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Protocols to Induce, Prevent, and Treat Post-traumatic Stress Disorder-like Memory in Mice: Optogenetics and Behavioral Approaches

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[Abstract] One of the cardinal features of post-traumatic stress disorder (PTSD) is a paradoxical memory alteration including both *emotional hypermnesia* for salient trauma-related cues and *amnesia* for the surrounding traumatic context. Interestingly, some clinical studies have suggested that contextual amnesia would causally contribute to the PTSD-related hypermnesia insofar as decontextualized, traumatic memory is prone to be reactivated in contexts that can be very different from the original traumatic context. However, most current animal models of PTSD-related memory focus exclusively on the emotional hypermnesia, *i.e.*, the persistence of a strong fear memory, and do not distinguish normal (adaptive) from pathological (PTSD-like) fear memory, leaving unexplored the hypothetical critical role of contextual amnesia in PTSD-related memory formation, and thus challenging the development of innovative treatments. Having developed the first animal model that precisely recapitulates the two memory components of PTSD in mice (emotional hypermnesia and contextual amnesia), we recently demonstrated that contextual amnesia, induced by optogenetic inhibition of the hippocampus (dorsal CA1), is a causal cognitive process of PTSD-like hypermnesia formation. Moreover, the hippocampus-dependent contextualization of traumatic memory, by optogenetic activation of dCA1 in traumatic condition, prevents PTSD-like hypermnesia formation. Finally, once PTSD-like memory has been formed, the re-contextualization of traumatic memory by its reactivation in the original traumatic context normalizes this pathological fear memory. Revealing the key role of contextual amnesia in PTSD-like memory, this procedure opens a therapeutic perspective based on trauma contextualization and the underlying hippocampal mechanisms.

Keywords: PTSD, Traumatic memory, Hippocampus, Emotional hypermnesia, Contextual amnesia, Mouse, Fear conditioning, Corticosterone

[Background] Post-traumatic stress disorder (PTSD) is a devastating stress-related condition that develops in the aftermath of a traumatic life event experienced as a threat of injury or death. Lifetime prevalence of PTSD is approximately 6-8% in the general population, but 20 to 30% of victims of severe

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traumatic events (*e.g.*, military combats, terrorist attacks, and rapes) develop this pathology, which can last several years post-trauma (American Psychiatric Association, 2013). Insofar as current treatments are mostly symptomatic, only effective for fewer than half of the patients, and include frequent side effects and residual symptoms, this pathology constitutes a major medical challenge. A cardinal feature of PTSD is a paradoxical memory alteration in which co-exist *emotional hypermnesia* based on salient/simple trauma-related cues and *amnesia* for peritraumatic contextual stimuli (Brewin *et al*., 1996; van Der Kolk, 1994, van Der Kolk *et al*., 2001; Brewin and Holmes, 2003). In addition, some clinical studies have suggested that contextual amnesia would causally contribute to the PTSD-related hypermnesia: decontextualized, traumatic memory would be out of control, and thus prone to be reactivated by any salient cue more or less related to the trauma and in contexts that may be very different from the original traumatic context (van Der Kolk *et al*., 2001; Brewin and Holmes, 2003; see also Jacobs and Nadel,1985). However, despite a precise clinical picture of this disorder, the lack of relevant animal models of this “pathological” fear memory has strongly limited our understanding of its underlying neurobiological mechanisms, thereby challenging the development of innovative treatments (for review, see Desmedt *et al*., 2015). Indeed, most current animal models of PTSD-related memory focus exclusively on emotional hypermnesia, *i.e.*, the persistence of a strong fear memory, which, however, can be perfectly adaptive *per se*. Thus, not only these models do not take into account the hypothetical critical role of contextual amnesia and its underlying hippocampal dysfunction in PTSD-related memory formation (Elzinga and Bremner, 2002; Bremner *et al*., 2003), but they do not even distinguish between a normal/adaptive and pathological/maladaptive (PTSD-like) fear memory (for a review entirely dedicated to this issue, see Desmedt *et al*., 2015).

We developed the first animal model that precisely recapitulates the two memory components of PTSD in mice (*i.e.*, emotional hypermnesia and contextual amnesia) and thus distinguishes “pathological” from “normal” fear memory (Kaouane *et al*., 2012). Combining contextual fear conditioning, in which a tone is presented but not predictive of a footshock, and post-training corticosterone (CORT) or saline (SAL) injection, we showed that compared to SAL-injected mice that display a normal conditioned fear response restricted to the predictive conditioning context, CORT-injected mice display an abnormally high fear response to the irrelevant tone (pathological *emotional hypermnesia*) and a reduced fear response to the context (*contextual amnesia)*. Using this model, we recently demonstrated that contextual amnesia, induced by optogenetic inhibition of dorsal CA1, is a causal cognitive process in the formation of PTSD-like hypermnesia, and that hippocampus-dependent contextualization of traumatic memory prevents PTSD-like hypermnesia formation and can restore a normal fear memory once PTSD-like memory has been formed (Al Abed *et al*., 2020).

Part I: Virus transfection and fiber implantation

Materials and Reagents

1. Glass capillaries (World Precision Instrument, catalog number: 1B150F-4)

2. Silicone catheter (Dominique DUTSCHER, Bernolsheim, catalog number: 351070)
3. Syringe 1 ml (Henke-Sass, Wolf, Tuttlingen, catalog number: 4010.200V0)
4. Syringe 5 ml (Henke-Sass, Wolf, catalog number: 4050.000V0)
5. Petri dish 100 × 15 mm (Sigma-Aldrich, catalog number: P5856-500EA)
6. Implantable Fiber Optic Cannulae (Thorlabs, catalog number: CFMLC12L02)
7. Optic fiber (Thorlabs, catalog number: FT200EMT)
8. Patch cable (Thorlabs, catalog number: M83L01)
9. Ceramic split mating sleeve (Thorlabs, catalog number: ADAL1)
10. Parafilm (Heathrow Scientific, catalog number: HEA234526A)
11. Surgical blades (Swann-Morton, Sheffield, catalog number: B.S.2982)
12. Screws (MicroFastenings, catalog number: M0.6x1.5)
13. Needle 26 G × ½" (Terumo Medical, catalog number: NN-2613R)
14. Needle 23 G × 1¼" (Henke-Sass, catalog number: 4710006030)
15. Cotton swab (The Lab Depot, catalog number: 394305)
16. Stitching kit and sutures (Péters Surgical, catalog number: 87001F)
17. 6-well culture plate (Corning, Falcon®, catalog number: 353046)
18. Microscope slide (Thermo Fisher Scientific, catalog number: LCSF02)
19. Cover slips (Knittel Glass, catalog number: VD12450Y1A.01)
20. Brush (Henry Schein France, catalog number: 878-7825)
21. 1 L Solvent Bottle (Thermo Fisher Scientific, catalog number: 045900)
22. Whatman® paper filter (GE Healthcare, catalog number: 1213125)
23. Young adult (3- to 4-mo-old) C57BL/6 male mice (Charles River, France)
24. AAV vectors:
    a. AAV-CAMKIIa-hChR2(H134R)-EYFP (University of North Carolina (UNC) Vector Core, see Addgene plasmid # Plasmid #26969 for vector map)
    b. AAV-CAMKIIa-ArchT-GFP (University of North Carolina (UNC) Vector Core, see Addgene plasmid # Plasmid #37807 for vector map)
    c. AAV-CAMKIIa-GFP (University of North Carolina (UNC) Vector Core, see Addgene plasmid # Plasmid #64545 for vector map)
25. Isoflurane 1,000 mg/g (4% induction and 1-2% for maintenance, Iso-Vet)
26. Povidone-iodine (Betadine)
27. Super glue (Loctite)
28. Liquid fix glue: Methylmethacrylate (Sigma-Aldrich, catalog number: M55909-25 ML)
29. Super-Bond C&B dental cement (Sun Medical, catalog number: P021E/0A)
30. Metacam: Méloxicam 1.5 mg/ml analgesic (Boehringer Ingelheim)
31. Lurocaine: Lidocaïne 20 mg (Vetoquinol, France)
32. 70% ethanol solution (UNIVAR, catalog number: UN1170)
33. Lacrigel: eye ointment (Europhta, France)
34. Sulmidol: Sulfapyridine 100 mg (MSD, santé animale, France)

35. Hydrogen peroxide (Sigma-Aldrich, catalog number: H1009)
36. Sodium phosphate dibasic, Na_2HPO_4 (Sigma-Aldrich, catalog number: S0876)
37. Sodium phosphate monobasic, NaH_2PO_4 (Sigma-Aldrich, catalog number: S0751)
38. Sodium chloride, NaCl (Sigma-Aldrich, catalog number: 433209)
39. Paraformaldehyde (PFA) (Sigma-Aldrich, catalog number: 441244)
40. Sodium Hydroxide solution 1.0 N, NaOH (Sigma-Aldrich, catalog number: S2770)
41. FluorSave reagent (Merck, catalog number: 345789)
42. Sodium Pentobarbital (0.1 ml/10 g mouse body weight, Bioveta)
43. 0.1 M PBS (see Recipes)
44. 0.2 M PB (see Recipes)
45. PFA 4%/0.1 M PB solution (see Recipes)

Equipment

1. Small scissors (World Precision Instruments, catalog number: 504615)
2. Bone scraper (World Precision Instruments, catalog number: 503759)
3. Drill (RWD Life Science, catalog number: 78001)
4. Fine tip forceps (World Precision Instruments, catalog number: 501975)
5. Needle holder with Suture Scissors (World Precision Instruments, catalog number: 500023)
6. Screwdriver (World Precision Instruments, catalog number: 501635)
7. Compact Power and Energy Meter Console (Thorlabs, catalog number: PM100D)
8. Anesthesia system for isoflurane (Datex Ohmeda ISO Isoflurane Anesthesia Vaporizer Tec 7)
9. Pipette puller (Sutter Instrument, model: P97)
10. Picospritzer (Parker Hannifin)
11. Hair clipper (PHYMEP, model: Contura Shaver)
12. Heating pad (Tem Sega, model: THERM250)
13. Mouse stereotaxic apparatus (KOPF INSTRUMENTS, model: 942)
14. Binocular loupe (Leica Microsystems, model: Leica S6E)
15. Perfusion pump (Cole-Parmer, model: Master Flex® L/S®)
16. Vibratome (Leica Biosystems, model: Leica VT 1000S)
17. Microscope (ZEISS, model: Axio Imager A2)
18. The Mouse Brain in Stereotaxic Coordinates, 2001
19. TTL pulse generator (Imetronic, Pessac, France)

Procedure

A. Preparation of the glass pipettes
   1. Pull the glass pipette using the puller and then break the tip back to a tip diameter between 10-20 µm.

2. Fill the glass pipette with virus solution (2 µl per mouse): Place a drop of virus on parafilm paper, then place the pipette on the stereotaxic frame and connect it to a silicone catheter. Under binocular control, gently lower the pipette until it touches the drop and gently aspirate the drop with a syringe of 5 ml, be careful not to aspire in air, and then remove the syringe to stop aspiration.
3. Graduate the glass pipette every 1 mm, corresponding to a volume of 1 µl.
4. Store the glass pipette filled with the virus and the Super Bond container at 4°C.

B. Virus injection procedure

1. Weight the mouse to monitor the health of the animal post-surgery. Place the mouse in the anesthetic induction box (4% isoflurane) until respiration slows down (5 min). Then transfer it onto a heating pad and fix it in the ear bar of the stereotaxic frame connected to the anesthesia mask (1% to 2%); always monitor the breathing rate, if the rhythm increases or decreases abnormally, it is necessary to adjust the flow of the anesthetic gas. Toe pinch the mouse to confirm anesthesia.
2. Once anesthesia is confirmed, do intraperitoneal injection of Metacam (0.2 ml diluted at 1:20), inject 0.1 ml of Lurocaine locally under the surface of the scalp, and apply eye ointment.
3. Shave the head of the mouse, then use a cotton swab with Betadine to clean the scalp. Make an incision in the scalp horizontally, remove tissues on top of the skull with a bone scraper, and dry the skull by applying Hydrogen peroxide (30%) with a cotton swab.
4. Confirm the orientation of the head by ensuring the Bregma and Lambda are aligned. Mark the Bregma and then attach a piece of metal tubing (like a needle) to the stereotaxic probe holder to make a mark on the skull at the coordinates of the bilateral injections sites that target the:
   a. dCA1: two bilateral sites, 1.8 and 2.5 mm posterior to the Bregma and 1.3 and 2 mm lateral of the midline, respectively.
   b. dCA2 and dCA3: 2.0 mm posterior to the Bregma and 2.5 mm lateral of the midline.
5. Finally, drill holes using a homemade hand drill or an electric drill.
6. Position the glass pipette containing the virus in the stereotaxic frame just above the first hole so that its tip touches the surface of the skull. Very gently lower the pipette down to -1.4 mm for dCA1 and -2 mm for dCA2/dCA3.
7. Wait 2 min after the descent of the pipette and then, using a syringe connected to the catheter (Figure 1), inject 0.2 µl of the virus by applying a positive pressure with hand and check the glass pipette graduations. Wait again for 2 min.
   Note: Remove the syringe just after injections; otherwise, the injection continues.

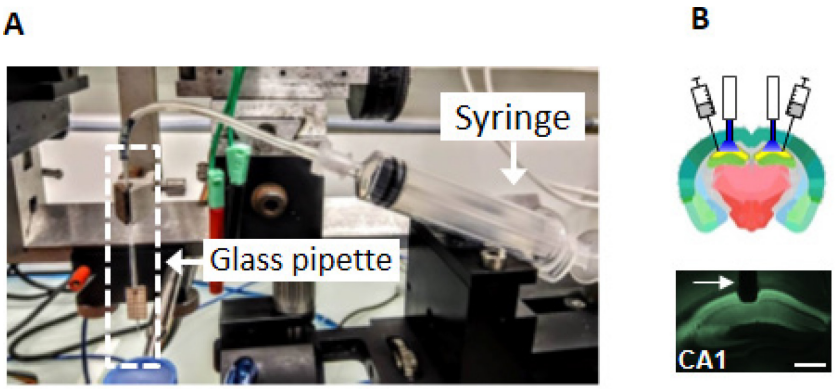

Figure 1. Image of the viral infection setup and histological control. A. The glass pipette containing the virus, maintained by the stereotaxic holder, can be visualized on the left part of the image. It is connected to the syringe via soft tubing. **B.** Top panel: schematic of the brain atlas (Allen brain atlas) representing viral infection and implantation location. Bottom panel: photograph (10×) of the viral infection (GFP in green) and implantation (arrow) above the pyramidal cell layer of the dorsal CA1 subfield of the hippocampus. Scale bar: 500 μm.

8. Very gently lift the pipette and repeat the procedure with the other four coordinates.
9. Clean the skull with Betadine, then suture the skin with 2-3 stitches using the needle holder. Apply Betadine, Sulmidol healing cream, and put back mouse in its home cage. Monitor each mouse for two weeks until fiber implantation: check the mouse activity and check its weight.

C. Fiber implantation

1. Two weeks later, perform a new surgery to implant optic fibers that target dCA1, dCA2, or dCA3.
2. Start by implanting screws to improve the stability of the fibers:
   a. Drill three small holes away from the coordinates of the fibers: one on the top left, one on middle top left, and one on middle bottom of the skull.
   b. Place the three screws and connect them by applying super glue gel to solidify different parts of the skull together. Apply a few drops of fix glue to dry the glue.
3. Place the optic fiber implant in the ‘ceramic split mating sleeve’ to hold the implant and above the first hole in the skull the targets either the:
   a. dCA1: anteroposterior -1.8 mm, lateral ± 1.3 mm.
   b. dCA2: anteroposterior -1.8 mm, lateral ± 2.1 mm.
   c. dCA3: anteroposterior -1.85 mm, lateral ± 2.45 mm
4. Then clean the coagulated blood and slowly lower the implant to the desired depth; dCA1 (1.4 mm), dCA2 (1.7 mm), and dCA3 (1.9 mm), then apply Super Bond cement and wait 15 min until the cement solidifies. Gently lift the fiber holder and repeat the procedure with the other location by ensuring that there is enough cement between the two fibers.

5. Suture the skin with two stitches using the needle holder. Apply Betadine, Sulmidol healing cream, and put mouse back in its cage. Monitor each mouse for 10 to 12 days until behavioral testing: check the mouse activity and its weight.

D. Injection sites and fiber implantation histological controls
   1. Euthanize the mice with an intraperitoneal injection of overdose Pentobarbital (0.1 ml/10 g mouse weight, 25 G needle, 1 ml syringe).
   2. Place the mouse on a dissection board, open the rib cage, and expose the heart. Insert the needle, attached to a catheter, in the left ventricle of the heart and clamp it.
   3. Snip the right atrium and turn on the peristaltic pump to perfuse ice-cold PFA 4%/PB 0.1 M solution perfusion (100 ml/mouse, at 10 ml/min).
   4. Open the skull to extract the brain. Pay attention to the dura. Immerse the brain in PFA solution and store at 4°C for 24 h.
   5. Make a small incision along the brain to mark the right side and glue the brain to the vibratome plate on the cerebellum side
   6. Cut coronal sections of 50 µm from -0.34 mm to -3.6 mm anteroposterior to collect the corresponding sections from the dorsal hippocampus (The Mouse Brain in Stereotaxic Coordinates, 2001).
   7. Using a brush, transfer the cuts to a slide and mount them with 3-4 drops of the Fluorsave reagent.

Part II: Optogenetic manipulations of dCA1 or dCA2/dCA3 activity in animals developing normally vs. PTSD-like memory

The behavioral procedure started after a 10- to 12-d recovery.

Materials and Reagents

1. Fiber-optic patch cords: diameter, 200 µm (Doric Lenses, catalog number: MFP_50/125/900-0.22_#.#_FC-FC)
2. Optic Rotary Joints (Doric Lenses, catalog number: FRJ_1x2i_FC-2FC_0.22)
3. Young adult (3- to 4-mo-old) C57BL/6 male mice (Charles River, catalog number: 027)
4. Ethanol 70%
5. Acetic acid 1% (Sigma-Aldrich, catalog number: 1005706)
6. Corticosterone (2-hydroxypropyl-β-cyclodextrin complex; Sigma-Aldrich, Catalog number: C2505)
7. NaCl (0.9%) (see Recipes)

Equipment

1. Laser (IkeCool OptoDuet 473/593 nm)
2. Fear conditioning chamber equipped for optogenetics (OptoPath platform; IMETRONIC, conditioning, and Tests in 2 distinct apparatus):
   a. A first chamber, round (20 cm diameter) with an opaque PVC floor, in a brightness of 100 lux and cleaned with 1% acetic acid before each trial is used for pre-exposure to allow the mice to acclimate and become familiar with the chamber later used for the tone re-exposure test (Figures 2A-2B left).
   b. A second chamber is used for the fear conditioning: it is squared (24 × 24 cm), with transparent walls, in a brightness of 100 lux (sum of the light in the chamber and from the experimental room), the doors of the apparatus are left opene to give access to the different visual-spatial cues in the experimental room (geometric shapes of different colors), and cleaned with 70% ethanol before each trial. The floor of the chamber consists of 19 stainless-steel rods (3 mm diameter), spaced 1 cm apart and connected to a shock generator (Figures 2A-2B right). This chamber is equipped for optogenetics: TTL pulse generator, a device that activates the laser.

Software

1. Fear conditioning cage associated software: POLY Fear (IMETRONIC)

Procedure

A. Induction of normal vs. PTSD-like fear memory

The contextual conditioning task (Figure 2A) is a classical fear conditioning, which here uses a discrete conditional stimulus (CS) that is never paired with an unconditional stimulus (US). This CS-US unpairing procedure is known to promote conditioning to the foreground context but not to the irrelevant (not predictive) salient CS. While a normal fear memory, which is characterized by a strong fear response to the conditioning context but not to the irrelevant tone, is observed in mice receiving post-conditioning (i.p.) saline injection (Veh-injected mice), PTSD-like memory is observed in mice submitted to post-conditioning corticosterone injection (CORT-injected mice): an abnormal fear response to the tone, mimicking the pathological emotional hypermnesia, is associated with a reduced fear response to the conditioning context that reveals a contextual amnesia (Kaouane *et al*., 2012). Using this model, we tested whether hippocampal inhibition during conditioning, which should impair context memorization, promotes the formation of PTSD-like memory in Vehicle (Veh)-injected mice, whereas hippocampal activation, expected to promote context memorization, may prevent PTSD-like memory formation in CORT-injected mice (Al Abed *et al*., 2020).

B. Procedure for acquisition of fear conditioning and optogenetic manipulations

1. Handle each mouse for 10 min daily for 2 days before fear conditioning (Figure 2A Day 0). The handling aims to accustom the mouse to the restraining required to connect the mouse to the optic fiber patch cords in the conditioning chamber. Put the mouse in a piece of fabric and gently habituate them to sustain the restrain by closing your hand on the animal, pressing on the implant, attaching the patch cord, *etc*.
2. The day before fear conditioning, place each mouse for 4 min into the round chamber (20 cm diameter) with an opaque PVC floor, in a brightness of 100 lux. Clean the box with 1% acetic acid before each trial (Figure 2A Day 1, Figure 2B left). This pre-exposure allows the mice to acclimate and become familiar with the chamber later used for the tone re-exposure test. Because the mice are pre-exposed to this chamber and are conditioned in a different environment (see below), it is referred to as safe familiar chamber in the rest of the protocol.
3. The acquisition of fear conditioning (Day 1) is performed in a different context, referred to as conditioning context: a squared conditioning chamber (24 × 24 cm; Figure 2B right), in a brightness of 100 lux, given access to the different visual-spatial cues in the experimental room. The floor of the chamber consisted of 19 stainless-steel rods (3 mm diameter), spaced 1 cm apart and connected to a shock generator.
    a. Clean the conditioning chamber with 70% ethanol before placing each animal; the odor is part of the contextual cues.
    b. Connect each animal to the fiber-optic patch cords and then place in the conditioning chamber for 4 min, during which it receives two tones CS (65 dB, 1 kHz, 15 s) and two footshocks US (squared signal 0.4 mA, 50 Hz, 1 s) according to a pseudorandom distribution, routinely used in our laboratory and known to promote the processing of contextual cues in the foreground (Desmedt *et al*., 1998; Calandreau *et al*., 2006 and 2010; Kaouane *et al*., 2012; Ducourneau *et al*., 2020). Specifically, 100 s after being placed in the chamber, the animal receives a shock; then, after a 20 s interval, a tone; finally, after a 30 s delay, the same tone and the same shock spaced by a 30 s interval are presented. After 20 s, place the animal back in its home cage. In this tone-shock unpairing procedure, as the tone is never followed by shock delivery, the animal identifies the conditioning context (set of static background contextual cues that constitutes the environment in which the conditioning takes place), and not the tone, as the right predictor of the shock.
4. Optogenetic manipulations (~6 mW per implanted fiber bilaterally conducted from the laser) are performed during the acquisition of fear conditioning or 5 min before, in Veh- or CORT-injected mice, depending on the group:
    a. Dorsal hippocampus inhibition in Veh-injected mice:
        i. Mice injected with the viral vector AAV-CamKIIa-ArchT-GFP or control vector AAV-CamKII2a-GFP, hence expressing the ArchT channel and GFP, respectively, in the dorsal hippocampus and chronically implanted with optic fibers in the dCA1 or dCA2 or

dCA3: to transitory inhibit the dCA1 (or dCA2 or dCA3) neuronal activity, activate the laser (continuous light deliver, 526 nm) during the 4 min acquisition of conditioning.

ii. Control experiment: Mice injected with the viral vector AAV-CamKIIa-ArchT-GFP or control vector AAV-CamKII2a-GFP, hence expressing the ArchT channel and GFP, respectively. To transitory inhibit the dCA1 neuronal activity, activate the laser during the same duration as the conditioning, *i.e.*, 4 min, (continuous light delivery, 526 nm), and perform 5 min before conditioning. This step is performed in the homecage, in an adjacent room, where mice from all groups are placed before the conditioning.

iii. Previous observations indicate that light stimulation *per se* does not impact the observed freezing behavior. Indeed, previous optogenetic experiments achieved in our laboratory clearly showed that the freezing behavior of control GFP mice (Laser ON) was not impacted by light stimulation of the dCA1 neurons (Sellami *et al.*, 2017). Consequently, the control mice in the present procedure are expressing ArchT channel and GFP in the dorsal hippocampus and are chronically implanted with optic fibers in the dCA1 but are submitted to the fear conditioning conditioning procedure with the laser OFF.

b. Dorsal hippocampus activation in CORT-injected mice:

i. Mice injected with the viral vector AAV-CamKIIa-ChR2-EYFP or control vector AAV-CamKII2a-EYFP, hence expressing the ChR2 channel and EYFP, respectively, in the dorsal hippocampus and chronically implanted with optic fibers in the dCA1: to transitory activate the dCA1 neuronal activity, activate the laser (light, at 473 nm, is delivered at 5 Hz: 5 ms laser on, 195 ms laser off) during the 4 min acquisition of conditioning.

ii. Mice injected with the viral vector AAV-CamKIIa-ChR2-EYFP or control vector AAV-CamKII2a-EYFP, hence expressing the ChR2 channel and EYFP, respectively, in the dorsal hippocampus and chronically implanted with optic fibers in the dCA1: to transitory activate the dCA1 neuronal activity, activate the laser (light, at 473 nm, is delivered at 5 Hz: 5 ms laser on, 195 ms laser off) 5 min before the beginning of conditioning during 4 min.

iii. Mice injected with the viral vector AAV-CamKIIa-ChR2-EYFP or control vector AAV-CamKII2a-EYFP, hence expressing the ChR2 channel and EYFP, respectively, in the dorsal hippocampus and chronically implanted with optic fibers in the dCA1 are submitted to the fear conditioning procedure with the laser OFF.

C. Post-conditioning systemic injection of corticosterone vs. NaCl

Corticosterone (2-hydroxypropyl-β-cyclodextrin complex; 2.5 mg/kg in a volume of 0.1 ml/10 g bodyweight) or vehicle (NaCl 0.9%) was administrated intraperitoneally (i.p.) immediately after the acquisition of fear conditioning. The complex of corticosterone with cyclodextrin allows dissolving this steroid in aqueous solutions. After the injection, animals were returned to their home cage. The dose of corticosterone was selected on the basis of previous results indicating that such dose (i) is

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in the range of concentrations induced by stress in the plasma (Pugh *et al.*, 1997; Cordero and Sandi, 1998) and (ii) effectively induces PTSD-like memory in mice when combined with fear conditioning using a relatively high footshock intensity (squared signal, 0.4 mA; Kaouane *et al.*, 2012).

D. Day 2: Memory tests

Twenty-four hours after the acquisition of fear conditioning, mice are submitted to two memory retention tests (Figure 2A, Day 2). During these two memory tests, animals are continuously recorded for off-line second-by-second scoring of freezing by an observer blind of experimental groups. The freezing behavior of animals is defined as a lack of all movement except for respiratory-related movements and was used as an index of conditioned fear response.

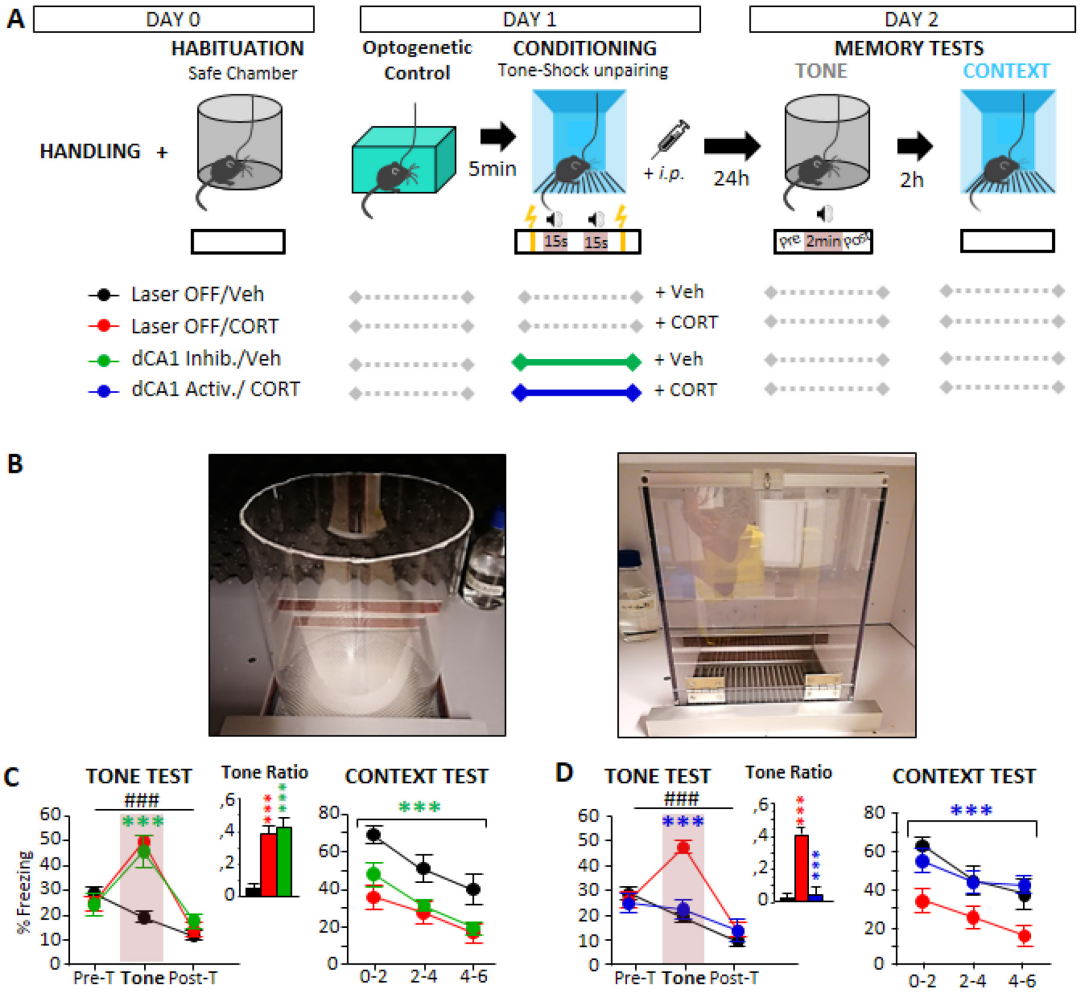

Figure 2. Inducing or preventing PTSD-like memory formation by dCA1 inhibition during stress and dCA1 activation in traumatic condition, respectively. A. Scheme illustrating the optogenetic approach, habituation, and handling (Day 0), contextual fear conditioning (Day 1), and memory tests (Day 2): re-exposure to the tone alone in the familiar (neutral) chamber (grey round chamber), then to the conditioning context 2 h later. PTSD-like memory formation (in CORT-injected

mice) has been associated with a hypofunction of the hippocampus, compared to normal memory (in Veh-injected mice). We hence investigated the causality of hippocampal activity in the formation of PTSD-like memory using optogenetic manipulation. **B.** Photographs of the two chambers used: the familiar safe chamber (round, left panel) and the context of conditioning (square, right panel). **C.** Compared to normal contextual fear memory, attested by no fear response to the tone (no increase of freezing to the tone, left panel) and high fear response to the context (right panel) in Vehicle-injected mice (Laser OFF, n = 15), PTSD-like memory in (Laser OFF, n = 13) CORT-injected mice is attested by a fear response specific to the tone (*repeated measures (RM)* of freezing during the tone test: $F_{2,24}$ = 35.908; P < 0.0001; tone ratio vs. 0; P < 0.0001) associated with decreased fear to the context. A similar difference between the groups is observed when NaCl is replaced by HBC as vehicle [data not shown]. dCA1 inhibition during conditioning produces PTSD-like memory in Veh-injected mice (n = 13; *RM* x *laser condition*: $F_{4,76}$ = 20.184; P < 0.0001). Although all groups display relatively high pre-tone freezing levels in the familiar chamber, these levels are lower than those expressed in the conditioning chamber (left vs. right: all F > 6.29, all P < 0.028). **D.** Compared to control (laser OFF; n = 11) CORT-injected (PTSD-like) mice, CORT-injected mice submitted to dCA1 activation (n = 11) during conditioning display a normalized (contextual) fear memory (*RM* x *laser condition*: $F_{2,40}$ = 15.684; P < 0.0001. Data are presented as mean ± SEM. ***P < 0.005. ###: block × condition interaction (P < 0.005). Adapted from Al Abed *et al.* (2020).

1. Mice are first submitted to the tone re-exposure test in the safe familiar chamber (*i.e.*, round chamber). Clean the testing chamber with 1% acetic acid before placing each animal. The tone test lasts for 6 min, with a tone presentation of 2 min. Quantify three successive sections of the behavioral responses: one before (first 2 min), one during tone presentation (next 2 min), and one after (2 last min). Conditioned response to the tone is expressed by the percentage of freezing during the tone presentation compared to the levels of freezing expressed before and after tone presentation (repeated measures on three blocks of freezing). The strength and specificity of this conditioned fear are attested by a ratio that considers the percentage of freezing increase to the tone with respect to a baseline freezing level (*i.e.*, pre- and post-tone periods mean). A strong and specific conditioned fear of the discrete tone CS implies a lower level of freezing when the shock is not expected (*i.e.*, 2 min before and 2 min after the tone presentation) compared to the freezing level expressed during the tone presentation (high ratio value). The tone ratio is calculated as follows: [% freezing during tone presentation−(% pre-tone period freezing + % post-tone period freezing)/2]/[% freezing during tone presentation + (% pre-tone period freezing + % post-tone period freezing)/2].
2. Two hours later, mice are submitted to the context re-exposure test: they are placed for 6 min in the conditioning chamber. Clean the conditioning chamber with 70% ethanol before placing each animal. Freezing to the context is calculated as the percentage of the total time spent freezing during the successive three blocks of 2-min periods of the test. While the first block is the critical block attesting difference between animals that are conditioned to the conditioning

context and those that are not or less, the following two blocks are presented in order to assess a gradual extinction of the fear responses in the absence of shock.

3. Normal contextual fear memory is attested by a high conditioned fear to the conditioning context (right predictor of the shock) together with an absence of conditioned fear to the non-predictive tone. In contrast, a maladaptive PTSD-like fear memory is attested by an opposite pattern of results indicating the erroneous selection of the tone instead of the context as predictor of the shock: an abnormal fear response to the tone associated with a decreased conditioned fear to the context (Kaouane *et al.,* 2012).
4. After the retention tests, mice are euthanized to verify the injections and the fibers implantation sites (see Part I Procedure D).
5. Results obtained from the retention tests are presented in Figures 2C and 2D (figure adapted from Al Abed *et al.*, 2020).

Part III: Recontextualizing traumatic memory by its reactivation in the original traumatic context cures PTSD-like memory

Materials and Reagents

1. Fiber-optic patch cords: diameter, 200 µm (Doric Lenses, catalog number: MFP_50/125/900-0.22_#.#_FC-FC)
2. Optic Rotary Joints (Doric Lenses, catalog number: FRJ_1x2i_FC-2FC_0.22)
3. Young adult (3- to 4-mo-old) C57BL/6 male mice (Charles River, catalog number: 027)
4. Ethanol 70%
5. Acetic acid 1% (Sigma-Aldrich, catalog number: 1005706)
6. Corticosterone (2-hydroxypropyl-β-cyclodextrin complex; Sigma-Aldrich, Catalog number: C2505)
7. NaCl (0.9%) (see Recipes)

Equipment

1. Laser (IkeCool OptoDuet 473/593 nm)
2. Fear conditioning chamber equipped for optogenetics (OptoPath platform; IMETRONIC, conditioning, and Tests in 2 distinct apparatus):
   a. A first chamber, round (20 cm diameter) with an opaque PVC floor, in a brightness of 100 lux and cleaned with 1% acetic acid before each trial is used for pre-exposure to allow the mice to acclimate and become familiar with the chamber later used for the tone re-exposure test.
   b. A second chamber is used for the fear conditioning: it is squared (24 × 24 cm), in a brightness of 100 lux, giving access to the different visual-spatial cues (geometric shapes

of different colors) in the experimental room and cleaned with 70% ethanol before each trial. The floor of the chamber consisted of 19 stainless-steel rods (3 mm diameter), spaced 1 cm apart and connected to a shock generator. This chamber is equipped for optogenetics: TTL pulse generator, a device that activates the laser.

c. The fear conditioning chamber is also used for the re-contextualization of traumatic memory on day 3. During this procedure, the tone is presented in the chamber.

Software

1. Fear conditioning cage associated software: POLY Fear (IMETRONIC)

Procedure

A. The re-contextualization of PTSD-like memory promotes its normalization.

A decontextualized memory of the trauma, associated with the systematic avoidance of conscious recollection of the trauma, would contribute to the intrusive re-experiencing of the trauma in safe situations (*i.e.*, flashbacks) characterizing PTSD (van Der Kolk *et al.*, 2001; Bremner *et al.*, 2003; Brewin and Holmes, 2003). Accordingly, treatment of PTSD would imply a switch from a decontextualized intrusive traumatic memory to a normal, contextualized fear memory. We thus reasoned that re-exposure to the most emotionally laden traumatic cue (*i.e.*, the tone in our study) in the traumatic context could not only reactivate the traumatic memory but also promote the re-contextualization of the trauma and thereby cure PTSD-like memory.

1. Cf. Part II, Procedure A, 1-3, and Post-conditioning injections of corticosterone or NaCl (Day 1)
2. Cf. Part II, Procedure A, Memory tests (Day 2), 1-3.
3. Two days after fear conditioning and one day after the classical memory tests (Day 3), re-expose the (Veh- and CORT-injected) animals to the tone cue *in* the conditioning context and record three successive sessions of the behavioral responses: one before (first 2 min), one during (next 2 min), and one after (2 last min) tone presentation. Therefore, the first 2 min (pre-tone) allows us to assess the level of conditioned fear to the conditioning context alone, while the conditioned response to the tone is assessed during the next 2 min, both by the percentage of freezing during the tone presentation and by the tone ratio described above.
4. Three control conditions are designed:
   a. Veh- and CORT-injected mice are re-exposed to the tone alone (in the familiar context)
   b. Veh- and CORT-injected mice are re-exposed to the conditioning context alone
   c. Veh- and CORT-injected mice are re-exposed both to the tone and to the context but spaced out of 2 h
5. On day 4 assess the quality of the fear memory resulting from these different re-exposure conditions to trauma-related stimuli by using the classical memory tests used on day 2.

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a. Animals from each re-exposure condition are first re-exposed to the tone cue in the familiar context.
b. Animals from each re-exposure condition are then re-exposed to the conditioning context 2 h later.

6. While an abnormal fear response to the tone together with a low conditioned fear to the context would confirm PTSD-like memory, an absence of conditioned fear to the tone together with a high conditioned fear to the context would attest for a normalization of traumatic memory (Figure 3, adapted from Al Abed *et al.*, 2020).

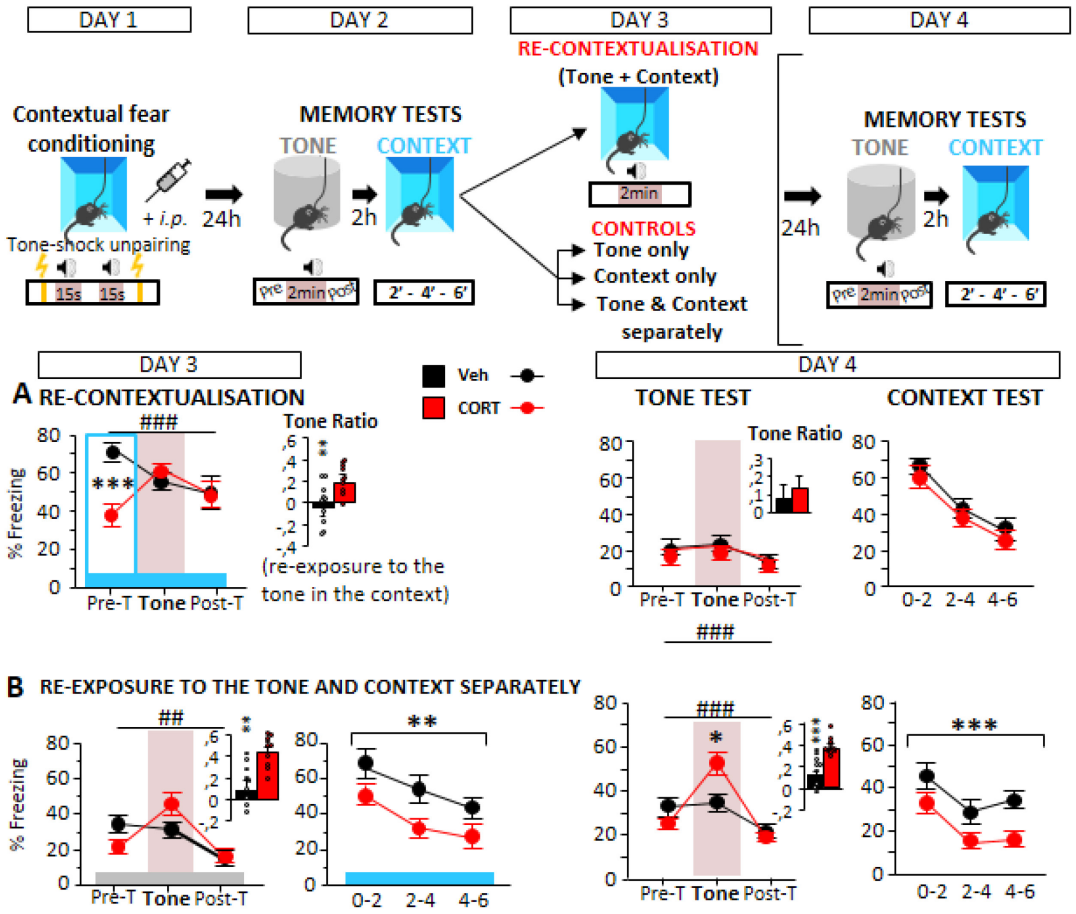

Figure 3. Switching from PTSD-like memory to normal fear memory by trauma re-contextualization. Top panel, scheme illustration of the behavioral procedure: fear conditioning (Day 1), memory tests (Day 2), trauma re-contextualization by complete re-exposure to trauma-related cues (*i.e.*, to the tone in the conditioning context vs. partial re-exposure to the tone alone, context alone, or tone and context spaced out of 2 h; Day 3), memory tests (Day 4). **A.** Curative effect of trauma re-contextualization with a switch from PTSD-like memory on Day 3 to normal contextual memory on Day 4: complete re-exposure to trauma-related cues in CORT-injected mice abolishes the abnormal fear response to the tone and normalizes the conditioned fear to the context (*Veh vs. CORT*: ns). **B.** Partial re-exposure to trauma-related cues (*i.e.*, tone and context spaced out of 2 h) on Day 3 does not modify the PTSD-like memory profile in CORT-

injected mice, which still display an abnormal fear response to the tone together with a low conditioned fear of the context on Day 4 (minimum significance in the tone test: *repeated measures* × *Veh/CORT:* $F_{2,36} = 4.755$; $P = 0.0147$; in the context test: *Veh/CORT*: $F_{1,18} = 4.570$; $P = 0.0465$). Similar results are obtained after partial re-exposure to the tone alone or to the context alone (data not shown, cf. Al Abed *et al.*, 2020). CORT-injected mice discriminate the two contexts used, as attested by their higher freezing levels in the conditioning context than in the familiar chamber (first 2 min, 2a vs. 2b; $F_{1,17} = 7.166$; $P = 0.0159$). The blue and grey bars symbolize the conditioning context and the familiar/safe chamber, respectively; Pre-T: Pre-Tone; Post-T: Post-Tone. Data are presented as mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$. #: block x condition interaction ($P < 0.05$); ##$P < 0.01$; ###$P < 0.005$. Adapted from Al Abed *et al.*, 2020.

B. The optogenetic inhibition of dCA1 neurons during the re-contextualization of traumatic memory suppresses its normalization.
   1. Cf. Part I: Virus transfection and fiber implantation.
   2. Replicate Part III, Procedure A, 1-3 in CORT-injected mice only.
   3. When placing the CORT-injected animals in the conditioning chamber for the re-contextualization procedure, connect each animal to the fiber-optic patch cords for optogenetic manipulations (~6 mW per implanted fiber bilaterally conducted from the laser).
      a. In mice expressing ArchT channel and GFP in the dorsal hippocampus and chronically implanted with optic fibers in the dCA1, transitory inhibit the dCA1 neuronal activity by activating the laser (continuous light deliver, 526 nm) during the 6 min re-contextualization procedure.
      b. In control mice expressing ArchT channel and GFP in the dorsal hippocampus and chronically implanted with optic fibers in the dCA1, maintain the laser OFF during the re-contextualization procedure (Figure 4, adapted from Al Abed *et al.*, 2020).

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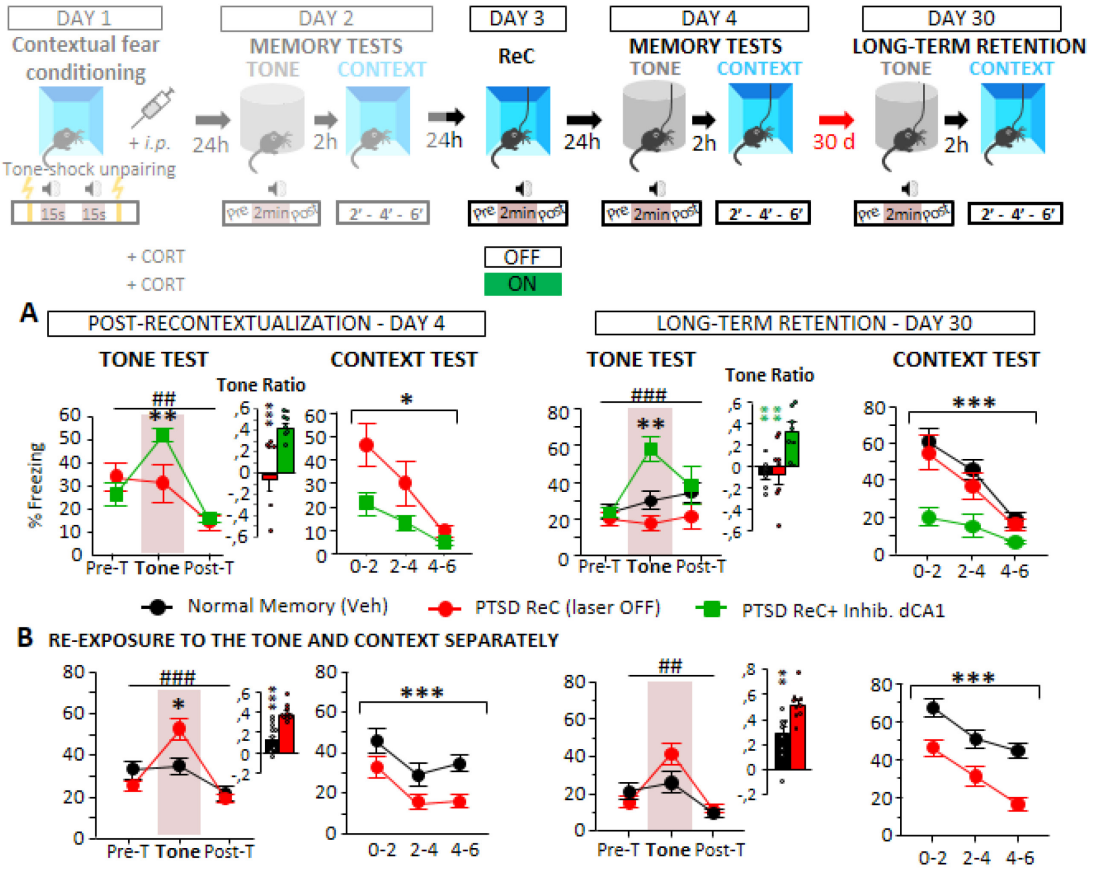

Figure 4. Trauma re-contextualization is hippocampus-dependent and is persistent in the long-term. Top panel, scheme illustration of the behavioral procedure: fear conditioning (Day 1), memory tests (Day 2), trauma re-contextualization (ReC) by complete re-exposure to trauma-related cues (*i.e.*, to the tone in the conditioning context; short-term: Day 4, long-term: Day 30) in control CORT-injected and CORT-injected mice exposed to dCA1 inhibition during re-exposure, memory tests (Day 4). **A.** dCA1 inhibition during complete re-exposure to trauma-related cues on Day 3 blocks the curative effects of such re-exposure: compared to their control (PTSD-like) re-exposed with Laser OFF (ReC; $n = 6$), PTSD-like re-exposed animals with Laser ON ($n = 7$) still display an abnormal fear response to the tone (*repeated measures* x *laser condition:* $F_{2,24} = 6.452$; $P = 0.0057$) together with a low conditioned fear to the context (*laser condition:* $F_{1,12} = 6.922$; $P = 0.0219$) on Day 4 and Day 30 (right panel). **B.** The remained PTSD-like memory subsequent to partial re-exposure to trauma-related cues (tone and context spaced out of 2 h) persists in the long-term (minimum significance in the tone test: *repeated measures* x *Veh/CORT:* $F_{2,34} = 7.935$; $P = 0.0015$; in the context test: *Veh/CORT*: $F_{1,17} = 4.607$; $P = 0.0466$, *repeated measures* x *Veh/CORT:* $F_{2,34} = 4.266$; $P = 0.0022$). Similar results are obtained after partial re-exposure to the tone alone or to the context alone (data not shown here, cf. Al Abed *et al.*, 2020). Statistical significance was assessed by repeated measures (three 2 min-blocks) 2-sided ANOVA with *post-hoc* test when appropriate. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$. #: block x condition interaction ($P < 0.05$); ##$P < 0.01$; ###$P < 0.005$. Pre-T: Pre-Tone; Post-T: Post-Tone. Data are presented as mean ± SEM.

C. PTSD-like and normalized fear memory are persistent

1. Replicate Part III, Procedures A and B.
2. To assess the persistence of the PTSD-like memory and normalized fear memory resulting from the different re-exposure conditions to trauma-related stimuli, submit the animals to the classical memory tests used (re-exposure to the tone alone, then to the conditioning context alone) 30 days after fear conditioning. The curative effect of trauma re-contextualization on PTSD-like memory by complete re-exposure to trauma-related cues (*i.e.*, tone in the conditioning context on day 3) was shown to be long lasting (Figure 4A, right panel). Similarly, animals that still displayed PTSD-like fear memory on day 4 after having been subjected to the inefficient partial re-exposure (*i.e.*, to the tone only, context only, or tone and context spaced out of 2 h), or to the optogenetic inhibition of dCA1 cells during the re-contextualization session, still display a PTSD-like memory on day 30 (Figures 4A and 4B, right panels).

Data analysis

Statistical analyses were performed using analysis of variance (ANOVAs) followed by Fisher's PLSD *post hoc test* when appropriate. Analyses were performed using StatView software. Statistical significance was considered at $P < 0.05$. All experiments were successfully replicated at least once.

All data is available in the main text or the supplementary information of the original paper (Al Abed *et al.*, 2020). Source Data that support the findings of this study are available from the corresponding author upon reasonable request. Source data that support the findings of this study are available at this address: https://doi.org/10.6084/m9.figshare.12644849.

Recipes

1. 0.1 M PBS
   Na_2HPO_4 11.36 g
   NaH_2PO_4 2.4 g
   NaCl 0.9 g
   Add ddH_2O to make 1 L solution and store it at 4°C
2. 0.2 M PB
   Na_2HPO_4 22.72 g
   NaH_2PO_4 4.8 g
   Add ddH_2O to make 1 L solution and store it at 4°C
3. PFA 4%/0.1 M PB solution
   To store the solution, you need to prepare 8% PFA solution:
   a. Mix 80 g of PFA powder in 600 ml of dH_2O and heat to about 70°C until the powder is dissolved (at least 120 min)
   b. Add a few drops of NaOH solution to make the solution transparent

c. Filter the solution using a Whatman® paper filter

d. Adjust the volume to 1 L with dH_2O and store it at 4°C (for a maximum 15 d)

When you need to use PFA 4%/0.1 M PB solution, dilute the solution with 0.2 M PB

4. NaCl (0.9%)

   NaCl 0.9 g

   Add ddH_2O to make 1 L solution and store it at 4°C

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This protocol was used to obtain the data published in Nature Communications (Al Abed *et al.*, 2020).

Competing interests

The authors declare no conflict of interest.

Ethics

All experimental procedures were conducted in accordance with the European Directive for the care and use of laboratory animals (2010-63-EU) and the animals care guidelines issued by theanimal experimental committee of Bordeaux University (CCEA50, agreement number A33-O63-099; authorization N°01377).

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