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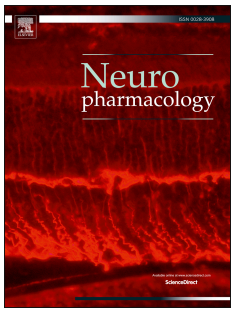

Taking advantage of fear generalization-associated destabilization to attenuate the underlying memory via reconsolidation intervention

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Taking advantage of fear generalization-associated destabilization to attenuate the underlying memory via reconsolidation intervention

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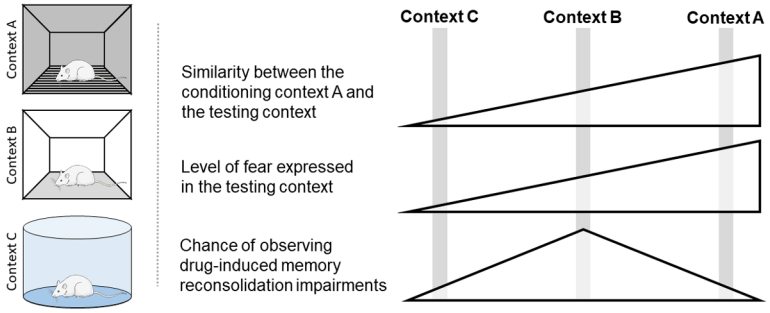
ABSTRACT

Upon retrieval, an aversive memory can undergo destabilization and reconsolidation. A traumatic-like memory, however, may be resistant to this process. The present study sought to contribute with a strategy to overcome this potential issue by investigating whether generalized fear retrieval is susceptible to destabilization-reconsolidation that can be pharmacologically modified. We hypothesized that exposure to a context that elicits moderate generalization levels would allow a malleable memory state. We developed a fear conditioning protocol in context A (cxt-A) paired with yohimbine administration to promote significant fear to a non-conditioned context B (cxt-B) in rats, mimicking the enhanced noradrenergic activity reported after traumatic events in humans. Next, we attempted to impair the reconsolidation phase by administering clonidine (CLO) immediately after exposure to cxt-A, cxt-B, or a third context C (cxt-C) neither conditioned nor generalized. CLO administered post-cxt-B exposure for two consecutive days subsequently resulted in decreased freezing levels in cxt-A. CLO after cxt-B only once, after cxt-A or cxt-C in two consecutive days, or independently of cxt-B exposures did not affect fear in a later test. A six-hour-delay in CLO treatment post-cxt-B exposures produced no effects, and nimodipine administered pre-cxt-B exposures precluded the CLO action. We then quantified the Egr1/Zif268 protein expression following cxt-B exposures and CLO treatments. We found that these factors interact to modulate this memory destabilization-reconsolidation mechanism in the basolateral amygdala but not the dorsal CA1 hippocampus. Altogether, memory destabilization can accompany generalized fear expression; thus, we may exploit it to potentiate reconsolidation blockers' action.

Exploiting memory destabilization accompanying fear generalization to potentiate the action of reconsolidation blockers



1. INTRODUCTION

The generalization from experience is an adaptive cognitive function that allows the adoption of appropriate responses in similar future situations (Shepard, 1987; Herszage and Censor, 2018). For instance, once a stimulus is associated with an aversive event, a similar fear response will be elicited when the subject is faced with a novel stimulus resembling that one already experienced. This phenomenon is termed fear generalization (Onat and Büchel, 2015; Whalley, 2016). It is noteworthy that classical conditioning in animals has long offered, for translational research, notions about behavioral and neurobiological aspects involved in fear generalization (Jasnow et al., 2017; Flandreau and Toth, 2018). In this procedure, a set of features from the environment is assumed to represent the conditioned stimulus; therefore, the degree of generalization will depend on the number of features of the original set present at the test session (Rescorla, 1976). Specifically, the higher the ratio of common features between training and testing, the higher the generalization (Pearce, 1987).

Fear generalization has also received scientific attention because of its association with posttraumatic stress disorder (PTSD) (Dymond et al., 2015; Perusini and Fanselow, 2015; Lopresto et al., 2016). A distorted perception of imminent danger – overgeneralization – after experiencing highly threatening situations can result in inappropriate emotional responses even in safe contexts (Perusini and Fanselow, 2015), making the understanding of this phenomenon of great clinical/therapeutic importance. In this case, the Pavlovian conditioning procedure is used to examine the exaggerated generalization of fear observed in PTSD in the laboratory setting (Lissek and van Meurs, 2015; Careaga et al., 2016; Vanvossen et al., 2017; Zinn et al., 2020). These animal studies' focus has frequently been on mimicking key aspects reported around traumatic

events in humans. For instance, the noradrenergic system's overactivation is implicated in traumatic memory formation in humans (Southwick et al., 1999; Hendrickson and Raskind, 2016). Similarly, fear conditioning paired with the post-acquisition administration of yohimbine, an α_2-adrenergic receptor antagonist that activates noradrenergic neurons, generates a strong memory in rats with maladaptive properties, such as to be generalizable to unrelated, neutral stimuli (Gazarini et al., 2013, 2014).

Under appropriate conditions, a consolidated aversive memory returns to a labile state upon retrieval and is thus destabilized, requiring a subsequent restabilization phase called reconsolidation (Misanin et al., 1968; Przybyslawski et al., 1999; Nader et al., 2000). This destabilization-reconsolidation process after retrieval appears to occur only with the availability of new information (Rodriguez-Ortiz et al., 2005; Hupbach et al., 2007; Tronson and Taylor, 2007). It allows memories to change adaptively and can offer an opportunity for adjusting potentially detrimental features of maladaptive emotional memories (Cain et al., 2012; Parsons and Ressler et al., 2013; Beckers and Kindt, 2017; Walsh et al., 2018). Both pharmacological and behavioral interventions carried out within this period have been shown to attenuate the aversive content of memories upon reactivation in animal and human studies (Przybyslawski et al., 1999; Monfils et al., 2009; Schiller et al., 2010; Schwabe et al., 2012; Steckler and Risbrough, 2012). However, PTSD-like memories may be less susceptible or even resistant to destabilization and reconsolidation (Gazarini et al., 2014; Kindt and van Emmerik, 2016). If fear generalization results from retrieving the original aversive memory, it would be interesting to investigate whether exposure to a context that elicits moderate generalization levels could induce a malleable memory state that can be pharmacologically modified.

Based on the above, the present study aimed to investigate whether fear expressed in a non-conditioned but generalized context is accompanied by sufficient destabilization of the underlying memory. To reach this aim, we developed a fear conditioning protocol in context A (cxt-A) paired with the administration of yohimbine to provoke significant fear to the non-conditioned context B (cxt-B) in rats, mimicking the enhanced noradrenergic activity reported after traumatic events in humans (Southwick et al., 1999; Hendrickson and Raskind, 2016). Of note, administering yohimbine around a stressful event has been shown to strengthen associative fear memory traces and trigger broader fear generalization in humans (Soeter and Kindt, 2011, 2012). Next, we attempted to impair the reconsolidation phase by administering the α_2-adrenergic receptor agonist clonidine, which attenuates the noradrenergic tonus associated with aversive memory reconsolidation (Gamache et al., 2012; Gazarini et al., 2013, 2014; Troyner et al., 2018; Troyner and Bertoglio, 2020), immediately after exposure to cxt-A, cxt-B, or a third context C (cxt-C) that was neither conditioned nor similar enough to the cxt-A to elicit generalized fear. We also used immunohistochemistry for Egr1/Zif268 protein to assess the relative contribution of the dorsal CA1 hippocampus and the basolateral amygdala to the process of memory destabilization-reconsolidation associated with cxt-B exposures since it is engaged upon conditioned context exposure (Hall et al., 2001; Lee et al., 2004; Besnard et al., 2014; Espejo et al., 2016; Couto-Pereira et al., 2019). Our findings suggest that we can use fear generalization-associated destabilization to attenuate the underlying memory via reconsolidation intervention. Clonidine treatment modulates related plasticity in the basolateral amygdala but not the dorsal hippocampus.

2. MATERIALS AND METHODS

2.1 Animals

Experiments were performed in male Wistar rats (bred and raised in the animal house of Federal University of Santa Catarina) aged 13-16 weeks and kept grouped (five per cage) on a 12 h light/dark cycle, with lights switched on at 7:00 AM. Animals were provided with food and water *ad libitum*. The Institutional Ethical Committee for the Care and Use of Laboratory Animals from our University approved this study in compliance with Brazilian legislation and the National Institutes of Health guide for laboratory animals' care and use.

2.2 Drugs

The α_2-adrenergic receptor antagonist yohimbine hydrochloride (1.0 or 2.0 mg/kg i.p.; Tocris, USA) was administered immediately after contextual fear memory acquisition to enhance the noradrenergic activity, generate a PTSD-like memory, and provoke persistent generalized fear expression (Gazarini et al., 2013, 2014). The α_2-adrenergic receptor agonist clonidine hydrochloride (0.3 mg/kg i.p.; Sigma-Aldrich, USA) was administered at a putative fear memory reconsolidation-impairing dose (Gamache et al., 2012; Gazarini et al., 2013, 2014; Troyner et al., 2018; Troyner and Bertoglio, 2020). The antagonist of L-type voltage-gated calcium channels (LVGCCs) nimodipine (15 mg/kg i.p.; Sigma-Aldrich, USA) was administered before cxt-B exposures to disrupt memory destabilization (Flavell et al., 2011; Haubrich et al., 2015; da Silva et al., 2016; Ortiz et al., 2019). In either case, the phosphate-buffered saline (PBS) 0.01 M served as the vehicle for drug administration.

2.3 Fear conditioning apparatus, behavioral procedures, and data collection

Most aspects mentioned in this section were conducted as fully described elsewhere (Gazarini et al., 2013, 2014; Franzen et al., 2019). We used three different chambers placed in acoustically isolated separate rooms and maintained at a constant temperature of 22 ± 1°C. The conditioning chamber (35 x 20 x 30 cm), termed cxt-A, was made of aluminum sidewalls and Plexiglas front wall and ceiling-door. The grid floor, made of parallel stainless-steel bars, was attached to a scrambled shocker (Insight, Brazil) to provide controlled electrical shocks, as subsequently detailed. The second chamber (30 x 30 x 30 cm), termed cxt-B, was made of glass and had a grid lid and transparent walls and floor. The third chamber, termed cxt-C, was a cylindrical and opaque plastic container (internal diameter: 30 cm; height: 20 cm) without a lid. The design of the non-conditioned contexts (cxt-B and cxt-C) has a decreasing gradient of similarity to cxt-A as follows: cxt-B > cxt-C. A 2.5-W white-light bulb supplied illumination (70 lux) while ventilation fans supplied the background noise (55 dB). Behavioral testing was performed from 1:00 to 6:00 PM.

2.3.1 Contextual fear conditioning followed by yohimbine treatment

This procedure was similar to that used by Gazarini et al. (2014). In all experiments, animals were transported from the housing room, individually placed in the context-to-be-conditioned (cxt-A), and left undisturbed for a 3 min period (familiarization session). The next day each animal returned to cxt-A, and after an initial 30 s delay, it received three shocks (US; 0.7 mA, 60 Hz, 3 s duration at an inter-shock interval of 30 s). Animals remained in this chamber for an additional 30 s, then were removed and systemically administered with yohimbine or its vehicle solution (1.0 ml/kg). After that,

they returned to their respective home cage.

2.3.2 Experimental design/timeline, test sessions, and data collection

A scheme of each experiment's design/timeline is shown above the graph depicting the corresponding data. The question to be addressed in each case is detailed in the results section.

In Test A, the animals were exposed to the conditioned cxt-A for 3 min in the US's absence. In Test B and Test C, animals were exposed for 3 min to the unpaired cxt-B and cxt-C, respectively. When the same type of test was performed more than once, the corresponding number succeeded the letter designating it (e.g., Test A_1).

The behavior of each animal was videotaped during the experimental sessions. The time spent freezing was subsequently and continuously quantified (in seconds) by a trained observer (inter- and intra-observer reliabilities ≥ 90%) blind to the experimental groups and expressed as the total time percentage. Freezing, defined as the ceasing of all body and head movements except the flank movements related to breathing, was used as a fear memory index (Blanchard and Blanchard, 1969).

2.4 Immunohistochemistry for early growth response protein 1 (Egr1; also known as Zif268 - zinc finger protein 268)

Ninety min after Test B_2 and treatment with vehicle or clonidine, animals were anesthetized and perfused transcardially with a solution of NaCl 0.9% and heparin 0.05%, followed by 4.0% of paraformaldehyde in PBS 0.1 M (pH = 7.4). Each brain was removed and post-fixed in 4.0% paraformaldehyde for 24 h. Brains were transferred initially to a 50% ethanol solution (v/v) for 48 h, then to a 70% ethanol solution (v/v)

where they were kept until further processing in ethanol, xylol, and paraffin inclusion (Leica tissue processer TP1020). After paraffin embedment, serial 8.0 µm thick coronal brain sections were cut in a microtome (Leica RM2255), collected, and fixated in gelatinized glass slides for microscopy. Fixated sections were deparaffinized and washed in PBS 0.01M (pH = 7.4). Endogenous peroxidase was inactivated by a 20 min treatment in 3.0% of H_2O_2 solution in methanol at room temperature. Antigen retrieval was made with a 0.05% trypsin solution in PBS at 37 °C for 10 min. After washing in cold PBS, sections were incubated overnight in a humidified chamber at 4 °C with mouse IgG kappa binding protein conjugated to Horseradish Peroxidase (1:100 in PBS 0.01M; Santa Cruz Biotechnology, USA), washed in cold PBS, and then incubated at room temperature in mouse monoclonal Egr1 antibody (1:100 in PBS; Santa Cruz Biotechnology, USA) for 120 min. After washing in cold PBS, sections were incubated with an avidin-biotin complex (1:500 in PBS-T; Vector Laboratories, USA) in a humidified chamber for 60 min at room temperature. They were then washed in PBS and stained with a solution containing 3030-diaminobenzidine (DAB 0.2% in DMSO), H_2O_2, and PBS for 2 min. Brain sections were dehydrated and coverslipped with ERV mount (EasyPath – Erviegas, Brazil).

Quantification of Egr1/Zif268-positive cells in 0.4 mm^2 of the dorsal CA1 hippocampus and the basolateral amygdala (2.2 and 3.2 mm posterior to Bregma) was performed manually using the ImageJ® software (NIH, USA). Each brain region of interest was analyzed in triplicate (three samples were taken from each animal), and the mean was calculated. A cell was determined as Egr1/Zif268-positive if the typical DAB-brown marker colored it.

2.5 Statistical analysis

The sample size determined by power analysis using G*Power 3.1.9.3 was of six to eight animals per group (α = 0.05, β = 0.10, and standardized effect size or Cohen's *d* = 1.0, which was based on a pilot study).

After ensuring the assumptions of normality with Shapiro-Wilk's W test and homogeneity of variance with Levene's test, freezing times from each type of test were subjected to a mixed analysis of variance (ANOVA) or a two-sample unpaired Student t-test. The number of Egr1/Zif268-expressing cells was analyzed using a two-way ANOVA (post-cxt-B treatments considered a single independent factor). The Newman-Keuls test was used for *post-hoc* multiple comparisons. The statistical significance level was set at $p < 0.05$. TIBCO Statistic® 13.5 was used for statistical analysis, and GraphPad Prism 8.02 was used for graphing.

The effect size was calculated using the formula for Hedges' *g* to reflect the mean-difference between two groups ($n \leq 20$ per group) that could be dissimilar in size. A $g \geq 0.8$ was considered a large effect size (Ellis, 2010).

3. RESULTS

3.1 Validating a procedure to induce generalized fear expression

Contextual fear conditioning associated with post-training yohimbine treatment induces a PTSD-like memory and provokes generalized fear expression over a month in rats (Gazarini et al., 2013, 2014). Based on this, we assessed two yohimbine doses to select ones capable of reducing the ability to restrict fear to the conditioned context under our experimental conditions (Experiment 1). Twenty-four animals were randomly

allocated to three groups based on the treatment (vehicle, n = 8; yohimbine 1.0 mg/kg, n = 8; or yohimbine 2.0 mg/kg, n = 8) given immediately after the cxt-A-shock pairing. Tests A_1, B_1, A_2, and B_2, were performed two, three, nine, and ten days later, respectively (Fig. 1A).

For Tests A freezing time, a mixed ANOVA showed significant effects of repeated testing [$F(1,21) = 14.6$; $p = 0.001$], but not treatment [$F(2,21) = 1.25$; $p = 0.31$] or interaction between repeated testing and treatment [$F(2,21) = 1.88$, $p = 0.18$]. As shown in figure 1B, all groups behaved similarly during Tests A_1 and A_2, a result in line with that reported by Gazarini et al. (2014).

For Tests B freezing time, a mixed ANOVA showed significant effects of treatment [$F(2,21) = 6.27$, $p = 0.007$], but not repeated testing [$F(1,21) = 0.81$, $p = 0.38$] or their interaction [$F(2,21) = 0.11$, $p = 0.90$]. As shown in figure 1B, Newman-Keuls *post-hoc* tests showed that animals treated with 2.0 mg/kg of yohimbine presented higher levels than controls during Test B_1 ($p = 0.02$; Hedges' *g* effect size = 2.16) and Test B_2 ($p = 0.05$; $g = 1.38$), indicating that the ability to restrict fear to the conditioned cxt-A was significantly reduced over ten days. Based on this, we selected this yohimbine dose to potentiate fear generalization in subsequent experiments.

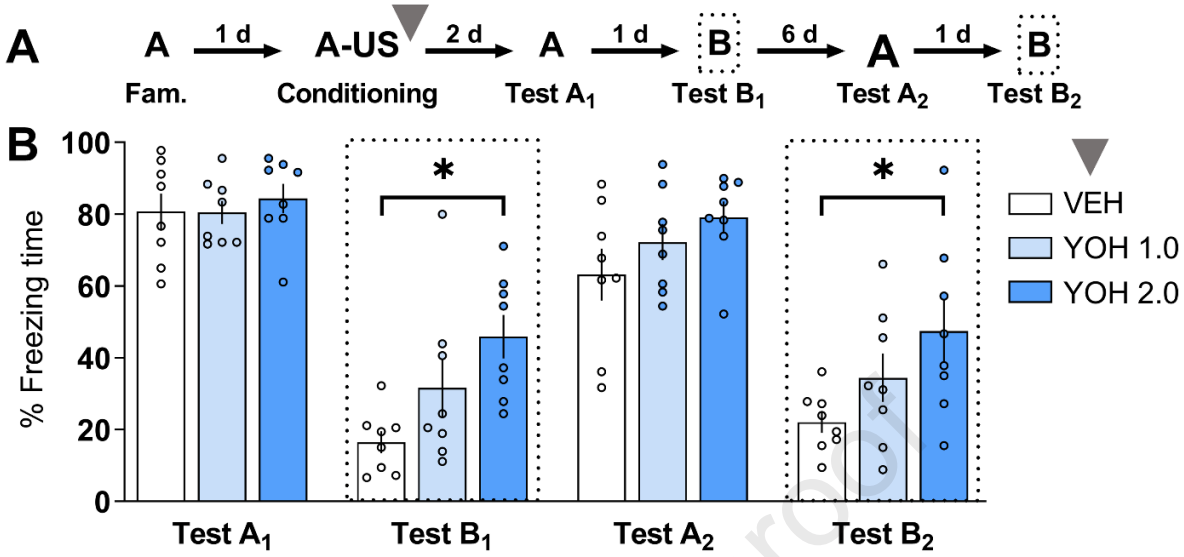


Figure 1. Contextual fear conditioning (A-US) associated with yohimbine (YOH) treatment provokes generalized fear expression over ten days. (**A**) Experiment's 1 design. (**B**) Bar graph showing the freezing times presented by animals treated with vehicle (VEH) or YOH (1.0 or 2.0 mg/kg i.p.) immediately after fear conditioning when later exposed twice to the conditioned context A (Tests A_1 and A_2) and the non-conditioned context B (Tests B_1 and B_2). The YOH 2.0 group could not restrict freezing to the conditioned since it had higher values than the VEH group during Tests B_1 and B_2. The arrowhead indicates the moment of the treatment. Data are expressed as individual units and mean ± S.E.M. (n per group = 8). The * denotes a statistically significant difference ($p \leq 0.05$) from the respective control group (mixed ANOVA followed by the Newman-Keuls test).

3.2 How can we take advantage of fear generalization-associated destabilization to tackle the underlying memory?

The PTSD-like memory generated by yohimbine treatment immediately after contextual fear conditioning has been reported to be resistant to the reconsolidation blocker clonidine (CLO) administered immediately after exposure to the conditioning

context (Gazarini et al., 2014). This lack of sensitivity to CLO's action could be due to the absence of the mismatch (novelty) necessary for memory destabilization upon retrieval (Rodriguez-Ortiz et al., 2005; Tronson and Taylor, 2007). We investigated herein whether exposure to our generalized context is sufficiently novel to induce mismatch and, consequently, trigger the destabilization of yohimbine-related memory, which would allow the impairing effects of CLO on reconsolidation (Experiment 2). Eighteen fear-conditioned and yohimbine-treated animals were randomly allocated to two groups based on the treatment (vehicle, n = 9; or 0.3 mg/kg of CLO, n = 9) given immediately after a single cxt-B exposure (Test B_1). Tests A and B_2 were performed on the following days (Fig. 2A).

For Tests B freezing time, a mixed ANOVA showed no significant effects of treatment [$F(1,16) = 0.45$, $p = 0.51$], repeated testing [$F(1,16) = 0.95$, $p = 0.34$] or their interaction ($F_{1,16} = 0.20$, $p = 0.66$). There were also no significant treatment effects for Test A freezing time [$t(16) = 1.30$; $p = 0.21$; Fig. 2B]. As shown in figure 2B, the CLO and vehicle groups behaved similarly during Test B_1, Test A, and Test B_2, which indicates that a single post-cxt-B CLO treatment did not affect fear expressed at a later test in the conditioned cxt-A. These results suggest that a single exposure to generalized cxt-B was unable to trigger memory destabilization.

Next, we tested whether repeated post-cxt-B CLO treatments attenuate fear response at test in the conditioned cxt-A (Experiment 3). This is based on a previous study reporting that two post-reactivation CLO treatments greatly impaired the original fear memory reconsolidation (Gamache et al., 2012). Sixteen fear-conditioned and yohimbine-treated animals were randomly allocated to two groups based on the treatment (vehicle, n = 8; or CLO, n = 8) given immediately after each one of two cxt-B

exposures (Tests B_1 and B_2). Tests A_1, B_3, and A_2 were performed one, two, and eight days later (Fig. 2C).

For Tests B freezing time, a mixed ANOVA showed significant effects of repeated testing [$F(2,28) = 4.43$; $p = 0.02$], but not treatment [$F(1,14) = 0.14$; $p = 0.71$] or their interaction [$F(2,28) = 0.01$; $p = 0.99$]. As shown in figure 2D, the CLO and vehicle groups behaved similarly during Tests B_1, B_2, and B_3. For Tests A freezing time, a mixed ANOVA showed significant effects of treatment ([$F(1,14) = 23.7$; $p = 0.0003$], but not repeated testing [$F(1,14) = 4.46$; $p = 0.06$] or their interaction [$F(1,14) = 0.45$; $p = 0.51$]. As shown in figure 2D, *post-hoc* tests showed that CLO-treated animals presented lower levels than controls during Test A_1 ($p = 0.003$; $g = 1.58$) and Test A_2 ($p = 0.03$; $g = 1.68$), suggesting that cxt-B exposures induced sufficient memory destabilization, which in turn allowed the impairing effects of CLO on reconsolidation.

To support our hypothesis that cxt-B exposures can induce memory destabilization, we tested whether CLO treatment after each one of two cxt-A exposures attenuates fear response in the subsequent exposure to this conditioned context (Experiment 4). Eighteen fear-conditioned and yohimbine-treated animals were randomly allocated to two groups based on the treatment (vehicle, n = 9; or CLO, n = 9) given immediately after Tests A_1 and A_2. Tests A_3 and B were performed on the following days (Fig. 2E).

For Test B freezing time, there were no significant treatment effects [$t(16) = 0.92$; $p = 0.37$; Fig. 2F]. For Tests A freezing time, a mixed ANOVA showed significant effects of repeated testing [$F(2,32) = 36.5$; $p = 0.00001$], but not treatment [$F(1,16) = 0.52$; $p = 0.48$] or their interaction [$F(2,32) = 0.01$; $p = 0.99$]. As shown in figure 2F, the CLO and vehicle groups behaved similarly during Tests A_1, A_2, and A_3. These results indicate that

post-cxt-A CLO treatments did not impair the contextual fear memory reconsolidation, suggesting an inability of the conditioning context to trigger the predictor error necessary for memory destabilization in yohimbine-treated animals, which are in line with those reported by Gazarini et al. (2014). Of note, the overall values during Test A_3 were inferior to those from Test A_1 (p = 0.0001), probably because the extinction learning has already started after a total of nine min (three sessions of three min) of cxt-A exposure without shock presentation, as reported by Franzen et al. (2019).

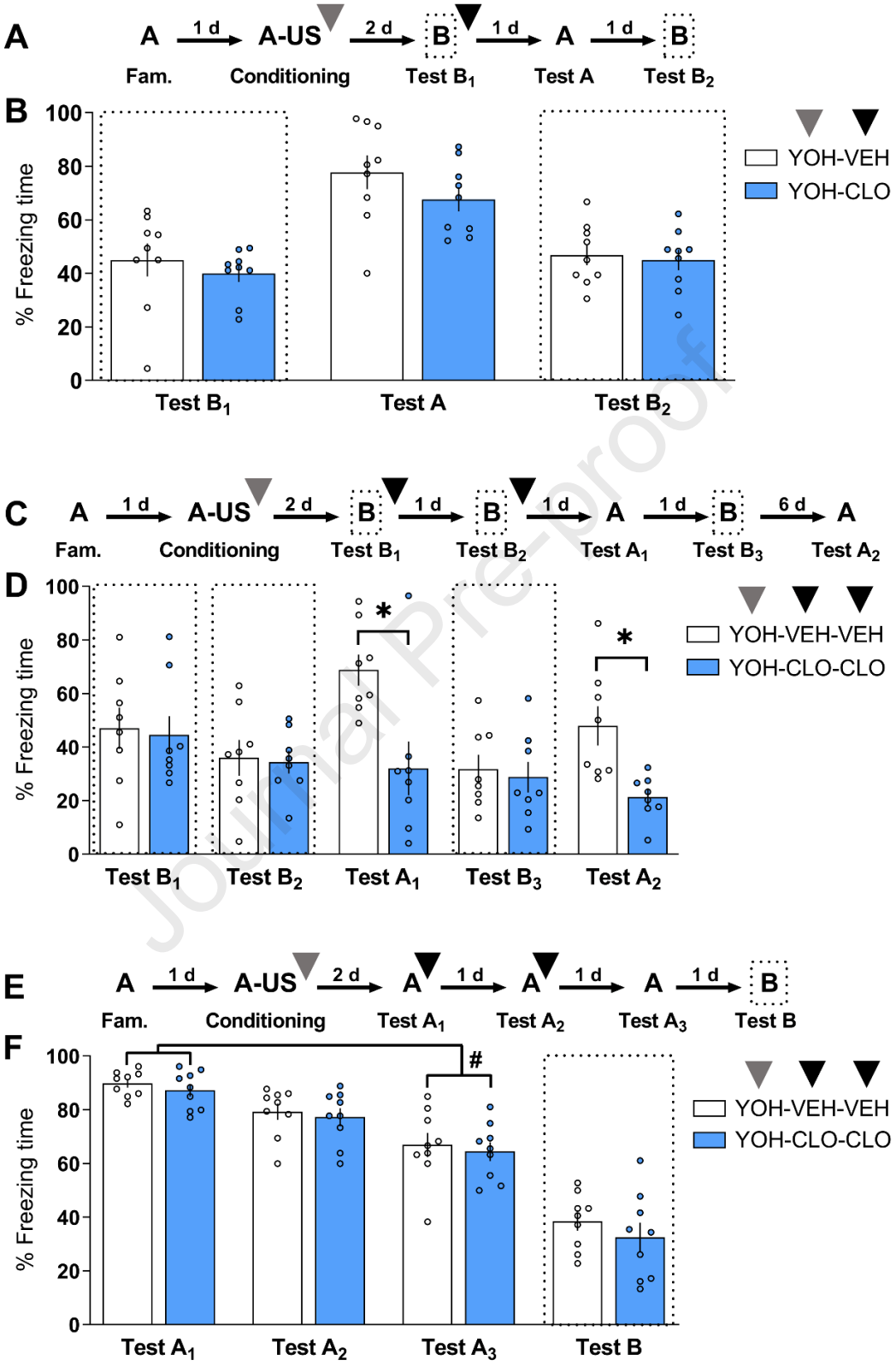


Figure 2. Effects of clonidine (CLO; 0.3 mg/kg i.p.) given immediately after one context

(cxt) B exposure (*upper graph*), each one of two cxt-B exposures (*middle graph*), or each one of two cxt-A exposures (*lower graph*), on memory reconsolidation and generalized fear expression in animals treated with yohimbine (YOH; 2.0 mg/kg i.p.) immediately after contextual fear conditioning. (**A**) Experiment's 2 design. (**B**) A single post-cxt-B CLO treatment produced no changes since drug-treated animals behaved similarly to controls during Tests A and B_2. (**C**) Experiment's 3 design. (**D**) Two post-cxt-B CLO treatments impaired the reconsolidation phase since drug-treated animals presented lower values ($p < 0.05$) than VEH-treated animals during Tests A_1 and A_2. (**E**) Experiment's 4 design. (**F**) Two post-cxt-A CLO treatments produced no changes since drug-treated animals behaved similarly to controls during Tests A_3 and B. The overall values during Test A_3 were inferior ($p < 0.05$) to those from Test A_1, suggesting that extinction learning has already started. Arrowheads indicate the moment of the treatments. Data are expressed as individual units and mean ± S.E.M. (n per group in experiment 2 = 9; n per group in experiment 3 = 8; and n per group in experiment 4 = 9). The * denotes a statistically significant difference from the respective control group, and $^{\#}$ denotes a between-session difference (mixed ANOVA followed by the Newman-Keuls test).

3.3 The absence of memory destabilization precludes the possibility of impairing the reconsolidation phase with clonidine

When an amnesic agent is administered in the absence of the reactivation session, no effects on fear expression have been shown in a later test (Nader et al., 2000; Bustos et al., 2006; Stern et al., 2012; Troyner and Bertoglio, 2020). We investigated whether CLO effects seen on Test A depend on the generalized context's exposures in yohimbine-treated animals (Experiment 5). Twenty animals were fear-conditioned and treated with yohimbine. Two days later, they were divided randomly into two groups, and for two consecutive days, received either vehicle (n = 10) or CLO (n =

10), and then returned to the home cage without the session of cxt-B exposure. Tests A and B were performed on the following days (Fig. 3A). As shown in figure 3B, there were no significant treatment effects for freezing time in either case, Test A [$t(18) = 0.05$; $p = 0.96$] and Test B [$t(18) = 0.68$; $p = 0.50$].

To corroborate the abovementioned findings, we tested whether CLO treatment after each one of two exposures to the non-generalized cxt-C interferes with fear response to the conditioned cxt-A (Experiment 6). Sixteen fear-conditioned and yohimbine-treated animals were randomly allocated to two groups based on the treatment (vehicle, n = 8; or CLO, n = 8) given immediately after the two cxt-C exposures (Tests C_1 and C_2). Tests A and C_3 were performed on the following days (Fig. 3C). For Test A freezing time, there were no significant treatment effects [$t(14) = 0.06$; $p = 0.95$; Fig. 3D]. For Tests C freezing time, a mixed ANOVA showed significant effects of repeated testing [$F(2,28) = 6.39$; $p = 0.006$], but not treatment [$F(1,14) = 0.001$; $p = 0.98$] or their interaction [$F(2,28) = 1.41$; $p = 0.26$]. As shown in figure 3D, the CLO and vehicle groups behaved similarly during Tests C_1, C_2, and C_3.

Based on results from experiments 5 and 6, the absence of memory destabilization, either by omitting exposure to the generalized cxt-B or exposure to the non-generalized cxt-C, precludes the possibility of inducing impairments in memory reconsolidation with clonidine.

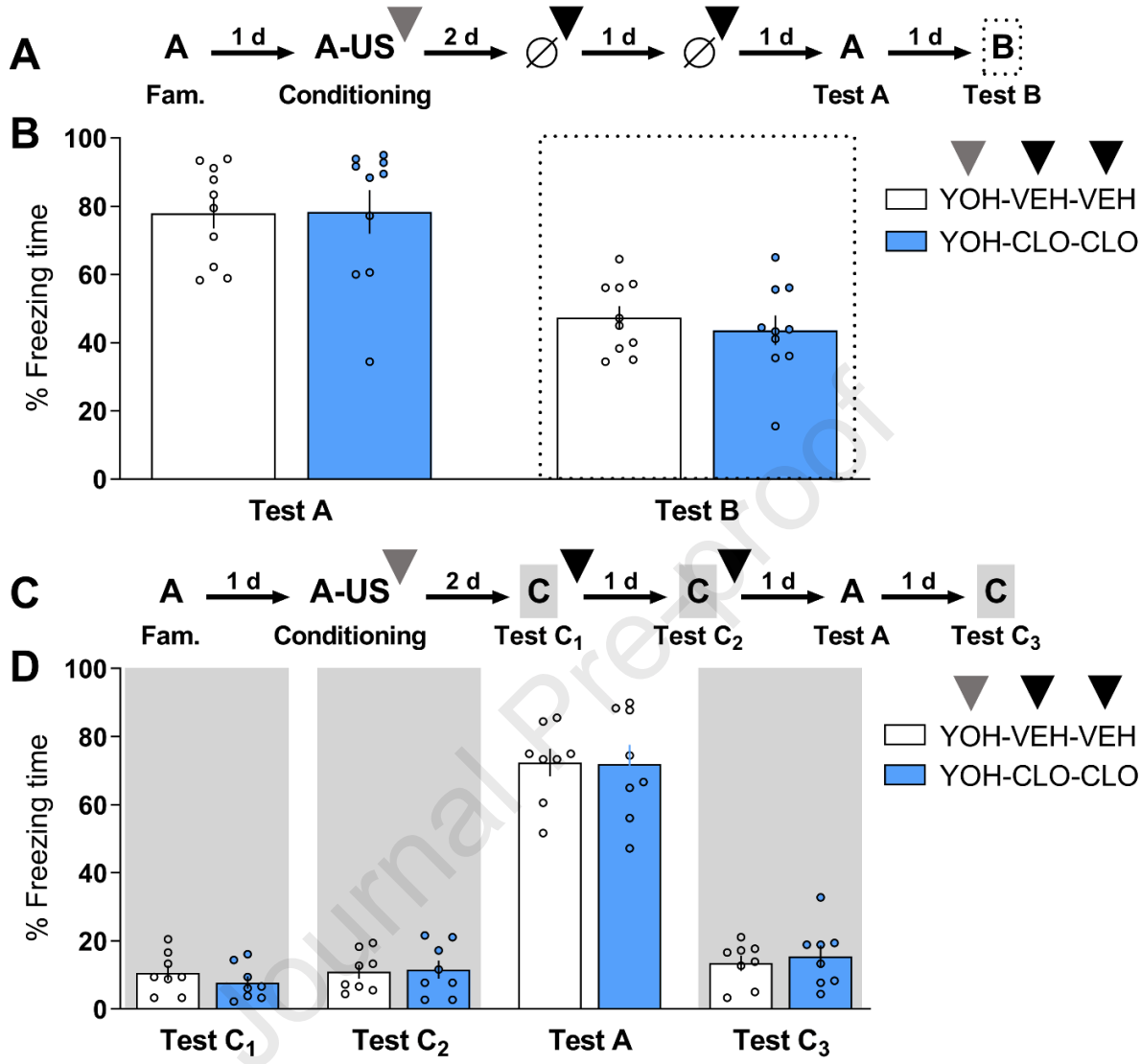


Figure 3. Effects of clonidine (CLO) given immediately after omitting each one of two cxt-B exposures (*upper graph*) or after each one of two cxt-C exposures (*lower graph*) on memory reconsolidation in animals treated with yohimbine (YOH) immediately after contextual fear conditioning. (**A**) Experiment's 5 design. (**B**) CLO induced no changes since drug-treated animals behaved similarly to controls during Tests A and B. (**C**) Experiment's 6 design. (**D**) CLO induced no changes since drug-treated animals behaved similarly to controls during Tests A and C_3. Arrowheads indicate the moment of the treatments. Data are expressed as individual units and mean ± S.E.M. (n per group in experiment 5 = 10; and n per group in experiment 6 = 8). There were no statistically significant differences.

3.4 The possibility of impairing the reconsolidation phase with clonidine depends on the time elapsed between cxt-B exposure and treatment

The impairing effects produced by pharmacological interventions on fear memory reconsolidation happen when performed within a limited time window (usually < 6 h) after reactivation in the conditioned context (Bustos et al., 2006; Stern et al., 2012; Gazarini et al., 2013; Troyner et al., 2018). However, the existence of a similar temporal frame following fear generalization-associated memory destabilization is unknown. Based on this, we tested whether delayed CLO treatment after each one of two cxt-B exposures still reduces the fear response to the conditioned cxt-A (Experiment 7). Sixteen fear-conditioned and yohimbine-treated animals were randomly allocated to two groups based on the treatment (vehicle, n = 8; or CLO, n = 8) given six hours after the two cxt-B exposures (Tests B_1 and B_2). Tests A and B_3 were performed on the following days (Fig. 4A).

For Tests B freezing time, a mixed ANOVA showed significant effects of repeated testing [$F(2,28) = 8.3$; $p = 0.01$], but not treatment [$F(1,14) = 0.01$; $p = 0.79$] or their interaction [$F(2,28) = 0.01$; $p = 0.99$]. As shown in figure 4B, the CLO and vehicle groups behaved similarly during Tests B_1, B_2, and B_3. For Test A freezing time, there were no significant treatment effects [$t(14) = 0.04$; $p = 0.97$]. These results indicate that the possibility of CLO inducing memory reconsolidation impairments is restricted to a time window shorter than six hours when given after generalized fear expression.

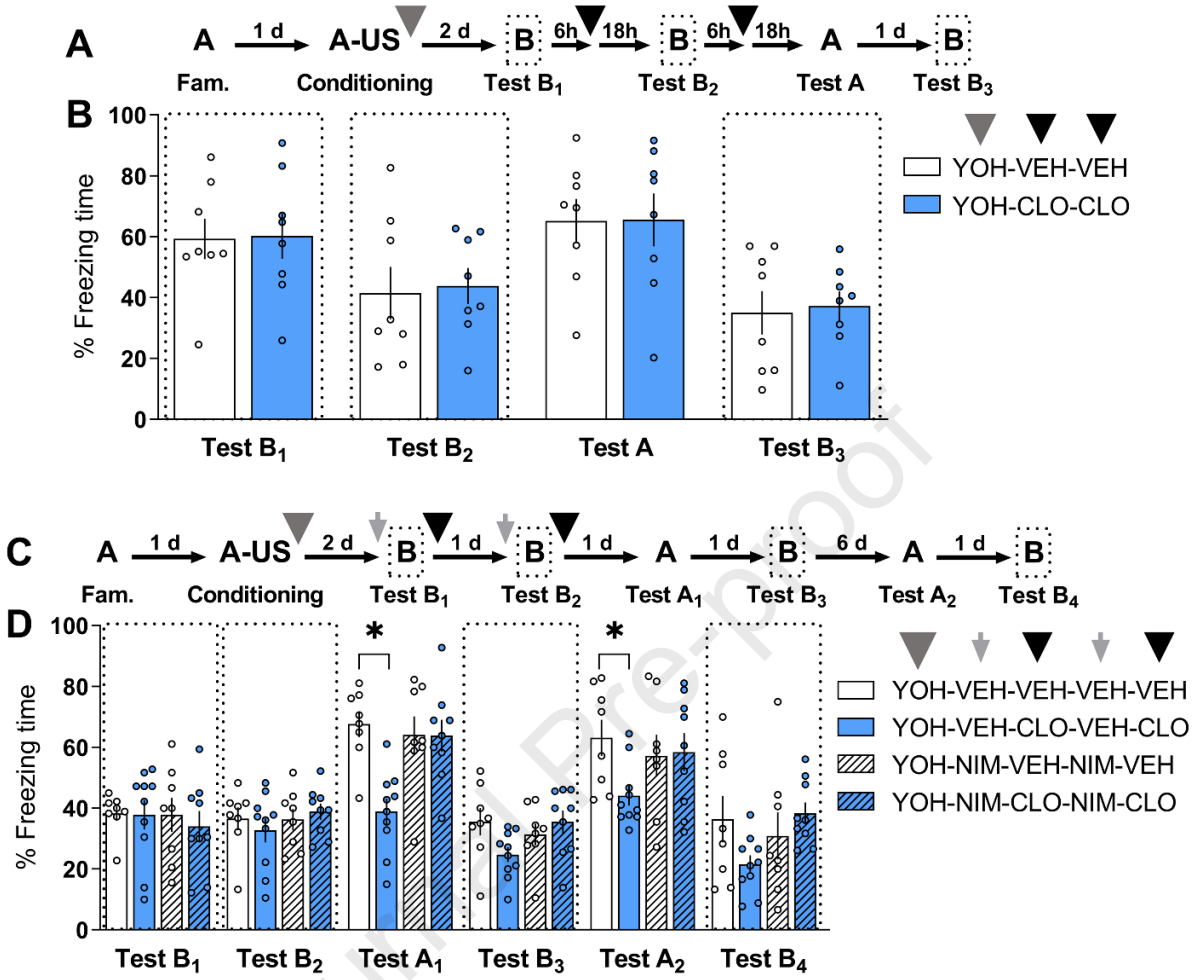


Figure 4. Effects of either a six hours-delay in treatment with clonidine (CLO) after each one of two context B exposures (*upper graph*) or treatment with nimodipine (NIM; 15 mg/kg i.p.) before each one of two context B exposures (*lower graph*) on memory reconsolidation and generalized fear expression in animals treated with yohimbine (YOH) immediately after contextual fear conditioning. (**A**) Experiment's 7 design. (**B**) CLO produced no effects since drug-treated animals behaved similarly to controls during Tests A and B_3. (**C**) Experiment's 8 design. (**D**) CLO impaired the reconsolidation process since the YOH-VEH-CLO-VEH-CLO group presented lower values ($p < 0.05$) than the VEH-VEH-VEH-VEH-VEH group during both Tests A_1 and A_2. This difference, however, no longer happened in NIM-pretreated animals. Arrowheads indicate the moment of the treatments. Data are expressed as individual units and mean ± S.E.M. (n per group in experiments 7 and 8 = 8-10). The * denotes a statistically significant

difference from the respective control group (mixed ANOVA followed by the Newman-Keuls test).

3.5 Disrupting memory destabilization before generalized fear expression prevents the impairing effects of clonidine on reconsolidation

The post-reactivation amnesia produced by pharmacological interventions requires the occurrence of memory destabilization upon retrieval (Ben Mamou et al., 2006; Lee et al., 2008; Milton et al., 2013), an event involving the activation of L-type voltage-gated calcium channels (LVGCCs) in the brain (Suzuki et al., 2008). Based on this, we investigated whether activation of LVGCCs is necessary for CLO-induced impairments in memory reconsolidation (Experiment 8). Thirty-five fear-conditioned and yohimbine (YOH)-treated animals were randomly allocated to four groups (n = 8-10 per group) based on the pretreatment [vehicle (VEH) or the LVGCCs antagonist nimodipine (NIM) 15 mg/kg] and the treatment [VEH or clonidine (CLO)] given 30 min before and immediately after, respectively, each one of two cxt-B exposures (Tests B_1 and B_2). Tests A_1, B_3, A_2, and B_4, were performed one, two, eight, and nine days later, respectively (Fig. 4C).

For Tests A freezing time, a mixed ANOVA showed significant effects of treatment [$F(1,31) = 7.4$; $p = 0.01$], but not pretreatment [$F(1,31) = 3.0$; $p = 0.09$] or repeated testing [$F(1,31) = 0.87$; $p = 0.36$]. There were significant effects of interaction between pretreatment and treatment [$F(1,31) = 8.1$; $p = 0.008$], but not pretreatment and repeated testing [$F(1,31) = 1.1$; $p = 0.30$], treatment and repeated testing [$F(1,31) = 0.80$; $p = 0.38$] or among pretreatment, treatment and repeated testing [$F(1,31) = 0.46$; p

= 0.50]. As shown in figure 4D, *post-hoc* tests showed that the YOH-VEH-CLO-VEH-CLO group presented lower levels than the YOH-VEH-VEH-VEH-VEH group during Test A_1 ($p = 0.007$; $g = 2.25$) and Test A_2 ($p = 0.05$; $g = 1.41$), which corresponds to Experiment 3 results (Fig. 2D). However, since YOH-NIM-VEH-NIM-VEH and YOH-NIM-CLO-NIM-CLO groups behaved similarly, the impairing effects of clonidine on reconsolidation were prevented in nimodipine-pretreated animals.

For Tests B freezing time, a mixed ANOVA showed no significant effects of repeated testing [$F(3,93) = 2.2$; $p = 0.09$], pretreatment [$F(1,31) = 0.63$; $p = 0.43$] or treatment [$F(1,31) = 0.63$; $p = 0.43$]. There were also no significant effects of interaction between pretreatment and treatment [$F(1,31) = 2.7$; $p = 0.11$], pretreatment and repeated testing [$F(3,93) = 0.79$; $p = 0.50$], treatment and repeated testing [$F(3,93) = 0.14$; $p = 0.93$], or among pretreatment, treatment and repeated testing [$F(3,93) = 2.2$; $p = 0.09$]. As shown in figure 4D, all groups behaved similarly during Tests B_1, B_2, B_3, and B_4, thus the nimodipine pretreatment produced no changes in ongoing freezing expressed during Tests B_1 and B_2.

3.6 Cxt-B exposures and clonidine treatments interact to modulate the basolateral amygdala Egr1/Zif268 protein expression in yohimbine-treated animals

The process of fear memory destabilization-restabilization upon exposure to the conditioned context engages Egr1/Zif268 protein expression in the rodent dorsal CA1 hippocampus and the basolateral amygdala (Hall et al., 2001; Lee et al., 2004; Besnard et al., 2014; Espejo et al., 2016; Couto-Pereira et al., 2019). We investigated herein whether cxt-B exposures and CLO treatments influence the number of Egr1/Zif268-expressing neurons in the abovementioned brain regions (Experiment 9). Forty-three

animals were randomly allocated to seven groups based on the experimental condition: (i) home cage (n = 5); (ii) animals treated with vehicle after fear conditioning and each one of two cxt-B exposures (the VEH-VEH-VEH group; n = 5); (iii) animals treated with yohimbine after fear conditioning, and vehicle after each one of two cxt-B exposures (the YOH-VEH-VEH group; n = 5); (iv) animals treated with vehicle after fear conditioning, CLO after the first, and vehicle after the second cxt-B exposure (the VEH-CLO-VEH group; n = 6); (v) animals treated with yohimbine after fear conditioning, CLO after the first, and vehicle after the second cxt-B exposure (the YOH-CLO-VEH group; n = 7); (vi) animals treated with vehicle after fear conditioning, and CLO after each one of two cxt-B exposures (the VEH-CLO-CLO group; n = 8); and (vii) animals treated with yohimbine after fear conditioning, and CLO after each one of two cxt-B exposures (the YOH-CLO-CLO group; n = 7). Animals were euthanized immediately after their removal from the vivarium (home cage group) or 90 min after the second treatment and had their brain collected and subsequently processed for Egr1/Zif268 immunohistochemistry (Fig. 5A). Home cage data were used only as a reference for normalization of the number of Egr1/Zif268-expressing neurons in the other groups. Results were analyzed as the percentage of home cage values (see Table 1 for raw data).

For dorsal CA1 hippocampus, ANOVA showed significant effects of pretreatment [$F(1,32) = 84.6$; $p = 0.000001$], but not treatment [$F(2,32) = 0.10$; $p = 0.91$] or their interaction [$F(2,32) = 0.36$; $p = 0.70$]. As shown in figure 5B, all yohimbine-treated groups presented more Egr1/Zif268-positive cells than respective control groups ($p \leq 0.003$; $g \geq 3.07$), which suggests that generalized fear retrieval during cxt-B exposure was accompanied by increased dorsal CA1 hippocampus plasticity regardless of the

treatment given after that.

For basolateral amygdala, ANOVA showed significant effects of pretreatment [$F(1,32) = 27.1$; $p = 0.00001$], treatment [$F(2,32) = 4.5$; $p = 0.02$], and their interaction [$F(2,32) = 12.0$; $p = 0.0001$]. As shown in figure 5C, the YOH-CLO-CLO group presented more Egr1/Zif268-positive cells than the others ($p \leq 0.0003$; $g \geq 1.86$), which indicates that cxt-B exposures and CLO treatments interact to modulate the basolateral amygdala plasticity.

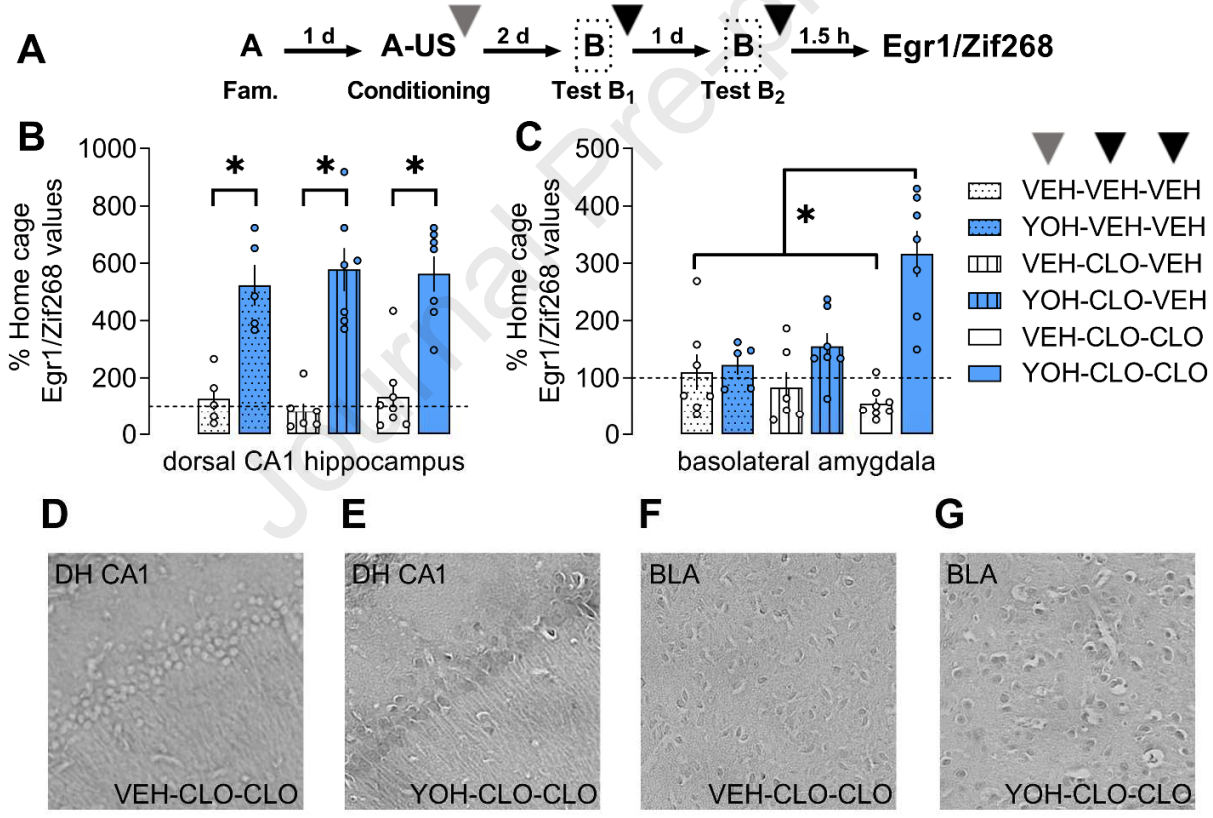


Figure 5. Effects of clonidine (CLO) treatment after one or two context (cxt) B exposures on the number of Egr1/Zif268-expressing cells in the dorsal CA1 hippocampus (*left graph*) and the basolateral amygdala (*right graph*) of fear-conditioned animals treated with vehicle (VEH) or yohimbine (YOH) shortly after that. Results were normalized to the

home cage control group. (**A**) Experiment's 9 design. (**B**) All YOH-treated groups presented high ($p < 0.05$) values than respective control groups, suggesting that increased dorsal CA1 hippocampus plasticity accompanies fear to the generalized cxt-B regardless of the treatment given after that. (**C**) The YOH-CLO-CLO group presented higher ($p < 0.05$) values than the others did, suggesting that differences in freezing times between CLO and VEH groups during Tests A seen in both experiments 3 and 8 (Figs. 2D and 4D, respectively) are accompanied by altered basolateral amygdala plasticity. Arrowheads indicate the moment of the treatments. Data are expressed as individual units and mean ± S.E.M. (n per group = 5-8). The * denotes a statistically significant difference from the respective control group (ANOVA followed by the Newman-Keuls test). (**D-G**) Middle zoom level photomicrograph of each one of the brain regions in selected experimental groups.

4. DISCUSSION

After showing that fear conditioning paired with yohimbine administration significantly increased fear generalization to cxt-B, we investigated whether such fear generalization is accompanied by sufficient destabilization of the underlying memory for clonidine treatment to impair its reconsolidation. There were no differences between drug-treated animals and controls when exposed to the conditioned cxt-A (Test A) after a single cxt-B exposure followed by clonidine treatment, which agrees with that recently reported with the use of the positive allosteric modulator of $GABA_A$ receptors midazolam (Alfei et al., 2020a,b). In contrast, a result indicating sufficient memory destabilization was found when two cxt-B exposures followed by clonidine treatments were carried out. Moreover, in line with the known role of brain L-type voltage-gated calcium channels (LVGCCs) in memory destabilization (Suzuki et al., 2008), we showed that treatment with nimodipine, a selective antagonist of LVGCCs, before each one of two cxt-B

exposures precludes the clonidine-induced action on reconsolidation. This result is consistent with contextual and cued fear conditioning findings concerning the conditioned stimulus (Flavell et al., 2011; Haubrich et al., 2015; da Silva et al., 2016; Ortiz et al., 2019). A six-hour-delay in clonidine treatment following each one of two cxt-B exposures also prevented its impairments in reconsolidation, probably because the neural/molecular events required for memory restabilization have already finished at that time point. Together, these results highlight the mechanistic and temporal similarities between memory destabilization-reconsolidation processes upon conditioned context exposure in animals not treated with yohimbine post-conditioning (Gazarini et al., 2013, 2014) and upon generalized context exposure in animals treated with yohimbine post-conditioning (current study). They also demonstrate that the conditioning context is not decisive for inducing destabilization since the aversive memory can return to a labile state upon retrieval in a context sharing enough elements with it (Maren et al., 2013; Ferrer Monti et al., 2016; Alfei et al., 2020a,b).

The process of generalization is proposed to result from the use of previously acquired information. Tulving and Thompson (1973) postulated that previous experiences influence both processing and understanding of current stimuli, which attributes to the memory a role similar to perception. Thus, the generalization from experience would be adaptive, allowing the adoption of appropriate responses in similar future situations (Shepard, 1987; Heszage and Censor, 2018). At the brain level, during exposure to a generalized stimulus, those features/patterns shared with the original one can activate common neuronal circuits, leading to the expression of a generalized response (Lopresto et al., 2016; Robertson, 2018). For instance, the generalization of fear correlates with overlapping yet distinct recruitment of lateral amygdala neurons that

would later be activated in response to a non-associated but generalized stimulus (Ghosh and Chattarji, 2015). Considering that generalized fear expression results from the original aversive memory retrieval, the impairing effects of clonidine on reconsolidation were attained because of the sufficient destabilization after cxt-B exposures.

The view that memory destabilization can accompany generalized fear expression is supported by the fact that clonidine administered either in the absence of cxt-B exposures or after each one of two exposures to the non-conditioned and non-generalized cxt-C produced no behavioral changes during Test A in yohimbine-treated animals. These results suggest the absence of memory destabilization, which precludes the possibility of clonidine impairing the reconsolidation phase. They are also in line with fear conditioning studies in rodents showing that the administration of reconsolidation blockers immediately after exposure to a non-generalized context had no effects at test in the conditioned context (Bustos et al., 2009, 2010; Stern et al., 2012; Gazarini et al., 2013; da Silva et al., 2020; Troyner and Bertoglio, 2020). Using a contextual fear-conditioning paradigm, it was recently reported that rats systemically treated with midazolam after exposure to a generalized context spent less time freezing than controls at subsequent test in the *same* context (Alfei et al., 2020b). Since the levels of generalized fear reached in our study were considerably lower than those observed in theirs, a possible explanation for the absence of clonidine effects during Test B_2 could be a floor effect, which would mask the observation of a potential drug-induced reduction in such response. Alternatively, a significant difference could require more than two cxt-B exposures to be revealed, an idea supported by data from Experiment 8 since animals treated with clonidine tended to spend less time freezing than controls

during Test B_4 (Fig. 4D).

Studies of fear memory reconsolidation have shown that exposure to the conditioning context can induce the process of destabilization-reconsolidation (Nader et al., 2000; Debiec et al., 2002; Lee et al., 2004; Bustos et al., 2006; Stern et al., 2012). It has been suggested that the availability of new information during retrieval could be a necessary condition for the readjustment or update of established memories (Rodriguez-Ortiz et al., 2005; Hupbach et al., 2007; Tronson and Taylor, 2007). This phenomenon is related to the concept of prediction error (a discrepancy between what is expected and what is experienced) or mismatch that has been deemed critical for the destabilization and subsequent memory updating (Pedreira et al., 2004; Sevenster et al., 2012; Alfei et al., 2015; Fernández et al., 2016). Here, clonidine administered after each one of two cxt-A exposures induced no memory changes in yohimbine-treated animals. A similar pattern of results has been associated with the formation of strong and more generalized aversive memories resistant to the onset of the destabilization-reconsolidation process (Gazarini et al., 2013, 2014; Espejo et al., 2016). Based on the above, we proposed that, although both cxt-A and cxt-B exposures can elicit memory retrieval, only the generalized context has the degree of novelty necessary to induce the prediction error-related memory destabilization. It is worth noting that the fear generalization-associated destabilization was observed even after a second exposure to the cxt-B, which indicates that it still has attributes that would provide the novelty necessary to induce the destabilization-reconsolidation process (Zinn et al., 2020). Another explanation is that the strength of the memory retrieval determines malleability or susceptibility to destabilization. This would follow an inverted U-shaped curve such that there is a mid-level of fear retrieval that is optimal for destabilization. Exposure to an

entirely novel context (cxt-C) is insufficient to reactivate the original memory at all, so the clonidine treatment is ineffective. Exposure to the conditioning context (cxt-A) produces a much more robust fear response, so the clonidine treatment is insufficient to cause disruption. This raises the question of whether a higher dose of clonidine or weakening of the fear response via extinction before treatment would alter the outcome. The difference in fear levels when the animals were exposed to cxt-A *versus* cxt-B and the corresponding noradrenergic tonus could also contribute to the varying effects of clonidine on reconsolidation observed. The neural circuit and mechanisms underpinning memory destabilization upon cxt-B exposures may differ from those engaged after cxt-A exposures. Of relevance to the present discussion are data showing that the formation of a reconsolidation-resistant fear memory entails noradrenergic inputs from the locus coeruleus to the basolateral amygdala. However, if these projections are silenced, it can later undergo destabilization and reconsolidation (Haubrich et al., 2020). Based on this, it is possible that in our case, the clonidine-induced attenuation of the noradrenergic tonus had a more significant influence on the neural assemblies supporting the yohimbine-potentiated memory that were reactivated upon cxt-B exposures. Such action was sufficient to update the memory content to a less aversive form during reconsolidation, as observed subsequently during Test A.

Some studies have reported no effects despite the amnesic treatment (midazolam) after a generalized performance in a non-associated context (Bustos et al., 2019, 2010; Alfei et al., 2020a,b). In these cases, the generalized response was high, reaching levels similar to those observed during exposure to the conditioned context. The novelty during exposure to the generalized context was possibly not enough to drive memory into a transient state of potential modification. We propose that, once the non-

associated stimulus can trigger memory destabilization after retrieval, there is an inverse relationship between fear generalization and the opportunity for memory reconsolidation interference. Classical conditioning assumes that the conditioned stimulus is represented by a set of features from the environment. Therefore, the degree of generalization will depend on the number of features of the original set present during the test session (Pearce, 1987). The more in-common features between training and testing sessions, the higher the generalization and, thus, the lower the probability of observing a change in the original response. Future studies are guaranteed to examine how the generalization gradient interrelates with the mismatch.

The process of destabilization-reconsolidation of fear memories in rodents exposed to the *conditioned* context engages Egr1/Zif268 protein expression in the dorsal CA1 hippocampus (Hall et al., 2001; Lee et al., 2004; Besnard et al., 2014; Couto-Pereira et al., 2019). Only the yohimbine-treated animals presented an increase in the number of Egr1/Zif268-expressing cells in the dorsal CA1 hippocampus after two cxt-B exposures, regardless of the treatment given shortly after that. This result would mean that this up-regulation is not necessarily associated with the process of memory destabilization-reconsolidation, but could instead represent a higher number of dorsal CA1 hippocampal cells recruited to retrieve a more generalized and strong fear memory, which is line with a significant positive correlation between indices of generalization at behavioral and neuronal levels shown in the lateral amygdala (Ghosh and Chattarji, 2015).

Pre-retrieval administration of the glutamate NMDA partial agonist D-cycloserine was reported to reverse both yohimbine-induced fear generalization and resistance to fear memory destabilization-reconsolidation (Gazarini et al., 2014). Similarly, while pre-

conditioning stress was associated with destabilization-resistant fear memory formation and lack of up-regulation of Egr1/Zif268 in the basolateral amygdala upon exposure to the conditioned context, local D-cycloserine infusion restored the Egr1/Zif268 expression, memory destabilization, and susceptibility to reconsolidation impairment (Espejo et al., 2016). Increasing the basolateral amygdala levels of GluN2B-containing NMDA receptors, which are required for fear memory destabilization (Ben Mamou et al., 2006; Wang et al., 2009; Milton et al., 2013), was also sufficient to enable the modification of resistant fear memories via reconsolidation (de Solis et al., 2019). Here, the yohimbine group treated with clonidine after two cxt-B exposures presented not only a greater number of Egr1/Zif268-expressing cells in the basolateral amygdala than the yohimbine group treated with vehicle post-cxt-B but also attenuated fear later at test in the conditioning cxt-A. Considering that destabilization of fear memories after conditioned context exposure engages Egr1/Zif268 protein expression in the basolateral amygdala (Espejo et al., 2016; Couto-Pereira et al., 2019), our results suggest that clonidine treatment restored the basolateral amygdala plasticity and original memory malleability upon retrieval. In this regard, since a neural circuit including both the dorsal CA1 hippocampus and the basolateral amygdala is necessary for successful contextual fear memory retrieval and reconsolidation (Lux et al., 2017), the yohimbine-induced enhanced noradrenergic activity during consolidation might have disrupted it, causing deficits in memory destabilization that would later be attenuated by clonidine treatment following cxt-B exposures. This assumption is supported by a recent study showing that activation of the noradrenaline-locus coeruleus system during strong fear memory encoding increased the molecular mechanisms of stability at the expense of lability in the basolateral amygdala (Haubrich et al., 2020).

The ability to adjust a memory in response to an ever-changing environment provides a benefit and reveals the functional relevance of its dynamic nature. Traumatic experiences, however, can limit this flexibility because the underlying memory often differs from the normal aversive memory in qualitative and quantitative aspects and, therefore, it may be less prone to attenuation by extinction- and reconsolidation-based interventions (Singewald et al., 2015; Careaga et al., 2016; Kida, 2019). Accumulating evidence from animal fear conditioning studies has indicated that potentiating memory destabilization upon retrieval in the conditioned context allows the impairing effects of several reconsolidation blockers (Bustos et al., 2010; Gazarini et al., 2014; Espejo et al., 2016). However, memory destabilization through exposure to the actual learning situation is not always possible or suitable. In this context, the present study contributes with an effective strategy to overcome the often-reported resistance to pharmacological interventions targeting the reconsolidation of PTSD-like memories since memory destabilization can accompany generalized fear expression. Thus, we may exploit it to potentiate the action of reconsolidation blockers. Our results also emphasize how important is the basolateral amygdala for maintaining a reconsolidation-resistant abnormal aversive memory similar to that underlying the PTSD.

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The authors declare no competing financial interests concerning the work described.

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7. AUTHOR CONTRIBUTIONS

L.J.B. and M.G. conceptualized and administrated the study; L.J.B., M.G., and V.A.M. planned the experiments; F.N.M, J.M.F., and F.T. carried out the experiments; M.G., J.M.F, and F.T. performed the analysis of data; M.G., V.A.M., and L.J.B. drafted the manuscript, and L.J.B. designed the figures; all authors discussed the results and contributed to the final manuscript.

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Table 1. Raw data for counts of Egr1/Zif268-expressing cells in the seven groups from Experiment 9. Data are expressed as mean ± SEM in 0.4 mm^2 (n = 5-8/group)

Brain region / Group	dorsal CA1 hippocampus	basolateral amygdala
Home cage	9 ± 3	36 ± 18
VEH-VEH-VEH	11 ± 3	40 ± 11
YOH-VEH-VEH	45 ± 6	45 ± 6
VEH-CLO-VEH	7 ± 2	30 ± 10
YOH-CLO-VEH	49 ± 6	56 ± 8
VEH-CLO-CLO	11 ± 4	20 ± 3
YOH-CLO-CLO	48 ± 5	115 ± 15

Legend: VEH = vehicle; YOH = yohimbine; CLO = clonidine

HIGHLIGHTS

- Post-fear conditioning yohimbine treatment promotes memory generalization;
- Such memory does not undergo destabilization after conditioned context exposure;
- However, it is malleable upon non-conditioned but generalized context exposure;
- Then, it can be pharmacologically modified via reconsolidation intervention;
- This situation engages Egr1/Zif268 protein expression in the basolateral amygdala.