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Enhancing a declarative memory in humans: The effect of clonazepam on reconsolidation☆

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ABSTRACT

A consolidated memory recalled by a specific reminder can become unstable (labile) and susceptible to facilitation or impairment for a discrete period of time. This labilization phase is followed by a process of stabilization called reconsolidation. The phenomenon has been shown in diverse types of memory, and different pharmacological agents have been used to disclose its presence. Several studies have revealed the relevance of the GABAergic system to this process. Consequently, our hypothesis is that the system is involved in the reconsolidation of declarative memory in humans. Thus, using our verbal learning task, we analyzed the effect of benzodiazepines on the re-stabilization of the declarative memory. On Day 1, volunteers learned an association between five cue- response-syllables. On Day 2, the verbal memory was labilized by a reminder presentation, and then a placebo capsule or 0.25 mg or 0.03 mg of clonazepam was administered to the subjects. The verbal memory was evaluated on Day 3. The volunteers who had received the 0.25 mg clonazepam along with the specific reminder on Day 2, exhibited memory improvement. In contrast, there was no effect when the drug was given without retrieval, when the memory was simply retrieved instead of being reactivated or when short-term memory testing was performed 4 h after reactivation. We discuss the GABAergic role in reconsolidation, which shows a collateral effect on other memories when the treatment is aimed at treating anxiety disorders. Further studies might elucidate the role of GABA in the reconsolidation process associated with dissimilar scenarios.

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1. Introduction

The consolidation theory establishes that memories are labile during a time window after acquisition, but as time progresses, memories become stable and resistant to amnesic agents. Several studies using behavioral, pharmacological and molecular approaches in diverse species, from nematodes to humans, have shown that consolidation is an evolutionarily conserved process that initially requires RNA and protein synthesis (Bailey et al., 1996; Davis and Squire, 1984; Dudai, 2002; Kandel, 2001; McGaugh, 2000; Squire and Alvarez, 1995). However, the notion of immutable memories after consolidation has been challenged. Since the pioneer study of Misanin et al. (1968), a growing number of reports have shown that old memories become labile and again become susceptible to amnesic agents after a specific reminder is presented. Such susceptibility decreases over time and leads to a re-stabilization phase, usually referred to as reconsolidation.

In humans, reconsolidation has been reported in a procedural motor-skill task (Walker et al., 2003), Pavlovian fear conditioning (Kindt et al., 2009; Schiller et al., 2010) and in a verbal learning task (Forcato et al., 2007; Hupbach et al., 2007). Previously, our group has not only reported that declarative human memories undergo reconsolidation (Forcato et al., 2007), but we have also described boundary conditions necessary to trigger labilization (Forcato et al., 2011, 2010, 2009). Our paradigm consists of learning a verbal material (lists of five pairs of nonsense syllables) acquired by a training process (L1-training) on Day 1. After this declarative memory is consolidated, it can be labilized when a specific

☆ In memoriam of my science mentor Dr Héctor Maldonado.

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[1] We regret to announce that our colleague and friend, Prof Maldonado, passed away during the publication of this study.

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reminder is presented. Then, this memory passes through a stabilization process. To reveal the presence of this process, the method selected was a second learning process (L2-training), which interfered with the re-stabilization phase. The time window for the interference was determined by demonstrating that at 6 h after labilization, the memory was still sensitive to an amnesic agent. Conversely, 10 h after reactivation, the memory was impervious to the interfering agent (Forcato et al., 2007). Furthermore, the labilization–reconsolidation was only triggered under certain circumstances. When the reminder was formed by the context cues and one cue syllable, without giving the subjects the opportunity to write down the response syllable (cue-reminder), the labilization–reconsolidation was triggered. In contrast, when the subjects had the possibility to write down the response syllable (cue-response reminder), the memory was evoked but not labilized. Thus, as in other paradigms, the presence of a mismatch and the discrepancy between expected and current events in the reminder determine the occurrence or absence of reconsolidation (Lee, 2009; Pedreira et al., 2004).

Furthermore, it is well known that Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in both the central nervous system (CNS) and the peripheral nervous system (Erdo et al., 1986). A considerable amount of evidence from different studies, using a variety of paradigms and tasks, supports a role for the GABAA receptor in diverse behavioral outcomes (Chapouthier and Venault, 2002; Paredes and Agmo, 1992). A large body of evidence from studies of human memory indicates that the use of benzodiazepines produces anterograde amnesia (Brown et al., 1982; Curran, 1991; Uzun et al., 2010; Venault et al., 1986;). In addition, there is general consensus that benzodiazepines do not produce retrograde amnesia (Ghoneim and Mewaldt, 1975; McNamara and Skelton, 1991; Savic et al., 2005). Moreover, in humans, retrograde memory-enhancing effects have been found (Hinrichs et al., 1984), and declarative memory retrieval has been improved by low doses of benzodiazepines (Delgado et al., 2005; File et al., 1999; Fillmore et al., 2001).

Regarding the role of GABA in the reconsolidation phase, the results obtained with different paradigms and animal models reveal the relevance of the GABAergic system to this process (Bustos et al., 2009, 2006; Carbo Tano et al., 2009; Zhang and Cranney, 2008). Thus, in mammals, previous researchers have demonstrated an amnesic effect following midazolam (MDZ) administration during the labilization–reconsolidation process of a contextual fear conditioning paradigm in rats (Bustos et al., 2006). These results support the view that stimulating $GABA_A$ receptor sites via this short-acting benzodiazepine selectively disrupts the reconsolidation process of a contextual fear memory. In line with these results and using the same paradigm, Zhang and Cranney (2008) revealed a reconsolidation impairment induced by the systemic administration of midazolam immediately after reactivation. Additionally, in this case, the effect of the drug did not differ between high- and low-anxiety rats. Interestingly, based on the well-known pharmacological actions of ethanol as a positive modulator of $GABA_A$ receptors (Lister, 1987; Weiss and Porrino, 2002), a recent study showed that ethanol, administered after the reactivation of a contextual fear memory, enhanced the performance of treated animals at testing (Nomura and Matsuki, 2008). Moreover, this effect appeared to be mediated by the GABAergic system because the administration of picrotoxin, a $GABA_A$ receptor antagonist, inhibited the memory enhancement produced by ethanol. Thus, this study supports the hypothesis that ethanol enhances contextual fear memories following labilization via the activation of $GABA_A$ receptors.

Therefore, it can be strongly argued that GABA transmission is implicated in memory reconsolidation. In this framework, we hypothesized that the GABAergic system is involved in the reconsolidation of a declarative memory in humans.

Taking into consideration that benzodiazepines, widely used for the treatment of anxiety disorders, increase the activity of $GABA_A$ receptors, we determine the role of the GABAergic system by analyzing the effect of benzodiazepines on the re-stabilization of declarative memory.

To accomplish the main goal of this study, we selected clonazepam, a long-acting benzodiazepine (half-life 20–50 h), which has a fast onset, high effectiveness, low toxicity and does not produce adverse reactions. The selected doses were low enough to avoid undesirable effects, such as excessive sedation during the course of the experiment.

Taking into account the absence of an emotive charge in our paradigm and the doses selected, we wondered if this type of modulation during re-stabilization would induce a memory enhancement, as has been shown in other reports (Nomura and Matsuki, 2008).

Thus, in this study, the volunteers learned an association between five cue-syllables and five respective response-syllables. Twenty-four hours later, the paired associated verbal memory was labilized by exposing the subjects to the cue-reminder and by administering a capsule of 0.25 mg or 0.03 mg clonazepam (CLZ) or a placebo (PLC). On Day 3, the list-memory was evaluated by presenting the 5 cue-syllables twice and allowing the subjects to respond with the response syllables. Memory improvement was observed when the volunteers received 0.25 mg of CLZ in conjunction with the specific reminder on Day 2. In contrast, there was no effect when the drug was administered without retrieval, when the memory was simply retrieved instead of being reactivated or when short-term memory testing was performed 4 h after reactivation.

We have therefore demonstrated, for the first time, that the daily dose of benzodiazepine prescribed to treat anxiety (0.25 mg of CLZ) enhances a reactivated declarative memory in humans. These results strongly suggest that the positive modulation of GABAsites was the factor that changed the strength of the previous consolidated memory.

Our results add new evidence regarding the role of the GABAergic system on mnemonic processes, showing that the effects critically depend on the characteristics and parametric conditions of the paradigm and agents used.

2. Materials and methods

2.1. Subjects

Two hundred and four undergraduate and graduate students volunteered for the study. To evaluate clonazepam's strengthening effect on declarative memory reconsolidation, only the subjects that achieved at least 45% of correct responses during the last four trials of the training session (9/20 correct responses) were included. Additionally, subjects were excluded for any of the following reasons: those who drank alcohol during the period of the experiment, those who wrote the syllables down, those who slept during the daytime after the reminder and drug administration, and/or those who missed some step in the protocol of the experiment.

The final sample was composed of 104 volunteers, 42 (40%) men and 62 (60%) women, with ages ranging between 20 and 35 and with a mean of 23 years old.

Before their participation in the experiment, subjects signed a written informed consent form approved by the Ethics Committee of the Fundación para la Lucha contra las Enfermedades Neurológicas de la Infancia (FLENI).

2.2. Procedure

The experiments were conducted in a dark room using a personal computer. Each subject was provided with earphones and seated facing a monitor placed in front of a large screen on the wall.

The subjects were required to learn a list of five pairs of nonsense syllables presented on the monitor screen. The List was associated with a specific context (light projected on the large screen, an image on the monitor screen; and sound coming through the earphones). The selection of this enriched context was based on previous reports. That is, it was demonstrated by Forcato et al. (2007) that the presentation of contextual cues enhanced performance during the testing session.

Each experiment consisted of a training session (Day 1), a treatment session (Day 2) and a testing session (Day 2 or 3). As in previous research, the experimental room and the contextual cues of the enriched environment associated with the list were identical for the three sessions.

2.2.1. The training session

The training session consisted of the presentation of 10 trials separated by a 4-s intertrial interval. In the first trial, the List was shown, and in the successive trials the subjects were required to write down the corresponding response-syllable for each cue-syllable presented. The List was composed of five pairs of nonsense cue-response-syllables in *rioplatense* Spanish: **ITE**-OBN, **ASP**-UOD, **FLI**-AIO, **NEB**-FOT, **COS**-GLE (bold type: cue-syllable; regular type: response-syllable) (Fig. 1A).

Each training trial consisted of a **context period** (red lighting, a picture of a forest and classical music) followed by the presentation of the syllables (Fig. 1B). The context period was composed of a fixed sequence of three cumulative steps: first, light alone was projected on the large screen for 2 s; second, an image was displayed on the monitor's screen along with the light for 2 s; and third, sound coming from the earphones was added to the image plus the light for 4 s. The context persisted during the entire trial. The **syllable period** started with the presentation of a cue-syllable on the left side of the monitor screen and an empty response-box on the right. Each cue-syllable was taken at random from a list of five pairs. Subjects were given 5 s to write down the corresponding response-syllable. Once that period was finished, three situations were possible: first, if no syllable was written, the correct one was shown for 4 s; second, if an incorrect syllable was written, it was replaced by the correct one, which was shown for 4 s; and third, if the correct response was given, it remained on the screen for an additional 4 s. Immediately after that, another cue-syllable was shown, and the process was repeated until the list was over. Altogether, the trial lasted 53 s (8 s for the context period and 45 s for the syllable presentation). The training session lasted 10 min.

Before the training session, the program consisted of 4 trials, similar to structure of those in the training but with another context and two different pairs of nonsense-syllables.

2.2.2. The treatment session

During the treatment session, a reminder of the training session was presented to the subjects. Different types of reminders were presented.

2.2.2.1. Types of reminder

2.2.2.1.1. Cue-reminder (Rc). This trial included the context of the list. Immediately after the presentation of the context period, as expected, a cue-syllable appeared on the left side of the monitor and the response-box on the right. However, 2 s later a notice displayed on the monitor announced that the session had to be suspended, thus not allowing any of the subjects to write down the response-syllable (Fig. 1C top diagram).

2.2.2.1.2. Cue-response-reminder (Rcr). This trial included the context of the list and immediately after the context period, a cue-syllable appeared and the subjects were allowed to answer with the respective response-syllable. After that, a notice displayed on the monitor announced that the session had to be suspended. It was previously demonstrated that this type of reminder does not trigger memory labilization–reconsolidation (Forcato et al., 2009) (Fig. 1C bottom diagram).

Additionally, a capsule of PLC or 0.25 mg or 0.03 mg CLZ was administered to the volunteers. **RIVOTRIL**® clonazepam capsules contain 0.25 mg or 0.03 mg of clonazepam and the following excipients: lactose, maize starch, potato starch, talc, magnesium stearate, iron oxide red and iron oxide yellow. The drug was processed and fractionated specifically for this experiment by Fleni's pharmacy.

2.2.3. Testing session

The testing session consisted of a testing trial to evaluate the retention of the syllables. We decided to examine the performance at testing according to three categories: correct response, incorrect response and no response.

Response categories:

Correct response: the response-syllable corresponded to the cue-syllable.

Incorrect response: the response-syllable was incomplete (i.e., 2 letters instead of 3), the 3-letter syllable was not the correct one, but it belonged to the List or the response-syllable was not included in the List.

No response: no response-syllable was written down.

2.3. Experimental groups

2.3.1. Experiment 1

Rc-PLC group ($n = 11$). Day 1: Subjects received the training session – Day 2: The Cue-reminder was presented and a **placebo** capsule was administered – Day 3: Subjects were tested.

Rc-CLZ 0.03 group ($n = 10$). Day 1: Subjects received the training session – Day 2: The Cue-reminder was presented and a **0.03 mg clonazepam** capsule was administered – Day 3: Subjects were tested.

Rc-CLZ 0.25 group ($n = 11$). Day 1: Subjects received the training session – Day 2: The Cue-reminder was presented and a **0.25 mg clonazepam** capsule was administered – Day 3: Subjects were tested.

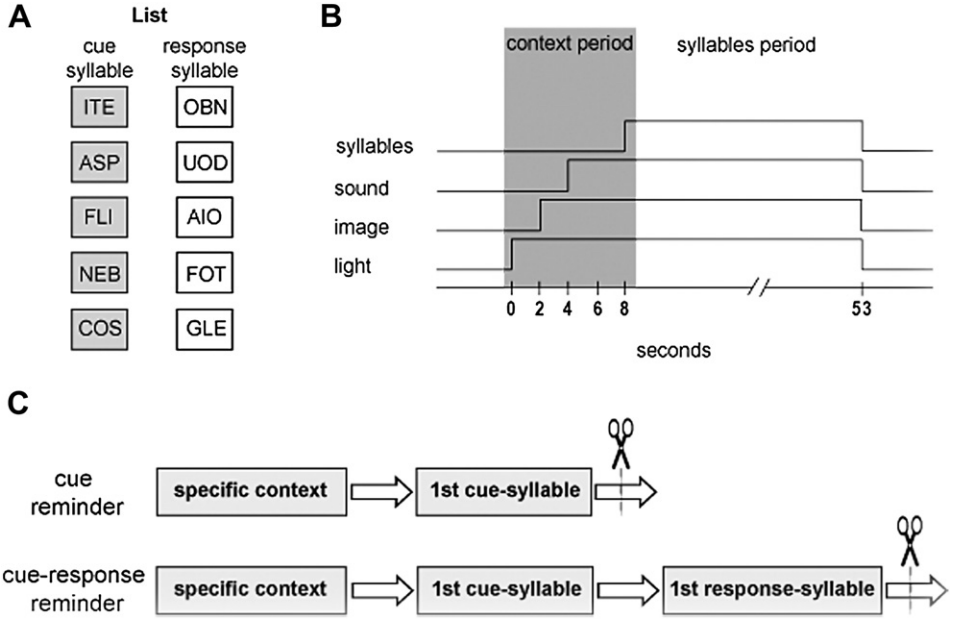

Fig. 1. Experimental Protocol. A) Paired-associated memory. The List presented in the training and testing sessions. B) Training trial. The context period was formed by the specific combination of a light (color illumination of the room), an image (a picture on the monitor) and sound (music melody from earphones), and by a syllable period: eight seconds after the stimuli presentation, five pairs of cue-response syllables were presented successively and in a random order. C) Types of reminders. (*Top diagram*) The cue reminder (Rc) included the specific context, then, a one cue-syllable was presented, after which the trial was abruptly interrupted, not allowing the subject to answer with the respective response-syllable. (*Bottom diagram*) The cue-response reminder (Rcr) included the specific context, and then, a one cue-syllable was presented and subjects were allowed to write down the first response-syllable. Next, the trial was interrupted. Scissors stand for the full-stop of each type of reminder.

2.3.2. Experiment 2

NR-PLC group ($n = 10$). Day 1: Subjects received the training session – Day 2: No reminder was presented and a placebo capsule was administered – Day 3: Subjects were tested.

CT group ($n = 10$). Day 1: Subjects received the training session – Day 2: Subjects did not attend the clinic – Day 3: Subjects were tested.

2.3.3. Experiment 3

NR-CLZ 0.25 group ($n = 11$). Day 1: Subjects received the training session – Day 2: No reminder was presented and a 0.25 mg clonazepam capsule was administered – Day 3: Subjects were tested.

Rcr-CLZ 0.25 group ($n = 11$). Day 1: Subjects received the training session – Day 2: The Cue-response reminder was presented and a 0.25 mg clonazepam capsule was administered – Day 3: Subjects were tested.

Rc-CLZ 0.25 group ($n = 11$). Day 1: Subjects received the training session – Day 2: The Cue-reminder was presented and a placebo capsule was administered – Day 3: Subjects were tested.

2.3.4. Experiment 4

Rc-PLC SHORT TERM ($n = 10$). Day 1: Subjects received the training session – Day 2: The Cue-reminder was presented and a placebo capsule was administered – 4 h later memory was tested.

Rc-CLZ 0.25 SHORT TERM ($n = 10$). Day 1: Subjects received the training session – Day 2: The Cue-reminder was presented and a 0.25 mg clonazepam capsule was administered – 4 h later memory was tested.

2.4. Statistics

2.4.1. Training session

The rate of correct responses per training-trial was reported and analyzed with a repeated measures one-way analysis of variance (ANOVA). Additionally, data from the mean percentage of correct responses at the last four trials were analyzed with ANOVA.

2.4.2. Testing session

The results were reported as the mean number of correct responses, incorrect responses and no responses. The data of each type of response were first analyzed with one-way analysis of variance (ANOVA), followed by post-hoc comparisons (FISHER, $\alpha = 0.05$).

3. Results

3.1. A therapeutic dose of clonazepam affects the reconsolidation of a declarative memory

To evaluate the effect of two different doses of CLZ on memory reconsolidation, a three-day experiment with three groups was performed (Fig. 2A). On Day 1, subjects learned a list of five pairs of cue-response syllables (training session). On Day 2, they received a treatment session. This session began for all groups with the presentation of a cue-reminder (Rc). The cue-reminder was formed by the specific context plus one cue-syllable, without enough time to write down the response-syllable. This type of reminder triggers the labilization–reconsolidation process (Forcato et al., 2009). Immediately after the interruption of the session, the volunteers received the capsule designated for each group. Thus, we administered PLC to the cue-reminder placebo group (Rc-PLC), 0.03 mg of CLZ to the cue-reminder CLZ 0.03 mg group (Rc-CLZ 0.03) and 0.25 mg of CLZ to the cue-reminder CLZ 0.25 mg group (Rc-CLZ 0.25). Finally, all the subjects underwent the testing session on Day 3.

3.1.1. Clonazepam improves performance on Day 3

Uniformity between groups at training was revealed by an ANOVA of repeated measures (Fig. 2B. $F(2.28) = 0.91$, $p = 0.415$) as well as no group trial interaction ($F(16.224) = 0.978$, $p = 0.481$), reaching the same average percentage of correct responses during the last four training trials (Fig. 2B inset, $F(2.28) = 0.393$, $p = 0.679$).

The performance on Day 3 of each group was estimated by analyzing the three categories of responses made at testing: correct-response, incorrect-response and no-response. The subjects that received 0.25 mg of CLZ on Day 2 exhibited better performance than those receiving PLC or 0.03 mg of CLZ (Fig. 2C). Specifically, the Rc-CLZ 0.25 group wrote down more correct response syllables than the Rc-PLC group during the testing trial (one-way ANOVA $F(2.28) = 5.454$, $p = 0.010$; LSD post-hoc comparison $p = 0.004$). Furthermore, the Rc-CLZ 0.03 group presented the same level of correct responses as the Rc-PLC group (LSD post-hoc comparison $p = 0.525$); the comparison between the 2-doses-of-CLZ-treatment groups revealed that the Rc-CLZ 0.25 group wrote down a significantly higher number of correct responses than the Rc-CLZ 0.03 group (LSD post-hoc comparison $p = 0.0206$). Notably, the increase in correct responses was due to a decrease in incorrect responses (one-way ANOVA $F(2.28) = 4.668$, $p = 0.018$, LSD post-hoc comparison $p = 0.019$ between the Rc-PLC and Rc-CLZ 0.25 groups, $p = 0.009$ between the Rc-CLZ 0.25 and Rc-CLZ 0.03 groups and $p = 0.708$ between the Rc-PLC and Rc-CLZ 0.03 groups) rather than a decrease in no responses (one-way ANOVA $F(2.28) = 1.107$, $p = 0.344$). This first result strongly suggests that 0.25 mg of CLZ administered after reactivation improved the performance at testing of a consolidated declarative memory.

Visiting the hospital for the experiment and receiving a capsule could be a stressful situation which, in turn, might modify the performance of the subjects at testing. Therefore, to evaluate the existence of those unspecific effects, we performed an additional experiment that included two groups: a no-reminder placebo group (NR-PLC group) and a control group (CT group). For both groups, the training and testing sessions were identical to those used in the first experiment. However, the CT group did not undergo a treatment session; namely, the volunteers did not visit the hospital on Day 2. Instead, in the NR-PLC group, the volunteers visited the hospital and received the placebo capsule. The performance of both groups was comparable during training (Fig. 3B, ANOVA of repeated measures $F(1.18) = 3.897$, $p = 0.064$, group trial interaction $F(8.1344) = 0.887$, $p = 0.529$, Fig. 3B inset, one-way ANOVA for the last four training trials $F(1.18) = 1.938$, $p = 0.181$). Both groups showed the same level of correct responses at testing (Fig. 3C, $F(1.18) = 0.243$, $p = 0.628$). Moreover, the incorrect-response and no-response categories remained equivalent for both groups (one-way ANOVA $F(1.18) = 1.296 = 0.270$ and $F(1.18) = 1.328$, $p = 0.264$, respectively). Thus, visiting the hospital for the experiment and receiving a capsule did not modify the level of correct responses written down by the volunteers at testing.

Taken together, these results support the view that the improved performance at testing, observed in experiment 1, was due to the administration of a therapeutic dose of CLZ (0.25 mg). This effect was not present when administering a placebo or a lower dose of clonazepam, and visiting the hospital and/or taking a capsule had no effect on performance at testing.

3.2. The improving effect of clonazepam depends on the labilization of a declarative memory

It could be speculated that the results obtained might also be observed under two different conditions: first, when the drug is given without retrieval, and second, when the memory is simply retrieved instead of being reactivated. The standard protocol for demonstrating that the drug effect depends on the labilization process is to compare a group exposed to the reminder with another that was not. However, our model offers different reminders (Forcato et al., 2009) to distinguish between pure retrieval (the memory is simply evoked) or retrieval plus labilization (the memory is evoked and reactivated). Indeed, we have shown that the omission of one of its parametrical conditions (Pedreira et al., 2004) – such as the mismatching component in the reminder of a cue-response group, Rcr – retrieves the memory but prevents the labilization of the target memory (Forcato et al., 2009).

A

EXPERIMENT-1	DAY 1	DAY 2	DAY 3
Rc-PLC Group	TR	Rc-placebo capsule	TS
Rc-CLZ 0.03 Group	TR	Rc-clonazepam 0.03 capsule	TS
Rc-CLZ 0.25 Group	TR	Rc-clonazepam 0.25 capsule	TS

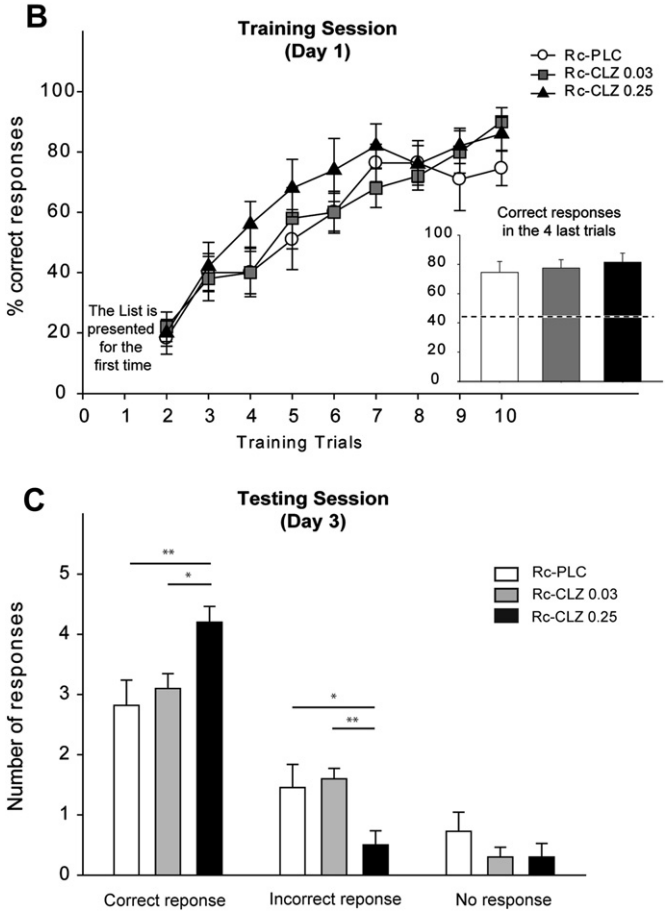

Fig. 2. Experiment 1. Clonazepam improves performance at testing. A) Experimental protocol. TR stands for training session, Rc for the cue reminder, TS for testing session, PLC for placebo and CLZ for clonazepam. Groups differ in the pill received on Day 2. The first group received a cue-reminder presentation and a placebo pill (Rc-PLC group), the second group received a cue-reminder presentation and a 0.03 mg clonazepam pill (Rc-CLZ 0.03 group), and the last group received a cue-reminder presentation and a 0.25 mg clonazepam pill (Rc-CLZ 0.25 group) B) Learning curves. Percentage of correct responses per trial on Day 1. For the first trial, the list is presented for the first time. Inset. Inclusion criteria. Mean percentage of correct responses in the four last training trials. The white bar stands for Rc-Plc group ($n = 11$), the gray bar for the Rc-CLZ 0.03 group ($n = 10$), the black bar for the Rc-CLZ 0.25 group ($n = 10$) and the dotted line indicates 45% correct responses, the inclusion criteria. C) Testing session. Number of correct responses, incorrect responses and no response +/− SEM on Day 3. $^{*}p < 0.05$; $^{**}p < 0.01$.

Thus, to evaluate the strengthening effect of CLZ on the target memory, we performed a three-day experiment with three groups (Fig. 4A). On Day 1, all groups learned the list of syllable-pairs (List). For the treatment session (Day 2), the no-reminder group (NR-CLZ) received a CLZ capsule of only 0.25 mg, the cue-response-reminder group (Rcr CLZ) was exposed to the cue-response reminder and immediately after that a 0.25 mg CLZ capsule was administered, and the cue-reminder group (Rc-CLZ), similar to the group in the previous experiment, received the cue reminder and the 0.25 mg CLZ capsule. Finally, all the subjects were tested on Day 3.

3.2.1. The administration of clonazepam without reactivation or its administration after retrieval on Day 2 does not enhance performance on Day 3

The NR-CLZ group, the Rcr-CLZ group and the Rc-CLZ group performed similarly in the training sessions (Fig. 4B, $F(2.30) = 0.274$, $p = 0.762$ interaction $F(16.24) = 1.073$, $p = 0.381$ Inset: $F(2.30) = 0.235$, $p = 0.792$). However, volunteers that received the cue-reminder plus the drug showed a higher number of correct responses than the Rcr-CLZ and NR-CLZ groups on Day 3. Specifically, significant differences were revealed at testing for the Rc-CLZ group (Fig. 4C, one-way ANOVA $F(2.30) = 3.363$, $p = 0.048$; LSD post-hoc comparison $p = 0.041$ and $p = 0.026$, respectively). In contrast, the subjects that received 0.25 mg of CLZ alone or a cue-response reminder plus the drug made a similar number of correct responses during the testing trial on Day 3 (LSD post-hoc Comparison $p = 0.832$). As seen in the above experiments, the improvement observed for the Rc-CLZ group was due to a decrease in incorrect responses (one-way ANOVA $F(2.30) = 3.363$, $p = 0.048$ LSD post-hoc comparison $p = 0.026$ between NR-CLZ and Rc-CLZ groups, $p = 0.041$ between Rc-CLZ and Rcr-CLZ groups and $p = 0.833$ between NR-CLZ and Rcr-CLZ groups). All groups maintained the same level of no responses (one-way ANOVA $F(2.30) = 0.897$, $p = 0.418$).

A

EXPERIMENT-2	DAY 1	DAY 2	DAY 3
NR-PLC Group	TR	placebo capsule	TS
CT Group	TR	----------	TS

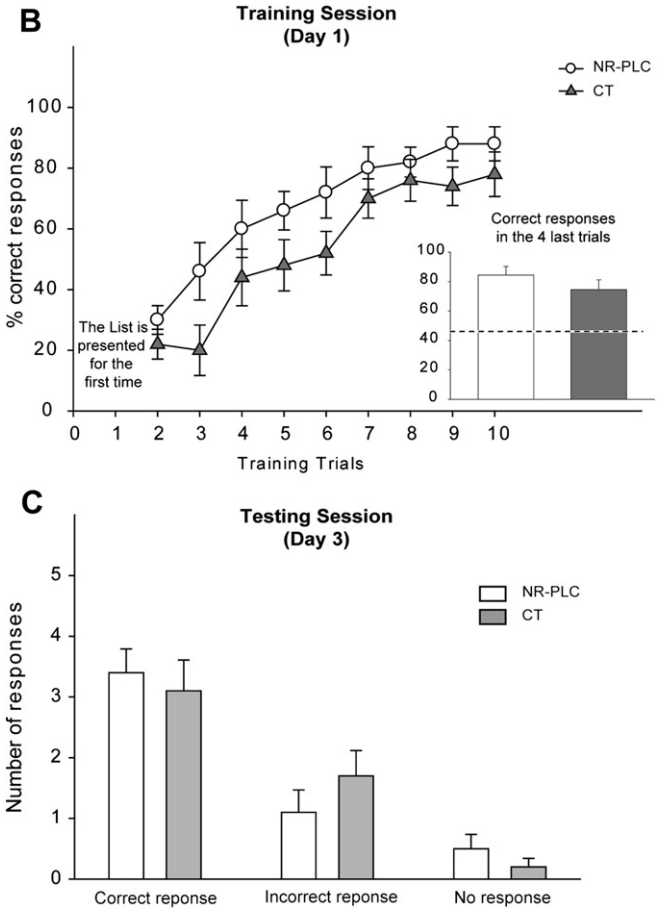

Fig. 3. Experiment 2. No effects are observed due to hospital attendance and administration of a placebo capsule. A) Experimental protocol. TR stands for training session, NR for no reminder, PLC for placebo, CT for control and TS for testing session. Groups differ in the treatment received on Day 2. The first group received a placebo capsule without any reminder presentation (NR-PLC group), and the second group did not receive a treatment session (CT group). B) Learning curves. Percentage of correct responses per trial on Day 1. For the first trial, the List is presented for the first time. Inset. Inclusion criteria. Mean percentage of correct responses in the four last training trials. The white bar stands for NR-PLC group ($n = 10$), the gray bar for the CT group ($n = 10$) and the dotted line indicates 45% correct responses, the inclusion criteria. C) Testing session. The number of correct responses, incorrect responses and no response +/− SEM on Day 3.

Therefore, in the absence of retrieval or as a consequence of only retrieval on Day 2, CLZ did not improve memory retention. The improving effect depended on the presentation of the reminder that induced the destabilization of the declarative memory, this being the only situation in which 0.25 mg of CLZ affected memory re-stabilization. Considering the pharmacokinetics of CLZ, the drug could be expected to not have been completely cleared at testing. However, the drug effect was only evident when it was administered close to the reactivation session. Thus, it is plausible to discard specific retrieval improvement (Rcr-CLZ) or an unspecific effect on memory retrieval (NR-CLZ) during testing.

3.3. *Memory facilitation by clonazepam is not expressed before reconsolidation takes place*

Evidence for reconsolidation has been accumulated rapidly in the last decade. At this point, it is clear that two different steps are included in the so-called reconsolidation. The first step is reactivation, which requires a destabilization of the consolidated memory, and then a process of re-stabilization occurs, which returns the memory to a stable state. To conclude that memory reconsolidation effects are at play, it is necessary to show that manipulation post-reactivation is not effective shortly after the treatment but unfolds over time, after the memory has become stable again (Nader et al., 2001). Thus, to estimate the strengthening effect of CLZ on the target memory, the testing session was performed four hours after the treatment session. In previous research, we demonstrated that the time window for re-stabilization in this paradigm emerged between 6 and 10 h after reactivation (Forcato et al., 2007). Consequently, in this experiment, the testing session was performed when the memory was still unstable and when the second step of the reconsolidation, that is, the re-stabilization, was taking place. Hence, we carried out a two-day experiment which involved two groups (Fig. 5A). On Day 1, subjects learned a list of syllable-pairs (List). On Day 2, they received a treatment session. The cue-reminder short-term 0.25 mg CLZ group (Rc-CLZ-ST) was exposed to the cue-reminder and given a CLZ capsule, and the cue-reminder short-term

A

EXPERIMENT-3	DAY 1	DAY 2	DAY 3
NR-Clz 0.25 Group	TR	clonazepam capsule	TS
Rcr-Clz 0.25 Group	TR	Rcr-clonazepam capsule	TS
Rc-Clz 0.25 Group	TR	Rc-clonazepam capsule	TS

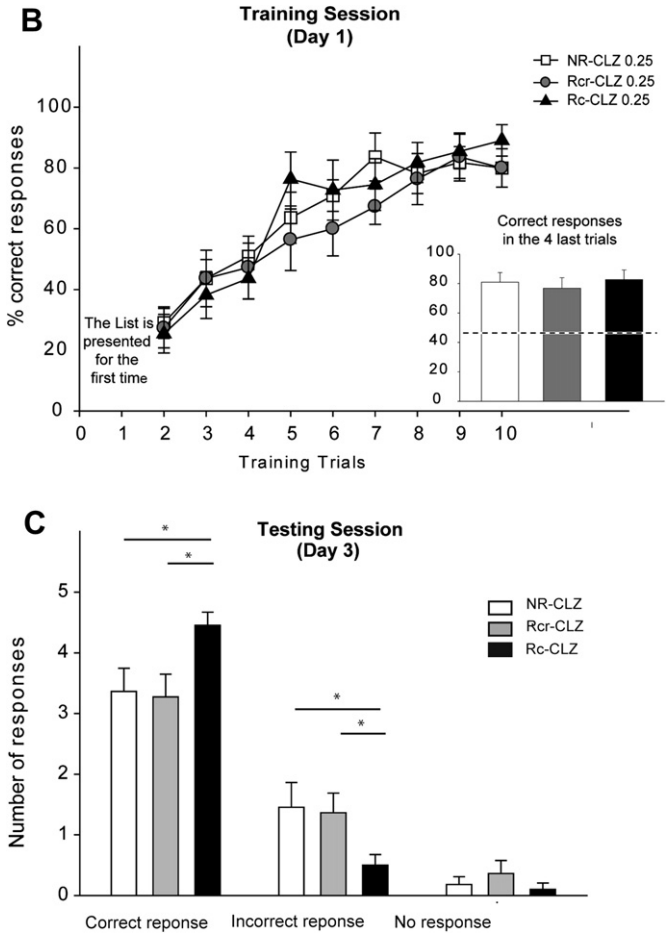

Fig. 4. Experiment 3. The clonazepam improvement effect depends on the labilization of the declarative memory. A) Experimental protocol. A three-day experiment. TR stands for training session, Rc for the cue reminder, Rcr for the cue response reminder, NR for no reminder, TS for testing session and CLZ for clonazepam. Groups differ in the reminder received on Day 2. The first group received a 0.25 mg clonazepam capsule without any reminder presentation (NR-CLZ 0.25 group), the second group received a cue response-reminder presentation and a 0.25 mg clonazepam capsule (Rcr-CLZ 0.25 group) and the last group received a cue-reminder presentation and a 0.25 mg clonazepam capsule (Rc-CLZ 0.25 group). B) Learning curves. Percentage of correct responses per trial on Day 1. For the first trial, the List is presented for the first time. Inset. Inclusion criteria. Mean percentage of correct responses in the four last training trials. The white bar stands for NR-CLZ 0.25 group ($n = 11$), the gray bar for the Rcr-CLZ 0.25 group ($n = 11$), the black bar for the Rc-CLZ 0.25 group ($n = 11$) and the dotted line indicates 45% correct responses, the inclusion criteria. C) Testing session. Number of correct responses, incorrect responses and no response +/− SEM on Day 3. $^{*}p < 0.05$.

placebo group (Rc-PLC-ST) received the cue-reminder and was administered the placebo. Both groups were tested four hours after the presentation of the reminder.

3.3.1. Clonazepam does not enhance performance when it is evaluated 4 h after the reminder presentation

As in previous experiments, a similar rate of correct responses given by all groups during training was revealed by an ANOVA of repeated measures (Fig. 5B. $F(1.18) = 0.043$, $p = 0.837$), and no group trial interaction was found ($F(8.144) = 1.349$, $p = 0.224$). Moreover, no significant differences were disclosed between groups for correct responses for the last four training trials (Fig. 5B inset, $F(1.18) = 0.000$, $p = 1.000$).

The subjects that received the PLC or the CLZ wrote down a similar number of correct responses in the testing trial that was carried out four hours after reactivation. In particular, no significant differences were revealed at testing between the three response categories (Fig. 5C, one-way ANOVA $F(1.18) = 0.444$, $p = 0.513$ for correct response, $F(1.18) = 0.000$, $p = 1.000$ for incorrect response and $F(1.18) = 1.532$, $p = 0.232$ for no response). Therefore, as demonstrated in other paradigms and animal models, when the effect of the treatment was evaluated shortly after reactivation, the memory remained labile, but it could be expressed during this short-term testing. Here, the treatment with CLZ immediately after the cue-reminder, which triggered the labilization process on Day 2, did not improve the performance of the destabilized memory when tested 4 h after the reminder. In agreement with the general observation based on the results obtained with diverse tasks and species (Lee et al., 2006), the effect of the treatment depends on the completion of the second phase, that is, the re-stabilization process

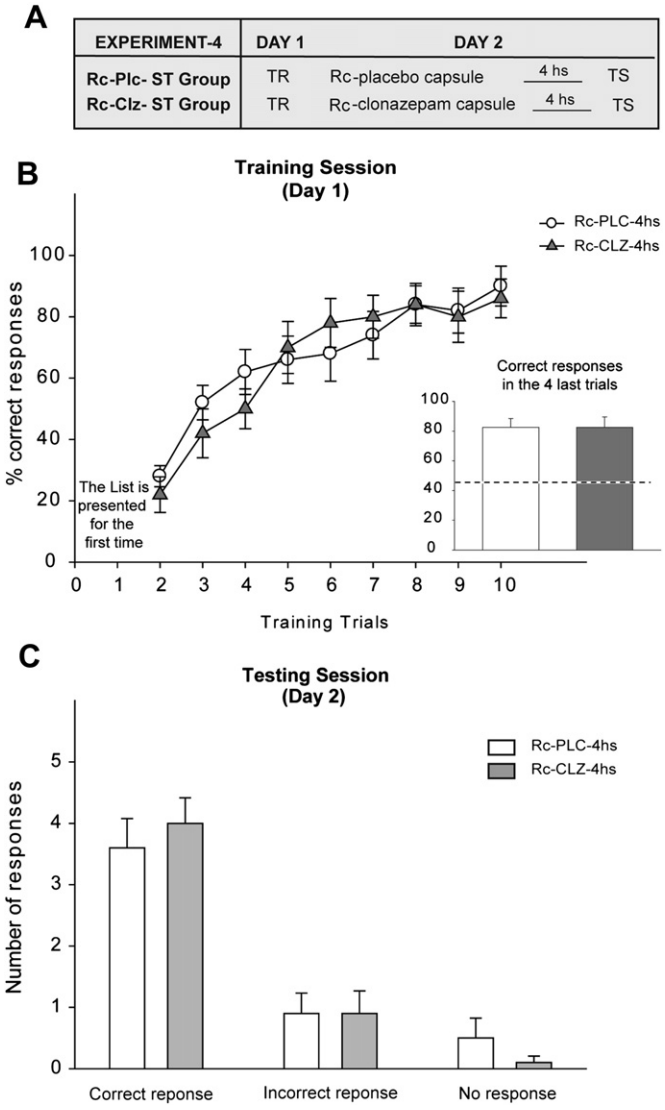

Fig. 5. Experiment 4. Memory facilitation by clonazepam is not expressed before reconsolidation takes place. A) Experimental protocol. A two-day experiment. TR stands for the training session, Rc for the cue reminder, TS for the testing session, ST for the short term testing session (4 h after reminder presentation), PLC for placebo and CLZ for clonazepam (0.25 mg). Groups differ in the pill received on Day 2. The first group received a cue-reminder presentation and a placebo pill (Rc-PLC-ST group), and the second group received a cue-reminder presentation and a 0.25 mg clonazepam pill (Rc-CLZ-ST group). B) Learning curves. Percentage of correct responses per trial on Day 1. For the first trial, the List is presented for the first time. Inset. Inclusion criteria. Mean percentage of correct responses in the four last training trials. The white bar stands for Rc-PLC-ST group ($n = 10$), the gray bar for the Rc-CLZ-ST Group ($n = 10$) and the dotted line indicates 45% of correct responses, inclusion criteria. C) Testing session. Number of correct responses, incorrect responses and no response +/− SEM on Day 3.

(Nader et al., 2000). Additionally, this experiment showed that the performance of the drug-treated volunteers was comparable to that of the placebo-treated group, despite the short period between the administration of the capsule and the testing session, which provides evidence against a CLZ effect on retrieval only.

4. Discussion

The central conclusion of this paper is that, when memory is labilized by the presentation of the proper reminder, the administration of 0.25 mg clonazepam strengthens a reactivated human declarative memory (experiment 1). This statement is supported by the lack of an enhancing effect on the original memory in the absence of the reactivation session or when a cue-response reminder, which prevents memory labilization, is presented (experiment 3). Together, these results support the view that mere retrieval does not affect memory stability, which remains unaffected under different interfering agents (Forcato et al., 2009; Frenkel et al., 2005; Pedreira et al., 2004) Furthermore, we can conclude that the observed improving effect of clonazepam only occurs when the parametrical conditions of reconsolidation are fulfilled, specifically, the incongruence between actual and expected events (Forcato et al., 2009; Pedreira et al., 2004). Using a different declarative memory paradigm, Hupbach et al. (2007) demonstrated that memory updating depends on the reconsolidation process. In this report, they included three different components in the reminder. First, they asked the subjects to describe the experimental procedure for the original learning event; second, they used the same room for the presentation of the new information; and finally, they had the same individual administer all the sessions. In a subsequent report (Hupbach et al., 2008), they demonstrated that updating

occurred in the same spatial context condition, even though the other components were altered. Moreover, they also demonstrated that in familiar environments, the diagnostic value of the spatial context for a specific episode was greatly diminished (Hupbach et al., 2011). All in all, these results are in accord with our research, in that they show not only the relevance of contextual cues but also, in our paradigm, the execution of the response, that is, that the writing down of the response-syllable is the principal component in the reminder structure (Forcato et al., 2009). This relevance is a consequence of the principal role of the mismatch component that triggers the reconsolidation process (Lee, 2009).

In addition, the observed effects cannot be explained by other factors such as capsule intake, interaction with physicians and/or hospital attendance for the treatment session (Day 2) as there were no significant differences in the response levels between the group that received the treatment session (Day 2) and the group that only attended the hospital for training and testing sessions (Experiment 2). Finally, memory enhancement only occurs after reconsolidation (Forcato et al., 2007) because similar performance levels were obtained when the test took place four hours after administering the drug (experiment 4).

With regards to the different types of verbal responses observed at testing, the analysis of the three possible categories revealed that the improvement in correct responses was due to a decrease in incorrect responses instead of a decrease in no responses.

Notably, however, the rates of no response were low enough in all groups to prevent a thorough analysis of the effect of clonazepam on this category.

Regarding the retrieval process, some experiments carried out in rats showed an enhancement effect from benzodiazepines in this phase in a fear-conditioning paradigm (Harris and Westbrook, 1998) and in an active avoidance task (Obradovic et al., 2004). However, pure retrieval deficits have also been observed in both humans and animals (Lombardi et al., 1997; Pompeia et al., 1996). In this research, it was possible to analyze the effect of the drug administered after retrieval or reactivation, using a specific reminder in each case: a cue-response reminder or a cue reminder, respectively. Our results indicate that memory enhancement was only observed after reactivation. Supporting this statement, the benzodiazepine used showed no effect on memory retrieval, even though clonazepam is a long-acting drug.

As we mentioned above, two different steps can be determined in the so-called reconsolidation. The first step is reactivation, which implies a destabilization of the consolidated memory, and then a process of re-stabilization occurs, which returns the memory to a stable state. Recent studies have begun to identify the molecular mechanisms underlying the re-stabilization of the reactivated memory (Merlo and Romano, 2008; Taubenfeld et al., 2001), and as a result, the memory is insensitive to disruptive agents. In this context, when the testing session is performed in the time window of the second step, the original memory emerges intact, and only when the evaluation is accomplished out of the re-stabilization time window is the effect of the treatment revealed (Hupbach et al., 2007; Nadel and Land, 2000; Pedreira et al., 2002). Consequently, in the present research, the short-term testing allowed us to infer that the drug affects memory re-stabilization, leaving memory expression intact 4 h after reactivation. In line with these results, we have shown in a previous study that repeated reactivation strengthens the declarative memory. This effect is revealed 24 after reactivations and not revealed immediately after reminder presentation (Forcato et al., 2011) However, future experiments might consider administering the drug outside the time window determined for the re-stabilization – i.e., between 6 and 10 h after reactivation – as this type of manipulation would provide further evidence to support its effect on the second step.

The volunteers showed the same number of correct responses when the memory was tested 4 h after CLZ or PLC administration. This result strongly suggests that the drug effect depends on memory re-stabilization (Nader et al., 2000; Pedreira et al., 2002). Moreover, this result also indicates that CLZ does not affect the performance of volunteers per se, in spite of the presence of the drug during short-term testing. In line with this absence of a non-specific effect, a similar number of correct responses appear when this long-lasting drug is present during long-term testing in the groups that only evoked or did not retrieve the original memory the day before (experiment 3). Furthermore, these groups wrote down a similar number of correct responses as the group that received a placebo instead the CLZ capsule (experiment 1). The strengthening effect is exclusive for the groups that combined the labilization of the target memory with the benzodiazepine administration and were evaluated outside the time window of re-stabilization.

To date, little is known about reconsolidation functionality from a biological standpoint. One of the functionalities proposed suggests that the labilization–reconsolidation process strengthens the original memory (Alberini, 2007; Inda et al., 2011). To study this function, we previously performed a series of experiments (Forcato et al., 2011) that allowed us to demonstrate that the memory was improved when at least a second reminder was presented in the time window of the first labilization–reconsolidation process, prompted by the earlier reminder and not as a consequence of retrieval only. A speculative analysis of these results suggests the possibility that successive reactivations trigger repetitive labilization processes, which in turn implies successive re-stabilization processes. Consequently, the second re-stabilization is mounted on a previous re-stabilization, resulting in a repeated activation of molecular pathways, which lead to either a higher expression of the macromolecules necessary or an increasingly available number them for the recovery of the stable state. This previous report raises the possibility of strengthening this type of memory by specific manipulations in the reconsolidation time-window.

In the present report, the protocol included the presentation of one proper reminder, making the previous interpretation untenable. Here, with regards to the pharmacological intervention, we propose that the improvement observed when the GABAergic system is stimulated via clonazepam should be interpreted in relation to the drug's effects, that is, lower levels of anxiety. Under this condition, the re-stabilization takes place in a different scenario, which in turn creates better conditions for re-storage, resulting in a strengthened memory. Moreover, we speculate that the low doses that were administered could ensure an anxiolytic effect, thereby avoiding major inhibition by $GABA_A$ stimulation, which in turn would compromise the re-stabilization of the original memory.

Moreover, it is worth noting that a large body of evidence in animals and humans has demonstrated traces of anterograde when benzodiazepines were administered to affect short- or long-term memories (Ghoneim and Mewaldt, 1975; McNamara and Skelton, 1991; Sanger et al., 1986; Savic et al., 2005; Tang et al., 1995; Venault et al., 1986). Additionally, some reports postulate that benzodiazepines can produce retrograde memory facilitation by improving the recall of information acquired before drug administration (Fillmore et al., 2001; Hinrichs et al., 1984).

Considering this background when examining our experimental design, during re-stabilization, clonazepam might impair the consolidation of new information, which could affect the re-storage of the original memory, and consequently, the target memory would gather strength during the reconsolidation process.

In conclusion, both interpretative frameworks postulate that benzodiazepines diminish the degree of interference during the reconsolidation process, thereby resulting in memory improvement.

The results obtained here showed that it is possible to improve the expression of a neutral declarative memory with benzodiazepines when the consolidated memory is reactivated. Future testing should explore whether similar effects could be found when the paradigm includes emotional memories. Particularly negative emotional burdens bear a close resemblance to those dysfunctional memories associated with anxiety-related disorders, such as PTSD, panic disorder or phobias.

It was established that decreased GABAergic activity plays a central role in the pathogenesis of anxiety disorders (Enna, 1984; Khozhenko, 2009; Kowalski et al., 2007; Lydiard, 2003), therefore, increasing GABAergic neurotransmission by means of benzodiazepine administration is widely accepted as an effective treatment for anxiety disorders (Ravindran and Stein, 2010). However, in this study, the declarative memory was improved. Therefore, when designing new therapies, it is important to consider that, for example, anxiety levels diminish while the declarative component of the maladaptive memory survives or even strengthens.

As we mentioned above, studies in human memory indicate that the use of benzodiazepines produces anterograde but not retrograde amnesia (Curran, 1991; Uzun et al., 2010; McNamara and Skelton, 1991; Savic et al., 2005). Conversely, some putative retrograde memory-enhancing effects have been found (Hinrichs et al., 1984), and declarative memory retrieval has been improved by low doses of benzodiazepines (Delgado et al., 2005; File et al., 1999; Fillmore et al., 2001).

With respect to the reconsolidation processes using contextual fear learning in animal models, different reports showed that the administration of $GABA_A$ agonists impairs the re-stabilization of the reactivated memory (Bustos et al., 2006; Carbo Tano et al., 2009; Zhang and Cranney, 2008). However, administering ethanol in the same type of paradigm improved the rats' performance at testing, an effect that was dependent on the $GABA_A$ receptor (Nomura and Matsuki, 2008). Our results are in line with this strengthening effect. We speculate that the low dose of CLZ administered in our research and the ethanol dose used in the Nomura and Matsuki report produce a similarly moderated activation of the GABAergic system and avoid major inhibition through $GABA_A$ stimulation, which in turn would compromise the re-stabilization of the original memory, as has been demonstrated in previous research (Bustos et al., 2006; Carbo Tano et al., 2009). Further studies employing different doses or paradigms might elucidate the role of GABA in the reconsolidation processes that are associated with dissimilar scenarios.

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