The role of prelimbic and anterior cingulate cortices in fear memory reconsolidation and persistence depends on the memory age

Thiago Rodrigues da Silva, Jeferson Machado Batista Sohn, Roberto Andreatini, and Cristina Aparecida Stern

Department of Pharmacology, Federal University of Parana, Curitiba 81531-980, Brazil

Reconsolidation is a time-limited process under which reactivated memory content can be modified. Works focused on studying reconsolidation mainly restrict intervention to the moments immediately after reactivation and to recently acquired memories. However, the brain areas activated during memory retrieval depend on when it was acquired, and it is relatively unknown how different brain sites contribute to reconsolidation and persistence of reactivated recent and remote fear memories. Here, we sought to investigate the participation of prelimbic (PL) and anterior cingulate cortices (ACC) in remote fear memory reconsolidation and persistence, respectively.

PL cortex is a key area in the retrieval of remote memories (Frankland et al. 2006; DeNardo et al. 2019; Makino et al. 2019), although there are studies showing that independent of the memory age, the hippocampus is also recruited (Makino et al. 2019). Similarily, it has been suggested that the reconsolidation of recent and remote fear memories depends on the hippocampus and cortical regions, respectively (Lee 2010; Einarsson and Nader 2012; Stern et al. 2014; Inaba et al. 2016; Vanvossen et al. 2017). More specifically, it has been shown that the reconsolidation of remote fear memories depends on the activity of the anterior cingulate (ACC) and prelimbic cortex (PL; Einarsson and Nader 2012; Stern et al. 2014), areas that integrate the medial prefrontal cortex (Vertes 2006) and that are directly or indirectly connected with the dorsal hippocampus (Debiec et al. 2002; Restivo et al. 2009; Goshen et al. 2011; Tayler et al. 2013; Aceti et al. 2015; Dolleman-van der Weel et al. 2019).

Memory reconsolidation is a postretrieval/reactivation process suggested as the mechanism under which memories can be updated or changed (Sara 2010). After retrieval and reactivation, the consolidated memory trace is destabilized and reconsolidated thereafter, being susceptible to interventions during this period. Changes in memory reconsolidation are observed at short-term moments, such as 1 d after intervention and are long-lasting (Nader et al. 2000; Nader and Einarsson 2010; Alberini 2011; Stern et al. 2012). This feature may open an avenue for treating mental disorders, such as posttraumatic stress disorder (Gisquet-Verrier and Le Dorze 2019; Lisboa et al. 2019). The reconsolidation time window is suggested to last up to 6 h (Nader et al. 2000; Stern et al. 2012) however, most studies restrict the drug administration to moments immediately after reactivation of recent fear memories (Stern et al. 2012; Wan et al. 2014; Amiri et al. 2015; Murkar et al. 2019).

Some authors have suggested that reactivation of a recent memory might contribute to memory persistence, a mechanism required to sustain long-term memories that involve late waves of protein synthesis (Nakayama et al. 2013, 2015; da Silva et al. 2016, 2020; Krawczyk et al. 2019). Behaviorally, amnesia induced by interference in memory persistence is observed in a long-term manner, that is, 7 d after memory reactivation, and not in the short-term, such as 1 d later (Nakayama et al. 2015; da Silva et al. 2020). For instance, it has been found that inhibiting PKC into the rats PL cortex for 6, 9, or 12 h, the protein synthesis in the basolateral amygdala 9.5 h, or ERK1/2 in the mice dorsal hippocampus 3 h after memory reactivation, respectively, impaired memory persistence without affecting the reconsolidation (Nakayama et al. 2013; Krawczyk et al. 2019; da Silva et al. 2020).

The brain areas activated during memory retrieval depend on when it was acquired. Evidence suggest that recent memories are more dependent on hippocampus activity to be retrieved than remote memories, which are dependent on cortical activity (Frankland et al. 2006; DeNardo et al. 2019; Makino et al. 2019), although there is evidence showing that independent of the memory age, the hippocampus is also recruited (Makino et al. 2019). More specifically, it has been shown that the reconsolidation of remote fear memories depends on the activity of the anterior cingulate (ACC) and prelimbic cortex (PL; Einarsson and Nader 2012; Stern et al. 2014), areas that integrate the medial prefrontal cortex (Vertes 2006) and that are directly or indirectly connected with the dorsal hippocampus (Debiec et al. 2002; Restivo et al. 2009; Goshen et al. 2011; Tayler et al. 2013; Aceti et al. 2015; Dolleman-van der Weel et al. 2019).

Corresponding author: cristina.stern@ufpr.br

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Tamoxifen (TMX) is an estrogen receptors modulator (Wang et al. 2002; Eigeliene et al. 2016) and inhibitor of protein kinase C (PKC) activity, both in rodents and humans (Abrial et al. 2013). Among the downstream-induced effects of PKC activation, the AMPA receptors trafficking and enhancement of BDNF (brain-derived neurotrophic factor) expression are of relevance to sustaining long-term memory (Giese and Mizuno 2013). PKC activity in the PL cortex is necessary for both, reconsolidation and persistence of a reactivated fear memory (da Silva et al. 2020). Given systemically and immediately or 6 h after memory reactivation, TMX impaired the persistence of a contextual recent fear memory without affecting the reconsolidation (da Silva et al. 2016). However, it is unknown whether remote fear memory is susceptible to interferences in reactivated memory persistence and which brain areas are involved in reactivation-induced persistence of fear memory. Thus, we hypothesized that the reconsolidation and persistence of recent or remote reactivated fear memories would be differently coordinated by the PL cortex and ACC. Then, we evaluated the short- (1 d after) and long-term (7 d after) effects of TMX infused into the PL or ACC immediately or 6 h after recent or remote fear memory reactivation.

**Results**

**The effect of tamoxifen infused into the PL cortex immediately or 6 h after reactivation of recent fear memory**

Animals conditioned to Context A were randomly allocated in two groups and received vehicle or TMX (0.25 µg/0.2 µL/side) into the PL cortex immediately (n = 9–11) after memory reactivation. Repeated-measures ANOVA showed a significant effect of Context A reexposures [F(2,36) = 24.58; P = 0.00001], treatment [F(1,18) = 21.40; P = 0.0002], and the interaction between Context A reexposures and treatment [F(2,36) = 10.62; P = 0.0002]. As shown in Figure 1C, there are no differences among groups during memory retrieval and Test A1 (P = 0.11). However, during Test A2 (P = 0.001; Hedges’ g effect size g = 3.224), the Tukey post-hoc test showed a significant difference between control and TMX-treated groups, suggesting an impairment in reactivated-memory persistence.

The effect of tamoxifen infused into the ACC immediately or 6 h after reactivation of recent fear memory

Animals conditioned to Context A were randomly allocated in two groups and received vehicle or TMX (0.25 µg/0.2 µL/side) into the ACC immediately (n = 8/group) after memory reactivation. Repeated-measures ANOVA showed a significant difference of Context-A reexposures [F(2,28) = 36.30; P = 0.00001], however, no significant difference of treatment [F(1,14) = 0.94; P = 0.3753] or the interaction between Context A reexposures and treatment was observed [F(2,28) = 0.15; P = 0.85]. As observed in Figure 2B, TMX-treated group present similar percentages of freezing time along the sessions, suggesting no effect in memory reconsolidation. There was a reduction of freezing time in Test A2 when compared with memory retrieval, suggesting an extinction of fear memory in both groups.

An independent group of conditioned animals was randomly allocated in two groups and received vehicle or TMX (0.25 µg/0.2 µL/side) into the ACC 6 h (n = 7/group) after memory reactivation. Repeated-measures ANOVA showed a significant difference of Context A reexposures [F(2,24) = 16.92; P = 0.00003], however, no significant difference of treatment [F(1,12) = 0.09; P = 0.76] or the interaction between Context A reexposures and treatment was observed [F(2,24) = 0.06; P = 0.94]. As observed in Figure 2C,
TMX-treated group present similar percentages of freezing time along the sessions, suggesting no effect in memory reconsolidation. There was a reduction of freezing time in Test A2 when compared with memory retrieval, suggesting an extinction of fear memory in both groups.

The effect of tamoxifen infused into the PL cortex immediately or 6 h after reactivation of remote fear memory

Animals conditioned to Context A were randomly allocated in two groups and received vehicle or TMX (0.25 µg/0.2 µL/side) into the PL cortex immediately (n = 7–9) after remote memory reactivation, conducted 21 d after fear conditioning. Repeated-measures ANOVA showed significant effect of Context A reexposures [F(2,28) = 29.14; P = 0.00001], treatment [F(1,14) = 15.15; P = 0.001], and an interaction between Context A reexposures and treatment [F(2,28) = 26.52; P = 0.00001]. As shown in Figure 3B, all groups presented similar levels of freezing during memory retrieval. During Test A1 (P = 0.0007; Hedges’ g effect size g = 5.046) and Test A2 (P = 0.0001; Hedges’ g effect size g = 2.099), the Tukey post-hoc test showed significant difference between control and TMX-treated groups and this effect lasted for at least 1 wk, suggesting an impairment of remote memory reconsolidation.

Figure 2. Recent fear memory reconsolidation and persistence do not rely on the ACC. (A) The diagram represents the experimental design. Animals were familiarized to Context A, that was paired with three footshocks (US) a day later. After 24 h, immediately or 6 h after recent memory reactivation, the animals received bilateral infusion of either vehicle (Veh) or TMX (0.25 µg/0.2 µL) intra-ACC. One and seven days later, the animals were submitted to Tests A1 and A2 to assess TMX effects on memory. (B) Effects of TMX when given into ACC cortex immediately after reactivation of recent fear memory. TMX-treated animals did not show significant differences in freezing behavior when compared to respective controls during Test A1 and Test A2. (C) Effects of TMX when given into ACC cortex 6 h after reactivation of recent fear memory. TMX-treated animals did not show significant differences in freezing behavior when compared to respective controls during Test A1 and Test A2. Values are expressed as mean ± S.E.M. The hashtag indicates a statistically significant difference (P ≤ 0.05) from the reactivation session. (Repeated-measures ANOVA followed by the Tukey test.)

Figure 3. Remote fear memory reconsolidation relies on the PL cortex. (A) The diagram represents the experimental design. Animals were familiarized to Context A, that was paired with three footshocks (US) a day later. After 21 d, immediately or 6 h after remote memory reactivation, the animals received a bilateral infusion of either vehicle (Veh) or TMX (0.25 µg/0.2 µL) intra-PL cortex. One and seven days later, the animals were submitted to Tests A1 and A2 to assess the TMX effects on memory. (B) Effects of TMX when given into PL cortex immediately after reactivation of remote fear memory. TMX-treated animals presented less freezing time than controls during Test A1 and Test A2, suggesting an impairment of remote memory reconsolidation. (C) Effects of TMX when given into PL cortex 6 h after reactivation of remote fear memory. TMX-treated animals did not show significant differences in freezing behavior when compared to respective controls during Test A1 and Test A2, suggesting that the persistence of a reactivated remote memory is independent of the PL cortex. Values are expressed as mean ± S.E.M. The asterisk indicates a statistically significant difference (P ≤ 0.05) from the respective control group. The hashtag indicates a statistically significant difference (P ≤ 0.05) from Veh of Test A2 compared to the memory reactivation section (Repeated-measures ANOVA followed by the Tukey test).
An independent group of conditioned animals was randomly allocated in two groups and received vehicle or TMX (0.25 µg/0.2 µL/side) into the PL cortex 6 h (n=10/group) after remote memory reactivation, conducted 21 d after fear conditioning. Repeated-measures ANOVA showed significant effect of Context-A reexposures [F(2,36)=24.50; P=0.0001], however, no significant difference of treatment [F(1,18)=0.73; P=0.40] or the interaction between Context-A reexposures and treatment was observed [F(2,36)=0.52; P=0.59]. As observed in Figure 3C no change in freezing behavior was observed, suggesting that persistence of a reactivated remote memory is independent of the PL cortex.

The effect of tamoxifen infused into the ACC cortex immediately or 6 h after reactivation of remote fear memory

Animals conditioned to Context A were randomly allocated in two groups and received vehicle or TMX (0.25 µg/0.2 µL/side) into the ACC immediately (n=7–8) after remote memory reactivation, conducted 21 d after fear conditioning. Repeated-measures ANOVA showed significant effect of Context A reexposures [F(2,26)=19.14; P=0.00001], treatment [F(1,13)=25.06; P=0.0002] and an interaction between Context A reexposures and treatment [F(2,26)=3.4285; P=0.04770]. As shown in Figure 4B, all groups presented similar levels of freezing during memory reactivation. During Test A1 (P=0.027; Hedges’ g effect size g=1.569) and Test A2 (P=0.001; Hedges’ g effect size g=2.213) the Tukey post-hoc test showed a significant difference between control and TMX-treated groups, suggesting an impairment of remote memory reconsolidation.

An independent group of conditioned animals was randomly allocated in two groups and received vehicle or TMX (0.25 µg/0.2 µL/side) into the PL 6 h (n=9/group) after remote memory reactivation, conducted 21 d after fear conditioning. Repeated-measures ANOVA showed significant effect of Context-A reexposures [F(2,32)=15.14; P=0.00002], treatment [F(1,16)=5.76; P=0.02], and the interaction between Context-A reexposures and treatment [F(2,32)=3.14; P=0.046]. As observed in Figure 4C the TMX-treated group presented less fear behavior than control in Test A2 (P=0.02; Hedges’ g effect size g=1.391) suggesting that persistence of reactivated remote memory is dependent of the ACC.

Discussion

Our results showed that TMX administered into the PL cortex immediately after memory retrieval impaired the reconsolidation of recent and remote fear memory; the treatment with TMX into the PL cortex 6 h after memory reactivation impaired the persistence of the recent fear memory, but not the persistence of remote memory; the TMX infusion into the ACC 6 h after memory retrieval impaired the reconsolidation of remote fear memory without affecting the recent memory; the treatment with TMX into the ACC 6 h after memory reactivation impaired the persistence of the remote fear memory, but not the persistence of the recent fear memory.

Rats that received TMX into the PL cortex immediately after memory reactivation presented less freezing behavior than controls on the following day, suggesting an impairment in fear memory reconsolidation. This result agrees with studies showing the participation of this area in reconsolidation of recent fear memories by inhibiting mTOR, blocking the CB1 or the alpha-1 adrenergic receptors, by inactivating it with muscimol or activating it with DREADDs or with a NMDA receptor agonist (Do Monte et al. 2013; Stern et al. 2014, 2015; Levin et al. 2017; Vanvossen et al. 2017; Ye et al. 2017). Importantly, the TMX effect lasted for at least 1 wk, suggesting no spontaneous recovery of fear memory, a result that agrees with reports showing no spontaneous recovery of fear memory 1 wk after impairing memory reconsolidation (Duvarcı and Nader 2004; Bustos et al. 2006; Stern et al. 2012). The reconsolidation time-window is suggested to last up to 6 h after reactivation (Nader et al. 2000; Bustos et al. 2006; Stern et al. 2012). Indeed, the TMX infusion into the PL 6 h after memory reactivation did not change the freezing behavior when the animals underwent Test A1 (24 h later), confirming previous studies showing that at this time-point interferences in memory reconsolidation are not observed (Nader et al. 2000; Stern et al. 2012). However, a reduction in freezing behavior, but not significant, was observed in Test A1 of TMX-treated rats. Since only one dose of TMX was tested,
mediated signaling and inhibits PKC (Horgan et al. 1986; blocks protein synthesis, while TMX interferes with estrogen-to account for the different results observed, since we have adopt-(Alberini 2011; Espejo et al. 2016), however, this factor is unlikely boundary condition to memory labilization and reconsolidation conditioning training or previous stress has been suggested as a fi

tion of a recent fear memory did not change the freezing behav-

tion in this area disrupted remote contextual fear memory reconso-
lidation (Einarsson and Nader 2012), however, it disagrees with a study showing that protein synthesis inhibition in the ACC imme-
diately after remote memory reactivation is resistant to the reconsolidation-impairing effect (Frankland et al. 2006). This dif-
ference might be related to the treatment adopted, since TMX in-
terferes with specific intracellular signaling pathways (Zarate and Manji 2009) and anisomycin disrupts the general protein synthe-
sis; the species used, that is, rats versus mice; and the memory age since Frankland et al. (2006) tested a 36-d old memory and here a 21-d old fear memory was tested. Together, the present re-
sults provide further evidence on the role of the ACC in remote fear memory reconsolidation.

When TMX was infused into ACC 6 h after remote fear memory reactivation, no effect was observed on the following day, a re-
sult consistent with a study showing that the blockade of AMPA/kainate receptors in the ACC 6 h after remote memory reactivation did not affect freezing expression 24 h later (Einarsson et al. 2015). Here, a significant reduction in freezing behavior of TMX-treated rats was observed 7 d later, suggesting that persistence of reactivat-ed remote fear memory depends on the ACC, providing evidence that memory retrieval may trigger mechanisms underpinning re-

ote, a similar result was observed after the systemic administration of TMX, the inhibition of PKC or PKMζ in the PL cortex, or the inhibition of protein synthesis in the basolateral amygdala (Nakayama et al. 2013; da Silva et al. 2016, 2020). Together, these results provide further evidence that the PL is re-
cruited for recent fear memory reconsolidation and persistence of reactivat

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To explore this, the effect of TMX infused in this region immediately or 6 h after re-
cent memory reactivation was investigated. The infusion of TMX into the ACC immediately after memory reactivation did not change the freezing behavior when compared to controls 1 or 7 d later, suggesting no impairment in memory reconsolidation and persistence. This result is in line with works showing that the infusion of anisomycin into the ACC of mice after reactiva-
tion of a recent fear memory did not change the freezing behav-

bition triggers independent mechanisms underlying memory recon-
solidation and/or persistence, however, evidence on these aspects are still incipient.

It has been suggested that the reconsolidation of recent memories is less dependent on cortical regions such as the ACC (Do-Monte et al. 2013; Makino et al. 2019). To explore this, the effect of TMX infused in this region immediately or 6 h after re-
cent memory reactivation was investigated. The infusion of TMX into the ACC immediately after memory reactivation did not change the freezing behavior when compared to controls 1 or 7 d later, suggesting no impairment in memory reconsolidation and persistence. This result is in line with works showing that the infusion of anisomycin into the ACC of mice after reactiva-
tion did not change the freezing behavior (Frankland et al. 2006). However, our result contrasts with findings showing that anisomycin given into the rat ACC immedi-
ately after reactivation of a recent fear memory impaired memory reconsolidation (Einarsson and Nader 2012). The intensity of conditioning training or previous stress has been suggested as a boundary condition to memory labilization and reconsolidation (Alberini 2011; Espejo et al. 2016), however, this factor is unlikely to account for the different results observed, since we have adopt-
ed a weaker fear conditioning protocol than Einarsson and Nader (2012). Another difference is the drug used, since anisomycin blocks protein synthesis, while TMX interferes with estrogen-mediated signaling and inhibits PKC (Horgan et al. 1986; O’Brien et al. 1990). The PKC role in memory reconsolidation has been addressed. For instance, inhibiting PKC in the dorsal hippocampus or the PL cortex immediately after memory retriev-

al impaired recent fear memory reconsolidation (Bonini et al. 2007; da Silva et al. 2020). However, the hippocampal contribu-
tion to recent memory reconsolidation is greater than that of the cortical areas such as the ACC (Webb et al. 2017). Together, our findings reinforce previous results suggesting that the ACC is not involved in recent fear memory reconsolidation and sug-

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medial prefrontal cortex, and that remote memory reconsolidation, but not its persistence, overlap in PL.

It is well accepted that ACC activity sustains the expression of remote memories (Frankland et al. 2004). However, since the effect of TMX 6 h after reactivation of remote fear memory in the ACC was not tested in the absence of the Test A1, we cannot exclude the possibility that TMX effect is a long-term effect in fear memory retrieval, as observed by other authors (Parsons and Davis 2011). The connections between the ACC, dorsal hippocampus and entorhinal cortex are important not only to sustain remote fear memories, but it is also necessary for memory consolidation (Insel and Takehara-Nishiuchi 2013; Tayler et al. 2013; Bero et al. 2014). For instance, optogenetic inhibition of the medial prefrontal cortex inhibited the entorhinal-hippocampal circuit activation and impaired long-term memory consolidation, suggesting that early cortical activity is critical for a stimulus-induced hippocampal activation and memory consolidation (Bero et al. 2014). At a recent, but not at a remote time point, spine density or c-Fos expression is higher in the hippocampus than in ACC of fear conditioned rats, whereas an inverse temporal pattern is observed in ACC, suggesting that gradual changes occur modifying the connectivity between the hippocampus and the ACC after the formation and reactivation of the remote memory (Restivo et al. 2009; Aceti et al. 2015). These studies align with the hypothesis that enhancement of ACC activity along time supports remote fear memories expression. However, some studies suggest that the CA1 region of the hippocampus is required for remote memory reactivation since inhibiting the protein synthesis in the hippocampus impaired a remote memory reconsolidation (Debiec et al. 2002; Goshen et al. 2011). It remains to be investigated whether these connections contribute to the TMX effect observed in the persistence of remote memory and to the dissociation of the ACC and the PL cortex participation in the reconsolidation and persistence processes.

The effects of TMX observed here agree with works showing the role of PKC in memory reconsolidation and persistence (Bonini et al. 2007; da Silva et al. 2016, 2020). However, its potential effect as a modulator of estrogen receptors cannot be excluded. The E α and Eb eta estrogen receptors are expressed in the medial prefrontal cortex (Almey et al. 2014), ample evidence supports their role in memory processing (Tuscher et al. 2015) and the TMX consolidation impairing effect of inhibitory avoidance depended on E α receptors (Lichtenfeld et al. 2017). Furthermore, studies comparing males and females have suggested differences in fear memory consolidation, extinction, and labilization/reconsolidation processing (Chang et al. 2009; da Silva et al. 2016; Franzen et al. 2019). Given systemically, TMX did not impair fear memory persistence in estrous females, although it similarly impaired memory persistence in proestrous and diestrous females and in males (da Silva et al. 2016). However, it is yet unknown whether and which estrogen receptor located in the medial prefrontal cortex is involved in TMX-induced effects in recent and remote fear memory reconsolidation and persistence.

In conclusion, our findings provide further evidence on the role of the PL and the ACC in recent and remote fear memory reconsolidation and suggest that as memory ages, the persistence of a reactivated fear memory becomes independent of the PL cortex and dependent of the ACC.

Materials and Methods

Animals

Adult male Wistar rats, 3 mo old, weighing between 290–320 g (from the Biological Sciences Sector of the Federal University of Paraná) were used. The animals were housed in plastic home cages in groups of four to five per cage with access to food and water ad libitum. The animals were kept in controlled temperatures of 22±2°C and a 12-h light–dark cycle (7:00 a.m.–19:00 p.m.). The experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals and the ARRIVE Guidelines (National Research Council 2011), and after the approval of the experimental protocol by the Ethics Committee for the care and use of laboratory animals of the Biological Sciences Sector of the Federal University of Paraná (authorization number 1011).

Drug

Tamoxifen (TMX; 0.25 µg/0.2 µL Sigma) a nonselective PKC inhibitor and a selective estrogen response modifier, was dissolved in saline containing 20% dimethyl sulfoxide, that alone was used as control (DMSO; Talebi et al. 2010).

Stereotaxic surgery and drug infusion

Rats were acclimated in the laboratory vivarium for 24 h and then were anesthetized with ketamine (75 mg/kg; Carlier, Brazil) and xylazine (15 mg/kg; Sespo, Brazil), associated with local anesthesia (3.0% lidocaine with norepinephrine 1:50000; Dentsply, Brazil). After anesthesia, rats were positioned in a stereotaxic frame and two stainless-steel guide cannulas (length: 11 mm; outer diameter: 0.7 mm) were implanted bilaterally aiming at the PL cortex or ACC following the coordinates (AP=+11.8 mm interaural, ML=±0.6 from central suture, DV=−1.8 from the skull) of The Rat Brain in Stereotaxic Coordinates (Paxinos and Watson 2009) and with two screws and dental acrylic they were fixed to the skull. A stylet containing 20% dimethyl sulfoxide, that alone was used as control (DMSO; Talebi et al. 2010).
was introduced inside each guide cannula to prevent obstruction. After the surgery, the animals received 0.4 mL of ibuprofen orally (20 mg/mL, Natulab, Brazil), and returned to the home cage.

After 10 d, the animals underwent the protocol of contextual fear conditioning. Immediately or 6 h after memory reactivation, rats received a bilateral infusion with dental needles introduced through guide cannulas until their tips were 1.5 mm (PL cortex) or 1.2 mm (ACC) below the cannula end.

The drug was injected over 1 min (0.2 µL/side) using two 5.0 µL Hamilton syringes connected to an automatic infusion pump (Insight, Brazil). A polyethylene catheter was inserted between the upper end of the dental needles and the syringes. To monitor the flow of the drug, an air bubble was displaced inside the polyethylene. The needles were kept for additional a 45 sec after the end of the injections to prevent backflow.

After the end of experiments, the animals were anesthetized as mentioned above, the methylene blue was infused through the guide cannulas, the animals were killed, and the brains were removed to confirm the drug infusion sites. Brain slices (50 µm thick) were obtained on a vibratome (Leica), and slides were mounted, stained with nissl, and the infusion site was ascertained. The animals were included in the statistical analysis when the infusion was bilateral in the PL or ACC cortices (Fig. 5).

General procedures
To avoid possible circadian influences on learning and memory processing, the experiments were performed between 1:00 and 5:00 P.M. and conducted similarly to previous studies (da Silva et al. 2020). All animals were moved and acclimated to the experimental room for 30 min before each session. The experimental rooms were kept under controlled temperature (21 ± 2°C) and brightness (~78 lux).

Contextual fear conditioning was done in Context A, a chamber, made of gray aluminum sidewalls, with a transparent acrylic top cover and front wall (26 × 31.5 × 21 cm; Insight, Brazil), the floor was composed of stainless steel-bars (3 mm in diameter and spaced 0.9 mm) connected to a shock generating source. Context B (34 × 26 × 33 cm), an unpaired chamber, had transparent acrylic walls and floor, and a black top cover to provide different clues from the paired context. Context B was used to assess the generalization of fear.

Contextual fear conditioning protocol consisted of the following sessions: on the first day the animals were familiarized to Context A for 3 min. After 24 h, the animals were submitted to Context A, after the initial 30 sec the animals received the unconditioned stimulus (US) that consisted of three footshocks of 0.8 mA/3 sec in 30 sec intervals. After the last US, the animal remained for 30 sec in Context A and then returned to the home cage. When the recent memory was evaluated, the reactivation session was conducted 24 h after conditioning and the animals were exposed to Context A for 3 min without the US presentation. After this session, the animals received immediately or 6 h later, bilateral infusions of vehicle or TMX into the PL cortex or ACC. After 1 or 7 d, the animals were reexposed to Context A for 3 min, these sessions were named Test A1 and Test A2, respectively. To evaluate the remote memory, the animals were submitted to a reactivation session 21 d after fear conditioning (Stern et al. 2014). Then, the animals received immediately or after 6 h, bilateral infusions of vehicle or TMX into the PL cortex or ACC. One or 7 d later, the animals were submitted to Test A1, and Test A2, respectively. The chambers were cleaned with a 10% ethanol/water solution after each session.

The freezing behavior, characterized as the total absence of body and head movements, except those associated with breathing (Blanchard and Blanchard 1969) was evaluated. The sessions were recorded by a video camera and freezing time was quantified in seconds using a stopwatch by a blind and trained observer and expressed as a percentage of the total session time.

After the end of the behavioral experiments, to confirm the injection side, the rats were anesthetized using 1.0 mL/kg of a solution containing xylazine (10 mg/mL, Carlier) and chloral hydrate (2.3 mg/mL, Vetec) intraperitoneally (i.p.). Methylene Blue dye (0.2 µL/hemisphere) was injected through the guide cannulas for the subsequent evaluation of the locations where vehicle or TMX was infused.

Brains were removed and immersed in a 10% formalin solution. The brain slices (50 µm thick) were obtained in a vibratome (Leica), mounted on microscope glass slides, and the injection site was determined. Animals were included in the analysis when both sides of the PL cortex or the ACC were correctly marked by methylene blue.

Experimental design
Experiment 1: To evaluate whether TMX infused into the PL cortex interferes in the reconsolidation or persistence of a reactivated recent memory rats that underwent contextual fear conditioning were randomly allocated to receive a bilateral infusion of either vehicle or TMX (0.25 µg/0.2 µL/side) intra-PL cortex immediately or 6 h after the reactivation session (Fig. 1).

Experiment 2: To evaluate whether TMX infused into the ACC interferes in the reconsolidation or persistence of a reactivated recent memory rats that underwent contextual fear conditioning were randomly allocated to receive a bilateral infusion of either vehicle or TMX (0.25 µg/0.2 µL/side) intra-ACC immediately or 6 h after the reactivation session (Fig. 2).

Experiment 3: To evaluate whether TMX infused into the PL cortex interferes in the reconsolidation or persistence of a reactivated remote memory, rats that underwent contextual fear conditioning were randomly allocated to receive a bilateral infusion of either vehicle or TMX (0.25 µg/0.2 µL/side) intra-PL cortex immediately or 6 h after the reactivation session (Fig. 3).

Experiment 4: To evaluate whether TMX infused into the ACC interferes in the reconsolidation or persistence of a reactivated remote memory, rats that underwent contextual fear conditioning were randomly allocated to receive a bilateral infusion of either vehicle or TMX (0.25 µg/0.2 µL/side) intra-ACC immediately or 6 h after the reactivation session (Fig. 4).

Statistical analysis
The results are expressed as mean ± S.E.M. After ensuring the homogeneity of the data, the percentage of freezing time observed in Context A (reactivation session, Test A1, and Test A2) was analyzed by repeated-measures analysis of variance (ANOVA). The Tukey test was used for post-hoc comparisons when ANOVA achieved significance. The level of statistical significance was set at P < 0.05. For statistical analysis, Statistica 12 (StatSoft) was used, and GraphPad Prism 8 (GraphPad Prism) was used for graphing. The formula for Hedges’ g, which reflect the mean differences between two groups (n ≤ 20 per group) that could be dissimilar in size, was used to calculate the size effect. Large effect size was considered when the g value was ≥ 0.8 (Ellis 2010).

Competing interests statement
The authors declare no competing interests.

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Authors contributions: T.R.S.: conducted the experiments, C.A., S.R.A., J.M.B.S., and T.R.S.: data analysis and interpretation, C.A.S., R.A., J.M.B.S., and T.R.S.: elaborated the work design, interpreted the results, and wrote the paper.

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