The Amygdala Encodes Specific Sensory Features of an Aversive Reinforcer

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Supplementary Figures (pages 2-9)

Supplementary Methods and Materials (pages 10-13)

LA : lateral nucleus of the amygdala ■ : ACSF

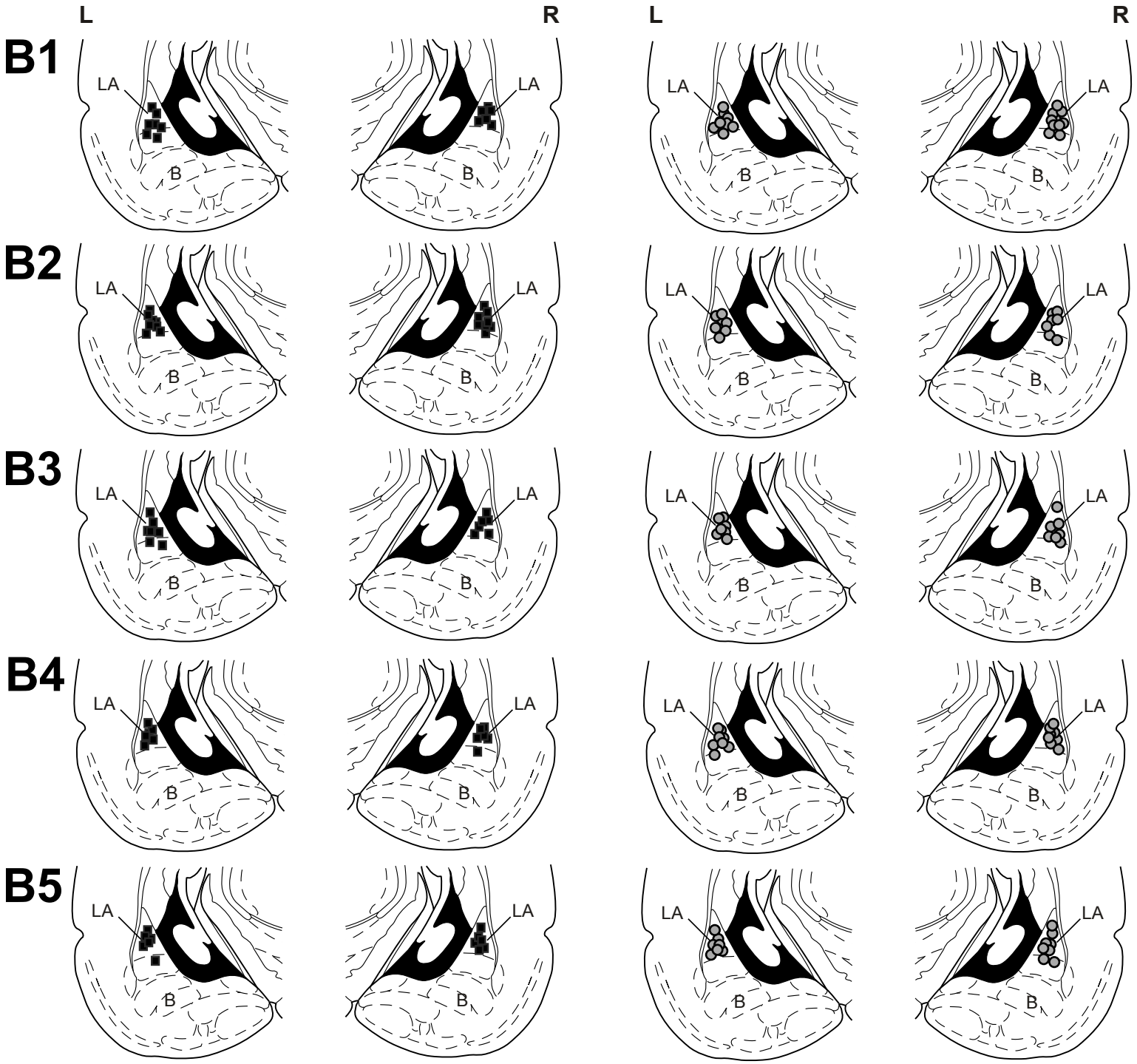
B : basal nucleus of the amygdala ◯ : Anisomycin

R : right; L: left (All sections depicted at around 3.8 mm posterior to Bregma)

Supplementary Figure 1a

Cannulae Placements

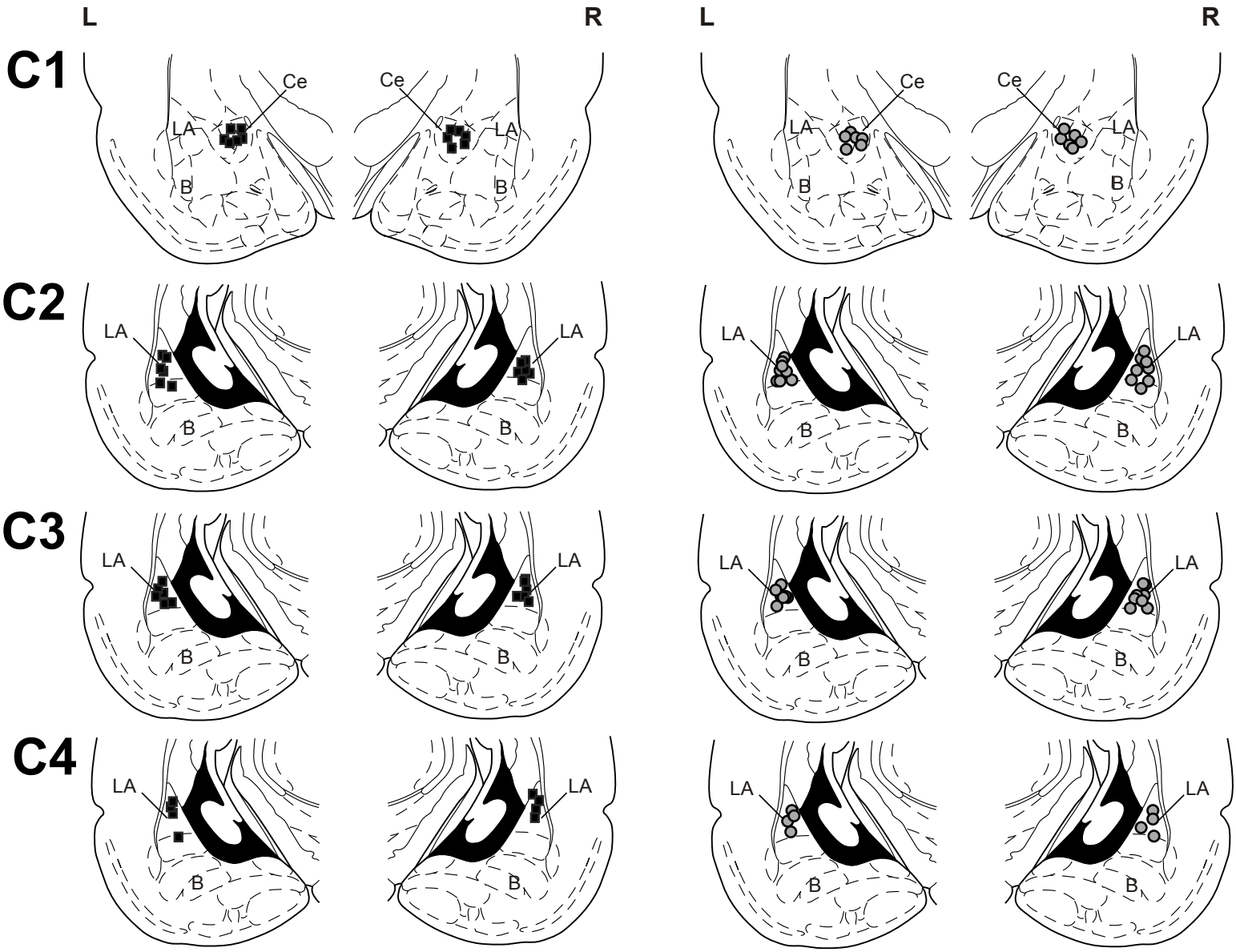
Black squares (animals infused with artificial cerebro-spinal fluid, ACSF) or grey circles (animals infused with Anisomycin, ANISO) indicate the location of the injecting cannula tip. **A1** - experiment described in Suppl. Fig. 2a; **A2** - experiment described in Suppl. Fig. 2b; **A3** - experiment described in Suppl. Fig. 2c; **A4** - experiment described in Suppl. Fig. 2d; **A5** - experiment described in Suppl. Fig. 2e.



Supplementary Figure 1b

Cannulae Placements

Black squares (animals infused with artificial cerebro-spinal fluid, ACSF) or grey circles (animals infused with Anisomycin, ANISO) indicate the location of the injecting cannula tip. **B1** - experiment described in Figure 1a; **B2** - experiment described in Figure 1b; **B3** - experiment described in Figure 1c; **B4** - experiment described in Figure 2a; **B5** - experiment described in Figure 2b.



LA : lateral nucleus of the amygdala
B : basal nucleus of the amygdala
Ce :central nucleus of the amygdala
R : right; L: left

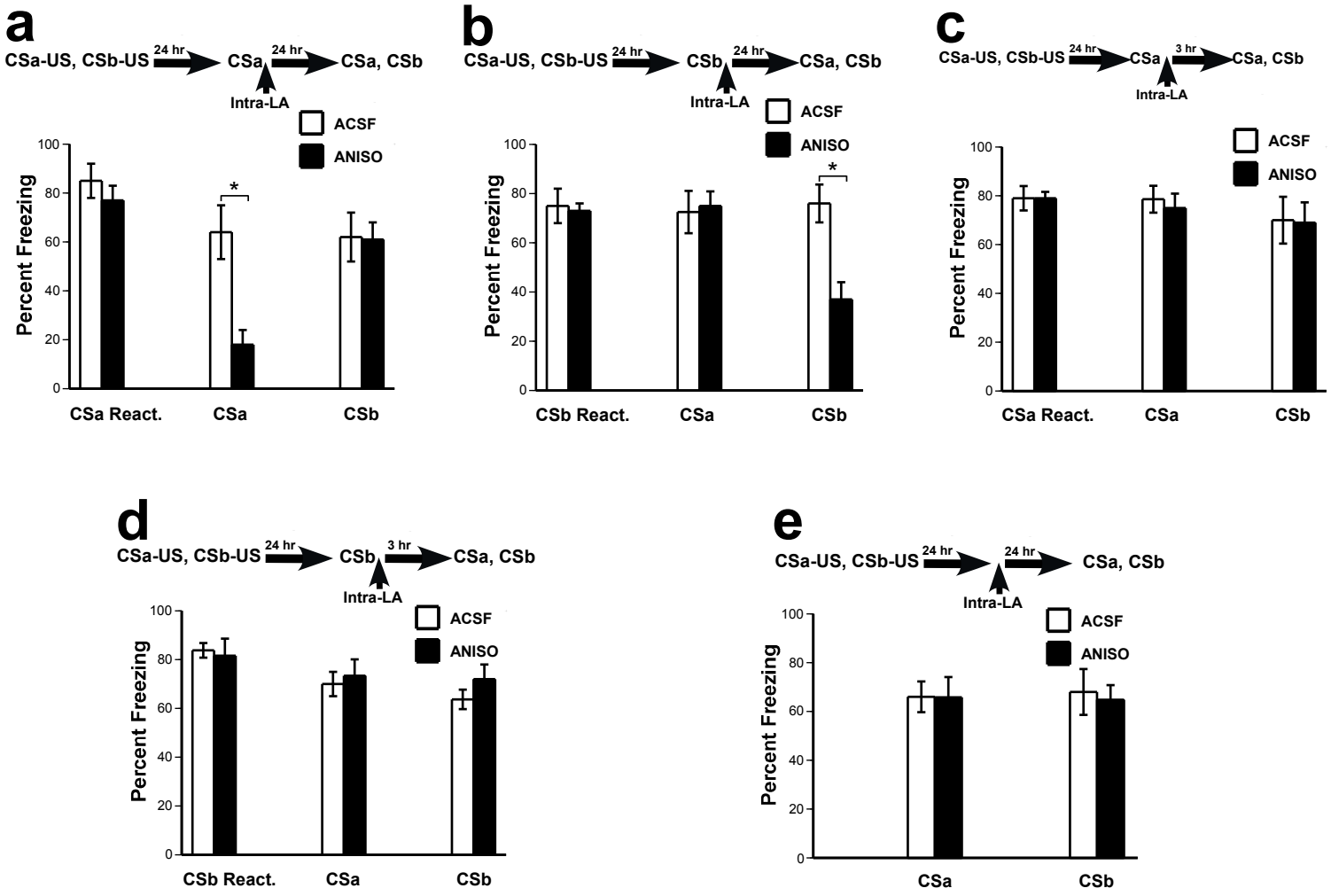
■ : ACSF
◯ : Anisomycin

(Section C1 depicted at around 2.8 mm posterior to Bregma)
(Sections C2-C4 depicted at around 3.8 mm posterior to Bregma)

Supplementary Figure 1c

Cannulae Placements

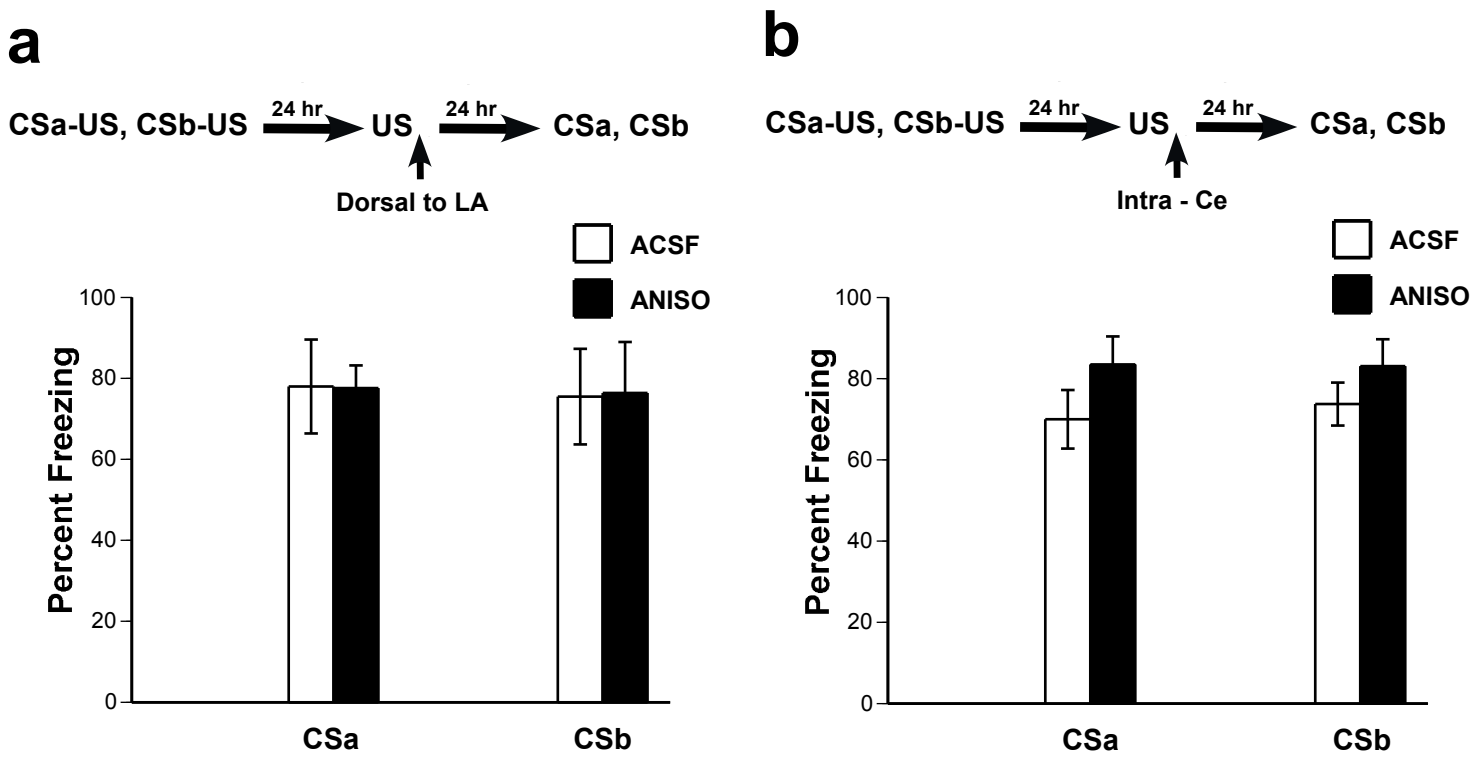
Black squares (animals infused with artificial cerebro-spinal fluid, ACSF) or grey circles (animals infused with Anisomycin, ANISO) indicate the location of the injecting cannula tip. **C1** - experiment described in Suppl. Fig. 3b; **C2** - experiment described in Suppl. Fig. 4a; **C3** - experiment described in Suppl. Fig. 4b; **C4** - experiment described in Suppl. Fig. 5.



Supplementary Figure 2

Protein Synthesis Dependent Memory Reconsolidation is Selective to the Reactivated Conditioned Stimulus

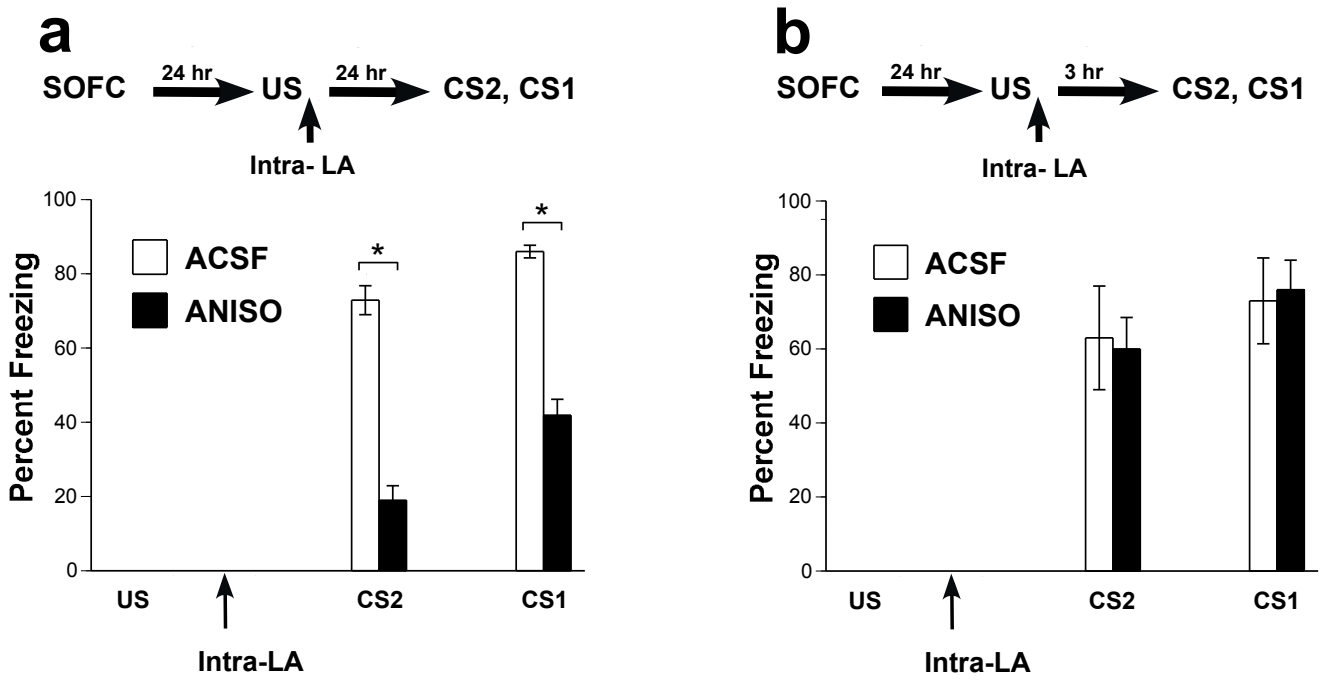
Using MAPK kinase inhibitor, we have recently demonstrated that reconsolidation of auditory fear conditioning is selective to the explicitly reactivated cue (Doyère et al., 2007). Here, we asked whether protein synthesis inhibitor anisomycin would also produce stimulus-selective disruption of reconsolidation of auditory fear conditioning (see: Supplementary Methods and Materials). Twenty-four hours after animals were trained in fear conditioning paradigm with two distinct conditioned stimuli: CSa (conditioned stimulus "a") and CSb (conditioned stimulus "b") paired with the same US (unconditioned stimulus), one CS was reactivated (**a** and **c**: CSa; **b** and **d**: CSb), whereas the other CS was not. Immediately after reactivation, animals received either Anisomycin (ANISO) or vehicle (ACSF) bilaterally in the lateral amygdala (LA). Fear memory to each CS was assessed through percent freezing (mean ± s.e.m.) either 24 hours (long-term memory test, LTM, **a**, **b** and **e**) or 3 hours (short-term memory test, STM, **c** and **d**) later. Anisomycin produced a significant reduction of freezing during LTM only to the reactivated CS (**a**, (ACSF n=7, ANISO n=11) Drug main effect $F(1,16)=4.8$, $p<.05$; CS Main Effect $F(1,16)=37.6$, $p<.001$; Drug x CS Interaction $F(1,16)=42.9$, $p<.001$; Tukey's HSD indicates drug effect for CSa ($p<.01$), but not CSb ($p=.99$) and **b**, (ACSF n=7, ANISO n=9) Drug main effect $F(1,14)=4.3$, $p=.057$; CS Main Effect $F(1,14)=19.3$, $p<.001$; Drug x CS Interaction $F(1,14)=27.7$, $p<.001$; Tukey's HSD drug effect for CSb ($p<.01$), but not CSa ($p=.99$)). The effect was not observed during STM (**c**, n=8 for both groups; No significant main effects or interaction (Drug x CS $p=.8$) and **d**, (ACSF n=6, ANISO n=7; No significant main effects or interaction (Drug x CS $p=.76$), nor when no CS was reactivated before the infusion (**e**, (ACSF n=6, ANISO n=6 No significant main effects or interaction; $p>0.78$). This pattern of results qualifies the effect as affecting the reconsolidation process in a highly selective way, and replicates with a protein synthesis inhibitor previous findings obtained with a MAPK kinase inhibitor (Doyère et al., 2007). Asterisks (*) indicate a significant difference between drug groups.



Supplementary Figure 3

Protein Synthesis Blockade outside the Lateral Amygdala Does Not Affect the Reconsolidation Process Triggered by the Unconditioned Stimulus

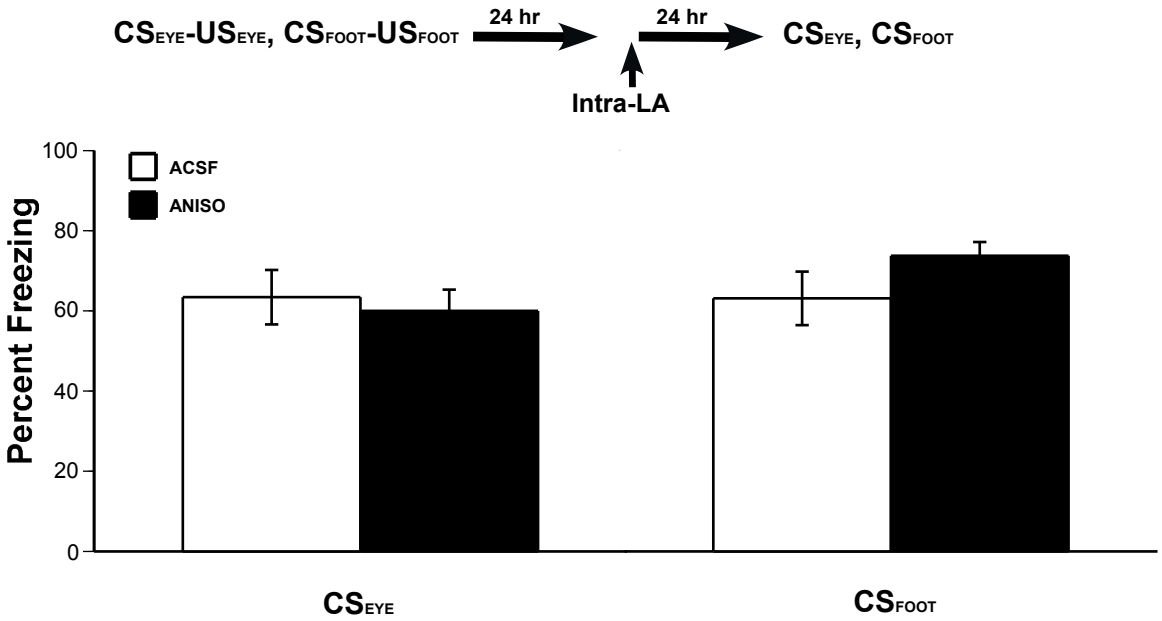
Twenty-four hours after animals were trained in fear conditioning paradigm with two distinct CSs paired with the same US, reactivation of the memory was triggered by a single US presentation (see: Supplementary Methods and Materials). Immediately after reactivation, animals received **a**: either Anisomycin (ANISO, n= 6) or vehicle (ACSF, n=7) bilaterally 2 mm dorsal to the lateral amygdala (LA). No deficit in fear memory was observed during LTM [No significant main effects or interaction $p>0.9$]; **b**: either Anisomycin (ANISO, n= 6) or vehicle (ACSF, n=6) bilaterally into the central nucleus of amygdala (CeA). No deficit in fear memory was observed during LTM [No significant main effects or interaction $p>0.9$]. The CeA was chosen in this control experiment as an infusion site because recent work from our lab (Wilensky et al., 2006) showed that intra-CeA protein synthesis blockade disrupts consolidation of auditory fear conditioning in rats.



Supplementary Figure 4

Exposure to the Unconditioned Stimulus Renders Both First- And Second-Order Fear Memories Protein Synthesis Dependent

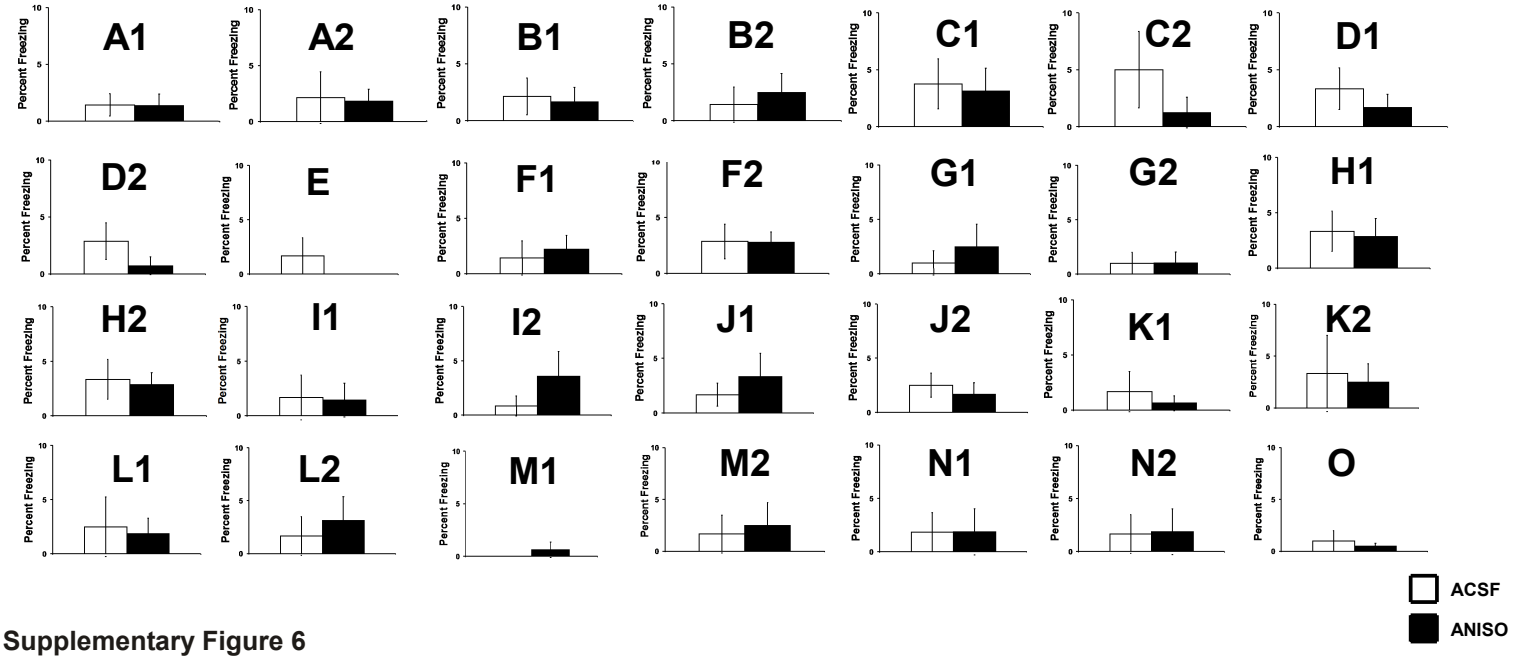
Rats underwent a second-order fear conditioning (SOFC) and then were exposed to a single US, followed immediately by intra-LA infusion of ANISO or ACSF (see: Supplementary Methods and Materials). Post-reactivation memory for both the first-order (CS1) and the second-order (CS2) CSs was tested either twenty-four hours (**a**, LTM, n=8 and n= 6 for ANISO and ACSF respectively) or three hours (**b**, STM, n=8 and n= 6 for ANISO and ACSF respectively) later. Protein synthesis inhibition in the LA after US reactivation disrupted both first and second-order long-term fear memories (**a**, Drug main effect $F(1,12)=51.0$, $p<.001$; CS main effect $F(1,12)=12.5$, $p<.01$; interaction n.s. $p=.39$). It has no effect on post-reactivation STM [**b**, CS main effect $F(1,12)=15.0$, $p<.01$; Drug main effect ($p=.98$) and interaction ($p=.35$) were n.s.]. These findings may suggest that both directly or indirectly associated memories undergo protein synthesis-dependent reconsolidation after the presentation of the US. Asterisks (*) indicate a significant difference between drug groups (No reactivation control experiment for this study has been published in: Debiec et al, 2006).



Supplementary Figure 5

Protein Synthesis Blockade in the Amygdala without Explicit Exposure to the Conditioning Cue (either CS or US) Has No Effect on Memory

Twenty-four hours after animals were trained in fear conditioning paradigm with two distinct CSs paired with two distinct USs (CSFOOT - USFOOT and CSEYE - USEYE, respectively), animals received bilateral intra-LA infusions of either Anisomycin (ANISO, n= 4) or vehicle (ACSF, n=4). bilaterally 2 mm dorsal to the lateral amygdala (see: Supplementary Methods and Materials). No deficit in fear memory was observed during LTM [No significant main effects or interaction $p>0.9$].



Supplementary Figure 6

Exposure to the Conditioning Context Alone Produces Minimal or No Freezing Responses

No significant freezing was measured before exposure to CSa during reactivation session (**A1**), as well as before the exposure to the first CS on LTM (long term memory) test (**A2**); freezing responding to auditory cues in this experiment are presented in Suppl. Fig. 2a. No significant freezing was measured before exposure to CSb during reactivation session (**B1**), as well as before exposure to the first CS on LTM test (**B2**); freezing responding to auditory cues in this experiment are presented in Suppl. Fig. 2b. No significant freezing was measured before exposure to CSa during reactivation session (**C1**), as well as before exposure to the first CS on STM (short term memory) test (**C2**); freezing responding to auditory cues in this experiment are presented in Suppl. Fig. 2c. No significant freezing was measured before exposure to CSb during reactivation session (**D1**), as well as before exposure to the first CS on STM test (**D2**); freezing responding to auditory cues in this experiment are presented in Suppl. Fig. 2d. No significant freezing was measured before exposure to the first CS on LTM test (**E**) (rats infused with anisomycin in this experiment showed no freezing to the context); freezing responding to auditory cues in this experiment are presented in Suppl. Fig. 2e. No significant freezing was measured before exposure to US during reactivation session (**F1**), as well as before exposure to the first CS on LTM test (**F2**); freezing responding to auditory cues in this experiment are presented in Figure 1a in the main text. No significant freezing was measured before exposure to US during reactivation session (**G1**), as well as before exposure to the first CS during memory retention test 4 weeks later (**G2**); freezing responding to auditory cues in this experiment are presented in Figure 1b in the main text. No significant freezing was measured before exposure to US during reactivation session (**H1**), as well as before exposure to the first CS on STM test (**H2**); freezing responding to auditory cues in this experiment are presented in Figure 1c in the main text. No significant freezing was measured before exposure to US during reactivation session (**I1**), as well as before exposure to the first CS on LTM test (**I2**); freezing responding to auditory cues in this experiment are presented in Suppl. Fig. 3a. No significant freezing was measured before exposure to US during reactivation session (**J1**), as well as before exposure to the first CS on LTM test (**J2**); freezing responding to auditory cues in this experiment are presented in Suppl. Fig. 3b. No significant freezing was measured before exposure to US during reactivation session (**K1**), as well as before exposure to the first CS on LTM test (**K2**); freezing responding to auditory cues in this experiment are presented in Suppl. Fig. 4a. No significant freezing was measured before exposure to US during reactivation session (**L1**), as well as before exposure to the first CS on STM test (**L2**); freezing responding to auditory cues in this experiment are presented in Suppl. Fig. 4b. No significant freezing was measured before exposure to US during reactivation session (**M1**), as well as before exposure to the first CS on LTM test (**M2**) (rats infused with vehicle in this experiment showed no freezing to the context); freezing responding to auditory cues in this experiment are presented in Figure 2a in the main text. No significant freezing was measured before exposure to US during reactivation session (**N1**), as well as before exposure to the first CS on LTM test (**N2**); freezing responding to auditory cues in this experiment are presented in Figure 2b in the main text. No significant freezing was measured before exposure to the first CS on LTM test (**O**); freezing responding to auditory cues in this experiment are presented in Suppl. Fig. 5.

Supplementary Methods and Materials

Subjects

Subjects were male Sprague-Dawley rats (Hiltop Laboratories, Scottdale, PA) weighing 250-300g at the beginning of the procedures. Rats were housed individually in plastic Nalgene cages and maintained on a 12/12 hr light/dark cycle. Food and water were provided *ad libitum*. All procedures were in accordance with the *NIH Guide for the Care and Use of Experimental Animals*, and were approved by the *New York University Animal Care and Use Committee*.

Surgery

Surgical procedures were conducted as described before (Dębiec et al., 2006, Doyère et al., 2007). Under Nembutal anaesthesia (45 mg/kg; i.p.), rats were implanted bilaterally with 22-gauge stainless guide cannulae aimed at the lateral nuclei of the amygdala (LA), the central nuclei of the amygdala (CeA) or 2mm dorsal to the LA. All coordinates were taken from Paxinos and Watson (1986). Coordinates for intra-LA were: 3.0 mm posterior to bregma, 5.3 mm lateral to the midline and 8.0 mm ventral to the skull surface. Coordinates for intra-CeA cannulae were: 2.8 mm posterior to bregma, 4.2 mm lateral to the midline and 6.5 mm ventral to the skull. For cannulae implanted 2 mm dorsal to the LA, anterior-posterior and lateral coordinates were the same as for intra-LA implants and the ventral coordinate was 6mm. The guide cannulae were fixed to screws in the skull using acrylic dental cement. A dummy cannula was inserted into each guide cannula to prevent clogging.

In the study using eyelid shocks, two stainless steel wire electrodes of 75 μm-diameter (stripped of insulation ~ 2 mm from the tip) were also threaded bilaterally subcutaneously over the nasal and temporal canthi of the orbicularis oculi muscles for delivery of the periorbital shock (Blair et al., 2005).

Postsurgical analgesics (2 mg/kg ketoprofen) were given daily for 3 days after all surgeries. Rats had at least one week to recover before the start of behavioural procedures.

Drug Infusions

Drug infusions were described in previous studies (Dębiec et al., 2006, Doyère et al., 2007). Using a 28-gauge injector cannulae and an infusion pump (Sage Instruments, USA), anisomycin or an equivalent volume of artifical cerebro-spinal fluid (ACSF) were injected were injected bilaterally into the LA (or 2mm dorsal to the LA) or Ce at a rate of 0.25 μl/min. Following drug infusion, injector cannulae were left in place for an additional minute to allow diffusion of the drug away from the cannula tip. Anisomycin (Sigma, St. Louis, MO) was dissolved in equimolar HCL, diluted with artificial cerebro-spinal fluid (ARCF), and adjusted to pH 7.4 with NaOH. The drug concentration was 125 μg/μl. The volume of anisomycin (or ACSF) infused intra-LA or 2 mm dorsal to the LA was 0.5 μl on each side (Dębiec et al., 2006, Doyère et al., 2007). The volume of anisomycin (or ACSF) infused intra-CeA was 0.2μl. Recent study from our lab (Wilensky et al., 2006) showed that bilateral intra-CeA infusions of 12.5μg of Anisomycin dissolved in 0.2μl of ACSF were sufficient to disrupt auditory fear conditioning consolidation processes.

Histology

At the termination of the experiment, rats were euthanized by an overdose of chloral hydrate (600 mg/kg) and perfused with 10% buffered formalin. Their brains were sectioned at 50μm thickness. The sections were stained using Cresyl violet and examined with light microscopy for cannula penetration. After histological verification, only animals that had both cannulae into the LA (or CeA, or 2mm dorsal to the LA as in the experiments described in Suppl. Fig. 3) were included in the present report (see: Suppl. Fig. 1).

Behavioral Apparatus and Stimuli

The apparatus and the tones used as two conditioned stimuli "a" and "b" (CSa, CSb) were described in earlier studies (Dębiec et al. 2006; Doyère et al. 2007). All procedures were conducted in a custom-made conditioning chamber (height x width x length: 28 x 26 x 29 cm). The walls of the chamber were constructed of stainless-steel bars, and the floor was a standard conditioning chamber grid floor used for delivering foot shock (Model E10-10, Coulbourn Instruments, Leigh Valley, PA). The conditioning chamber was enclosed within a temperature-regulated and ventilated acoustic isolation box. A diffuse light illuminated the chamber during the procedures. Behavior was recorded using a microvideo camera mounted within an isolation box.

CSa (or CS_{EYE}) was a 20-s series of acoustic pips (1 kHz, 50 –ms duration, 1-ms ramp, intensity 20 dB higher than the background noise, delivered at 1 Hz) and CSb (or CS_{FOOT}) was a 20-s series of frequency modulation sweeps (12.5-kHZ) carrier frequency, 50-Hz modulation frequency, 2.5-kHz modulation depth, 250-ms duration. In the second-order conditioning CSa was used as first-order stimulus (CS1) and CSb was used as the second-order stimulus (CS2).

The US (unconditioned stimulus) foot shock (US_{FOOT}) was a 0.5-s, 1.5-mA electric foot shock delivered through the grid floor (Model E10-10, Coulbourn Instruments, Leigh Valley, PA). The US eyelid shock (US_{EYE}) was a 1.2 s train of five very brief shock pulses (3 mA for 2 ms) delivered at a rate of 4 Hz to both eyes simultaneously. The US began immediately after the offset of the final pip.

Behavioral Procedures

General Procedures

In order to reduce the probability of conditioning to the training context (see: *The role of contextual cues in post-retrieval disruption of fear memories* below), prior to conditioning all rats received four 30 min sessions of habituation to the conditioning box as described in the previous study (Dębiec et al., 2006). During the last habituation session, all rats received habituation trials to the tones used as CSs: three exposures to CSa (or CS_{EYE}) followed by three exposures to CSb (or CS_{FOOT}). The intertrial interval (ITI) was variable (157 s on average).

All conditioning, memory reactivation, and memory retention sessions started with a 120s acclimation period to the chamber.

Freezing during the CS presentations was videotaped and scored off-line by an observer that was blind to the experimental (drug treatment and training) conditions during the memory retention test.

Fear conditioning

1. Two distinct CSs paired with the same US: This procedure is exactly the same as previously reported (Doyère et al., 2007). On the next day following the last habituation session, rats were placed in the chamber. Three CSa - US pairings intermixed with three CSb - US pairings were given. The US was delivered immediately after the end of each CS (ITI = 130 s on average).

2. Second-order fear conditioning (SOFC): This procedure is exactly the same as previously reported (Dębiec et al., 2006). On the next day following the last habituation session, rats were submitted to two days of first-order conditioning. Four CS1 (CSa)–US pairings were given on each day. The US was delivered immediately after the end of each CS (ITI =130 s on average). The next day, second-order conditioning was given: Four trials in which CS2 (CSb) was paired with CS1, with CS1 occurring immediately after CS2 (ITI = 130 s on average).

3. Two distinct CSs paired with two distinct USs: On the next day following the last habituation session, half of the animals received three CS_{FOOT} - US_{FOOT} pairings followed by three CS_{EYE} - US_{EYE} pairings. For the other half of the animals, the CS_{EYE} - US_{EYE} pairings were presented first. The US was delivered immediately after the end of each CS (ITI = 130 s on average).

Memory reactivation: Memory reactivation session took place 24 h after fear conditioning. A single stimulus: either CSa (CS1 in the SOFC) or CSb (CS2 in the SOFC) or US (US_{FOOT} or US_{EYE}) was then presented. When either CSa (CS1 in the SOFC) or CSb (CS2 in the SOFC) were played, freezing in response to the CS was measured and used to equate performance for groups that were to receive either drug or vehicle. Immediately after exposure to the stimulus, the rats received an infusion of drug or vehicle.

Memory retention tests: To measure short-term memory (STM) the retention test was given 3 h after reactivation. To measure long-term memory (LTM) the retention test was given approximately 24 h or 4 weeks after drug infusions. The memory retention test involved presentation of CSa (or CS_{EYE}) and CSb (or CS_{FOOT}); in each experimental group half of the rats received four CSa (or CS_{EYE}) trials followed 180 s by the four CSb (or CS_{FOOT}) trials. For the other half of rats, the order of presentation was reversed. Memory retention test used in the SOFC procedure were the same as in the previous study (Dębiec et al. 2006). In the SOFC, during memory retention test the four CS2 trials were presented first and then after 180 s, the four CS1 trials were presented.
An average of the four scores for each CS for each rat was used for the statistical analysis.

Statistical Analysis
Data were analyzed by using two- and three-factor ANOVA (STATISTICA, Ver. 7; StatSoft, Sulsa, OK) with CS test as a within-subject factor and all other variables as

between-subject factors. Significant effects were analyzed using a single interaction and a posthoc Tukey's honest significant difference test where appropriate.

The Role of Contextual Cues in Post-Retrieval Disruption of Fear Memories

During auditory fear conditioning, contextual cues may become part of associations. Contextual fear memories following an exposure to the conditioning context undergo protein synthesis-dependent reconsolidation processes (Dębiec et al., 2002; Suzuki et al., 2004). In order to minimize conditioning to the context, we administered extensive habituation to the context (see: Supplementary Information/Behavioral Procedures) using well-described latent inhibition effect (Lubow and Moore, 1959). In our previous studies (Dębiec et al., 2006; Doyère et al., 2007), we demonstrated that this amount of habituation to the context prevents observable effects of conditioning to the context. Indeed, analysis of freezing for 20 s before presentation of the stimulus (CS or US) during memory reactivation, and at the beginning of the memory retention test (20 s prior to an exposure to the first test US) demonstrates that levels of freezing to the context were very low (0 – 5 %) and equivalent for all experimental groups (see: Suppl. Fig. 6. Thus, it is very unlikely that freezing to the context in our experiments contributed to the differential amount of freezing measured during exposure to the explicit cue in the different groups.

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