution; rabbit polyclonal; Sigma), anti-GluA1 (1:10,000 dilution; rabbit polyclonal; Santa Cruz), anti-GluA2/3 (1:10,000 dilution; rabbit polyclonal; Santa Cruz) or anti-β-tubulin (1:40,000; rabbit polyclonal; Abcam) antibodies. Then, blots were washed with TTBS and incubated with HRP-coupled anti-IgG antibody. West-Pico enhanced chemiluminescence kit (GE Healthcare) was used to detect immunoreactivity. Blots were quantified using Amersham Imager 600 RGB system (GE Healthcare).

Electrophysiological recordings

Local field potential (LFP) were recorded continuously using a Cerebus Neural Signal Processor system (Blackrock Microsystems). Signals were amplified, digitized, filtered at cut-off frequencies of 0.3 Hz and 250 Hz and sampled at 1 kHz. Data were analyzed offline using built-in (Signal Processing Toolbox) and custom written routines in MATLAB

(RRID:SCR_001622). The CA1 pyramidal cell layer was identified by stereotaxic coordinates and standard electrophysiological parameters (Brankack et al., 1993; Bragin et al. 1995), as described in Radiske et al., 2017a. To evaluate hippocampal oscillatory activity during object exploration periods, LFPs were continuously recorded throughout training and test sessions in the NOR task. Digital video cameras fixed above the arenas were used for tracking the animal's position. Video data were acquired at 30 frames/s. Active exploration was defined as sniffing and touching the stimuli objects with the muzzle and/or forepaws, and was detected using ObjectScan system software (CleverSys Inc). LFP epochs of object A active exploration were extracted, merged and analyzed (Figure 6A shows a schematic diagram of the procedure). Data from adjacent exploration epochs separated by less than 0.5 s were merged and considered as a single event. Data from exploration periods < 0.5 s were excluded from analysis. We used the Welch periodogram method (3 s Hamming windows, 75% overlap) for power spectra computing. Band power of theta, beta and slow gamma were defined as the average power in the frequency range of 5-10 Hz, 13-20 Hz and 30-55 Hz, respectively. To determine power changes during object A exploration in training (TR) and test sessions, we normalized data from each subject using a power ratio that indicates power per unit frequency normalized by power during 60 s-long baseline epoch. Baseline LFPs were acquired in the recording cage 30 min before TR and test sessions. For cross-frequency coupling analysis, theta phases and slow gamma amplitude were computed from the Hilbert transform of the filtered versions of each frequency band. Theta phases were binned into 18 intervals of 20°. Mean gamma amplitude was computed for each theta phase bin and normalized by the sum of amplitude values over all bins. We used the modulation index (MI) to express coupling strength. MI 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 defined as the average MI values in the (5-10 Hz) x (30-55

Hz) regions of the comodulation maps. MIs were calculated from 10 s-long epochs of object A exploration with equalized theta and gamma power between training and test sessions, to ensure that changes in MI values were not a result of differences in theta or gamma power. A 10-s long period was chosen given that it is long enough to average out fluctuations in gamma amplitude that are not coupled to the theta cycle (Zhang et al., 2016). Slow gamma events, defined as time intervals when gamma power surpassed by 2 SD the time-averaged power, were identified and the theta phase associated with them was determined. Time intervals with power above 6 SD were excluded to avoid analysis of artefactual events. Episodes 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. To evaluate the effect of intra-CA1 ZIP infusion (1 nmol/µl) on spontaneous hippocampal oscillatory activity, we recorded LFPs for 30 min before drug infusion (baseline) and for ~60 min after intra-CA1 administration of VEH, ZIP or LID. In this analysis, we evaluated changes in theta, beta and the entire gamma range, defining gamma band power as the average power in the frequency range of 30-90 Hz. Band power and LFP root mean square (RMS) were computed using epochs of 5 min corresponding to baseline, 15 min and 60 min after drug infusion. To construct spectrograms, power spectrum was computed using sliding windows of 4 s and 2 s steps. Multi-unit activity (MUA) was estimated by bandpass filtering (500-3000 Hz) recordings sampled at 30 kHz, and computing RMS, as described in Sharma et al., 2012. To evaluate the effect of intra-CA1 drug infusions, we computed the ratio of post-infusion to pre-infusion hippocampal neural activity.

Data analysis

Statistical analyses were performed using GraphPad Prism 6 software (RRID:SCR_002798). Significance was set at $p < 0.05$. NOR data were analyzed using one-sample t test with theoretical mean = 0, unpaired Student's t test, or one-way/two-way ANOVA followed by

306 Bonferroni's multiple comparisons. MWM data were analyzed using one-sample t test with
307 theoretical mean = 25 and unpaired Student's t test. IA data were analyzed using two-tailed
308 Mann–Whitney U test. Data from immunoblots were analyzed using repeated-measures
309 ANOVA followed by Bonferroni's multiple comparisons test or Student's t test.
310 Electrophysiological data were analyzed using repeated-measures two-way ANOVA
311 followed by Bonferroni's multiple comparison test, Student's t-test, one-sample t-test with
312 theoretical mean = 1, or Rayleigh test.
313
314 *Cannula and electrodes placement*
315 To verify cannula placement, 2-4 h after the end of the behavioral tests, we infused 1 µl of
316 4% methylene-blue as described above, and 30 min thereafter animals were sacrificed. The
317 dye spread was taken as indication of the diffusion of the drug previously injected. Only data
318 from animals with correct cannula implants (96%) were included in statistical analysis. To
319 verify electrodes placement, animals were anesthetized and perfused intracardially with 4%
320 paraformaldehyde (pH 7.2). Immediately after that, brains were removed and left in 30%
321 sucrose for 48 h before cutting into coronal sections (50 µm). Relevant slices were stained
322 with cresyl violet to confirm electrode tracks.
323
324 **Results**
325 **ORM reconsolidation requires PKMζ activity in the dorsal hippocampus.**
326 To analyze the effect of hippocampal PKMζ inhibition on ORM reconsolidation, we trained
327 naïve adult male Wistar rats in a novel object recognition (NOR) task involving exploration of
328 two different, but behaviorally equivalent novel objects (objects A and B) in an open-field
329 arena. In this task, the hippocampus is engaged in reconsolidation only when the
330 consolidated recognition trace is reactivated in the presence of a novel object (Rossato et
331 al., 2007). Therefore, to induce hippocampus-dependent ORM reconsolidation, one day after
332 training we re-exposed the animals for five minutes to one of the objects presented during
333 the training session (object A) together with a novel object C to reactivate the ORM trace.

Immediately after that, the animals received bilateral intra-CA1 infusions of vehicle (VEH; saline), the PKMζ inhibitor, zeta inhibitory peptide (ZIP; 1 nmol/µl; Parsons and Davis, 2011), or its inactive analog (Scr-ZIP; 1 nmol/µl). ORM retention was tested 24 h later. To do that, we randomly assigned the animals to different experimental groups and exposed them to a novel object D in the presence of familiar object A, B or C. Rats that received VEH or Scr-ZIP immediately after ORM reactivation discriminated novel object D from objects A, B and C during the retention session, reflecting that they remembered objects A and B, which had been presented during the training session, and also that they acquired memory for object C during the reactivation session. Conversely, animals that received ZIP right after ORM reactivation did not discriminate objects A and C from object D, indicating that ZIP hampered retention of the memory for object A and blocked formation of the memory for object C. Memory for familiar object B, which was absent during the reactivation session, was unaffected by ZIP (Figure 1a; Test AD: $F(2, 25) = 9.334$, $p = 0.0009$ for treatment effect; $t(25) = 3.667$, $p < 0.01$ for VEH vs ZIP; $t(25) = 3.842$, $p < 0.01$ for Scr-ZIP vs ZIP; Test CD: $F(2, 25) = 7.078$, $p = 0.0037$ for treatment effect; $t(25) = 3.667$, $p < 0.01$ for VEH vs ZIP; $t(25) = 3.842$, $p < 0.01$ for Scr-ZIP vs ZIP). ZIP also hindered memory for objects A and C when given 6 h, but not 12 h, after reactivation (Figure 1b, Left panel; Test AD: $t(16) = 3.390$, $p < 0.01$; Test CD: $t(15) = 3.864$, $p < 0.01$). The amnesia caused by ZIP was independent of the time elapsed between training and reactivation sessions and between reactivation and retention test sessions (Figure 1c, Left panel; Test AD: $t(18) = 2.199$, $p < 0.05$; Test CD: $t(18) = 2.382$, $p < 0.05$; Right panel; Test AD, $t(18) = 5.243$, $p < 0.001$; Test CD, $t(18) = 4.316$, $p < 0.001$). ZIP did not affect memory for object A when given immediately after ORM reactivation with two familiar objects (Figure 1d), or when injected either after exploration of the training arena in the absence of stimuli objects (Figure 1e) or in the presence of two novel ones (Figure 1f). Intra-CA1 administration of ZIP immediately after memory reactivation in the presence of a novel object did not impair ORM retention when tested three hours later (Figure 1g). Moreover, pre-reactivation intra-CA1 infusion of muscimol (MUS; 0.1µg/µl) impeded both ORM retrieval and the amnesia caused by ZIP (Figure 1h; RA

session: t(46) = 6.734, p < 0.0001; Test session: F(1, 44) = 9.335, p = 0.0038 for pre-reactivation treatment effect; F(1, 44) = 6.975, p = 0.0114 for post-reactivation treatment effect; F(1, 44) = 13.90, p = 0.0005 for interaction; t(44) = 4.505, p < 0.001 for VEH-VEH vs VEH-ZIP; t(44) = 4.028, p < 0.01 for MUS-VEH vs VEH-ZIP; t(44) = 4.797, p < 0.001 for VEH-ZIP vs MUS-ZIP), while ORM reactivation in the presence of a novel object, but not in the presence of two familiar ones, increased PKMζ levels (Figure 1i; F(5, 15) = 4.300, p = 0.0126 for treatment effect; t(15) = 4.130, p < 0.01 TR vs 360 min post-RA). Importantly, ZIP had no effect on ORM when given in dorsal CA1 immediately, 24 h or 168 h after training in the absence of specific contextual stimuli (Figure 1j). These results demonstrate that ORM is immune to intra-hippocampal ZIP administration up to seven days posttraining, unless it is reactivated in the presence of a novel object. The amnesia caused by ZIP took more than three hours to develop, was contingent on ORM reactivation in the presence of a novel object, and specific for that object and the familiar one present during the reactivation session, suggesting that PKMζ is necessary to update ORM through reconsolidation but not to consolidate or maintain the dormant recognition trace.

ZIP does not affect spontaneous oscillatory activity or basal CaMKII autophosphorylation.

There is an ongoing debate over whether the effect of ZIP on memory is due to PKMζ inhibition or results from nonspecific mechanisms of action (Lisman, 2012; 2017). Actually, PKMζ knockout mice can form ZIP-sensitive memories (Volk et al., 2013; Lee et al., 2013), and ZIP induces seizures and reduces spontaneous hippocampal local field potentials (LFP) in anesthetized rats when used at concentrations 10 or more times higher than in our experiments (LeBlancq et al., 2016). At high concentrations, ZIP can also inhibit CaMKII and PKCι/λ (Pastalkova et al., 2006; Jalil et al., 2015), which can control AMPAR function and transport, and compensate for PKMζ in PKMζ knockout mice, respectively (Ren et al., 2013; Tsokas et al., 2016). At the dose we used in our experiments (1 nmol/µl), we did not observe seizure-like episodes following ZIP administration. Moreover, amnesic animals that received

ZIP after ORM reactivation were later able to acquire and express a spatial preference in the water maze (Figure 2a, Left panel - Training: $F(1, 16) = 1.427$, $p = 0.2496$ for treatment effect, $F(4, 64) = 31.45$, $p < 0.0001$ for session effect) and to learn an inhibitory avoidance response (Figure 2a, Right panel), two memory types that require the integrity of the hippocampal formation (Martin et al., 2005; Paratcha et al., 2000), suggesting that ZIP did not trigger any lingering deleterious effect on hippocampal function.

Analysis of spontaneous hippocampal oscillatory activity in freely moving animals after ZIP (1 nmol/µl) or lidocaine (LID, 4%) administration showed that LID reduced LFP power and root mean square values 15 min post-infusion but ZIP did not modify ongoing LFP (Figures 2b to 2e; Theta: $F(2, 18) = 20.49$, $p < 0.0001$ time effect, $F(2, 9) = 4.450$, $p = 0.0453$ treatment effect; $t(18) = 4.857$, $p < 0.001$ for LID 15' vs baseline; Beta: $F(2, 18) = 9.069$, $p = 0.0019$ time effect, $F(2, 9) = 1.940$, $p = 0.1993$ treatment effect; $t(18) = 3.465$, $p < 0.01$ LID 15' vs baseline; RMS: $F(2, 18) = 21.36$, $p < 0.0001$ for time effect, $F(2, 9) = 3.107$, $p = 0.094$ for treatment effect, $F(4, 18) = 7.893$, $p = 0.0007$ for interaction; $t(18) = 5.364$, $p < 0.001$ for LID 15' vs baseline). ZIP did not affect theta, beta and gamma peak frequencies (Figure 2g) or multi-unit activity either (Figure 2h). In addition, immunoblots of dorsal CA1 homogenates showed that intra-CA1 infusion of ZIP (1 nmol/µl) did not alter basal αCaMKII auto-phosphorylation levels (Figure 2i).

PKMζ knock-down but not PKCι/λ inhibition mimics the amnesic effect of ZIP.

We also found that intra-CA1 administration of the PKCι/λ inhibitor ICAP (0.01-1 nmol/ µl; Tsokas et al., 2016) immediately after ORM reactivation had no effect on retention (Figure 3a), suggesting that the amnesia caused by ZIP was indeed triggered by PKMζ inhibition. Supporting this claim, knocking-down hippocampal PKMζ expression with antisense oligodeoxynucleotides (ASO; 2 nmol/µl) mimicked the amnesic effect of ZIP. PKMζ scrambled antisense oligonucleotides (sASO; 2 nmol/µl) did not affect ORM (Figure 3b; $t(14) = 3.227$, $p = 0.0061$ for sASO vs ASO).

PKMζ acts downstream BDNF to control AMPAR recycling during reconsolidation.

ORM reconsolidation induces LTP in the hippocampus (Clarke et al., 2010), and PKMζ sustains both LTP and non-declarative LTM by regulating N-ethylmaleimide-sensitive factor (NSF)/GluA2-dependent AMPAR trafficking (Ling et al 2006; Migues et al., 2010). Because BDNF modulates PKMζ turnover (Kelly et al., 2007) to maintain hippocampal LTP even when protein synthesis was blocked (Mei et al., 2011), and rescues reactivated ORM from the amnesia induced by protein synthesis inhibitors (Radiske et al., 2017b), we posited that BDNF controls PKMζ function to regulate AMPAR synaptic insertion during ORM reconsolidation. In agreement with this hypothesis, we found that BDNF co-infusion (0.25 µg/µl) reversed the amnesia caused by post-reactivation intra-CA1 administration of the protein synthesis inhibitor anisomycin (ANI; 160 µg/µl), as previously shown (Radiske et al., 2017b), but not that induced by ZIP (Figure 4a; $F(5, 50) = 11.11$, $p < 0.001$ for treatment effect; $t(50) = 4.468$, $p < 0.001$ for VEH vs ANI; $t(50) = 4.051$, $p < 0.01$ for VEH vs ZIP; $t(50) = 3.289$, $p < 0.05$ for VEH vs BDNF-ZIP; $t(50) = 4.820$, $p < 0.001$ for ANI vs BDNF-VEH; $t(50) = 4.993$, $p < 0.001$ for ANI vs BDNF-ANI; $t(50) = 4.407$, $p < 0.001$ for ZIP vs BDNF-VEH; $t(50) = 4.575$, $p < 0.001$ for ZIP vs BDNF-ANI; $t(50) = 3.665$, $p < 0.01$ for BDNF-VEH vs BDNF-ZIP; $t(50) = 3.833$, $p < 0.01$ for BDNF-ANI vs BDNF-ZIP).

We also found that Pep2m (PEP; 5 pmol/µl), a peptide that disrupts NSF/GluA2 interaction and reduces AMPAR surface expression (Ralph et al., 2001), was amnesic when given in dorsal CA1 immediately after ORM reactivation in the presence of a novel object (Figure 4b; Test AD: $t(16) = 3.430$, $p = 0.0034$; Test CD: $t(16) = 3.084$, $p = 0.0071$). Conversely, post-reactivation intra-CA1 infusion of dynasore (DYN; 120 pmol/µl), a cell-permeable dynamin inhibitor that impedes AMPAR internalization (Ferreira et al., 2015) and increased GluA1 and GluA2 expression in a crude synaptosomal fraction (CSF) enriched in synaptic-associated proteins (Figure 4c; Left panel – GluA1: $t(3) = 3.879$, $p = 0.0303$ for VEH vs DYN; GluA2: $t(3) = 6.635$, $p = 0.007$ for VEH vs DYN; Right panel – $t(2) = 8.217$, $p = 0.014$ for HT vs CSF) reversed the amnesia caused by ZIP (Figure 4d; $F(3, 34) = 8.496$, $p = 0.0002$ for treatment effect; $t(34) = 3.962$, $p < 0.01$ for VEH vs ZIP; $t(34) = 4.152$, $p < 0.01$ for ZIP vs DYN; $t(34) =$

446 4.130, $p < 0.01$ for ZIP vs ZIP-DYN). Moreover, ORM reactivation in the presence of a novel
447 object, but not in the presence of two familiar ones, increased CSF-associated GluA1 and
448 GluA2 levels in dorsal CA1 (Figure 4e; GluA1: $F(5, 15) = 4.113$, $p = 0.0149$ for treatment
449 effect; $t(15) = 3.276$, $p < 0.05$ for TR vs RA180; GluA2: $F(5, 15) = 7.096$, $p = 0.0014$ for
450 treatment effect; $t(15) = 3.632$, $p < 0.05$ for TR vs RA360), an effect prevented by ZIP and
451 PEP (Figure 4f; GluA1: $F(3, 9) = 9.567$, $p = 0.0037$ for treatment effect; $t(9) = 3.529$, $p < 0.05$
452 for VEH vs TR; $t(9) = 4.108$, $p < 0.01$ for VEH vs ZIP; $t(9) = 4.985$, $p < 0.01$ for VEH vs PEP;
453 GluA2: $F(3, 9) = 8.365$, $p = 0.0057$ for treatment effect; $t(9) = 4.868$, $p < 0.01$ for VEH vs TR;
454 $t(9) = 3.019$, $p < 0.05$ for VEH vs ZIP; $t(9) = 3.421$, $p < 0.05$ for VEH vs PEP).
455
456 **Relearning after ZIP-induced amnesia recapitulates ORM consolidation.**
457 The question of whether the amnesia caused by reconsolidation impairment results from
458 permanent storage deficit or reversible retrieval failure remains unsolved (Lee et al., 2017).
459 Interestingly, research on the nature of the amnesia triggered by PKMζ inhibition has caused
460 similar dispute (Glanzman, 2013). We reasoned that if impairment of ORM reconsolidation
461 with ZIP erased the recognition trace, subsequent re-presentation of the forgotten object
462 should induce repetition of the consolidation process because the animal would have to
463 relearn that object anew. Therefore, treatments able to specifically hinder consolidation
464 should block ORM reacquisition upon retraining. On the contrary, if the amnesic effect of ZIP
465 were due to retrieval impairment, retraining should not induce repetition of consolidation, and
466 so consolidation blockers would not affect ORM reinstatement. To tackle this problem, we
467 took advantage of the fact that ORM consolidation, but not reconsolidation, requires protein
468 synthesis in the entorhinal cortex (Lima et al., 2009; Figures 5a and 5b). We found that
469 animals rendered amnesic by ZIP infused into dorsal CA1 after ORM reactivation reacquired
470 memory for forgotten object A upon retraining. However, consistent with the notion that
471 PKMζ inhibition erases the reactivated recognition trace, intra-entorhinal cortex injection of
472 ANI after retraining abolished relearning, as if memory for object A had to be consolidated
473 again (Figure 5c; Test 1: $t(34) = 4.286$, $p = 0.0002$ for VEH vs ZIP; Test 2: $F(1, 32) = 15.29$,

p = 0.0005 for pre-reactivation treatment effect; F(1, 32) = 14.32, p = 0.0006 for post-reactivation treatment effect; F(1, 32) = 6.661, p = 0.00146 for interaction; t(32) = 5.441, p < 0.001 for VEH-VEH vs ZIP-ANI; t(32) = 4.501, p < 0.001 for ZIP-VEH vs ZIP-ANI; t(32) = 4.590, p < 0.001 for VEH-ANI vs ZIP-ANI).

To further investigate if the amnesia caused by ZIP returned the amnesic brain to its pre-learning state, we analyzed whether relearning following ZIP-induced reconsolidation impairment recapitulated the electrophysiological correlates of ORM acquisition. It is known that declarative memory acquisition increases theta and slow-gamma power in the hippocampus, while retrieval is usually linked to theta/slow-gamma cross-frequency coupling (Chang and Huerta, 2012; Colgin, 2015; Trimper et al., 2017).

ORM acquisition augmented theta (5-10 Hz) and slow-gamma (30-55 Hz) band power without modifying beta oscillations (13-20 Hz), theta/slow-gamma coupling or slow-gamma amplitude distribution over theta phases in dorsal CA1 (Figures 6a to 6d; t(9) = 3.477, p = 0.0070 for theta power; t(9) = 3.316, p = 0.0090 for slow gamma power). On the contrary, ORM retrieval did not modify theta amplitude but increased beta (13-20 Hz) and slow gamma power (Figures 6a to 6d; t(4) = 4.453, p = 0.0112 for beta power; t(4) = 8.340, p = 0.0011 for slow gamma power). We also found that ORM retrieval induced strong theta/slow-gamma coupling (Figures 6e to 6g; t(8) = 3.033, p = 0.0162) with slow gamma events concentrated on the late ascending portion of the theta cycle (Figure 6h; 328.5° ± 38.84; non-uniform phase distribution, z = 3.98, p = 0.0186). In keeping with the hypothesis that impairing reconsolidation through PKMζ signaling disruption deletes the reactivated ORM trace, animals rendered amnesic with ZIP showed prominent theta and slow gamma oscillatory activity as well as low modulation indexes during a retraining session (Figures 6d and 6g; t(4) = 2.999, p = 0.0400 for theta power; t(4) = 10.86, p = 0.0004 for slow gamma power), resembling the oscillatory pattern typical of the ORM acquisition process. During active exploration periods, rats remained motionless or moved slowly (mean speed < 6 cm/s).

Discussion

Our findings confirm that PKMζ plays a prominent role in mnemonic processing and endorse the hypothesis that its inhibition deletes memory (Pastalkova et al., 2006). However, our data also indicate that hippocampal PKMζ activation mediates ORM reconsolidation rather than ORM maintenance. This is in agreement with a previous report showing that intra-hippocampal ZIP does not affect ORM consolidation (Hardt et al., 2010) but at odds with extensive literature showing that PKMζ is essential for storage of different memory types from mollusks to mammals (Sacktor, 2012).

ZIP has been crucial to study the participation of PKMζ on memory (Sacktor and Hell, 2017). Nonetheless, ZIP specificity has been questioned by studies showing that at high concentrations it impairs plasticity in PKMζ knockout mice, suppresses brain oscillations, and hinders CaMKII/PKCι/λ activity (Kwapis and Helmstetter, 2014). We overcame these confounders using a dose of ZIP much lower than that typically employed, and performing a series of control experiments showing that in our preparation ZIP did not alter spontaneous oscillatory activity or basal CaMKII autophosphorylation, and that its amnesic effect was mimicked by PKMζ antisense oligonucleotides but not by PKCι/λ inhibition.

Regardless of the suggestion that low doses of ZIP may selectively disrupt reactivated associations (Sacktor, 2012), it is unlikely that the differential effect of ZIP on ORM reconsolidation and consolidation that we observed was due to the low concentration of ZIP we used because reconsolidation is usually less sensitive than consolidation to metabolic inhibitors (Debiec et al., 2002). Moreover, it was previously shown that ZIP does not impair ORM consolidation when injected in the hippocampus at 10X the concentration we utilized in our experiments (Hardt et al., 2010).

The lack of effect of ZIP in ORM consolidation does not mean that the hippocampus does not participate in this process. Although it is true that, based mainly on lesions studies, it has previously been suggested that the involvement of the hippocampus in ORM is restricted to the processing of spatial and contextual information instead of recognition memory per se (Ainge et al., 2006), it has lately become clear that this region is critical for ORM

consolidation, even when all spatial and contextual components of the task are omitted (Stackman et al., 2016; Liu et al., 2016; Lymer et al., 2017; Canto de Souza et al., 2017; Cercato et al., 2017). This is particularly evident when rodents are exposed to two identical or two different but behaviorally equivalent objects in a familiar open-field arena. Under these training conditions, temporary inactivation of the hippocampus hinders ORM consolidation (de Lima et al., 2006), which requires protein synthesis, NMDAR, and proteasome activation (Rampon et al., 2000; Baker and Kim, 2002; Figueiredo et al., 2015), and increases hippocampal c-fos (Tanimizu et al., 2017) and extracellular glutamate levels (Cohen et al., 2013). In any case, we found that 1 nmol/µl ZIP was sufficient to prevent acquisition of the memory for a new object, provided that this object was presented alongside a familiar one. This result not only indicates that the dose of ZIP we employed is enough to inhibit PKMζ activity, but also that reconsolidation, and not consolidation mechanisms, brings about active ORM updating in the hippocampus, which is of the outmost importance, inasmuch as it has been reported that the opposite is true for non-declarative memories (Tronel et al., 2005). Nevertheless, the majority of literature demonstrates that ZIP causes amnesia in the absence of memory reactivation, suggesting that the synaptic plasticity involved in ORM is different from that in most other forms of memory. Indeed, unlike the rapid effect that it causes on the maintenance of other memory types (Pastalkova et al., 2006; Hardt et al., 2010), ZIP administration following ORM reactivation results in delayed amnesia. In fact, ORM processing, and in particular ORM reconsolidation, depends on the integrity of a rather specific set of pathways and networks that span various brain regions and their interactions (Winters et al., 2011; Rossato et al., 2013; 2015). For example, contrary to other memory types (Lee et al., 2004), ORM reconsolidation requires hippocampal BDNF signaling (Radiske et al., 2017b), which, as demonstrated here, controls integration of new information into active ORM through a protein synthesis-independent mechanism acting upstream PKMζ to modulate hippocampal AMPAR synaptic insertion, in agreement with findings showing that ORM reconsolidation induces synaptic potentiation in the hippocampus (Clarke et al., 2010) and that BDNF facilitates LTP in the absence of

protein synthesis through PKMζ (Mei et al., 2011; Schuette et al., 2016). Therefore, our results should not be generalized to indicate that all memories are sensitive to PKMζ inhibition only when reactivated. However, future studies might benefit from taking into account the possibility that, besides impairing memory maintenance, PKMζ inhibition can also affect memory reconsolidation. This suggestion may be particularly useful for studies dealing with recent memories, as memories become increasingly resistant to reconsolidation with age (Frankland et al., 2006), as well as for those analyzing memories that are sensitive to PKMζ blockers and reconsolidation inhibitors at the same posttraining times. It is important to stress that reconsolidation can result not only from exposure to obvious reminders but also to inconspicuous signals unable to produce discernible behavioral outputs (Gisquet-Verrier and Riccio, 2012; Soeter and Kindt, 2015). On this matter, the only other study that analyzed the involvement of PKMζ in reconsolidation thoroughly showed that retrieval protects conditioned taste aversion memory (CTA) from the amnesic effect of intra-insular cortex (IC) ZIP administration during the same amount of time it takes ZIP to be able to impair consolidation, supposedly as a result of reconsolidation induction (Levitan et al., 2016). It is necessary to point out though that the differences between CTA and ORM are so fundamental that such contradictory findings should come as no surprise. CTA is a nondeclarative memory prone to be extinguished upon retrieval (Maroun et al., 2012), whereas ORM is an inextinguishable declarative memory. Thus, CTA non-reinforced reactivation may induce two competing, opposite and overlapping processes requiring IC protein synthesis, and therefore, different to ORM, the effect of post-reactivation PKMζ inhibition on CTA persistence cannot be unequivocally attributed to either reconsolidation or extinction modulation. Moreover, CTA maintenance requires IC PKMζ activity late after training, but ORM consolidation is unaffected by hippocampal PKMζ inhibition, which precludes ORM reconsolidation and ZIP-sensitive mechanisms related to ORM persistence from being mutually exclusive, as proposed for CTA.

A great deal of effort has been dedicated to determine if forgetting caused by inhibition of memory maintenance mechanisms is due to storage or retrieval failure. Most studies on the

586 subject involve treating animals with agents that affect the mechanisms under scrutiny at
587 different post-learning or post-retrieval times and later, once the agent has washed-out,
588 verify the occurrence of amnesia, evidenced by impaired performance. If amnesia is
589 observed, then some intervention is given, usually in the form of a reminder cue, and
590 performance reassessed in a subsequent retention test. If memory recovers, retrieval failure
591 is assumed to be responsible for amnesia. On the contrary, absence of recovery is
592 commonly accepted as evidence of storage impairment. However, absence of recovery does
593 not unequivocally demonstrate memory erasure because it could also reflect failure to
594 retrieve an otherwise intact memory (Hardt et al., 2009). Besides, a memory that cannot be
595 retrieved at a given time can theoretically be retrieved later. Successful memory recovery
596 does not prove retrieval failure either, because reminder cues may cause apparent recovery
597 by promoting new learning indistinguishable form the original one. This matter is especially
598 important in the case of PKMζ inasmuch as it has been suggested that its effect on memory
599 maintenance results from memory erasure rather than retrieval impairment because the
600 amnesia caused by PKMζ inhibition persists even after removal of its inhibitor (Pastalkova et
601 al., 2006). To investigate this issue for the amnesia caused by the intrahipocampal
602 administration of ZIP following ORM reactivation in the presence of a novel object we used a
603 twofold strategy. Firstly, we exploited the fact that the rats' brain engages different
604 mechanisms to consolidate and to reconsolidate ORM. ORM consolidation requires protein
605 synthesis in EC whereas ORM reconsolidation does not. Therefore, we analyzed whether
606 rats that received intra-hippocampal ZIP after ORM reactivation were able to reacquire ORM
607 upon retraining and, if so, whether EC protein synthesis inhibition blocked reacquisition. The
608 result of this experiment was straightforward. Relearning was blocked by ANI given in EC
609 after retraining as if the amnesic animals were consolidating the forgotten ORM again.
610 Secondly, we analyzed whether ORM reacquisition recapitulates the eletrophysiological
611 signature of original learning. To do that, we took advantage of the fact that ORM acquisition
612 and retrieval are associated to different hippocampal oscillatory patterns. Again, the result
613 was clear-cut. During retraining, the oscillatory pattern of animals that received ZIP after

614 ORM reactivation was indistinguishable from that recorded during original training, as if
615 these animals were learning the forgotten object anew.
616 In conclusion, our results show that hippocampal PKMζ acts downstream BDNF to regulate
617 AMPAR recycling and ORM updating during reconsolidation and indicate that its inhibition
618 deletes the reactivated memory trace.
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941 **Legends**

942 **Table 1. Objects utilized in the NOR task have no natural significance and are**
943 **behaviorally equivalent for naïve adult male Wistar rats.** Mean exploration time and
944 discrimination index (±SEM) for naïve animals during spontaneous object exploration in the
945 training session of the NOR task. Total exploration time did not differ between object pairs (F
946 (9, 86) = 0.5161, P = 0.8592). Discrimination indexes for each object pair exploration are
947 shown (p# in one-sample Student's t-test with theoretical mean = 0).

948

949 **Table 2. Adult male Wistar rats trained in the NOR task discriminate between novel**
950 **and familiar objects during the whole retention test session.** Mean ±SEM discrimination
951 index (DI) and total exploration time for each consecutive minute of a 5-min-long ORM
952 retention test session in the presence of a familiar (A) and a novel object (C) carried out 24 h
953 after training in the presence of two different novel objects A and B. p# in one-sample
954 Student's t-test with theoretical mean = 0, n = 50.

955

956 **Figure 1. ZIP impairs ORM reconsolidation but does not affect ORM consolidation. A.**
957 Rats were trained in NOR using two different objects (A and B - Day 1 - TR) and 24 h later
958 (Day 2) submitted to an ORM reactivation session (RA) in the presence of familiar object A
959 and novel object C. Immediately after RA, animals received intra-CA1 infusions of vehicle
960 (VEH; saline), zeta inhibitory peptide (ZIP) or scrambled ZIP (Src-ZIP)**.** One day later (Day 3
961 - TEST), animals were exposed to a familiar object (A, B or C) and novel object (D) to
962 evaluate retention. Data (mean ± SEM) are presented as discrimination index during TR, RA
963 and TEST, n = 9-10 per group. Animals explored objects equally during TR (A=28.81 ± 1.14
964 s, B=30.6 ± 1.13 s, t (83) = 1.548, p = 0.125), and discriminated them during RA (A=21.29 ±
965 0.961 s, C=35.74 ± 1.285 s, t (83) = 19.10, p < 0.001). Total object exploration time did not
966 differ between TR, RA and TEST (F (2,249) = 1.257, p = 0.286). **B.** Rats were treated as in
967 A, but received intra-CA1 VEH or ZIP 6 h (left panel) or 12 h (right panel) after RA. Data
968 (mean ± SEM) are presented as discrimination index during TR, RA and TEST, n = 8-10 per

group. Animals explored objects equally during TR (Left panel: A=27.12 ± 1.68 s, B=28.85 ± 1.51 s, t (51) = 1.111, p = 0.2717; Right panel: A=31.87 ± 1.242 s, B=33.31 ± 1.31 s, t (57) = 0.967, p = 0.338), and discriminated them during RA (Left panel: A=21.84 ± 1.26 s, C=38.02 ± 1.76 s, t (51) = 12.61, p < 0.001; Right panel: A=25.60 ± 0.95 s, C=40.93 ± 1.38 s, t (57) = 16.33, p < 0.001. Total object exploration time did not differ between TR, RA and TEST (Left panel: F (2,153) = 2.357, p = 0.0981; Right panel: F (2,171) = 2.885, p = 0.0586). **C.** Rats were treated as in A, but RA was carried out 7 days after TR (left panel) or TEST was carried out 7 days after RA (right panel). Data (mean ± SEM) are presented as discrimination index during TR, RA and TEST, n = 9-10 per group. Animals explored objects equally during TR (Left panel: A=24.94 ± 1.24 s, B=26.86 ± 1.08 s, t (57) = 1.60, p = 0.115; Right panel: A=27.48 ± 1.07 s, B=28.09 ± 1.12 s, t (57) = 0.484, p = 0.63), and discriminated them during RA (Left panel: A=19.31 ± 0.71 s, C=31.14 ± 1.06 s, t (57) = 16.85, p < 0.001; Right panel: A=21.0 ± 0.77 s, C=35.23 ± 1.16 s, t (57) = 16.96, p < 0.001). Total object exploration time did not differ between TR, RA and TEST (Left panel: F (2,171) = 2.33, p = 0.101; Right panel: F (2,171) = 2.71, p = 0.067). **D.** Rats were treated as in A but RA was carried out in the presence of familiar objects A and B. Data (mean ± SEM) are presented as discrimination index during TR, RA and TEST, n = 10 per group. Animals explored objects equally during TR (A=26.89 ± 1.88 s, B=29.39 ± 1.71 s, t (19) = 1.35, p = 0.193), and did not discriminate them during RA (A=23.11 ± 1.7 s, C=24.52 ± 1.70 s, t (19) = 0.95, p = 0.354). Total object exploration time did not differ between TR, RA and TEST (F (2,57) = 1.95, p = 0.152). **E.** Rats were treated as in A but 24 h after training they explored the training arena in the absence of objects. Data (mean ± SEM) are presented as discrimination index during TR and TEST, n = 8-9 per group. Animals explored objects equally during TR (A=27.87 ± 1.30 s, B=29.37 ± 1.17 s, t (50) = 1.149, p = 0.256). Total object exploration time did not differ between TR and TEST (t (100) = 1.252, p = 0.214). **F.** Rats were treated as in A but 24 h after TR they explored two novel objects (C and D; TR2). Data (mean ± SEM) are presented as discrimination index during TR, TR2 and TEST, n = 8-10 per group. Animals explored objects equally during TR (A=31.25 ± 1.36 s, B=31.34 ± 1.43 s, t (70) = 0.0596, p = 0.953),

997 and TR2 (C=32.14 ± 1.56 s, D=30.54 ± 1.52 s, t (70) = 0.935, p = 0.353). Total object
998 exploration time did not differ between TR, TR2 and TEST (F (2,210) = 1.55, p = 0.215). **G.**
999 Rats were treated as in A but ORM was evaluated 3 h after RA. Data (mean ± SEM) are
1000 presented as discrimination index during TR, RA and TEST, n = 9-10 per group. Animals
1001 explored objects equally during TR (A=36.65 ± 3.06 s, B=34.98 ± 2.05 s, t (18) = 0.530, p =
1002 0.602), and discriminated them during RA (A=25.7 ± 2.00 s, C=42.88 ± 2.47 s, t (18) =
1003 10.07, p < 0.001). Total object exploration time did not differ between TR, RA and TEST (F
1004 (2,54) = 0.115, p = 0.892). **H.** Rats were treated as in A but 15 min before RA received intra-
1005 CA1 infusions of VEH or muscimol (MUS) and immediately after RA were given VEH or ZIP
1006 in dorsal CA1. Data (mean ± SEM) are presented as discrimination index during TR, RA and
1007 TEST, n = 12 per group. Animals explored objects equally during TR (A=22.55 ± 0.81 s,
1008 B=22.21 ± 0.73 s, t (47) = 0.402, p = 0.67). Animals that received VEH before RA (A=15.47
1009 ± 0.87 s, C=28.57 ± 1.39 s, t (23) = 7.65, p < 0.001), but not those given MUS (A=21.88 ±
1010 1.71 s, C=18.55 ± 1.46, t (47) = 1.62, p = 0.119), discriminated objects during RA. Total
1011 object exploration time did not differ between TR, RA and TEST (F (2,141) = 1.69, p =
1012 0.188). **I.** Rats were trained as in A and 24 h later submitted to RA in the presence of familiar
1013 object A and novel object C. At different times after RA animals were killed and the CA1
1014 region of the dorsal hippocampus dissected out and homogenized to determine PKMζ levels
1015 by immunoblot. PKMζ levels were also evaluated 360 min after ORM reactivation in the
1016 presence of familiar objects A and B (AB_{360}). Data are expressed as mean ± SEM, n = 4.
1017 TUB = β-tubulin. **J.** Rats were trained as in A and immediately (0 h; Left panel), 24 h (Center
1018 panel) or 168 h (Right panel) after training received intra-CA1 infusions of VEH or ZIP. ORM
1019 retention was evaluated 24 h later. Data (mean ± SEM) are presented as discrimination
1020 index during TR and TEST, n = 10-11 per group. Animals explored objects equally during TR
1021 (Left panel: A=22.86 ± 1.61 s, B=25.78 ± 2.84 s, t (19) = 1.257, p = 0.224; Center panel:
1022 A=30.7 ± 1.97 s, B=29.08 ± 1.8 s, t (19) = 0.84, p = 0.412; Right panel: A=25.78 ± 1.29 s,
1023 B=27.39 ± 1.15 s, t (21) = 1.265, p = 0.22). Total object exploration time did not differ
1024 between TR and TEST (Left panel: t (38) = 0.646, p = 0.523; Center panel: t (38) = 1.47, p =

0.149; Right panel: t (42) = 1.82, p = 0.076). In all graphs, dashed lines represent chance level; #p < 0.05 in one-sample Student's t-test with theoretical mean = 0; *p < 0.05, **p < 0.01 and ***p < 0.001 in unpaired t-test or Bonferroni's multiple-comparison test after one-way or two-way ANOVA.

Figure 2. ZIP does not affect spontaneous oscillatory activity or CaMKII autophosphorylation. A. Animals that received intra-CA1 infusions of vehicle (VEH; saline) or zeta inhibitory peptide (ZIP) immediately or 6 h after ORM reactivation were trained in the spatial version of the Morris water maze (MWM) or in the inhibitory avoidance task (IA) 48 h later. Bottom leftmost panel; escape latency during MWM training sessions (TR) shown in blocks of 8 trials/session. Bottom center panel; % of time spent in target quadrant (TQ) during a 60 s-long probe test carried out 24 h after the last training session. Data are presented as mean ± SEM, n = 9 per group. Bottom right panel; step-down latency during IA training (TR) and a retention test session (TEST) carried out 24 h later; data are expressed as median ± interquartile range, n = 8 per group **B.** LFP signals were simultaneously recorded from the hippocampus before (Baseline) and after intra-CA1 infusions of VEH, ZIP (1 nmol/µl) or lidocaine (LID, 4%). Five-min long recording periods were analyzed. **C.** Representative power spectrum density plots from animals that received VEH, ZIP or LID. **D.** Representative hippocampal raw LFP traces and spectrograms from animals that received VEH, ZIP or LID. Baseline, 15 min and 60 min post-treatment time points are shown. **E.** Normalized hippocampal theta, beta and gamma power and RMS values from animals that received intra-CA1 infusions of VEH, ZIP or LID. Baseline, 15 min and 60 min post-treatment time points are shown. **F.** Mean speed and traveled distance from animals that received intra-CA1 infusions of VEH, ZIP or LID. Baseline, 15 min and 60 min post-treatment time points are shown. **G.** Peak frequency of theta, beta and gamma oscillations from animals that received intra-CA1 infusions of ZIP. Baseline, 15 min and 60 min post-treatment time points are shown. **H.** Hippocampal multiunit activity (MUA) from animals that received intra-CA1 infusions of ZIP. Baseline, 15 min and 60 min post-treatment time points are shown.

Data from electrophysiological recordings are presented as mean ± SEM, n = 4 per group. **I.** Rats received intra-CA1 infusions of VEH or ZIP and 30 minutes thereafter were killed by decapitation, the CA1 region of the dorsal hippocampus dissected out and homogenized to determine total, pT286 and pT305 αCaMKII levels by immunoblot. Data are expressed as mean ± SEM, n = 4 per group. **p < 0.01 and ***p < 0.001 in paired t-test or Bonferroni's multiple-comparison test after one-way or two-way ANOVA, as appropriate.

Figure 3. PKMζ knockdown, but not PKCι/λ inhibition, mimics the amnesic effect of ZIP. A. Rats were trained in NOR using two different objects (A and B - Day 1 - TR) and 24 h later (Day 2) submitted to an ORM reactivation session (RA) in the presence of familiar object A and novel object C. Immediately after RA, animals received bilateral intra-CA1 infusions (1 µl/side) of vehicle (VEH) or the specific PKCι/λ inhibitor ICAP. One day later (Day 3 - TEST), animals were exposed to familiar object A and novel object D to evaluate ORM retention. Data (mean ± SEM) are presented as discrimination index during TR, RA and TEST, n = 8-9 per group. Animals explored objects equally during TR (A=25.96 ± 1.23 s, B=28.55 ± 1.68 s, t (33) = 1.15, p = 0.13), and discriminated them during RA (A=19.61 ± 1.30 s, C=32.73 ± 1.82 s, t (33) = 10.61, p < 0.001). Total object exploration time did not differ between TR, RA and TEST (F (2,99) = 0.201, p = 0.82). **B.** Rats were treated as in A but 20 min before RA received intra-CA1 infusions of PKMζ antisense oligodeoxynucleotides (ASO) or scrambled antisense oligodeoxynucleotides (sASO). Data (mean ± SEM) are presented as discrimination index during TR, RA and TEST, n = 8 per group. Animals explored objects equally during TR (A=27.1 ± 2.12 s, B=24.82 ± 3.04 s, t (15) = 1.102, p = 0.288), and discriminated them during RA (sASO - A=19.00 ± 3.32 s, C=39.06 ± 6.31 s, t (7) = 3.63, p < 0.01; ASO - A=17.74 ± 1.94 s, C=31.94 ± 3.16, t (7) = 5.84, p < 0.001). Total object exploration time did not differ between TR, RA and TEST (F (2,45) = 0.239, p = 0.788). In both graphs, dashed lines represent chance level; #p < 0.05 in one-sample Student's t-test with theoretical mean = 0; **p < 0.01 in unpaired t-test.

Figure 4. PKMζ acts downstream BDNF to regulate SPM-associated AMPAR levels during ORM reconsolidation. A. Rats were trained in NOR using two different stimuli objects (A and B - Day 1 - TR) and 24 h post-training (Day 2) submitted to a 5-min long ORM reactivation session (RA) in the presence of familiar object (A) and novel object (C). Immediately after RA, animals received intra-CA1 infusions of vehicle (VEH; saline), zeta inhibitory peptide (ZIP), anisomycin (ANI) [-BDNF] or co-infusions of BDNF with VEH, ZIP or ANI [+BDNF]. One day later (Day 3 - TEST), animals were exposed to a familiar object (A) and a novel object (D) for five extra minutes to evaluate ORM retention. Data (mean ± SEM) are presented as discrimination index during TR, RA and TEST, n = 9-10 per group. Animals explored objects equally during TR (A=28.04 ± 1.32 s, B=26.05 ± 1.04 s, $t(55) = 1.64$, $p = 0.107$), and discriminated them during RA (A=19.79 ± 0.764 s, C=33.93 ± 1.17 s, $t(55) = 17.16$, $p < 0.001$). Total object exploration time did not differ between TR, RA and TEST ($F(2,165) = 2.81$, $p = 0.063$). **B.** Animals were treated as in A, except that they received bilateral intra-CA1 infusions of VEH or Pep2m (PEP) immediately after RA. One day later, animals were exposed to a familiar object (A, B or C) and a novel object (D) for five minutes to evaluate ORM retention. Data (mean ± SEM) are presented as discrimination index during TR, RA and TEST, n = 8-9 per group. Animals explored objects equally during TR (A=28.75 ± 0.90 s, B=28.63 ± 0.96 s, $t(52) = 0.129$, $p = 0.898$), and discriminated them during RA (A=19.84 ± 0.73 s, C=33.72 ± 1.04 s, $t(52) = 18.43$, $p < 0.001$). Total object exploration time did not differ between TR, RA and TEST ($F(2,156) = 1.625$, $p = 0.20$). **C.** Left panel. Rats received intra-CA1 infusions of VEH or dynasore (DYN) and 30 minutes later were killed by decapitation, the CA1 region of the dorsal hippocampus dissected out, homogenized, and processed to purify a crude synaptosomal fraction (CSF) to determine GluA1 and GluA2 levels by immunoblot. Data are expressed as mean ± SEM, n = 4 per group. Right panel. Densitometry quantification and representative immunoblot showing the relative enrichment of PSD-95 in a CSF purified from dorsal CA1; HT=dorsal CA1 total homogenate. Data are expressed as mean ± SEM, n = 3 per group. **D**. Animals were treated as in A, except that immediately after RA they received intra-CA1 infusions of VEH, ZIP, DYN or a co-infusion of

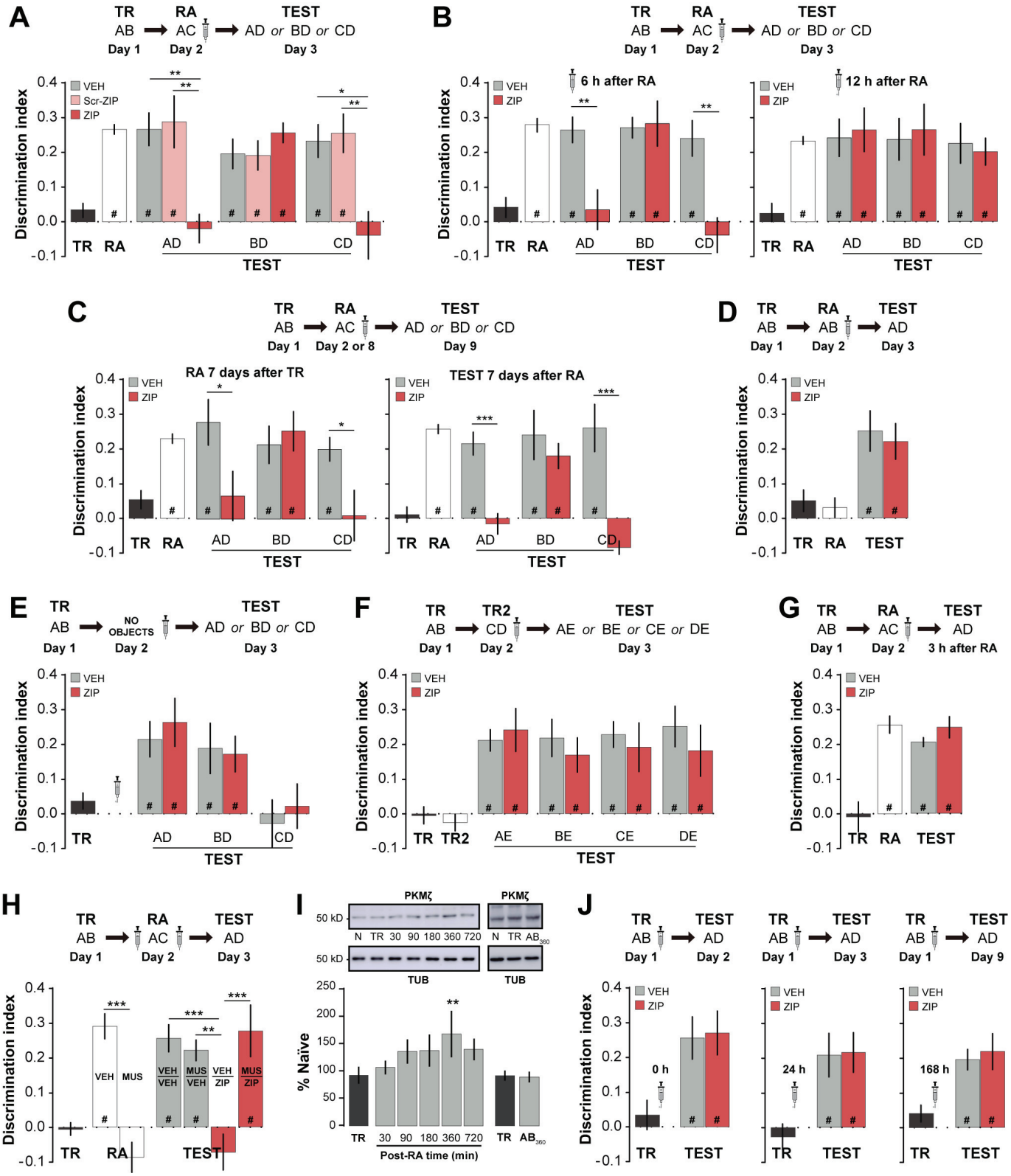
DYN and ZIP. Data (mean ± SEM) are presented as discrimination index during TR, RA and TEST, n = 9-10 per group. Animals explored objects equally during TR (A=31.04 ± 1.78 s, B=30.10 ± 1.50 s, t (37) = 0.584, p = 0.563), and discriminated them during RA (A=21.65 ± 1.56 s, C=38.62 ± 2.34 s, t (37) = 10.58, p < 0.001). Total object exploration time did not differ between TR, RA and TEST (F (2,111) = 2.455, p = 0.090). **E.** Rats were trained as in A and 24 hours later submitted to RA in the presence of a familiar object (A) and a novel object (C). At different times after RA (30, 90, 180, 360 or 720 min), animals were killed by decapitation and the CA1 region of the dorsal hippocampus dissected out, homogenized, and processed to purify a CSF to determine GluA1 and GluA2 levels by immunoblot (left panel). GluA1 and GluA2 levels were also evaluated 180 min and 360 min after ORM reactivation in the presence of two familiar objects (A and B; right panel). Data are expressed as mean ± SEM, n = 4 per group. **F.** Rats were treated as in E except that immediately after RA they received intra-CA1 infusions of VEH, ZIP or PEP and 180 min or 360 min later were killed by decapitation, the CA1 region of the dorsal hippocampus dissected out, homogenized, and processed to purify a CSF to determine GluA1 and GluA2 levels by immunoblot. Data are expressed as mean ± SEM, n = 4 per group. Dashed lines represent chance level. #p < 0.05 in one-sample Student's t-test with theoretical mean = 0. *p < 0.05, **p < 0.01 and ***p < 0.001 in paired t-test, unpaired t-test or Bonferroni's multiple-comparison test after one-way ANOVA, as appropriate. TUB = β-tubulin.

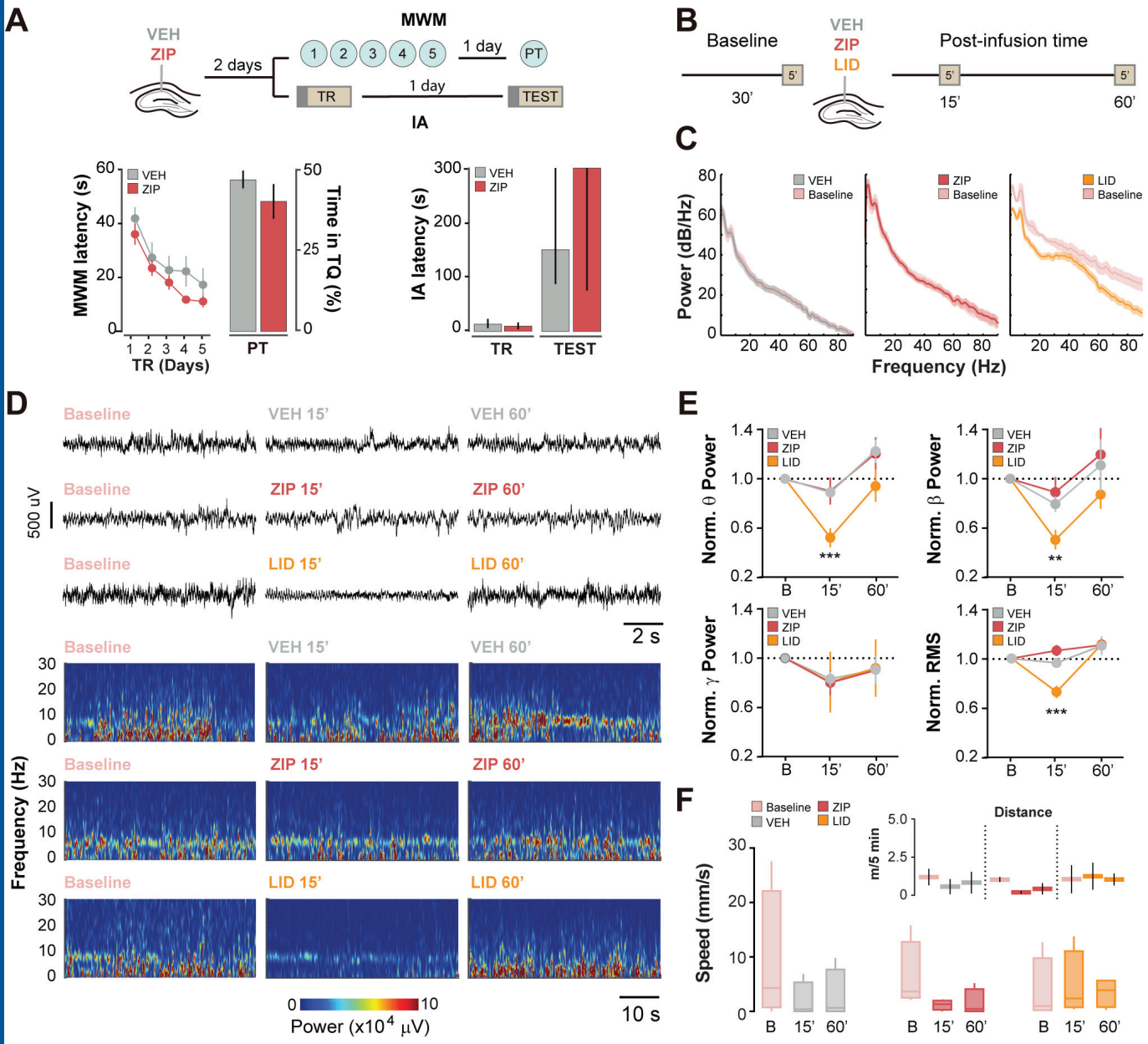
Figure 5. PKMζ inhibition erases the reactivated recognition trace. A. Rats were trained in NOR using two different stimuli objects (A and B - Day 1 - TR) and immediately thereafter received bilateral infusions of vehicle (VEH; saline) or anisomycin (ANI) into the entorhinal cortex (EC). One day later (Day 2 - TEST), animals were exposed to a familiar object (A) and a novel object (D) for five minutes to evaluate ORM retention. Data (mean ± SEM) are presented as discrimination index during TR and TEST, n = 12 per group. Animals explored objects equally during TR (A=28.7 ± 2.83 s, B=29.79 ± 3.05 s, t (23) = 0.480, p = 0.635). Total object exploration time did not differ between TR and TEST (t (46) = 0.222, p = 0.825).

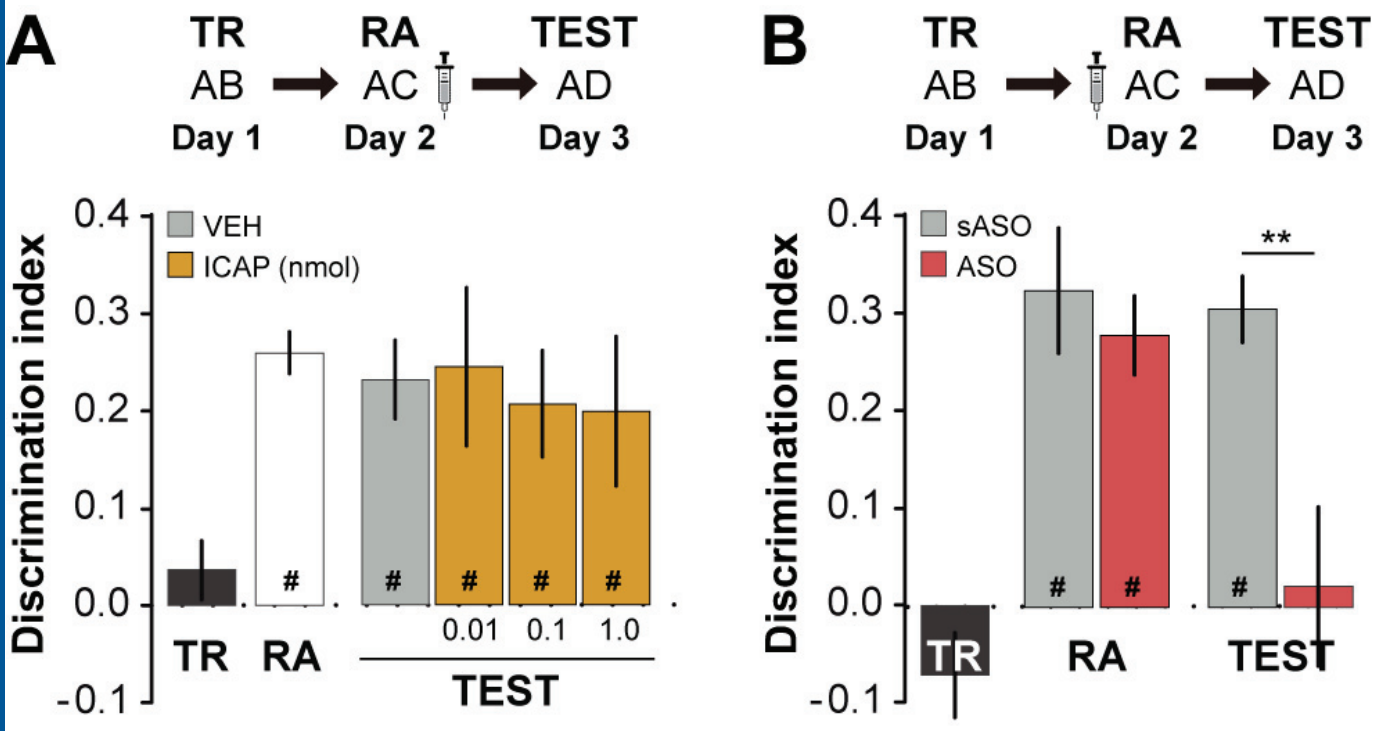
B. Rats were trained as in A and 24 h post-training (Day 2) submitted to a 5-min long ORM reactivation session (RA) in the presence of two familiar objects (A and B) or in the presence of familiar object (A) and a novel object (C). Immediately after RA, animals received bilateral intra-EC infusions of VEH or ANI. One day later (Day 3 - TEST), animals were exposed to a familiar object (A) and a novel object (D) for five minutes to evaluate ORM retention. Data (mean ± SEM) are presented as discrimination index during TR, RA and TEST, n = 11 per group. Animals explored objects equally during TR (Left panel: A=24.57 ± 2.56 s, B=23.54 ± 1.81 s, $t(21) = 0.371$, $p = 0.714$; Right panel: A=24.07 ± 2.59 s, B=23.37 ± 2.71 s, $t(21) = 0.432$, $p = 0.670$). Animals submitted to RA in the presence of familiar objects (AB) did not discriminate between them during that session (A=21.90 ± 0.66 s, B=20.05 ± 1.27 s, $t(21) = 1.50$, $p = 0.148$). Animals submitted to RA in the presence of familiar object A and novel object C discriminated the objects during that session (A=21.77 ± 2.34 s, B=32.87 ± 2.75 s, $t(21) = 5.47$, $p < 0.001$). Total object exploration time did not differ between TR, RA and TEST (Left panel: $F(2,63) = 1.81$, $p = 0.172$; Right panel: $F(2,63) = 0.721$, $p = 0.490$). **C.** Rats were trained as is A and 24 h post-training (Day 2) submitted to a 5-min long ORM reactivation session (RA) in the presence of a familiar object (A) and a novel object (C). Immediately after RA, animals received bilateral intra-CA1 infusions of VEH or zeta inhibitory peptide (ZIP). One day later (Day 3 - TEST 1), animals were exposed to a familiar object (A) and a novel object (D) for five extra minutes to evaluate ORM retention and, immediately thereafter, received bilateral infusions of VEH or ANI in EC. One day later (Day 4 - TEST 2), animals were exposed to familiar object (A) and novel object (E) for five minutes to evaluate ORM retention. Data (mean ± SEM) are presented as discrimination index during TR, RA, TEST1 and TEST2, n = 9 per group. Animals explored objects equally during TR (A=25.77 ± 1.16 s, B=29.59 ± 1.88 s, $t(35) = 1.98$, $p = 0.055$), and discriminated them during RA (A=20.66 ± 1.30 s, B=34.59 ± 1.79 s, $t(35) = 10.42$, $p < 0.001$). Total object exploration time did not differ between TR, RA, TEST1 and TEST2 ($F(3,140) = 0.105$, $p = 0.957$).

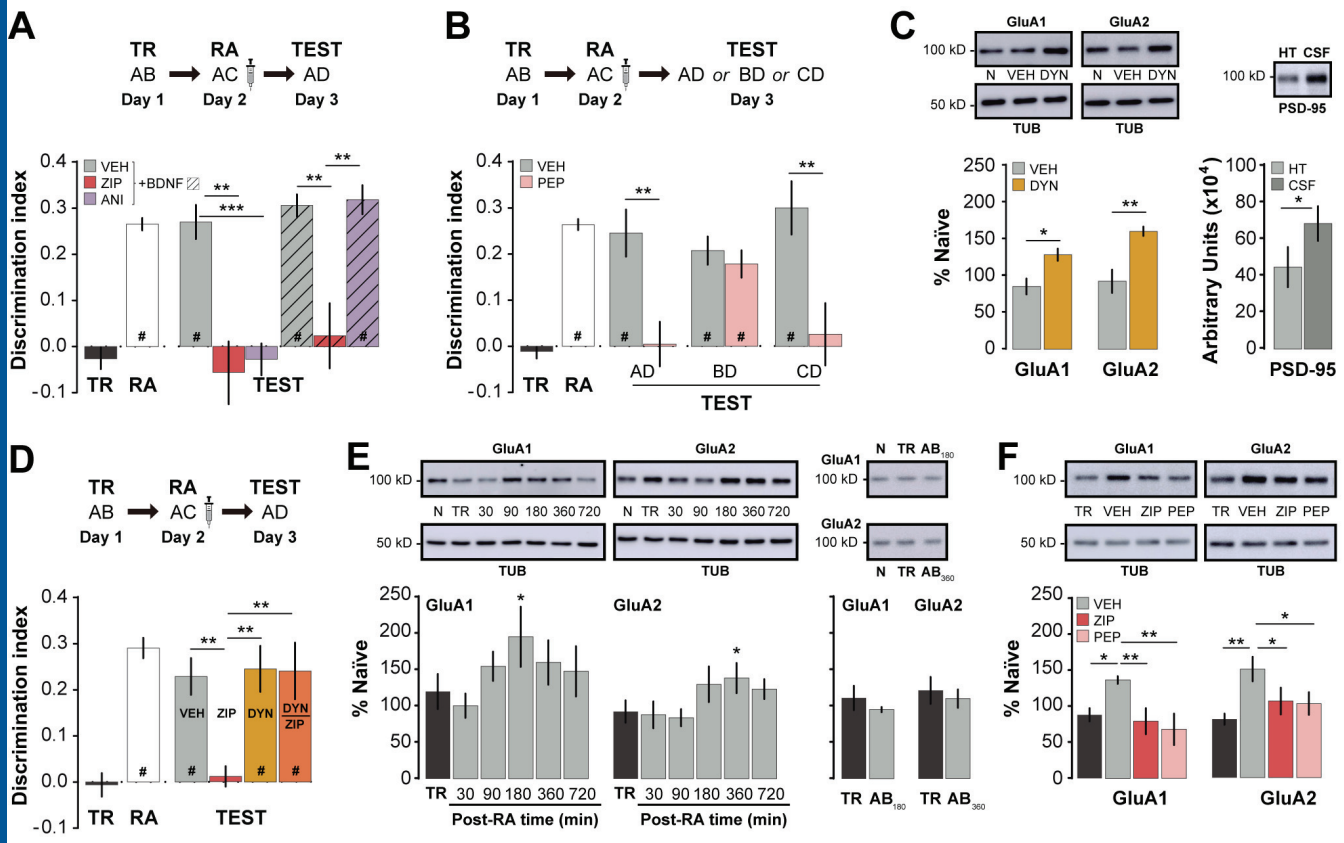
Figure 6. ORM reacquisition after ZIP-induced amnesia recapitulates the electrophysiological correlates of original learning. A. Rats were trained in NOR using two different stimuli objects (A and B - Day 1 - TR) and 24 h post-training (Day 2) submitted to a 5-min long ORM reactivation session (RA) in the presence of a familiar object (A) and a novel object (C). Immediately after RA, animals received bilateral intra-CA1 infusions (1 µl/side) of vehicle (VEH; saline) or zeta inhibitory peptide (ZIP; 1 nmol/µl). One day later (Day 3 - TEST), animals were exposed to a familiar object (A) and a novel object (D) for five extra minutes to evaluate ORM retention. Local field potential (LFP) signals from dorsal CA1 were recorded during TR and TEST. LFP activity from epochs of object A exploration was analyzed. We show a representative diagram of instantaneous speed (in grey) and periods of active exploration (object A in orange; object B in purple) during TR from one rat. **B.** Left panel: Discrimination index during TR and TEST (mean ± SEM). # $p < 0.05$ in one-sample Student's t-test with theoretical mean = 0. Dashed line represents chance level. Right panel: mean speed during exploration of object A in TR and TEST. During active exploration periods, rats remained motionless or moved slowly (mean speed < 6 cm/s). Mean speed did not change across TR and TEST. **C.** Mean power ratio (1-100 Hz) showing alterations in hippocampal oscillatory activity during TR and TEST; bold lines represent group mean and shaded areas represent SEM. **D.** Mean power ratio for theta (5-10 Hz), beta (13-20 Hz) and slow gamma (30-55 Hz) frequency bands during TR and TEST. *$p < 0.05$ in one-sample Student's t-test with theoretical mean = 1. **E.** Example of filtered dorsal-CA1 LFP recordings during object A exploration in TEST for VEH- and ZIP-treated animals. **F.** Representative phase-amplitude comodulograms during object A exploration in TR and TEST. **G.** Boxplot distribution of mean theta-slow gamma modulation indexes during object A exploration in TR and TEST. Modulation indexes were calculated using 10 s-long epochs in which theta and slow gamma power did not differ between TR and TEST. *$p < 0.05$, in unpaired Student's t test. **H.** Representative circular histograms showing the distribution of gamma events over theta phases during object A exploration in TR and TEST. TR in green; VEH in grey; ZIP in

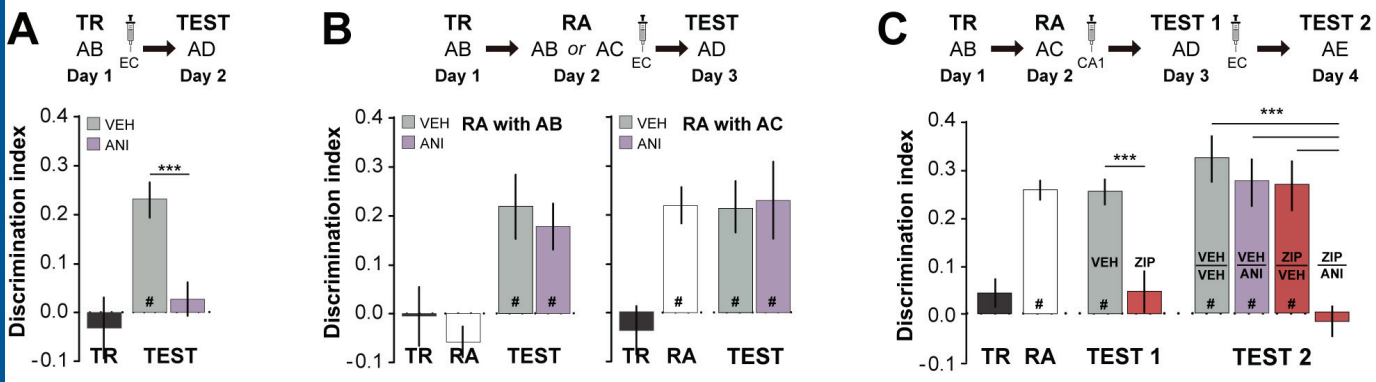
red; θ: theta; β: beta; ɣS: slow gamma; 0° was defined as the peak of the theta cycle; n = 5 per group.

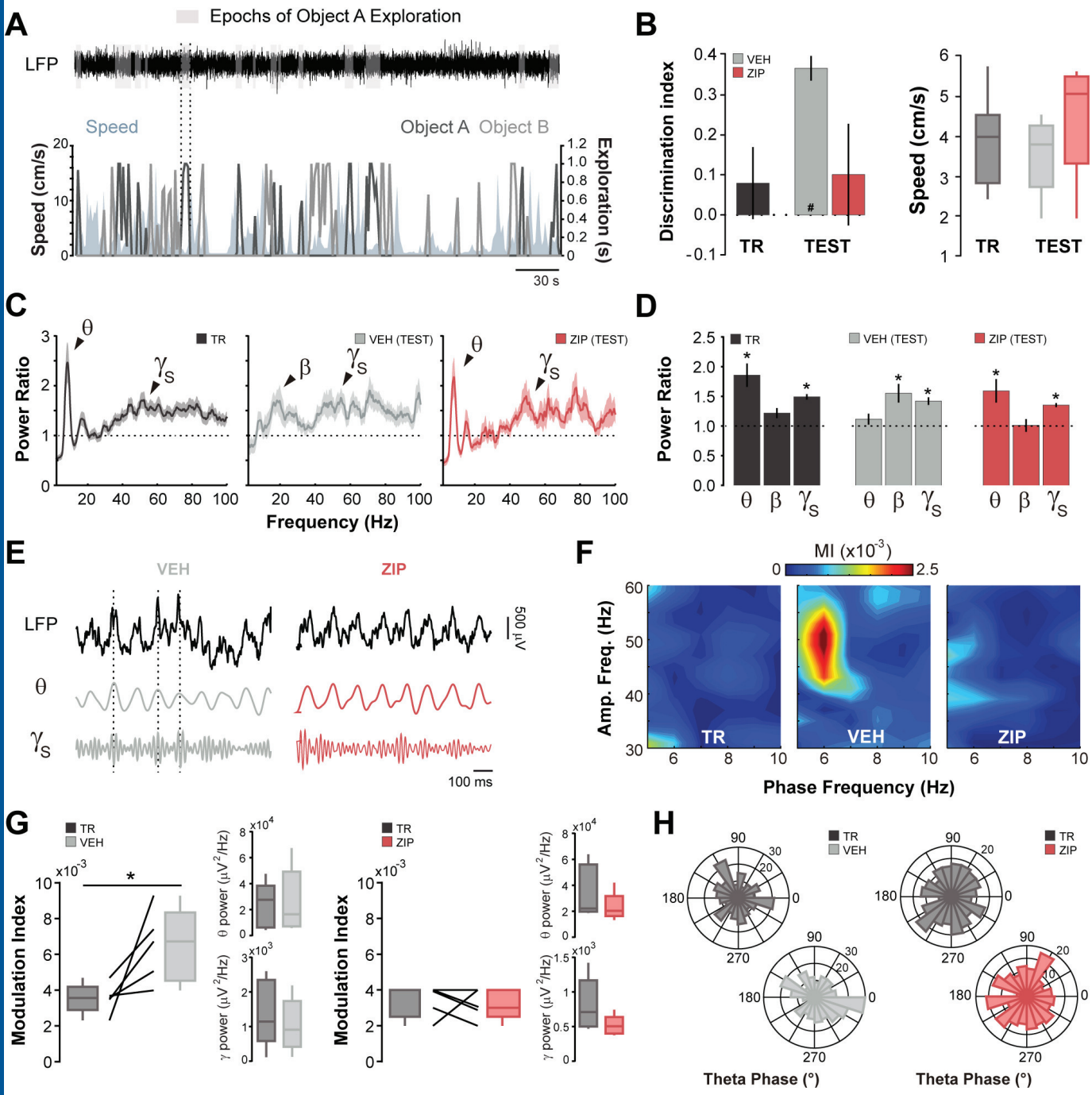












Object Pair	Object exploration time (s)			Discrimination Index	$p^{\#}$	n
	Object (1)	Object (2)	Total			
AB	28.69 ± 3.72	29.04 ± 4.54	57.73 ± 7.27	-0.0057 ± 0.0784	0.9431	9
AC	25.18 ± 4.11	22.87 ± 3.52	48.05 ± 7.31	-0.0467 ± 0.0685	0.5124	10
AD	24.20 ± 2.73	25.36 ± 2.58	49.57 ±4.58	0.0286 ± 0.0552	0.6166	10
AE	27.50 ± 3.68	26.19 ± 3.06	53.66 ± 5.60	-0.0300 ± 0.0745	0.6959	10
BC	26.14 ± 2.31	28.74 ± 3.65	54.88 ± 5.18	0.0271 ± 0.0564	0.6431	9
BD	28.59 ± 2.26	26.47 ± 1.51	55.06 ±2.88	-0.0286 ± 0.0481	0.5669	10
BE	26.79 ± 2.81	26.83 ± 4.35	53.63 ± 5.92	-0.0255 ± 0.0819	0.7632	9
CD	31.19 ± 3.13	29.83 ± 2.79	61.02 ± 4.72	-0.0192 ± 0.0626	0.7667	9
CE	29.86 ± 2.56	29.45 ± 3.72	59.30 ± 5.72	-0.0292 ± 0.0506	0.5777	10
DE	27.83 ± 3.62	25.44 ± 2.26	53.27 ± 5.56	-0.0255 ± 0.0391	0.5306	10

	TEST				
	1st min	2nd min	3rd min	4th min	5th min
DI	0.276 ± 0.043	0.312 ± 0.060	0.221 ± 0.059	0.209 ± 0.075	0.320 ± 0.067
p#	< 0.001	< 0.001	< 0.001	< 0.01	< 0.001
Object exploration time (s)	15.76 ± 0.65	13.55 ± 0.79	11.37 ± 0.75	10.92 ± 0.79	11.24 ± 0.89