

**LACK OF EFFECT OF PROPRANOLOL ON THE RECONSOLIDATION OF
CONDITIONED FEAR MEMORY DUE TO A FAILURE TO ENGAGE MEMORY
DESTABILISATION**

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ABSTRACT

The prospect of exploiting memory reconsolidation to treat mental health disorders has received great research interest, particularly following demonstrations that the β -adrenergic receptor antagonist propranolol, which is safe for use in humans, can disrupt the reconsolidation of pavlovian conditioned fear memories. However, recent studies have failed to replicate the effects of propranolol on fear memory reconsolidation, and have questioned whether treatments based upon reconsolidation blockade would be robust enough for clinical translation. It remains possible, though, that studies reporting no effect of propranolol on memory reconsolidation could be due to a failure to engage the memory destabilisation process, which is necessary for the memory to become susceptible to disruption with amnestic agents. Demonstrating that memory destabilisation has *not* been engaged is challenging when only using behavioural measures, but there are molecular correlates of memory destabilisation that can be used to determine whether memory lability has been induced. Here, we attempted to replicate the classic finding that systemic administration of propranolol disrupts the reconsolidation of a pavlovian auditory fear memory. Following a failure to replicate, we manipulated the parameters of the memory reactivation session to enhance prediction error in an attempt to overcome the boundary conditions of reconsolidation. On finding no disruption of memory despite these manipulations, we examined the expression of the post-synaptic density protein Shank in the basolateral amygdala. Degradation of Shank has been shown to correlate with the induction of memory lability, but we found no effect on Shank expression, consistent with the lack of observed behavioural effects.

Keywords: memory, reconsolidation, propranolol, fear, Shank, rat

HIGHLIGHTS

- Propranolol did not disrupt the reconsolidation of pavlovian fear memories
- Varying reactivation parameters to enhance prediction error did not change outcomes
- Molecular markers of destabilisation suggest that the memory did not become labile

INTRODUCTION

Since the rediscovery of memory reconsolidation at the beginning of the 21st century (Nader et al., 2000), there has been intense interest in exploiting this process to provide a novel form of treatment for mental health disorders, including post-traumatic stress disorder (Dębiec and Altemus, 2006; Dębiec and LeDoux, 2006). The potential development of reconsolidation-based interventions for mental health disorders was boosted by the finding that the β -adrenergic receptor antagonist, propranolol, can be used to disrupt the reconsolidation of conditioned fear memories in both rats (Dębiec and LeDoux, 2004) and humans (Kindt et al., 2009; Soeter and Kindt, 2015), as unlike the protein synthesis inhibitors used in early reconsolidation studies (Nader, Schafe and LeDoux, 2000), propranolol is approved for use in humans.

Recently, the robustness of reconsolidation-based interventions has been questioned (Treanor et al., 2017), with some high-profile failures to replicate previously observed amnesic effects of propranolol (Bos et al., 2014; Luyten et al., 2021; Schroyens et al., 2017; Thome et al., 2016) and behavioural interference (Chalkia et al., 2020; Luyten and Beckers, 2017). However, reconsolidation is a multi-stage process, involving first the destabilisation of the memory under appropriate 'reactivation' or 'reminder' conditions, usually involving a 'violation of expectations' (Pedreira et al., 2004), and the subsequent restabilisation of the memory in its updated form (Lee, 2009). It was originally thought – and still is by many – that propranolol was preventing the restabilisation of the memory when given in conjunction with memory reactivation (Dębiec and LeDoux, 2004; Milton et al., 2008). Given the recent questioning of propranolol's efficacy in disrupting memory reconsolidation, alternative mechanistic accounts have been proposed. Some studies have suggested that β -adrenergic receptor-mediated signalling is necessary for the destabilisation of memories (Lim et al., 2018; Otis et al., 2013; Otis and Mueller, 2017; Otis et al., 2015) or that it modulates memory retrieval (Leal Santos et al., 2021). However, this β -adrenergic receptor dependence of destabilisation appears to be associated with regions including the hippocampus and medial prefrontal cortex, rather than the basolateral amygdala, which is necessary for the reconsolidation of pavlovian memories (Duvarci et al., 2005; Dębiec and LeDoux, 2004; Lee et al., 2006; Milton et al.,

2008; Nader, Schafe and LeDoux, 2000). Alternatively, the failures to replicate the amnestic effects of propranolol could be due to the boundary conditions that control when a memory becomes destabilised and consequently susceptible to disruption with amnestic agents (de Oliveira Alvares and Do-Monte, 2021). However, it is difficult to conclude that memory destabilisation has not been induced from behavioural data alone without engaging in circular reasoning. Other measurements, such as the analysis of molecular markers of memory destabilisation, can be used to corroborate whether a failure to engage memory destabilisation accounts for a lack of amnestic effect (Cahill and Milton, 2019). One such marker is the post-synaptic density protein Shank, which is degraded in the hippocampus when contextual fear memories become destabilised during a reminder session (Lee et al., 2008) and in the perirhinal cortex during the destabilisation of object recognition memories (Wideman, 2019). If a reduction in the expression of Shank protein is indicative that a memory has destabilised, then sustained Shank expression can be used to infer that a specific reminder session has *not* engaged memory destabilisation.

Here, we initially aimed to replicate the findings of Dębiec and LeDoux (2004), that post-reactivation systemic administration of the β -adrenergic receptor antagonist propranolol could produce a persistent impairment in freezing in rats previously trained on auditory fear conditioning. However, following an apparent failure to replicate the amnestic effects of propranolol on fear memory reconsolidation, we varied numerous experimental conditions in an attempt to overcome any boundary conditions that were preventing memory destabilisation. Finally, we assessed the expression of Shank protein in the basolateral amygdala, a region known to support the reconsolidation of pavlovian fear memories (Doyère et al., 2007; Dębiec et al., 2006; Dębiec and LeDoux, 2004; Milton et al., 2013) to determine whether the reminder sessions were inducing memory destabilisation.

EXPERIMENTAL PROCEDURES

Subjects

Subjects were 213 Lister-Hooded rats (Charles River, Bicester, UK), weighing at least 200g at the start of experiments, housed in pairs or groups of four in a vivarium maintained at 21°C on a reversed light-dark cycle (lights on at 1900hrs). Water and food were available *ad libitum* except during behavioural procedures. All experimental procedures were conducted under Project Licences 80/2234 and PA9FBFA9F, in accordance with both the UK Animals (Scientific Procedures) Act 1986, amendment regulations 2012 and the EU legislation on the protection of animals used for scientific purposes (Directive 2010/63/EU) following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB).

Behavioural apparatus and procedures

All behavioural experiments were conducted in four conditioning chambers (Paul Fray Ltd., Cambridge, UK) as described previously (Lee et al., 2005). Rats were habituated to the apparatus for 2 hours on the day prior to conditioning to reduce fear to the context. On the day of conditioning, rats were placed in the chamber for an approximately 48-minute session, consisting of 35 ± 1 minutes of context exposure prior to a 60-second presentation of an auditory CS (usually an 80dB, 10Hz clicker, but for Experiments 4 and 5 an 80dB, 2.9kHz tone) that co-terminated with a 0.5-second, 0.5mA scrambled footshock. Following an intertrial interval of 5 ± 1 minutes, a second CS-US pairing was delivered (except for Experiment 5). The session terminated 5 ± 1 minutes following the final CS-US pairing. 24 hrs following fear conditioning, rats were returned to the same conditioning chambers for a 2-minute memory reactivation session, which consisted of 1 minute of context exposure followed by a 60-second presentation of the CS in the absence of the US. Retention of the fear memory was tested in two post-reactivation long-term memory (PR-LTM) tests, taking place 24hrs and 8 days after the memory reactivation session. These also consisted of 1 minute of context exposure, followed by a 60-second presentation of the CS in the absence of the

US. All sessions were video recorded and scored offline, manually by an experimenter blind to experimental condition.

The study comprised several variations of this standard fear conditioning procedure in separate cohorts of rats, as follows: (1) training in one context (Context A) and reactivating and testing the memory in a different context (Context B), with drug treatment given immediately after the memory reactivation session; (2) training, reactivating and testing the memory in the same context (Context A) with drug treatment given immediately after the memory reactivation session; (3) training, reactivating and testing the memory in the same context (Context A) with drug treatment given 30-minutes before the memory reactivation session; (4) training, reactivating and testing the memory in the same context (Context A) with drug treatment given immediately after the memory reactivation session, but with a tone CS rather than a clicker CS; (5) training, reactivating and testing the memory in the same context (Context A) with drug treatment given immediately after the memory reactivation session, but with a single tone-shock pairing. For Experiment 6, rats were conditioned in Context A, reactivated in Context A or B (or non-reactivated by remaining in the home cage), and 60 minutes later killed by exposure to a rising concentration of carbon dioxide before brains were extracted and rapidly frozen on dry ice for subsequent molecular analyses.

Where different contexts were used, Context A was the 'standard' conditioning chamber which had been cleaned with Distel High-Level Medical Surface Disinfectant (Fisher Scientific, UK). Context B was distinguishable from Context A in having: (i) red light rather than the white houselight; (ii) black and white wallpapers affixed to two of the chamber walls; (iii) a ginger scent (3 drops of Tisserand ginger essential oil, placed on a cotton pad out of reach of the rat at the bottom of the chamber), and (iv) background music playing in the room at 55dB ('Take It Easy' by The Eagles).

Drugs

The β -adrenergic receptor antagonist DL-propranolol (Merck Life Science UK Ltd., UK) was prepared fresh on the day of injection in 0.9% sterile physiological saline (Aqupharm, Animalcare UK) at a concentration of 10mg/ml, for intraperitoneal (i.p.) injection at 1ml/kg. Depending on

experimental group, 30 minutes prior or immediately after fear conditioning, rats received a habituation injection of 0.9% sterile physiological saline at 1 ml/kg. Propranolol or saline vehicle was administered i.p. either 30 minutes prior to the memory reactivation session, or immediately (within 5 minutes) afterwards, in accordance with the administration time of the previous day.

Sample preparation and western blotting

For Experiment 6, rats were killed by exposure to a rising concentration of carbon dioxide and neck dislocation, and the brains were rapidly removed, frozen on dry ice and subsequently stored at -80°C. Samples from the basolateral amygdala were microdissected using a 0.99mm diameter punching tool from 150µm brain sections. The punched tissue was individually disrupted with a Dounce tissue grinder (loose pestle, Wheaton) in 100µl of buffer (10mM HEPES, pH 7.9, 1.5mM MgCl₂, 10mM KCl, 1mM DTT, 1µg/ml Pepstatin A, 10µg/ml leupeptin, 0.5mM PMSF and 10µg/ml aprotinin) and centrifuged for 1000g for 10 minutes at 4°C. The supernatant (cytoplasmic protein extract) was transferred to a clean tube and stored at -80°C. The protein content of each sample was quantified using a spectrophotometer (NanoDrop). 7-8µg of samples were loaded and separated using a 7.5% SDS-PAGE and was electrotransferred to a nitrocellulose membrane (ThermoFisher Scientific, UK). Blots were probed with the following antibodies, which were tested to deliver a linear relationship between the amounts of protein in the blot and signal intensity: mouse anti-pan-Shank (clone N23B/49, MABN24, 1:100, Merck Millipore, UK) and mouse anti-β-actin (1:5000, AbCam, UK) diluted in Intercept® TBS blocking buffer (Li-Cor, UK) containing 0.05% Tween-20. The blots were subsequently imaged using an Odyssey CLx scanner (Li-Cor, UK) following probing of the blots with a fluorescent secondary antibody (IRDye® 680RD goat anti-mouse IgG(H+L), 1:20000, Li-Cor, UK) and scanning on the 700nm channel. Signal analysis and quantification was performed using ImageJ software (National Institutes of Health) by an experimenter blind to experimental condition. The amount of β-actin for each sample was used as a loading control for normalisation.

Statistical analysis

Data are presented as mean \pm s.e.m. unless otherwise stated. Behavioural data were analysed using mixed-effects repeated measures ANOVAs with analysis of the training, reactivation and test data being conducted separately. Data were tested for normality by inspection of Q-Q plots and the Kolmogorov-Smirnov test with the Lilliefors correction, and for homogeneity of variance using Levene's test. Mauchly's test of sphericity was not required as there were fewer than three levels for each within-subjects factor. For training, CS (CS vs. context) and Pairing (first vs. second) were within-subject factors, and prospective Drug group (vehicle vs. propranolol) was the between-subjects factor. For reactivation, CS was the within-subjects factor and Drug group the between-subjects factor. For test, CS and Session (PR-LTM1 vs. PR-LTM2) were the within-subject factors and Drug group was the between-subjects factor. Where significant interactions were observed, these were interrogated further with the use of pairwise comparisons. These were corrected for multiple comparisons with the use of the Šidák correction, which is a mathematically accurate version of the Bonferroni approximation, as recommended by Cardinal & Aitken (2006). Molecular data were analysed using fixed-effects univariate ANOVA with Drug group and experimental Group as between-subjects factors, using the same tests of normality and homogeneity of variance described above.

Data availability

All data accompanying this publication are available at the University of Cambridge data repository (<https://doi.org/10.17863/CAM.72114>).

RESULTS

Attempted replication of Debiec & LeDoux (2004)

Following training with two CS-US pairings in Context A, rats were reactivated with a single presentation of the CS in Context B (**Fig. 1a**). Immediately after the reactivation session, the rats

received 10 mg/kg DL-propranolol (i.p.), and were returned to the home cage. Rats were returned to Context B for memory retention tests conducted 24hrs (PR-LTM1) and 8 days (PR-LTM2) later. There were no differences in the acquisition of conditioned fear to the CS (**Fig. 1b**). All rats froze more to the CS than the context (CS: $F_{1,14} = 34.0$, $p < .001$, $\eta^2 = 0.71$) with an increase of freezing on presentation of the second CS, following the first being shocked (Pairing: $F_{1,14} = 44.2$, $p < .001$, $\eta^2 = 0.76$; CS x Pairing: $F_{1,14} = 6.88$, $p = .02$, $\eta^2 = 0.33$). The experimental groups were well-matched for conditioning, with no differences between the prospective drug groups (Drug: $F < 1$; CS x Drug: $F < 1$; Pairing x Drug: $F_{1,14} = 1.11$, $p = .31$). Although there was a CS x Pairing x Drug interaction that approached significance between the prospective groups ($F_{1,14} = 4.29$, $p = .057$), pairwise comparisons revealed that this was due to increased context freezing prior to the second CS (as compared to the first CS) in the prospective VEH group ($p = .007$) while contextual freezing remained low in the prospective PRO group ($p = .69$). There were no differences in the conditioned freezing to the CS between the prospective drug groups (all p 's $> .38$) or to the context (all p 's $> .081$). There were also no differences in the conditioned fear shown by the prospective drug groups in the memory reactivation session in Context B (Drug: $F < 1$), with both groups showing greater freezing to the CS than the context (CS: $F_{1,14} = 424$, $p < .001$, $\eta^2 = .97$; CS x Drug: $F_{1,14} = 3.85$, $p = .07$).

In contrast to previous reports (Dębiec and LeDoux, 2004), we did not observe reductions in conditioned freezing in rats that had received post-reactivation propranolol in either of the two post-reactivation long-term memory (PR-LTM) tests conducted 24hrs and 8 days later. All rats froze more to the CS than Context B (CS: $F_{1,14} = 92.7$, $p < .001$, $\eta^2 = 0.87$) across both PR-LTM sessions (Session: $F_{1,14} = 2.80$, $p = .12$). There were no differences in the levels of fear shown by vehicle-treated or propranolol-treated rats (Drug: $F < 1$; CS x Drug: $F < 1$; Session x Drug: $F < 1$; CS x Session x Drug: $F < 1$). Therefore, we did not replicate Debiec & LeDoux's (2004) previous finding that post-reactivation propranolol, administered systemically, could reduce subsequent conditioned fear.

Effects of post-reactivation propranolol on conditioned fear reactivated and tested in the same context

It is possible that the reactivation and testing of the fear memory in Context B, when it was acquired in Context A, may have promoted the formation of a new memory rather than reactivation of the original fear memory. Consistent with this hypothesis, consolidation of a new fear memory does not appear to be impaired by the administration of post-reactivation propranolol (Dębiec and LeDoux, 2004). Thus, we assessed the effect of post-reactivation propranolol on the reconsolidation of the fear memory when it was trained, reactivated, and tested in the same context (Context A; **Fig. 2a**).

Again, there were no differences in the acquisition of conditioned fear to the CS (**Fig. 2b**). All rats froze more to the CS than the context (CS: $F_{1,30} = 13.8$, $p = .001$, $\eta^2 = 0.32$) and showed increased freezing to the second CS presentation as compared to the first (Pairing: $F_{1,30} = 124$, $p < .001$, $\eta^2 = 0.81$; CS x Pairing: $F_{1,30} = 5.21$, $p = .03$, $\eta^2 = 0.15$). There were no differences in the freezing shown by the prospective drug groups (Drug: $F < 1$; CS x Drug: $F < 1$; Pairing x Drug: $F < 1$; CS x Pairing x Drug: $F < 1$). Rats in both prospective drug groups also showed similar levels of fear during the memory reactivation session (Drug: $F < 1$; CS x Drug: $F_{1,30} = 2.16$, $p = .15$), with greater freezing to the CS than the context (CS: $F_{1,30} = 310$, $p < .001$, $\eta^2 = 0.91$).

When conditioned fear was assessed in the PR-LTM tests, it was found again that post-reactivation propranolol did not affect subsequent conditioned freezing (Drug: $F < 1$; CS x Drug: $F < 1$; CS x Session x Drug: $F < 1$) with all rats showing high levels of fear to the CS, which were sustained across both PR-LTM sessions (CS: $F_{1,30} = 129$, $p < .001$, $\eta^2 = 0.81$; CS x Session: $F < 1$). Thus, maintaining the same context across training, reactivation and testing did not alter the susceptibility of the conditioned fear memory to disruption with propranolol.

Effects of pre-reactivation propranolol on conditioned fear reactivated and tested in the same context

Although it has been argued that post-reactivation manipulations are preferable for demonstrating reconsolidation-based effects (Schiller and Phelps, 2011), this is not universally accepted in the field (Finnie and Nader, 2012). It remains possible that the post-reactivation timing of propranolol led to the peak dose of drug in the brain being mistimed with the reconsolidation of the fear memory. Thus, we tested whether the administration of propranolol 30 minutes prior to memory reactivation would lead to subsequent impairment in the conditioned fear memory (**Fig. 2c**).

The rats assigned to the prospective drug groups were well-matched for acquisition of the conditioned fear memory (**Fig. 2d**), with all rats freezing more to the CS than the context (CS: $F_{1,55} = 22.5$, $p < .001$, $\eta^2 = 0.29$) and more to the second CS than the first CS (Pairing: $F_{1,55} = 110$, $p < .001$, $\eta^2 = 0.66$; CS x Pairing: $F_{1,55} = 30.2$, $p < .001$, $\eta^2 = 0.35$). There were no differences in the training performance of the prospective drug groups (Drug: $F < 1$; CS x Drug: $F < 1$; Pairing x Drug: $F < 1$; CS x Pairing x Drug: $F < 1$). During the reactivation session, conditioned freezing remained comparable across both the vehicle-treated and propranolol-treated rats (Drug: $F < 1$; CS x Drug: $F < 1$) and all rats froze more to the CS than the context (CS: $F_{1,55} = 364$, $p < .001$, $\eta^2 = 0.87$).

Pre-reactivation administration of propranolol did not affect conditioned freezing at the subsequent PR-LTM tests (Drug: $F < 1$; CS x Drug: $F < 1$; Session x Drug: $F_{1,55} = 3.17$, $p = .08$; CS x Session x Drug: $F < 1$). All rats froze to the CS more than the context (CS: $F_{1,55} = 278$, $p < .001$, $\eta^2 = 0.84$), with a decline in fear across the two test sessions (Session: $F_{1,55} = 18.3$, $p < .001$, $\eta^2 = 0.25$) that was specific to the CS (CS x Session: $F_{1,55} = 8.07$, $p = .006$, $\eta^2 = 0.13$; pairwise comparisons showed a reduction in freezing to the CS ($p < .001$) but not the context ($p = .19$) across the PR-LTM tests). Thus, pre-reactivation propranolol was not sufficient to disrupt the reconsolidation of the conditioned fear memory.

Effects of post-reactivation propranolol on weaker conditioned fear memories

Memory strength is a robust boundary condition on memory reconsolidation (de Oliveira Alvares and Do-Monte, 2021; Suzuki et al., 2004). Our conditioning procedure used a weaker footshock than Debiec & LeDoux (0.5mA for 0.5 seconds, compared to 1mA for 1 second) but two CS-US pairings rather than one. Furthermore, the current procedure used a 10Hz clicker as a CS, rather than a continuous 5kHz tone. We have previously observed that conditioned fear to tone CSs is lower than for clicker CSs (Wood, 2010). Thus, the lack of effect of propranolol on the reconsolidation of the fear memory could be due to the increased levels of conditioned fear produced by both the clicker CS and the increased number of pairings during training. We tested this hypothesis in two separate cohorts of rats: one cohort receiving two tone-shock presentations during training, and another receiving one tone-shock presentation during training (**Fig. 3**).

For the rats that received two tone-shock pairings, there were no differences between the prospective drug groups in acquisition of conditioned fear (**Fig. 3b**), though consistent with previous findings (Wood, 2010) freezing to the tone CS was numerically lower than to the clicker CS. To compare the effects of the tone and clicker CSs in supporting conditioned fear, the training data of rats receiving two tone ($n = 44$) or clicker ($n = 153$) CSs were compared. This revealed that although all rats froze more to the CS than the context (CS: $F_{1,195} = 473$, $p < .001$, $\eta^2 = 0.71$) and froze more to the second CS presentation than the first (Pairing: $F_{1,195} = 229$, $p < .001$, $\eta^2 = 0.54$; CS x Pairing: $F_{1,195} = 201$, $p < .001$, $\eta^2 = 0.51$), the freezing to the tone CS was lower than the freezing to the clicker CS (CS-ID: $F_{1,195} = 7.51$, $p = .007$, $\eta^2 = 0.037$). This did not seem to be due to any inherent differences in detectability of the CSs, as the levels of freezing to the first CS presentation (prior to shock delivery) were equivalent; it was only on presentation of the second CS that differences in conditioned freezing emerged (Pairing x CS-ID: $F_{1,195} = 10.5$, $p = .001$, $\eta^2 = 0.051$; pairwise comparisons revealed equivalent freezing to the first ($p = .57$) but not second ($p < .001$) CS presentation). This analysis supports our previous finding (Wood, 2010) that clicker CSs better support conditioned freezing compared to tone CSs.

All rats acquired fear to the tone CS across the two CS-US pairings (CS: $F_{1,30} = 36.8$, $p < .001$, $\eta^2 = 0.55$; Pairing: $F_{1,30} = 21.2$, $p < .001$, $\eta^2 = 0.41$; CS \times Pairing: $F_{1,30} = 9.10$, $p = .005$, $\eta^2 = 0.23$). Fear during training was equivalent across the prospective drug groups (Drug: $F < 1$; CS \times Drug: $F < 1$; Pairing \times Drug: $F < 1$; CS \times Pairing \times Drug: $F < 1$). Conditioned fear to the tone CS was also equivalent during the memory reactivation session, where rats in both drug groups froze equivalently to the CS (CS: $F_{1,34} = 168$, $p < .001$, $\eta^2 = 0.85$; Drug: $F_{1,30} = 1.37$, $p = .25$; CS \times Drug: $F < 1$).

However, there were also no differences in conditioned fear in the PR-LTM tests between the groups that had received vehicle or propranolol post-reactivation. All rats continued to freeze to the CS across both sessions (CS: $F_{1,30} = 42.5$, $p < .001$, $\eta^2 = 0.59$; Session: $F_{1,30} = 1.10$, $p = .30$; CS \times Session: $F_{1,30} = 2.21$, $p = .15$) but with no differences across the treatment groups (Drug: $F < 1$; CS \times Drug: $F < 1$; Session \times Drug: $F < 1$; CS \times Session \times Drug: $F < 1$). Thus, the reduced freezing produced by the use of a tone, rather than a clicker, CS did not reveal an effect of propranolol on reconsolidation of the conditioned fear memory.

To investigate further whether a weaker memory would make the memory susceptible to disruption with propranolol, a separate cohort of rats were trained with a single tone-shock pairing (**Fig. 3c**). Both prospective drug groups acquired the conditioned fear memory, with greater freezing to the CS than the context (CS: $F_{1,14} = 29.6$, $p < .001$, $\eta^2 = 0.68$) and no differences between the prospective drug groups (Drug: $F < 1$; CS \times Drug: $F < 1$). There were also no differences between the groups during the memory reactivation session (Drug: $F < 1$; CS \times Drug: $F < 1$), with all rats freezing more to the CS than the context (CS: $F_{1,14} = 68.9$, $p < .001$, $\eta^2 = 0.83$). However, during the PR-LTM sessions, there were no differences in fear between the groups that had received post-reactivation vehicle and propranolol (Drug: $F < 1$; CS \times Drug: $F < 1$; Session \times Drug: $F_{1,14} = 1.36$, $p = .26$; CS \times Session \times Drug: $F < 1$) with all rats freezing consistently to the CS across the two test sessions (CS: $F_{1,14} = 7.13$, $p = .018$, $\eta^2 = 0.34$; Session: $F < 1$; CS \times Session: $F_{1,14} = 2.48$, $p = .14$). Thus, training a weaker memory with a single tone-shock pairing did not reveal an effect of propranolol on the reconsolidation of the conditioned fear memory.

Effects of propranolol on the expression of pan-Shank

It is possible that the lack of effect of propranolol on the reconsolidation of conditioned fear memories was due to a failure to engage memory destabilisation mechanisms with the current reactivation procedures. In order to assess whether this was the case, we analysed the expression of pan-Shank, which is known to be degraded during memory destabilisation (Lee et al., 2008) in the basolateral amygdala (BLA) following treatment with propranolol under non-reactivated and reactivated conditions. Consistent with the behavioural studies reported above, one group received drug treatment 30 minutes before memory reactivation in Context A, two groups received drug treatment immediately after memory reactivation (in Contexts A and B, respectively) and non-reactivated animals were administered drug and directly returned to the home cage (**Fig. 4a**).

All rats acquired fear to the clicker CS during training (**Fig. 4b**; Pairing: $F_{1,52} = 118$, $p < .001$, $\eta^2 = 0.69$; CS x Pairing: $F_{1,52} = 14.8$, $p = .001$, $\eta^2 = 0.22$), with no differences between the prospective reactivation conditions (Group: $F_{3,52} = 1.34$, $p = .27$; CS x Group: $F < 1$; Pairing x Group: $F < 1$; CS x Pairing x Group: $F < 1$) or prospective drug groups (Drug: $F < 1$; CS x Drug: $F < 1$; Pairing x Drug: $F < 1$; CS x Pairing x Drug: $F < 1$) and no interactions (Drug x Group: $F_{3,52} = 1.57$, $p = .21$; CS x Drug x Group: $F_{3,52} = 1.03$, $p = .39$; Pairing x Drug x Group: $F < 1$; CS x Pairing x Drug x Group: $F_{3,52} = 1.34$, $p = .27$). For those rats that experienced a reminder session, freezing was consistently higher to the CS than the context (CS: $F_{1,33} = 229$, $p < .001$, $\eta^2 = 0.87$), with no differences in freezing between the groups treated with propranolol and vehicle (Drug: $F < 1$; CS x Drug: $F < 1$), or the different reactivation conditions (Group: $F < 1$; CS x Group: $F < 1$) and no interactions (Drug x Group: $F < 1$; CS x Drug x Group: $F < 1$). Thus, the groups were well-matched for fear memory acquisition and expression during the reminder session.

Consistent with the hypothesis that memory reactivation was not engaged with the current reactivation procedures, there were no differences in the BLA pan-Shank expression levels between vehicle- and propranolol-treated animals in any of the reactivation conditions (**Fig. 4c**; Drug: $F < 1$; Drug x Group: $F < 1$). Overall, pan-Shank expression was higher in rats that had received injections

prior to memory reactivation (Group: $F_{3,52} = 5.89$, $p = .001$, $\eta^2 = 0.25$; pairwise comparisons revealed that rats receiving drug pre-reactivation differed from all other groups (p 's $< .026$) which did not differ from each other (all p 's $> .64$). However, even in the pre-reactivation group there was no difference between vehicle- and propranolol-treated animals ($p = .76$). Thus, it appears likely that the specific reactivation conditions used – and varied – in these experiments were not sufficient to induce memory destabilisation, thereby accounting for the lack of effect of propranolol on subsequent conditioned fear expression.

DISCUSSION

In contrast to previous work (Dębiec and LeDoux, 2004) but consistent with recent reports (Luyten et al., 2021), we found no effect of propranolol on the reconsolidation of a pavlovian auditory fear memory in rats. This was despite variations in the parameters of the memory reactivation session that might relate to hypothesised boundary conditions, including a change in context at reactivation, the timing of the propranolol treatment and the strength of conditioning (manipulated by changes to both the CS and the number of USs). In all cases, propranolol had no effect on fear memory retrieval, or conditioned freezing in subsequent long-term memory tests. We hypothesised that the apparent lack of effect of propranolol on reconsolidation was a result of the parameters of the reminder session not inducing memory destabilisation. To test this hypothesis, expression of the post-synaptic density protein Shank was assessed in the basolateral amygdala in rats that had experienced the same reactivation conditions where propranolol had no effect. Shank has been shown previously to be degraded in the hippocampus when contextual fear memories destabilise (Lee et al., 2008) and the perirhinal cortex during the destabilisation of object recognition memories (Wideman, 2019). There were no differences in Shank expression across any of the reactivation conditions, and propranolol did not affect Shank expression, consistent with the hypothesis that the parameters of the reactivation sessions used in the behavioural experiments did not induce memory destabilisation.

It is unlikely that the lack of effect of propranolol was due to an insufficient dose, as the dose used (10 mg/kg) and the intraperitoneal route of administration have been used to disrupt pavlovian fear memories in other studies (Dębiec and LeDoux, 2004) and, indeed, in our own work targeting the reconsolidation of pavlovian drug memories (Milton, Lee and Everitt, 2008; Schramm et al., 2016). However, it has been observed previously with PTSD patients that propranolol is ineffective at inducing long-term reductions in fear memories when memory destabilisation has been unsuccessful – for example, through a lack of prediction error (Pedreira, Pérez-Cuesta and Maldonado, 2004) – even when similar reactivation procedures have been used to destabilise the memory in other patients (Kindt and van Emmerik, 2016). Furthermore, the lack of reduction of Shank protein across any of the reactivation conditions used in these experiments compared to non-reactivated controls, independent of any effects of propranolol, supports the hypothesis that memory destabilisation was not induced. Although we did not perform a time course analysis of Shank degradation, the 60-minute time point analysed is within the range of times in which reduced Shank expression is observed in both the hippocampus (Lee et al., 2008) and basolateral amygdala (Jarome et al., 2011). These data are relevant not only in terms of their implications for the boundary conditions that control reconsolidation, but also for the effects of propranolol on memory destabilisation. Previous research with drug-associated memories has indicated that propranolol may acutely impair the retrieval of memories (Otis, Dashew and Mueller, 2013; Otis and Mueller, 2017; Otis, Werner and Mueller, 2015), and potentially also memory destabilisation (Lim et al., 2018; Wideman, 2019). Though the effects on drug-associated memory retrieval appear to be mediated primarily by the medial prefrontal cortex (Otis, Dashew and Mueller, 2013; Otis and Mueller, 2017), other studies have shown propranolol prevents the destabilisation of contextual fear memories and associated changes in hippocampal neuronal excitability (Lim et al., 2018), and functional coupling between the basolateral amygdala and other limbic structures (Leal Santos et al., 2021). However, as the reactivation procedures used in these experiments did not appear to induce memory destabilisation, it is not surprising that propranolol was ineffective at modulating this effect. Even if β -adrenergic signalling is necessary for memory destabilisation, antagonising these receptors in the absence of

destabilisation would not be predicted to have any behavioural effect. It may also be the case that different brain regions are differentially dependent on β -adrenergic signalling for memory destabilisation and restabilisation. For instance, a recent study (Leal Santos et al., 2021) found differential effects on the expression of the immediate early gene *c-fos* in the hippocampus, prefrontal cortex and amygdala subnuclei during the destabilisation of contextual fear memories. While c-Fos expression in the prefrontal cortex, dentate gyrus and lateral amygdala was reduced by a systemic propranolol injection prior to contextual fear memory reactivation, c-Fos expression in the basolateral amygdala was *increased* (Leal Santos et al., 2021). Our molecular analyses indicated a small increase in Shank expression in the basolateral amygdala when injections were given prior to the reminder session (as compared to non-reactivated controls), but did not show any modulation of Shank expression with β -adrenergic receptor antagonism.

The current data clearly indicate that the previously described ‘boundary conditions’ on reconsolidation cannot fully account for failures to replicate (Luyten et al., 2021). The most commonly cited boundary conditions are memory age and memory strength (Bermudez-Rattoni and McGaugh, 2017). Here, all memories were recently consolidated (i.e. 24 hours old), and weakening of the memory was not sufficient to induce destabilisation. Modulation of the context in which the CS was presented also did not produce a sufficient ‘violation of expectations’ (Pedreira, Pérez-Cuesta and Maldonado, 2004) to induce memory destabilisation. It is not clear why the parameters of these reminder sessions were not sufficient to induce memory lability – particularly when similar parameters have been used successfully in other studies (Dębiec and LeDoux, 2004; Lee et al., 2005; Lee et al., 2006; Merlo et al., 2018; Merlo et al., 2014; Milton et al., 2013) – but these data do show the value of corroborating molecular analyses to interpret behavioural effects (Cahill and Milton, 2019). Considering the complex dynamics underlying memory retrieval, destabilisation and extinction (de Oliveira Alvares and Do-Monte, 2021; Vavřková et al., 2020), it is possible that minor differences in the reactivation procedures used, alongside other extraneous factors such as strain differences (Luyten et al., 2021), were sufficient to keep the memory out of the ‘reconsolidation window’ and thereby prevent any effects of propranolol on the persistence of the

memory. While supporting the value of ‘null findings’ and their reporting in the literature, we also argue for the importance of corroborating non-behavioural measures to determine whether failures to replicate reconsolidation-based effects are, in fact, due to a failure to engage reconsolidation at all.

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FIGURE LEGENDS

Figure 1. Attempted replication of Debiec & LeDoux (2004). **(A)** Schematic of experimental procedures. Rats were trained to associate a clicker CS with a footshock US in Context A. 24hrs later, they were reminded of the CS in Context B and received i.p. administration of DL-propranolol (PRO) or saline vehicle (VEH) immediately after the reminder session. Rats were tested on post-reactivation long-term memory (PR-LTM) tests in Context B 24hrs after the reminder session, and 7 days after the first test. **(B)** Freezing to the CS (solid lines, circular markers) and context (Cxt; dotted lines, square marker) during the two CS presentations during conditioning (CS1 and CS2), during the reminder session ('React') and the two PR-LTM tests. Group sizes: VEH, $n = 8$; PRO, $n = 8$. Individual data points: circles, freezing to CS; crosses, freezing to context; grey, VEH; magenta, PRO.

Figure 2. Propranolol did not disrupt the reconsolidation of pavlovian conditioned fear memory when the reminder session occurred in the same context as training. **(A)** Schematic of experimental procedures, with propranolol being administered immediately after the reminder session in Context A. **(B)** There were no differences in conditioning, freezing during the reminder session, or freezing in the PR-LTM test sessions between rats receiving propranolol (PRO) or vehicle (VEH) immediately after the reminder session. Group sizes: VEH, $n = 16$; PRO, $n = 16$. **(C)** Schematic of experimental procedures, with propranolol being administered 30 minutes prior to the reminder session in Context A. **(D)** There were no differences in conditioning, freezing during the reminder session, or freezing in the PR-LTM sessions between rats receiving PRO or VEH prior to the reminder session. Group sizes: VEH, $n = 29$; PRO, $n = 28$. Individual data points: circles, freezing to CS; crosses, freezing to context; grey, VEH; magenta, PRO.

Figure 3. Propranolol did not disrupt the reconsolidation of weaker pavlovian conditioned fear memories. **(A)** Schematic of experimental procedures, with the use of a tone rather than clicker CS. **(B)** Although freezing to the tone was lower than to the CS with the same number of CS-shock pairings, there were no differences in conditioning, freezing during the

reminder session, or freezing in the PR-LTM test sessions between rats receiving propranolol (PRO) or vehicle (VEH) immediately after the reminder session. Group sizes: VEH, $n = 16$; PRO, $n = 16$.

(C) When a single tone-shock pairing was experienced during conditioning, there were no differences in conditioning, freezing during the reminder session, or freezing in the PR-LTM test sessions between rats receiving PRO or VEH immediately after the reminder session. Group sizes: VEH, $n = 8$; PRO, $n = 8$. Individual data points: circles, freezing to CS; crosses, freezing to context; grey, VEH; magenta, PRO.

Figure 4. Neither reactivation nor propranolol administration affected the expression of

Shank protein in the basolateral amygdala. (A) Schematic of experimental procedures. Rats

were fear conditioned in Context A, and separate groups underwent either a reminder session in Context A with propranolol (PRO) or vehicle (VEH) administered 30 minutes before ('Pre') or

immediately after ('Post') the reminder session, or a reminder session in Context B with PRO or VEH administered immediately afterwards, or were culled straight from the home cage without a

reminder session ('NRe'). **(B)** There were no differences in conditioning or, where measured, freezing during the reminder session in any of the rats destined for molecular analyses. Individual

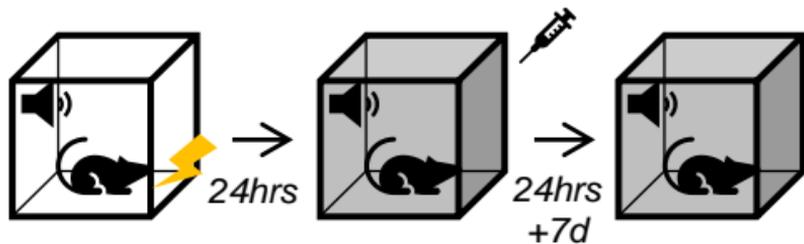
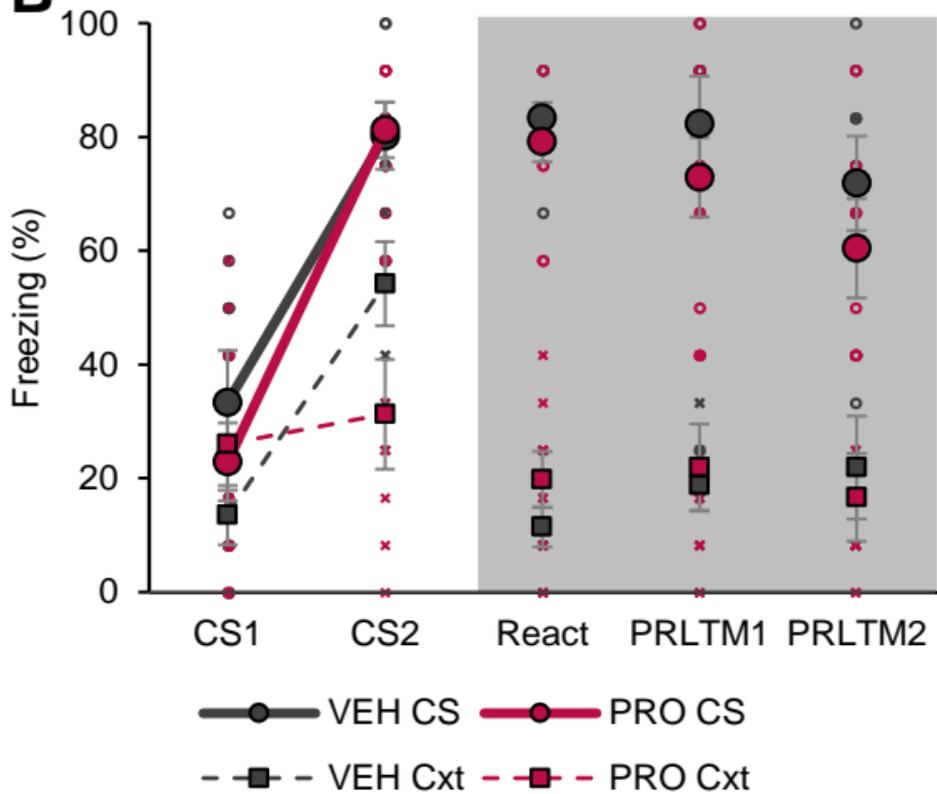
data points: circles, freezing to CS; crosses, freezing to context; grey, VEH; magenta, PRO. **(C)**

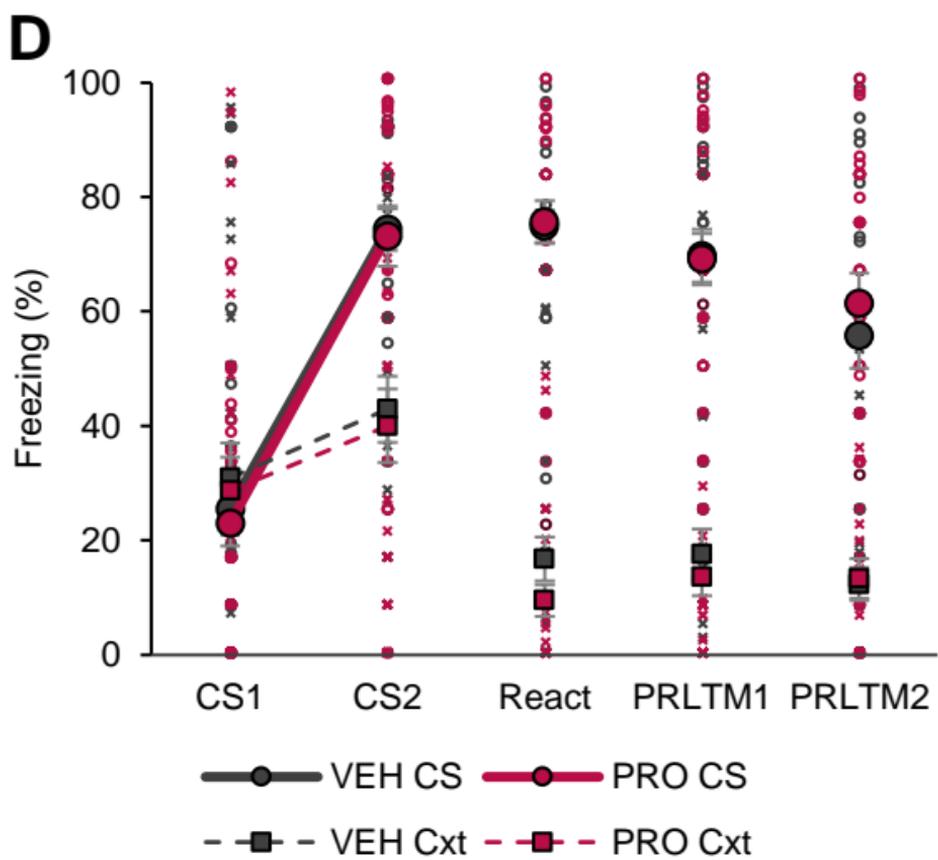
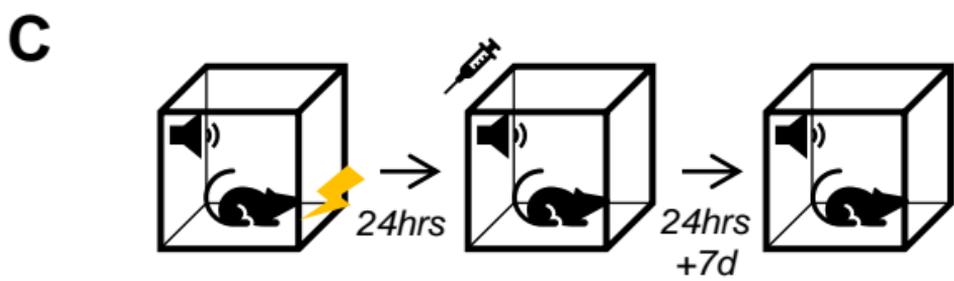
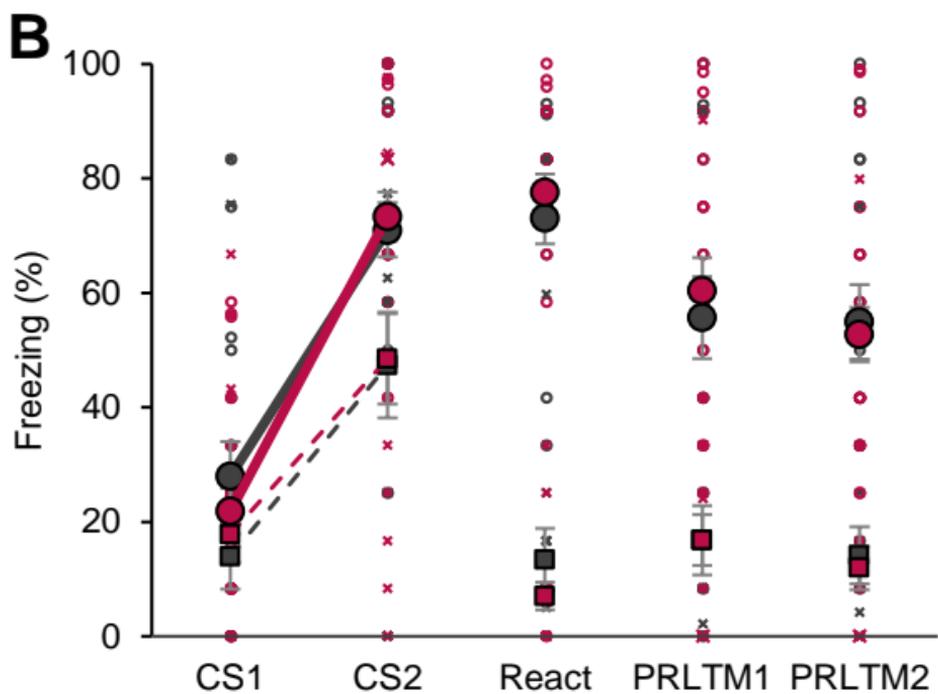
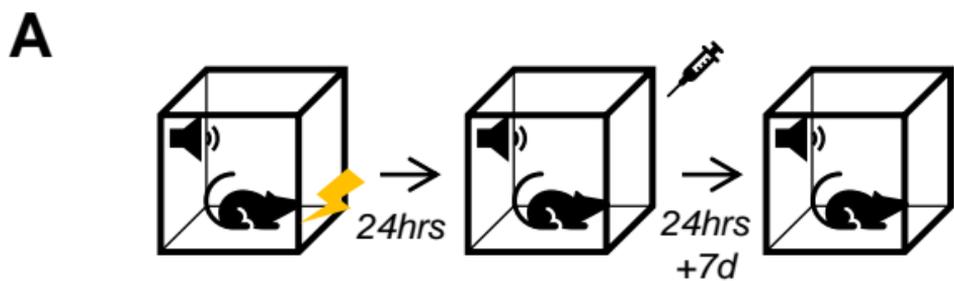
There were no differences in Shank expression in the basolateral amygdala between rats that

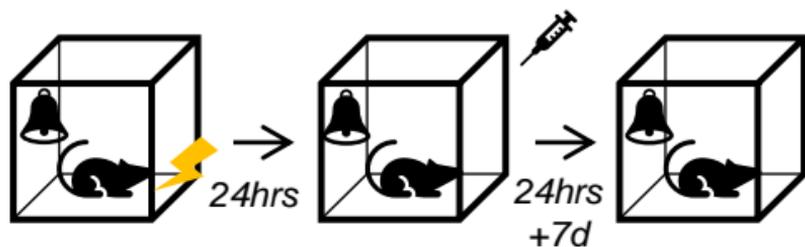
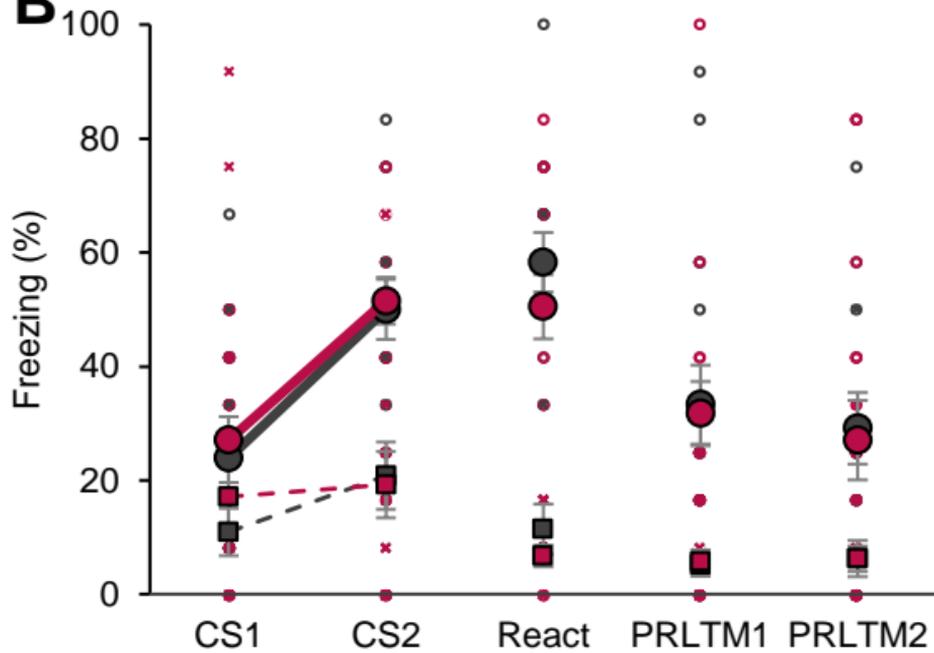
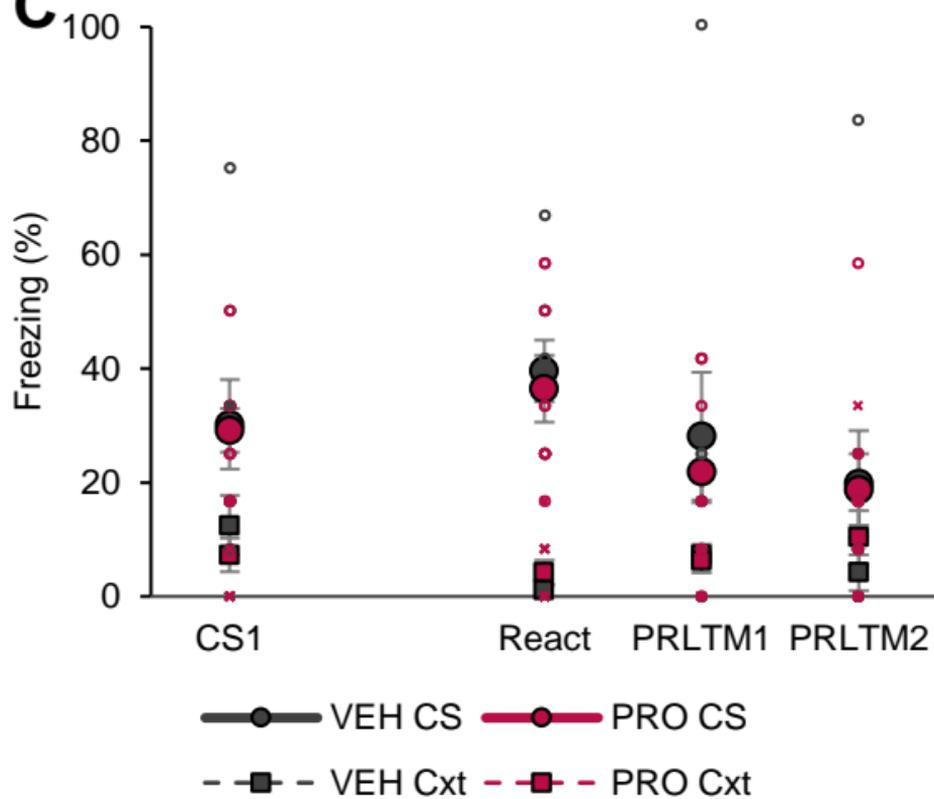
received PRO or VEH in any reminder (or non-reactivated) condition, and a small increase in Shank expression relative to non-reactivated controls for rats that received injections prior to the

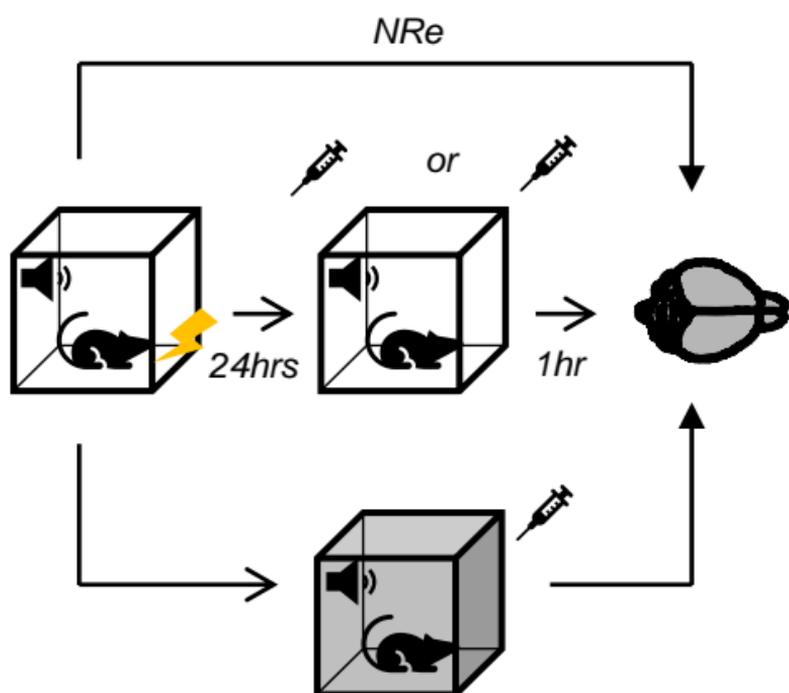
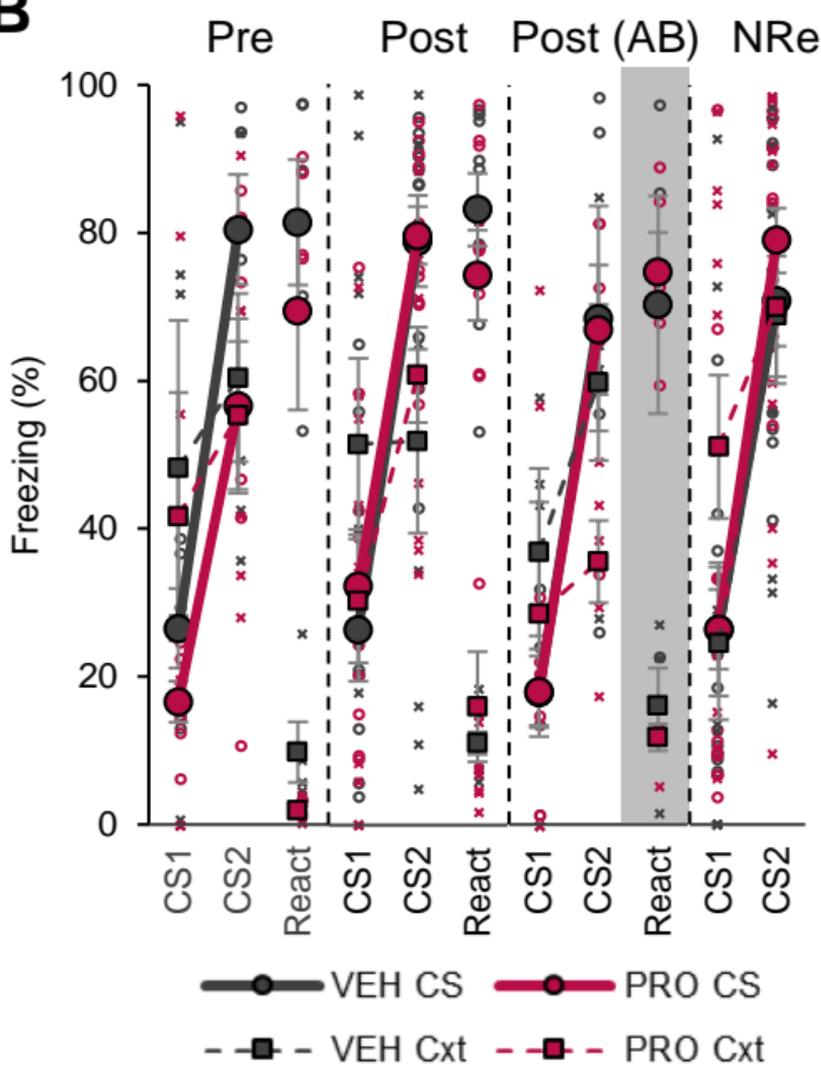
reminder session. Individual data points: grey circles, VEH; magenta circles, PRO. Group sizes: NRe VEH, $n = 10$; NRe PRO, $n = 11$; Pre VEH, $n = 5$; Pre PRO, $n = 6$; Post VEH, $n = 9$; Post PRO, $n = 10$;

Post (AB) VEH, $n = 4$; Post (AB) PRO, $n = 5$.

A**B**



A**B****C**

A**B****C**