Research

Enhanced noradrenergic activity potentiates fear memory consolidation and reconsolidation by differentially recruiting α 1- and β -adrenergic receptors

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Consolidation and reconsolidation are phases of memory stabilization that diverge slightly. Noradrenaline is known to influence both processes, but the relative contribution of α 1- and β -adrenoceptors is unclear. The present study sought to investigate this matter by comparing their recruitment to consolidate and/or reconsolidate a contextual fear memory trace under enhanced noradrenergic activity induced by yohimbine. We report that this α 2-adrenoceptor antagonist was able to potentiate fear memory trace consolidation or reconsolidation when administered immediately after acquisition or retrieval, respectively, resulting in increased freezing expression. In either case, generalization of this response to an unpaired context was also seen when it achieved a ceiling level in the paired context. These effects endured for over 7 d and relied on action at central rather than peripheral sites, but were prevented when a memory trace was not acquired, when memory reactivation was omitted, or when administration of yohimbine was delayed until 6 h after acquiring or retrieving the memory trace. The β -adrenoceptor antagonist propranolol was able to prevent the above-mentioned effects of yohimbine, while pretreatment with the α 1-adrenoceptor antagonist prazosin blocked only its facilitating effects on memory reconsolidation. These results highlight a differential participation of α 1- and β -adrenoceptors in fear memory processing. Moreover, it was shown that the α 2-adrenoceptor agonist clonidine, as opposed to yohimbine, mitigates fear expression by weakening memory consolidation or reconsolidation.

Noradrenaline was initially associated with memory processing by Kety (1972), who proposed it could induce lasting changes in the brain that could sustain memories over time. As confirmed later on by Harley (1987) and others, its neurotransmission indeed strengthens memory-related synaptic plasticity such as long-term potentiation, allowing memories to be formed and maintained in a more intense and enduring manner, a notion particularly valid for those with emotional content (van Stegeren 2008; Sara 2009; Joëls et al. 2011). Like other types of memory, an emotional memory has to be consolidated to allow its later retrieval (Dudai 2004). On such an occasion, an established memory can become labile again after being reactivated briefly, requiring a new phase of stabilization known as reconsolidation to be reestablished (Nader et al. 2000; Sara 2000). Accumulating evidence has indicated that noradrenaline acts during these gradual stages to fine-tune the strength and/or persistence of a memory (Gold and van Buskirk 1978; Lewis 1979; McGaugh 1983; Roulet and Sara 1998; LaLumiere et al. 2003; Tronel et al. 2004; Debiec et al. 2011; Soeter and Kindt 2011; Guzmán-Ramos et al. 2012).

The facilitating role of noradrenaline in consolidation and/or reconsolidation of emotional memories charged with positive or negative valence has mostly been attributed to the activation of β -adrenergic receptors (Cahill et al. 1994; Przybylski et al. 1999; Sara et al. 1999; McGaugh and Roozendaal 2002; Gelinas and Nguyen 2005; Milton et al. 2008; Furini et al. 2010; Debiec et al. 2011; Schutsky et al. 2011). Whether α 1-adrenergic receptor stimulation interferes with these memory phases, however, is still unclear. A role for this subtype of adrenergic receptor in long-term potentiation induction has been reported (Pussinen and Sirviö 1998), but few studies have corroborated its participation in con-

solidation or reconsolidation of spatial (Puumala et al. 1998) or emotional memories (Ferry et al. 1999; Bernardi et al. 2009, but see Lazzaro et al. 2010). As α 1- and β -adrenergic receptors recruit different sets of intracellular signaling cascades (Hein 2006) and seem to be heterogeneously expressed within the brain regions involved in memory consolidation and reconsolidation (Nicholas et al. 1996), one would anticipate a differential contribution by them to both memory phases, a premise consistent with convergent evidence now indicating that memory consolidation and reconsolidation diverge slightly in terms of micro and macro aspects (Dudai and Eisenberg 2004; Lee et al. 2004; Besnard et al. 2012). To the best of our knowledge, however, a study primarily aimed at investigating this subject is still lacking. Of potential relevance to this matter is the usefulness of noradrenergic-enhancing drugs such as yohimbine, an antagonist of α 2-adrenergic receptors that counteracts the inhibitory action mediated by these receptors and stimulates the locus coeruleus (Ivanov and Aston-Jones 1995). Stimulation of the locus coeruleus is known to increase noradrenaline release in several brain regions (Abercrombie et al. 1988; Crespi 2009), particularly those necessary for emotional memory processing (Sara 2009). Moreover, as yohimbine acts as an indirect sympathomimetic agent, the endogenous neurotransmitter is the one that ultimately intensifies the noradrenergic transmission. From a physiological perspective such an outcome would seem to be more advantageous than that arising from selective and potent agonists for α - or β -adrenergic receptors, because noradrenaline binds and activates these adrenoceptors differently from the latter compounds (Zhang et al. 2004).

Based on these facts, the present study sought to investigate the relative contributions of α 1- and β -adrenergic receptors in consolidating and/or reconsolidating an emotional memory trace under enhanced noradrenergic activity induced by yohimbine in rats evaluated in a contextual fear conditioning paradigm. The working hypothesis was that each one of these memory steps could be

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Article is online at <http://www.learnmem.org/cgi/doi/10.1101/lm.030007.112>.

associated with a differential recruitment of these adrenoceptors. A possible inability to restrict fear to the appropriate context was also assessed throughout the experiments because this maladaptive response has also been related to noradrenergic-mediated signaling mechanisms (Debiec et al. 2011; Soeter and Kindt 2012). We demonstrate that: (1) yohimbine is able to intensify the expression of freezing in a paired context by potentiating memory consolidation or reconsolidation, (2) the $\alpha 2$ -adrenergic receptor agonist clonidine can induce just the opposite effects, (3) the facilitating effects of yohimbine are long lasting and seem to recruit $\alpha 1$ - and β -adrenergic receptors differently, and (4) yohimbine is able to induce fear generalization when memory consolidation and reconsolidation are potentiated separately or together.

Results

Experiment I: Enhanced noradrenergic activity potentiates consolidation of a fear memory trace

To investigate whether yohimbine could potentiate the consolidation of a fear memory trace, 22 rats were randomly allocated to two independent groups ($n = 10$ –12/group) based on systemic treatment (vehicle or 1.0 mg/kg of drug) given immediately after pairing Context A to a single foot shock, the unconditioned stimulus (US).

Repeated-measures ANOVA showed a significant main drug treatment effect for freezing time in the paired context ($F_{(1,20)} = 35.9$; $P < 0.00001$). As shown in Figure 1A, yohimbine-treated animals presented significantly more freezing than controls when reexposed to Context A 1 d and 8 d later (Tests A₁ and A₂), suggesting that under yohimbine's influence the encoding of a fear memory trace was potentiated in an enduring manner. Figure 1A also depicts the freezing times of these groups when exposed to a neutral and unpaired Context B. Repeated-measures ANOVA showed

Figure 1. (A) Potentiating effect of yohimbine (YOH) on contextual fear memory trace consolidation in rats. After a familiarization (Fam.) session, animals had Context A paired with a single foot shock (US), and then received vehicle or YOH (1.0 mg/kg i.p.). When reexposed to the paired context 1 d and 8 d later (Tests A₁ and A₂), drug-treated animals spent more time freezing than controls. This difference also occurred in an unpaired context (Test B₂). (B) Lack of evidence that YOH is acting as a US to induce a fear memory formation when the foot shock was omitted under our experimental conditions. (C) Delayed YOH treatment spares consolidation of a fear memory trace from potentiation. The arrowhead indicates the moment of drug treatment. Bars represent the percentage of total time spent freezing. Values are expressed as mean $\pm$ S.E.M. Asterisks indicate a significant difference ($P < 0.05$) from respective controls (repeated-measures ANOVA followed by Newman–Keuls test); (n.s.) nonsignificant difference.

Table 1. At the dose of 1.0 mg/kg, yohimbine is anxiogenic to rats exposed to the elevated plus-maze

Behavioral measure	Vehicle (n = 7)	Yohimbine (n = 8)	Statistical analysis (Student's <i>t</i> -test)
%OAT	15.6 $\pm$ 4.9	4.9 $\pm$ 1.7*	$t_{(13)} = 4.69$; $P < 0.05$
%OAE	30.6 $\pm$ 4.5	15.4 $\pm$ 4.9*	$t_{(13)} = 5.04$; $P < 0.05$
EAE	8.6 $\pm$ 0.7	7.5 $\pm$ 1.0	$t_{(13)} = 0.73$; $P = 0.40$

Animals were systemically treated with this drug or its vehicle 30 min before testing. When compared to controls, yohimbine-treated animals expressed significantly more inhibitory avoidance to open-arms (%OAT and %OAE). This effect was observed in the absence of changes in enclosed arm-entries (EAE), a general exploratory activity index in this test of anxiety. Data are presented as mean $\pm$ S.E.M. (%OAT) Percentage of open-arms time; (%OAE) percentage of open-arms entries; (EAE) enclosed arm-entries. Asterisks indicate a significant difference ($P < 0.05$) from vehicle group. For further details of elevated plus-maze procedure, see Gazarini et al. (2011).

a significant main drug treatment effect for this behavioral measure ($F_{(1,20)} = 7.4$; $P < 0.05$). No significant differences between groups were observed on the first exposure (Test B₁), but drug-treated animals displayed significantly more freezing than controls during Test B₂ performed a week later. This latter result indicates a tendency to fail in restricting fear expression to the appropriate context after the memory trace consolidation has been potentiated by yohimbine administration.

The anxiogenic-like effect of a drug can act by itself as an US to induce aversive memory formation in certain experimental conditions (Guittion and Dudai 2004; Cavalli et al. 2009). As an anxiogenic action of yohimbine is seen at the dose currently tested (Table 1), it could be reasoned that the augmentation of freezing time induced by this drug during reexposure to the paired Context A relied on this pharmacological property. To address this issue, 18 rats were allocated to two independent groups ($n = 8$ –10/group) that received either vehicle or yohimbine (1.0 mg/kg) immediately after being exposed to Context A without any foot shock presentation (“no pairing” session). As shown in Figure 1B, both groups behaved equally when reexposed to Context A (Test A₁: $t_{(16)} = 0.54$; $P = 0.48$), and when exposed to Context B as well (Test B₁: $t_{(16)} = 0.45$; $P = 0.51$), suggesting that yohimbine is potentiating consolidation rather than acting as an US under our experimental conditions.

Memory consolidation is a gradual process that takes up to 6 h after acquisition to be completed (Dudai 2004). To investigate whether the yohimbine-induced augmentation of freezing behavior observed later in the paired context was a specific interference with the consolidation phase, 20 rats received either vehicle or 1.0 mg/kg of drug ($n = 10$ /group) 6 h after a session of Context A–US pairing. As shown in Figure 1C, when drug administration was delayed, both groups expressed equally short freezing levels when reexposed to Context A (Test A₁: $t_{(18)} = 0.20$; $P = 0.66$) or exposed to Context B (Test B₁: $t_{(18)} = 0.54$; $P = 0.47$). These results indicate that the fear memory trace facilitation induced by yohimbine was no longer seen when it was administered after completion of the consolidation process.

As shown in Table 2, there was a significant interaction between drug treatment (vehicle or yohimbine) and number of foot shocks delivered (zero or one) for freezing time in the paired context ($F_{(1,36)} = 22.3$; $P < 0.0001$). Shocked yohimbine-treated animals presented significantly more freezing than nonshocked yohimbine-treated animals when reexposed to Context A (Tests A₁). This result agrees with those in another study in which a similar protocol of weak training was adopted to produce a low level of that fear defensive response, in order to optimize the outcome of experimental manipulations hypothesized to have a facilitating role in memory processing (Maldonado et al. 2011). No

Table 2. Pairing Context A with one foot shock is able to induce a fear memory trace that, in turn, can later be potentiated by yohimbine

Number of foot shocks	Vehicle	Yohimbine 1.0 mg/kg
0	21.2 $\pm$ 5.2	16.5 $\pm$ 2.8
1	25.7 $\pm$ 4.7	70.7 $\pm$ 6.7*

Data are presented as mean $\pm$ S.E.M of freezing time displayed during re-exposure to the paired context (Test A₁). The asterisk indicates a significant difference ($P < 0.05$) from the respective control group (two-way ANOVA followed by Newman–Keuls test).

statistically significant differences between shocked and non-shocked vehicle-treated groups were found. Based on this result, one might question whether a single foot shock is sufficient to induce a fear memory trace. The administration of yohimbine only increased freezing time in the shocked group, which suggests that a contextual fear memory trace may occasionally be translated into a mild behavioral change that can reach statistical significance after drug-mediated potentiation of its associated memory trace.

Experiment 2: Enhanced noradrenergic activity potentiates reconsolidation of a fear memory trace

To investigate whether yohimbine could also potentiate the reconsolidation of a fear memory trace, 19 Context A–US paired rats were randomly allocated to receive either vehicle or 1.0 mg/kg of drug ($n = 9$ –10/group) just after the session of memory retrieval/reactivation in the paired context, and then reexposed to Context A in tests 1 d and 8 d later.

Repeated-measures ANOVA showed a significant drug treatment $\times$ Context A reexposures interaction for freezing time ($F_{(2,34)} = 11.7$; $P < 0.001$). As shown in Figure 2A, both groups displayed equally brief amounts of freezing during the retrieval/reactivation session, but yohimbine-treated animals expressed significantly more freezing than controls when reexposed to Context A 1 d and 8 d later (Tests A₁ and A₂), suggesting that the reconsolidation of a fear memory trace was reinforced under the influence of yohimbine, an effect that endured over 1 wk. As in the previous experiment, these animals were also exposed to an unpaired context. No significant drug treatment effects ($F_{(1,17)} = 0.13$; $P = 0.72$) were observed when they were exposed to Context B either 2 d or 9 d (Tests B₁ and B₂) after the memory trace retrieval/reactivation session. Accordingly, the groups expressed a similar short freezing time in either case (Fig. 2A).

As retrieval followed by the reactivation of a memory trace is necessary to trigger reconsolidation (Sara 2000), a complementary experiment was conducted to investigate the specificity of yohimbine's effects on the latter process. For this aim, 15 Context A–US paired rats were randomly allocated into two independent groups ($n = 7$ –8/group) based on the systemic treat-

ment (vehicle or 1.0 mg/kg of drug) given immediately after exposure to Context B, a neutral context different from the one used for US pairing (“no retrieval/reactivation” session). As shown in Figure 2B, no significant drug treatment effects were observed during reexposure to either Context A (Test A₁: $t_{(13)} = 0.43$; $P = 0.52$) or Context B (Test B₁: $F_{(1,13)} = 0.19$; $P = 0.66$). Together, these results confirm that yohimbine-induced facilitation in reconsolidation depends on prior memory trace retrieval/reactivation.

The process of reconsolidation shares with consolidation its gradual nature of stabilization over time, taking several hours to be completed (Nader et al. 2000; Dudai 2004). To confirm that facilitation of the fear memory trace induced by yohimbine was specific to reconsolidation, 20 Context A–US paired rats were randomly allocated to receive the drug or its vehicle ($n = 10$ /group) 6 h after retrieval/reactivation. Repeated-measures ANOVA showed neither a drug treatment $\times$ Context A reexposure interaction ($F_{(1,18)} = 0.60$; $P = 0.44$) nor significant main effects of these factors ($F_{(1,18)} = 0.30$; $P = 0.59$ and $F_{(1,18)} = 0.60$; $P = 0.44$, respectively). Accordingly, as shown in Figure 2C, yohimbine-treated animals behaved like controls, exhibiting short freezing times during the retrieval/reactivation session and Test A₁. This indicates that drug-induced facilitation of the fear memory trace was no longer seen when it was administered after completion of the reconsolidation process. These groups also had similar short freezing times on Test B₁ ($t_{(18)} = 0.15$; $P = 0.70$).

Experiment 3: Enhanced noradrenergic activity-induced potentiation of consolidation and/or reconsolidation of a memory trace results in generalized fear expression

To investigate whether yohimbine could reinforce both consolidation and reconsolidation of a contextual fear memory trace, 22 rats were administered with either vehicle or 1.0 mg/kg of drug

Figure 2. (A) Potentiating effect of yohimbine (YOH) on contextual fear memory trace reconsolidation in rats. After a familiarization (Fam.) session, animals had Context A paired with a single foot shock (US). On the next day, they were reexposed to Context A to retrieve/reactivate the memory trace, and then received vehicle (VEH) or YOH (1.0 mg/kg i.p.). In comparison to controls, drug-treated animals expressed more fear when reexposed to the paired context 1 d and 8 d later (Tests A₁ and A₂). No differences between groups were observed in an unpaired context (Tests B₁ and B₂). (B) Memory trace reactivation is necessary for YOH to potentiate reconsolidation. (C) Delayed YOH treatment spares reconsolidation of a fear memory trace from potentiation. The arrowhead indicates the moment of drug treatment. Bars represent the percentage of total freezing time. Values are expressed as mean $\pm$ S.E.M. Asterisks indicate a significant difference ($P < 0.05$) from respective controls (repeated-measures ANOVA followed by Newman–Keuls test).

($n = 10$ – 12 /group) immediately after a session of Context A–US pairing and after the memory retrieval/reactivation session.

Repeated-measures ANOVA showed a significant drug treatment effect for freezing time in the paired context ($F_{(1,17)} = 105$; $P < 0.00001$). As shown in Figure 3A, yohimbine-treated animals presented significantly longer freezing times than controls during retrieval/reactivation, replicating the facilitation in fear memory trace consolidation demonstrated in Experiment 1. When yohimbine was administered again, now following the retrieval/reactivation session, it induced a trend ($P = 0.10$) to further increase the freezing behavior expressed by drug-treated animals during Tests A_1 and A_2 performed 1 d and 8 d later. The possibility that this result could be related to a ceiling level ($\sim 85\%$) of freezing achieved by the earlier yohimbine-induced facilitation of memory trace consolidation, which in turn rendered any statistically significant further augmentation of the conditioned fear response

less prone to happen, will be examined later. Importantly, double yohimbine administration was also able to induce a robust and enduring augmentation in expression of this defensive response in an unpaired context. Accordingly, repeated-measures ANOVA showed a significant drug treatment effect on freezing time during exposures to Context B ($F_{(1,17)} = 55.4$; $P < 0.00001$). As shown in Figure 3A, yohimbine-treated animals expressed significantly more freezing than controls during Tests B_1 and B_2 performed 2 d and 9 d after the memory retrieval/reactivation session. These results indicate the occurrence of fear generalization.

In the preceding experiment, the additional administration of yohimbine after memory trace retrieval/reactivation induced fear generalization, but failed to intensify fear expression in the paired Context A. In an attempt to investigate this issue, 20 Context A–US paired rats were treated with vehicle or 1.0 mg/kg of drug ($n = 10$ /group) immediately after two consecutive memo-

ry retrieval/reactivation sessions performed 24-h apart. Repeated-measures ANOVA showed a significant drug treatment $\times$ Context A reexposure interaction for freezing time ($F_{(3,54)} = 52.5$; $P < 0.00001$). As shown in Figure 3B, both groups behaved equally on the first retrieval/reactivation session, but the group treated with yohimbine expressed significantly more freezing than the control group during the second session of memory trace retrieval/reactivation. On subsequent reexposure to the paired context (Test A_1), a significant increase in freezing time was observed in yohimbine-treated animals relative to that previously expressed by the same group, an effect that persisted for a week (Test A_2). Thus, in the absence of a prior ceiling level of freezing, the facilitated reconsolidation induced by yohimbine could, indeed, intensify fear expression in the paired context. Moreover, repeated-measures ANOVA showed no significant drug treatment effect on freezing time during exposures to the unpaired Context B ($F_{(1,18)} = 0.29$; $P = 0.6$) (Fig. 3B).

No fear generalization was observed in the former experiment after twice potentiating memory reconsolidation with yohimbine. To investigate whether this outcome results from a difference between memory consolidation and reconsolidation that is qualitative (i.e., fear generalization would only be induced when memory consolidation is potentiated) or quantitative (i.e., after the achievement of a ceiling freezing level, any further potentiation would result in fear generalization), 18 Context A–US paired rats were treated with vehicle or 1.0 mg/kg of drug ($n = 9$ /group) immediately after three consecutive memory retrieval/reactivation sessions performed 24-h apart. Repeated-measures ANOVA showed a significant drug treatment $\times$ Context A reexposure interaction for freezing time ($F_{(4,64)} = 106.8$; $P < 0.0000001$).

Figure 3. (A) Administration of yohimbine (YOH) during both consolidation and reconsolidation of a fear memory trace induces a generalized fear expression in rats. After a familiarization (Fam.) session, animals had Context A paired with a single foot shock (US), and then received vehicle (VEH) or YOH (1.0 mg/kg i.p.). On the next day, this treatment was repeated after the fear memory trace was reactivated by reexposing animals to Context A. Drug-treated animals presented more freezing than controls when tested not only in the paired (reactivation and Tests A_1 and A_2), but also in an unpaired context (Tests B_1 and B_2). (B) Potentiating the memory trace reconsolidation twice with yohimbine (YOH) intensifies fear expression in the paired context only. (C) Potentiating the memory trace reconsolidation three times with YOH also induces a generalized fear expression in rats. Arrowheads indicate the moment of drug treatment. Bars represent the percentage of total freezing time. Values are expressed as mean $\pm$ S.E.M. Asterisks indicate a significant difference ($P < 0.05$) from respective controls while hash symbols indicate a significant difference from the same group on the second reactivation (repeated-measures ANOVA followed by Newman–Keuls test).

As shown in Figure 3C, both groups behaved equally during the first session of memory retrieval/reactivation, but the yohimbine-treated animals presented significantly more freezing than controls in both the second and the third memory retrieval/reactivation sessions, with a gradual increase in freezing time over this period. On the next day, during reexposure to the paired context (Test A₁), no change in freezing time was observed in yohimbine-treated animals relative to that expressed previously by the same group, suggesting that the ceiling level of this defensive response was achieved after the last yohimbine administration. Drug-treated animals still displayed nearly the maximum level of freezing relative to respective controls during both Tests A₁ and A₂.

Moreover, triple yohimbine administration was able to induce a robust and enduring augmentation of freezing expression in the unpaired context. Repeated-measures ANOVA showed a significant drug treatment effect for freezing time in Context B ($F_{(1,16)} = 108.5$; $P < 0.0000001$). As shown in Figure 3C, yohimbine-treated animals expressed significantly more freezing than controls during Tests B₁ and B₂ performed 2 d and 9 d after the third session of memory retrieval/reactivation. These latter results suggest that fear generalization is a quantitative rather than a qualitative phenomenon associated with yohimbine-induced potentiation of a fear memory trace.

Experiment 4: α 1- and β -adrenergic receptors contribute differently to the enhanced noradrenergic activity-induced potentiation of fear memory trace consolidation and reconsolidation

The facilitating role of yohimbine in both fear memory trace consolidation and reconsolidation has been ascribed to its indirect ability to increase the brain's noradrenergic activity. If so, prevention of this action would be expected to result from pharmacologically antagonizing one or more different subtypes of adrenergic receptors. As either α 1- or β -adrenergic receptors can modulate emotional memory processing (Bernardi et al. 2009; Schutsky et al. 2011), their relative contributions to the aforementioned yohimbine effects were investigated using either the α 1-adrenergic receptor antagonist prazosin (0.5 mg/kg) or the nonselective β -adrenergic receptor antagonist propranolol (10 mg/kg). Thus, 108 rats were randomly allocated into 12 groups ($n = 8$ –11/group) based on both the systemic pretreatment given immediately after (vehicle, propranolol, or prazosin) and the treatment (vehicle or 1.0 mg/kg yohimbine) given 10 min after Context A–US pairing (for consolidation interference) or a retrieval/reactivation session (for reconsolidation interference).

Two-way ANOVA showed a significant interaction between drug pretreatment and treatment for freezing time ($F_{(2,48)} = 11.62$; $P < 0.0001$) in the former experimental design. As shown in Figure 4A, vehicle-pretreated animals administered with yohimbine after the pairing session expressed significantly more freezing than respective controls when reexposed to the paired Context A (Test A₁). This difference was also observed when the prazosin–yohimbine group was compared with the prazosin–vehicle group. On the other hand, in animals pretreated with propranolol, the potentiated consolidation induced by yohimbine was no longer observed relative to respective controls, or when compared with the vehicle–yohimbine treated group. A similar pattern of results was also observed during exposure to Context B (Test B₁). There was a significant interaction between drug pretreatment and treatment for freezing time ($F_{(2,48)} = 18.1$; $P < 0.01$), and vehicle-pretreated animals treated with yohimbine expressed more freezing than respective controls, but pretreatment with propranolol, not prazosin, prevented this effect.

Figure 4. Evidence for a differential participation of α 1- and β -adrenergic receptors in the potentiation of a fear memory trace consolidation and reconsolidation induced by yohimbine (YOH). (A) After pairing Context A with a single foot shock (US), animals were pretreated with vehicle (VEH) and 0.5 mg/kg adrenergic α 1-receptor antagonist prazosin (PRAZ) or 10 mg/kg adrenergic β -receptor antagonist propranolol (PROP). Ten minutes later, they received VEH or YOH (1.0 mg/kg). The VEH–YOH and PRAZ–YOH groups presented more freezing than respective controls when tested in either the paired (Test A₁) or the unpaired (Test B₁) context. The PROP–YOH group, however, was not different from the respective controls, showing a lower level of freezing relative to VEH–YOH-treated animals. (B) One day after pairing Context A with the US, an independent group of animals was reexposed to Context A to reactivate the fear memory trace, and then pretreated with VEH, PRAZ, or PROP. Ten minutes later, they received VEH or YOH. The VEH–YOH group froze more than respective controls when tested in the paired context (Test A₁). The PRAZ–YOH and PROP–YOH groups, however, were no longer different from respective controls, showing a lower level of freezing when compared to the VEH–YOH group. No differences between groups were observed in an unpaired context (Test B₁). Arrowheads indicate the moment of drug pretreatment and treatment. Bars represent the percentage of total freezing time. Values are expressed as mean $\pm$ S.E.M. Asterisks indicate a significant difference ($P < 0.05$) from respective controls, while hash symbols indicate a significant difference from the VEH–YOH group (repeated-measures ANOVA followed by Newman–Keuls test).

Altogether, these results indicate that β - rather than α 1-adrenergic receptors mediate the facilitating role of yohimbine in memory trace consolidation of a fear experience, and in subsequent expression of fear in a neutral context as well.

In the reconsolidation protocol, repeated-measures ANOVA showed a significant pretreatment vs. treatment vs. Context A re-exposure interaction for freezing time ($F_{(2,48)} = 12.2$; $P < 0.0001$). As shown in Figure 4B, all groups presented a similar low level of freezing in the reactivation session. Vehicle-pretreated animals administered with yohimbine after reactivation presented significantly more freezing than respective controls during Test A₁. In both prazosin- and propranolol-pretreated animals, however, yohimbine-induced enhancement of freezing was no longer seen relative to their respective controls, with an overall reduction in freezing time when compared to the vehicle–yohimbine treated group. These results indicate that both α 1- and β -adrenergic receptors play a role in the potentiation induced by yohimbine on fear memory trace reconsolidation. Moreover, all groups had equivalent low levels of freezing time during Test B₁ ($F_{(2,48)} = 0.67$; $P = 0.51$).

It is worth mentioning that α 2-adrenergic receptors are expressed not only in the central nervous system, but also in the periphery, such as in the adrenal glands controlling adrenaline release (Hein 2006). Even though adrenaline cannot cross the

blood–brain barrier, its action on β -adrenergic receptors present in the vagus terminal results in locus coeruleus stimulation and increased noradrenaline release in several brain regions related to aversive memory processing (Chen and Williams 2012). If that were the case here, pretreatment with nadolol, a nonselective β -adrenergic receptor antagonist that does not cross the blood–brain barrier (Cruickshank and Prichard 1987), would prevent the positive modulatory effects of yohimbine on memory trace consolidation and reconsolidation. As shown in Table 3, however, yohimbine was still able to induce its aforementioned effects on memory processing after blockade of peripheral β -adrenergic receptors, suggesting that enhanced noradrenergic activity reflects the antagonism of α 2-adrenergic receptors in central rather than peripheral sites.

Finally, as a further proof of the yohimbine effects mediated by α 2-adrenergic receptors, we provide results showing that activation of these receptors with clonidine impairs consolidation and reconsolidation of a fear memory acquired by pairing Context A with the delivery of three foot shocks (Table 4). As shown in Table 5, the effective dose of clonidine is devoid of lasting changes in anxiety and general exploratory activity parameters as assessed in the elevated plus-maze.

Discussion

When administered following Context A–foot shock pairing, yohimbine increased freezing time in animals reexposed to the paired context over 8 d, suggesting a potentiation in fear memory trace consolidation. Consonant with this premise is the facilitated encoding of a contextual memory seen in rats fear-conditioned after being submitted to physical restraint (Maldonado et al. 2011), a procedure also known to enhance noradrenergic transmission in the brain (Tanaka et al. 1982). Systemically administering adrenaline also increases noradrenaline release in the brain and facilitates the consolidation of emotion-driven memories (Gold and Van Buskirk 1975). Both yohimbine and adrenaline have been shown to strengthen the induction and maintenance of hippocampal LTP (Mondaca et al. 2004; Korol and Gold 2008). As this type of neuronal plasticity is believed to sustain memories and depend on noradrenergic activity (Harley 1991), it is suggested that the effects produced by yohimbine and adrenaline may rely at least in part on this mechanism. Moreover, yohimbine-treated animals presented a moderate but significant increase in freezing time when exposed to a neutral context 9 d after Context A–foot

Table 3. The potentiation of fear memory trace consolidation and reconsolidation induced by yohimbine (YOH) seems not to depend on peripheral enhancement of adrenergic activity

	VEH–VEH	VEH–YOH	NAD–VEH	NAD–YOH
Consolidation	(<i>n</i> = 8)	(<i>n</i> = 11)	(<i>n</i> = 9)	(<i>n</i> = 10)
Test A ₁	25.1 ± 2.8	73.2 ± 7.1*	27.2 ± 1.6	68.0 ± 1.9*
Test B ₁	9.4 ± 1.7	18.6 ± 2.6*	9.6 ± 1.4	15.3 ± 1.8*
Reconsolidation	(<i>n</i> = 8)	(<i>n</i> = 9)	(<i>n</i> = 9)	(<i>n</i> = 9)
Reactivation	23.5 ± 2.8	24.0 ± 1.4	21.3 ± 0.9	25.4 ± 2.2
Test A ₁	24.4 ± 2.1	55.2 ± 5.6*	21.1 ± 1.2	51.4 ± 2.0*
Test B ₁	10.0 ± 0.8	13.4 ± 2.4	10.5 ± 0.6	13.3 ± 0.7

Rats were systemically treated with vehicle (VEH) or the peripheral β -adrenergic antagonist nadolol (NAD; 10 mg/kg) just after the session of pairing Context A with a single foot shock (consolidation) or the reactivation session (reconsolidation), and received VEH or 1.0 mg/kg YOH 10 min later. The experimental design was similar to that used in Experiment 4. The statistically significant YOH-induced increase in freezing time was maintained regardless of the blockade of peripheral β -adrenergic receptors. Data are presented as mean ± S.E.M. Asterisks indicate a significant difference ($P < 0.05$) from the VEH–VEH group (repeated-measures ANOVA followed by Newman–Keuls test).

Table 4. Activating α 2-adrenergic receptors with clonidine significantly impairs both consolidation and reconsolidation of a contextual fear memory acquired by pairing Context A with three foot shocks

	Vehicle	Clonidine 0.1 mg/kg	Clonidine 0.3 mg/kg
Consolidation	(<i>n</i> = 10)	(<i>n</i> = 8)	(<i>n</i> = 9)
Test A ₁	73.2 ± 4.5	65.2 ± 5.2	50.3 ± 4.5*
Test B ₁	18.5 ± 2.5	17.2 ± 2.3	16.5 ± 1.5
Reconsolidation	(<i>n</i> = 10)	(<i>n</i> = 8)	(<i>n</i> = 8)
Reactivation	75.3 ± 1.8	73.4 ± 2.4	74.65 ± 1.7
Test A ₁	75.1 ± 1.6	64.1 ± 2.9	44.0 ± 6.4*
Test B ₁	16.8 ± 1.9	16.6 ± 1.3	14.9 ± 2.2

Rats were systemically treated with this drug (0.1 or 0.3 mg/kg) or its vehicle just after acquiring (consolidation) or reactivating (reconsolidation) the memory. The experimental design was similar to that used in Experiments 1 and 2, respectively. Data are presented as mean ± S.E.M. Asterisks indicate a significant difference ($P < 0.05$) from respective controls (repeated-measures ANOVA followed by Newman–Keuls test).

shock pairing. This result finds support in a previous study in which this drug elicited a certain degree of generalized fear expression in humans subjected to aversive conditioning (Soeter and Kindt 2012), and indicates that enhanced noradrenergic activity during fear memory encoding could lead to the expression of such maladaptive responses.

As systemic treatment with yohimbine has been shown to intensify the expression of unconditioned defensive responses, such as inhibitory avoidance (Table 1), the increase in freezing time seen during reexposure to Context A performed 24 h after its administration could alternatively be interpreted as a prolonged anxiogenic-like effect of this drug. Nevertheless, as significant concentrations of yohimbine in the rat brain only persist up to 8 h (Hubbard et al. 1988), this possibility is unlikely. Besides, this drug does not seem to be acting as an US under our experimental conditions since its facilitating action was no longer seen when animals were exposed to Context A but the foot shock was omitted (i.e., in the absence of a prior memory trace acquisition), reinforcing the specific effect of yohimbine on memory consolidation.

Yohimbine-induced potentiation of reconsolidation of a fear memory trace recalled a day after Context A–foot shock pairing was also shown in the current study. Since memory reconsolidation depends on briefly retrieving/reactivating its trace, one would expect no yohimbine-induced changes in freezing time in the absence of this condition. Indeed, when administered to animals after Context B exposure, yohimbine did not interfere with the level of fear on subsequent reexposure to Context A. This result confirms the specificity of the drug's effect on the memory reconsolidation phase, and adds further support for a key role of the noradrenergic system in reconsolidation of aversive memories (Przybylski et al. 1999; Debiec et al. 2011). It has previously been shown that administering the same yohimbine dose used here potentiates the extinction of an auditory fear memory in rats (Morris and Bouton 2007). Although there was a facilitating effect of this drug on memory processing in both studies, their behavioral outcomes were diametrically opposite: augmentation of freezing in our case and attenuation of the fear response in theirs. At least two aspects, namely the duration of the reactivation sessions (3 min vs. 10 min) and which memory is being considered (the original vs. that of extinction), may account for this divergence. It has been shown that whereas a brief (1.5–5 min) reexposure to the paired context without US presentation favors memory reconsolidation and preserves the fear response, a prolonged session (≥ 10 min) tends to cause extinction, resulting in

Table 5. At a memory-disrupting dose, clonidine has no effects on behavioral measures scored in the elevated plus-maze

Behavioral measure	Vehicle (n = 9)	Clonidine 0.3 mg/kg (n = 9)	Statistical analysis (Student's <i>t</i> -test)
%OAT	14.7 ± 3.3	12.5 ± 2.1	$t_{(16)} = 0.30$; $P = 0.59$
%OAE	34.4 ± 2.9	32.3 ± 1.4	$t_{(16)} = 0.39$; $P = 0.54$
EAE	6.4 ± 0.7	7.1 ± 0.5	$t_{(16)} = 0.60$; $P = 0.45$

Rats were systemically treated with vehicle or 0.3 mg/kg clonidine and tested 24 h later to verify the occurrence of any lasting locomotor or emotional changes that would coincide with the temporal window used in the experiment whose results are depicted in Table 4. No statistically significant changes in anxiety (%OAT and %OAE) or general exploratory activity (EAE) were observed. Data are presented as mean ± S.E.M. (%OAT). Percentage of open-arms time; (%OAE) percentage of open-arms entries; (EAE) enclosed arm-entries.

attenuation of fear responses (Bouton 2004; Lee et al. 2006; Stern et al. 2012). For these reasons, by reconsolidating the original fear memory under yohimbine's influence one would expect to see more freezing than before. By contrast, the fear response would be attenuated after longer reactivation of the original fear memory owing to its gradual suppression by the extinction memory, which could also be facilitated by yohimbine. Of note, based on its facilitating effects on fear extinction, this drug has been suggested as a potential pharmacological adjuvant to exposure-based psychotherapies for posttraumatic stress disorder (Cain et al. 2004, 2012, but see Holmes and Quirk 2010). In view of current findings, however, this approach should be reconsidered since it could backfire, potentiating reconsolidation rather than extinction of traumatic memories.

The susceptibility of memory consolidation and reconsolidation to noradrenergic manipulations has been shown to be restricted to a limited time window (Roullet and Sara 1998; Tronel et al. 2004). Accordingly, in the present study a facilitating effect of yohimbine on both consolidation and reconsolidation of a fear memory trace was observed when it was administered immediately after, but not 6 h later, Context A–foot shock pairing and a reactivation session, respectively. These results indicate that yohimbine's effects are specific to these memory stages, as no positive modulation was observed when the drug was given at a time point at which memory consolidation and reconsolidation had already been completed. Furthermore, the absence of changes in freezing time of animals reexposed to the paired context 18 h after being treated with yohimbine also rules out the possibility that a residual anxiogenic-like effect of this drug could explain the results observed.

As opposed to yohimbine, administering the α 2-adrenergic receptor agonist clonidine, which attenuates locus coeruleus discharge, noradrenergic activity, and LTP induction/maintenance (Abercrombie et al. 1988; Mondaca et al. 2004), disrupted both consolidation and reconsolidation of a contextual fear memory in a dose-dependent manner. Importantly, these effects are not attributable to a possible anxiolytic-like effect of this drug (Table 5). It is also thought that neither sedation nor locomotor activity impairments were confounding effects of clonidine in the dose-range tested because freezing time was reduced and not increased. The present results agree with those in which clonidine impaired consolidation of a two-way avoidance task (Gozzani and Izquierdo 1976) or reconsolidation of an auditory fear-conditioning (Gamache et al. 2012), and support the premise that the facilitating role of yohimbine in both these memory phases is ultimately caused by enhanced noradrenergic transmission through blockade of α 2-adrenergic receptors. If so, despite other effects of this drug being at least in part ascribed to its interaction with different receptors such as dopamine type 2 and serotonin type 1A (Holmes

and Quirk 2010), one would anticipate a role for α 1- and/or β -adrenergic receptors. In the present study, yohimbine's effect on fear memory trace consolidation was prevented by propranolol, a nonselective β -adrenergic receptor antagonist, but not by the nonselective β -adrenergic receptor antagonist nadolol, which does not cross the blood–brain barrier (Cruikshank and Prichard 1987). Such a finding implicating central rather than peripheral β -adrenergic receptors in the effect of yohimbine is consistent with results demonstrating that memory consolidation in rodents depends on their activation in the brain (Sara et al. 1999), particularly in the hippocampus, amygdala, and prefrontal cortex (Tronel et al. 2004; Furini et al. 2010; Guzmán-Ramos et al. 2012). Similarly, propranolol, but not nadolol, prevented increases in freezing time during exposures to the unpaired Context B. As yohimbine-induced fear generalization can also be attenuated in humans by propranolol administration (Soeter and Kindt 2012), our results and theirs suggest a role for β -adrenergic receptors in this inappropriate response. Overall, the requirement for β -adrenergic receptor activation seems to rely on its ability to modulate protein synthesis necessary for plastic changes related to memory formation (Gelinas and Nguyen 2005). Moreover, as reconsolidation also depends on protein synthesis (Nader et al. 2000), the participation of central β -adrenergic receptors in this memory phase would also be expected (Przybylski et al. 1999; Milton et al. 2008). Accordingly, herein their antagonism by propranolol rather than nadolol prevented yohimbine-induced facilitation of fear memory trace reconsolidation. Importantly, the inability of nadolol to prevent yohimbine-induced potentiating effects on memory supports a minor contribution of peripheral adrenergic receptors to the aforementioned effects.

Another prominent result was the prevention of yohimbine's effects on reconsolidation by prazosin, a selective α 1-adrenergic receptor antagonist, a finding that agrees with the involvement of that receptor in the reconsolidation of a conditioned place preference induced by cocaine in rats (Bernardi et al. 2009). Antagonism of α 1-adrenergic receptors, however, failed to prevent yohimbine-induced facilitation of contextual fear memory trace consolidation. Although the current study only tested the effects of a single dose of prazosin, it is worth remembering that it was the very same dose sufficient to prevent the effects of yohimbine on reconsolidation. As both α 1- and β -adrenergic receptors are expressed in interconnected brain regions related to learning and memory, such as amygdala, prefrontal cortex, and hippocampus (Nicholas et al. 1996), they could potentially be the areas in which these adrenergic receptors are activated after enhancing noradrenergic transmission with yohimbine. Of note, an unequal recruitment of these brain regions, which are heterogeneously exposed to noradrenaline (van Veldhuizen et al. 1994), has been documented during consolidation and reconsolidation of the same type of memory (Taubenfeld et al. 2001; Garcia-Delatorre et al. 2010). For instance, the prefrontal cortex can be differentially recruited during these memory phases depending on the arousal level induced by the task to be performed (Maroun and Akirav 2009). Of potential relevance to the present set of results is the higher expression of α 1-adrenergic receptors in the latter area relative to that found in hippocampus and amygdala (Rainbow and Biegon 1983; Nicholas et al. 1996). However, a key role of prefrontal cortex α 1-adrenergic receptors in memory reconsolidation is still unknown. Altogether, it is suggested that the facilitating role of yohimbine in consolidation and reconsolidation of a contextual fear memory trace engages a slightly different pattern of activation of adrenergic receptor subtypes, which possibly mirrors their encephalic location and respective densities.

Administering yohimbine during both consolidation and reconsolidation of a memory trace induces a ceiling level of freezing in the paired context and a generalization of this fear behavior to

an unpaired context. This latter effect seems to result from a maladaptive interference with fear memory processing induced by enhanced noradrenergic transmission. Of note, such a response mimics a feature of posttraumatic stress disorder (Jovanovic et al. 2009), which in turn has also been associated with excessive noradrenergic functioning (Pitman 1989; O'Donnell et al. 2004). Moreover, patients suffering from this anxiety disorder may present impairment in traumatic memory extinction (Cain et al. 2012). Interestingly, extinction impairments were also demonstrated in healthy humans after enhancing noradrenergic activity with yohimbine during aversive memory consolidation (Soeter and Kindt 2012), and in rats after activating β -adrenergic receptors in the amygdala during fear memory reconsolidation (Debiec et al. 2011).

Administering yohimbine after two consecutive sessions of fear memory trace retrieval/reactivation induces a cumulative increment in freezing expression in the paired context. This result supports the theory that an aversive memory constantly reprocessed under increased noradrenergic tone could be gradually potentiated in a persistent manner (Debiec 2012). Since fear generalization was not observed after double administration of yohimbine during memory reconsolidation, it could be argued that such a maladaptive response would result from interferences made exclusively on memory consolidation. Nevertheless, as fear generalization was achieved with a triple memory retrieval/reactivation protocol followed by yohimbine administration, this behavioral outcome is not associated with a qualitative difference between consolidation and reconsolidation processes. Rather, fear generalization seems to be a quantitative aspect of fear conditioning: when an asymptotic or ceiling level of freezing is obtained in the paired context, any further noradrenergic-mediated potentiation of either memory consolidation or reconsolidation resulted in an inability to restrict fear to the appropriate context. The quantitative nature of fear generalization achievement is supported by evidence from another study in which the administration of corticosterone induced cue generalization only when a higher level of freezing had already been reached in the paired context (Kaouane et al. 2012). It is unknown, however, whether potentiating a fear memory with drugs that do not interfere directly with signaling mechanisms implicated in stress and aversive memory processing also causes fear generalization.

In summary, enhancing noradrenergic activity allows a contextual fear memory trace to be consolidated and reconsolidated in a more intense and enduring manner, with substantial freezing expression in the paired context, and generalization of this response to the unpaired context as well. Importantly, these effects of yohimbine are time-specific, depend on memory trace acquisition and retrieval/reactivation, and rely differentially on the activation of both α 1- and β -adrenergic receptors in the brain. Although the limited efficacy of β -adrenergic receptor antagonists has put their clinical usefulness in check (Muravieva and Alberini 2010), our findings encourage further studies aimed at investigating the potential of drugs acting on α -adrenergic receptors, such as prazosin and clonidine, as possible pharmacological tools to attenuate/uncouple the negative valence associated with traumatic memories that can cause an inability to restrict fear to the appropriate context.

Materials and Methods

Animals

Experiments were performed in male Wistar rats (bred and raised by the animal house of the Federal University of Santa Catarina, Florianopolis, Brazil) weighing 300–350 g and aged 12–14 wk. The animals were housed in groups of four per cage (50 × 30 × 15 cm), kept on a 12-h light/dark cycle (lights on at 7:00 AM),

and received food and water ad libitum. All procedures were approved by the Institutional Ethical Committee for the Care and Use of Laboratory Animals of the Federal University of Santa Catarina (173/CEUA/PRPe/2011) in compliance with guidelines of the Brazilian Society of Neuroscience and Behavior and Brazilian legislation.

Drugs

Yohimbine hydrochloride (Tocris), clonidine hydrochloride (Sigma-Aldrich), propranolol hydrochloride (Sigma-Aldrich), nadolol (Sigma-Aldrich), and prazosin hydrochloride (Sigma-Aldrich) were dissolved in 0.9% NaCl, which alone served as the vehicle control. The yohimbine dose selection was based both on a pilot experiment, in which doses ranging from 0.5 to 2.5 mg/kg were tested, and on previously published studies showing its facilitating role in memory extinction and LTP induction (Mondaca et al. 2004; and Bouton 2007). The choice of propranolol, nadolol, and prazosin doses was based on pilot experiments or studies where these drugs prevented the behavioral effects of selective agonists for α 1- and β -adrenergic receptors (Do-Monte et al. 2010a,b). The clonidine range was selected based on studies showing its ability to reduce both the brain's noradrenaline efflux and the LTP in vivo (Abercrombie et al. 1988; Mondaca et al. 2004). All solutions were prepared immediately before use and injected intraperitoneally in a volume of 1.0 mL/kg.

Apparatus

Fear conditioning was performed in a rectangular chamber (35 × 20 × 30 cm), with aluminum sidewalls and a front wall and ceiling-door made of Plexiglas, which was designated herein as Context A. Its grid floor, made of stainless steel bars (3-mm diameter, spaced 9-mm apart center-to-center), was connected to a circuit board and a shock generator (Insight) to enable delivery of controlled electrical foot shocks as detailed in the section on general procedure. A second chamber (30 × 30 × 30 cm), designated herein as Context B, was made of glass and had a grid lid and transparent walls and floor to provide contextual cues as different as possible from those of foot shock paired with Context A, in order to allow the inference of a possible fear generalization in a neutral context. Context B was also used as a neutral context unable to induce fear memory reactivation.

General procedures and data collection

Behavioral testing was always carried out under low-intensity illumination (70 lux) during the diurnal phase (from 13:00 to 17:00 h). In all experiments, each animal was placed in Context A and allowed to freely explore it for 3 min, as an initial familiarization session, and returned to its home cage. On the next day, the animal was again placed in Context A for the weak training session, during which it received, after an initial 30-sec delay (pre-shock period), the unconditioned stimulus (a single electrical foot shock of 0.7 mA, 60 Hz, for 3 sec). The animal remained in this chamber for an additional 30 sec (post-shock period) before return to its home cage. In the reactivation session, the animal was reexposed to Context A for 3 min without presentation of the unconditioned stimulus, to induce the fear memory trace retrieval/reactivation. In Test A₁, the animal was reexposed to Context A for 3 min in the absence of unconditioned stimulus presentation, whereas in Test B₁ it was exposed to Context B (i.e., the neutral chamber; unpaired context), also for 3 min. All sessions were spaced 24-h apart. In some cases, each test session was repeated 7 d later, in order to evaluate memory persistence, and denominated Test A₂ and B₂, respectively. In Experiments 1 and 4A, the reactivation session was omitted because the objective was just to evaluate the fear memory consolidation. In Experiments 3B and 3C, however, additional reactivation sessions were added to evaluate the potentiating effect of yohimbine on the reconsolidation process, which took place two and three times, respectively. After each behavioral session, both chambers were cleaned with

a paper tissue soaked with 10% ethanol–water solution. The experimenter was unaware of the treatment condition in all studies.

Freezing behavior, a commonly used index of fear in rats (Fanselow 1980) and defined as a total absence of body and head movements, except those associated with breathing, was continuously recorded during the experimental sessions by a video camera. The freezing time in each period was quantified (in seconds) by a trained observer (inter- and intra-rater reliabilities $\geq 90\%$) blind to the experimental groups using a stopwatch and expressed as the percentage of total session time.

Statistical analysis

Results are expressed as mean $\pm$ S.E.M. After ensuring the assumptions of normality and homogeneity of variance, the percentages of freezing time observed in Context A (reactivation session, Test A₁ and/or Test A₂) and Context B (no reactivation session, Test B₁ and/or Test B₂) were submitted to separated one-way, factorial, or repeated-measures analysis of variance (ANOVA). Following significant ANOVA results, the Newman–Keuls test was used for post-hoc comparisons. In cases where no repetition in the same manuscript was done, unpaired two samples Student's *t*-test was adopted. The statistical significance level was set at $P < 0.05$.

Acknowledgments

This work was supported by Brazilian grants from Fundação de Amparo à Pesquisa e Inovação do Estado de Santa Catarina, Fundação de Amparo à Pesquisa do Estado de São Paulo, and Conselho Nacional de Desenvolvimento Científico e Tecnológico. We thank F.H.M. do Monte for constructive comments on the manuscript, and J.E. da Silva Santos and P. de Souza for kindly donating the clonidine.

Author contributions: All authors designed research; L.G. and C.A.J.S. performed research; L.G. and L.J.B. analyzed data; and all authors participated in the discussion and writing of the manuscript.

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Received December 12, 2012; accepted in revised form January 18, 2013.

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Learn. Mem. 2013, **20**:

Access the most recent version at doi:[10.1101/lm.030007.112](https://doi.org/10.1101/lm.030007.112)

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