

Short-term memory reactivation of a weak CS–US association promotes long-term memory persistence in conditioned odor aversion

Jorge Tovar-Díaz,¹ Jean-Pascal Morín,² Jorge Eduardo Ríos-Carrillo,²
Hilda Sánchez de Jesús,² and Gabriel Roldán-Roldán²

¹*School of Medicine and Psychology, Autonomous University of Baja California, Tijuana, Baja California 22427, México;* ²*Department of Physiology, Faculty of Medicine, National Autonomous University of México, Mexico City 04510, México*

In conditioned odor aversion (COA), the association of a tasteless odorized solution (the conditioned stimulus [CS]) with an intraperitoneal injection of LiCl (the unconditioned stimulus [US]), which produces visceral malaise, results in its future avoidance. The strength of this associative memory is mainly dependent on two parameters, that is, the strength of the US and the interstimuli interval (ISI). In rats, COA has been observed only with ISIs of ≤ 15 min and LiCl (0.15 M) doses of 2.0% of bodyweight, when tested 48 h after acquisition (long-term memory [LTM]). However, we previously reported a robust aversion in rats trained with ISIs up to 60 min when tested 4 h after acquisition (short-term memory [STM]). Since memories get reactivated during retrieval, in the current study we hypothesized that testing for STM would reactivate this COA trace, strengthening its LTM. For this, we compared the LTM of rats trained with long ISIs or low doses of LiCl initially tested for STM with that of rats tested for LTM only. Interestingly, rats conditioned under parameters sufficient to produce STM, but not LTM, showed a reliable LTM when first tested for STM. These observations suggest that under suboptimal training conditions, such as long ISIs or low US intensities, a CS–US association is established but requires reactivation in the short-term in order to persist in the long-term.

[Supplemental material is available for this article.]

The dynamic and malleable nature of memories is a well-studied phenomenon. Traditionally, for memory formation to occur, a set of processes collectively known as consolidation are thought to be needed in order to stabilize memories, making them susceptible to modification during this period (Dudai et al. 2015). More recently a slightly distinct theory, known as memory integration, was proposed according to which memories are rapidly formed during learning without the need for consolidation, but any relevant information around the event can be integrated modifying them (Gisquet-Verrier and Riccio 2019). Common to both theories though, is that memories alternate between an inactive and an active state and modifications can mostly occur during the active state, which lasts for some time after learning, or during its reactivation due to retrieval (Lee et al. 2017; Albo and Gräff 2018; Gisquet-Verrier and Riccio 2019). Thus, memory malleability is explained either because consolidation can be altered or because additional information can be integrated with the initial memory (Bailey et al. 1996; Dudai 2004; Wixted 2004; Alberini et al. 2006; Lee et al. 2008, 2017; McGaugh and Roozendaal 2009; Roesler and Schröder 2011; Dudai et al. 2015; Nader 2015; Crossley et al. 2019; Gisquet-Verrier and Riccio 2019).

In conditioned odor aversion (COA), an odorized tasteless solution (conditioned stimulus, CS) whose ingestion is followed by gastrointestinal malaise (unconditioned stimulus, US) is rejected in future encounters (conditioned response, CR). In most COA studies, a robust aversion has been observed only when the interstimulus interval (ISI) is ~ 5 min, and no significant aversion can

be seen when the ISI is > 15 min (Hankins et al. 1973; Palmerino et al. 1980; Ferry et al. 1995, 1996; Ferry and di Scala 1997; Ferry et al. 2006; Chapuis et al. 2007). This observation has been attributed to a short-lasting memory of the odor that becomes unavailable for its association with the US after ISI > 15 min. However, in all these instances the CR was measured 48 h after conditioning (LTM test), leaving up the possibility that CS–US association was formed but somehow did not last till the long-term. In keeping with this possibility, we previously reported a significant aversion during a test performed 4 h after conditioning (i.e., STM test) in rats trained with ISIs up to 60 min, three times longer than previously described (Tovar-Díaz et al. 2011). The LTM, however, was not tested so no further insight was provided regarding its persistence due to STM reactivation.

Thus, in the current paper we hypothesized that a STM test would reactivate the initial memory, allowing it to further consolidate/integrate the information and to persist in the LTM. To test this possibility, we trained independent groups of rats with reduced US intensities or prolonged ISIs in a standard two-bottle choice COA paradigm and tested them twice at 4 and 48 h after conditioning. Our findings suggest that COA takes place under milder US and longer ISIs than previously thought and reactivating this memory during the STM test promotes its persistence in the LTM test.

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Corresponding authors: jorge.tovar.diaz@uabc.edu.mx,
gabaergico@gmail.com

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Results

CS-US association at low US intensities and reactivation-induced long-term persistence of COA

In flavor aversion studies the intensity of the US has a direct influence on the magnitude of the CR (Nachman and Ashe 1973; Garcia et al. 1974; Andrews and Braveman 1975; Twining et al. 2016). Therefore, in the first set of experiments, we systematically reduced the intensity of the US while keeping the ISI fixed at 5 min. We decreased the dose of LiCl from 2.0% to 1.0% or 0.5% BW and tested independent groups at 4 h (STM test) or 48 h (LTM test) after conditioning. The groups tested for STM were tested again for LTM (LTM_{Reactivated} test) and compared with the LTM test-only groups (experimental design, Fig. 1A). Within the LTM test groups, only 2.0% LiCl induced a significant CR, in agreement with previous publications (one-way ANOVA, followed by Dunnett's post-hoc test: $P < 0.001$) (Fig. 1B). Interestingly, among the STM test groups, all doses produced a significant CR response (one-way ANOVA, followed by Dunnett's post-hoc test: $P < 0.001$) (Fig. 1C). Moreover,

when these animals were tested for LTM (LTM_{Reactivated} test groups), they still displayed a significant CR, regardless of US dose (one-way ANOVA, followed by Dunnett's post-hoc test: $P < 0.001$) (Fig. 1D). Furthermore, a linear regression analysis of the LTM groups revealed a direct relationship between US intensity and CR magnitude ($r^2 = 0.3399$, $P < 0.001$) (Fig. 1E), but not in the LTM_{Reactivated} groups, either when tested for STM or LTM ($r^2 = 0.01513$, $P > 0.05$ and $r^2 = 0.00089$, $P > 0.05$, respectively) (Fig. 1E) and the slope of the curve dropped significantly in comparison with LTM test ($F = 11.10$, $DFn = 2$, $DFd = 86$, $P < 0.0001$) (Fig. 1E), suggesting a role of STM-reactivation in maintaining the CR over time.

CS-US association at long ISIs and reactivation-induced long-term persistence of COA

Next, we reproduced our previous experiments with ISIs ranging from 5 to 60 min (Tovar-Díaz et al. 2011) on independent groups of rats tested at 4 h (STM test) or 48 h after conditioning (LTM test). The STM-tested groups were tested again for LTM (LTM_{Reactivated} test). The US intensity was kept at 2.0% BW of LiCl (Fig. 2A, experimental design). Among the groups tested for LTM only, a statistically significant CR was found at 5, 15, and 30 min (one-way ANOVA, followed by Dunnett's post-hoc test: $P < 0.001$, $P < 0.01$ and $P < 0.05$, respectively) (Fig. 2B). In the STM test, all groups showed a significant CR response (one-way ANOVA, followed by Dunnett's post-hoc test: $P < 0.001$) (Fig. 2C). Interestingly, this CR was also observed in all groups when tested for LTM_{Reactivated} test (one-way ANOVA, followed by Dunnett's post-hoc test: $P < 0.001$) (Fig. 2D). Importantly, a linear regression analysis of the LTM test revealed a significant effect of ISI length on CR ($r^2 = 0.2237$, $P < 0.01$, Fig. 2E). This effect was not observed in the LTM_{Reactivated} either when evaluated for STM or LTM_{Reactivated} ($r^2 = 0.01842$, $P > 0.05$ and $r^2 = 0.0003$, $P > 0.05$, respectively) (Fig. 2E) and the slope of the curve dropped significantly ($F = 4.994$, $DFn = 2$, $DFd = 107$, $P < 0.01$) in comparison with the LTM groups (Fig. 2E), again suggesting that STM-reactivation promoted the persistence of the CR.

Medicine-like effect or reactivation-induced long-term persistence of COA?

An alternative explanation to reactivation in experiments 1 and 2 is that during the STM test rats could associate the neutral odor (CS-) with the recovery of LiCl toxicosis (a medicine-like effect) (Green and Garcia 1971; Garcia et al. 1974), thus increasing its intake. To address this issue, we performed two additional experiments using the lowest LiCl dose or the longest ISI where no CR was observed in the LTM test (see Figs. 1B, 2B for 0.5% LiCl and 60-min ISI,

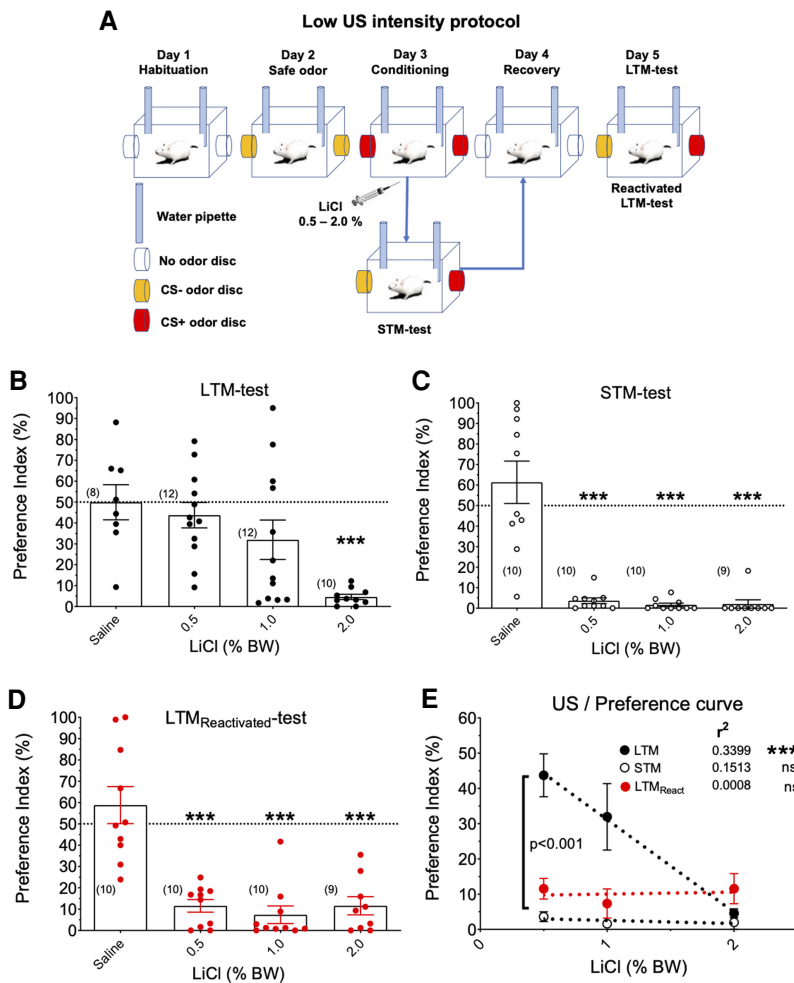


Figure 1. CS-US association at low US intensities and reactivation-induced long-term memory in COA. (A) Experimental protocol. (B) Preference Index (P.I.) of LTM test, no significant reduction was observed with 0.5 or 1.0% BW LiCl. (C) P.I. of STM test, a significant reduction was observed with all doses tested. (D) P.I. of LTM_{Reactivated} test (same groups previously tested for STM shown in C), a significant reduction was observed at all doses of LiCl tested. One-way ANOVA, followed by Dunnett's post-hoc test for all groups. (***) $P < 0.001$. (E) Linear regression analysis of all doses and conditions tested, only LTM test groups showed a significant LiCl dose-dependent reduction on P.I. and the slope of the curve was significantly different to STM and LTM_{Reactivated} groups. Group size in parentheses.

respectively). A STM test with only the CS+ present was carried out, followed by a CS-/CS+ LTM test (Fig. 3A, experimental design). If the low P.I. observed in the LTM_{Reactivated} condition is due to a medicine-like effect induced by the presence of the CS-, then presenting only the CS+ should have no effect on the LTM. As shown in Figure 3E, this was not the case as rats injected with LiCl of the ISI group showed a lower P.I. in comparison with saline-injected rats (*t*-test $P < 0.01$ for the 60 min ISI) (Fig. 3E). Although the group of LiCl 0.5% did not reach a significant effect ($P > 0.05$) (Fig. 3D), we believe it was due to the small size of the sample. When the raw intake was analyzed, both experimental groups showed a significantly different intake between CS- and CS+ in the LTM_{Reactivated} test, while their respective saline-injected controls ingested similar amounts of both odors (Supplemental Fig. 3).

Correlation between time to first test and CR magnitude: impaired retention rather than failed CS-US association

So far, we explored the CR only during the STM or during the LTM. Thus, to explore the persistence of the CR over time we ran a first test in independent groups at 8 or 12 h (intermediate-term memory [ITM] test) after conditioning. Then, to see the effect of reactivation we ran a second test 48 h after conditioning (LTM test) (we included the results of 4 h in these figures in order to perform a time-dependent analysis). Since the CR for the lower US intensities (0.5% and 1.0%) and the longer ISIs (15–60 min) was very strong during the STM test but absent during the LTM test, we expected a time-dependent decline during the first test. We also expected that the reactivation during the first test would maintain the strength of CR during the second test at least at the same level of the first test of the corresponding group.

Reduced US intensity

Among the different LiCl dosage groups, the one of 0.5% BW displayed a significant CR at 4 and 8, but not 12 h (one-way ANOVA followed by Dunnett's post-hoc test: $P < 0.001$ at 4 h; $P < 0.05$ at 8 h; $P > 0.05$ at 12 h) (Fig. 4A) and a linear regression analysis showed a significant decay over time ($r^2 = 0.431$, $P < 0.001$) (Fig. 4C). The LiCl 1.0% groups displayed a significant CR at all times tested (one-way ANOVA followed by Dunnett's post-hoc test: $P < 0.001$) (Fig. 4D), with significant decay over time ($r^2 = 0.1815$, $P < 0.01$) (Fig. 4F). The LiCl 2.0% groups also displayed a significant CR at all times tested (one-way ANOVA followed by Dunnett's post-hoc test: $P < 0.001$) (Fig. 4G), but no decay over time ($r^2 = 0.1152$, $P > 0.05$) (Fig. 4I). During the LTM_{Reactivated} tests, the LiCl 2% groups showed a significant CR at all times tested (one-way ANOVA followed by Dunnett's post-hoc test: $P < 0.001$) (Fig. 4H), without significant

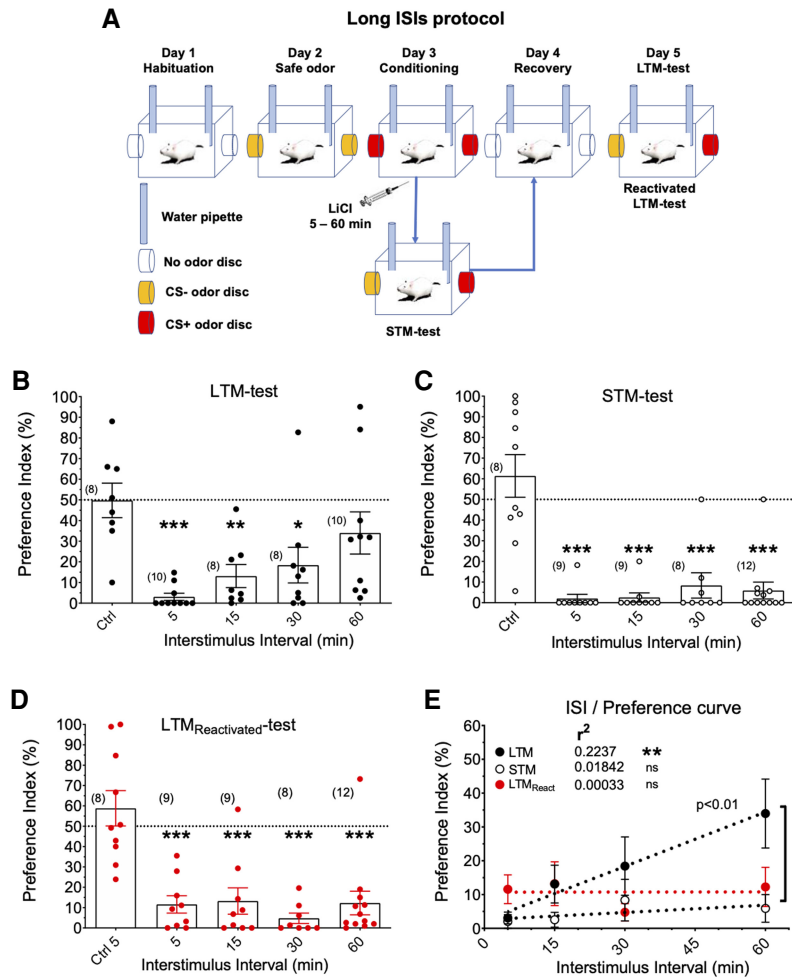


Figure 2. CS-US association at long ISIs and reactivation-induced long-term memory in COA. (A) Experimental protocol. (B) Preference Index (P.I.) of LTM test, no significant reduction was observed after 30 min. (C) P.I. of STM test, a significant reduction was observed on all ISIs tested. (D) P.I. of LTM_{Reactivated} test (same groups previously tested for STM shown in C), a significant reduction was observed at all ISIs tested. One-way ANOVA, followed by Dunnett's post-hoc test for all groups. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$. (E) Linear regression analysis of all ISI and all conditions tested, only LTM test groups showed a significant ISI length-dependent reduction on P.I. and the slope of the curve was significantly different to STM and LTM_{Reactivated} groups. Group size in parentheses.

decay over time ($r^2 = 0.0394$, $P > 0.05$) (Fig. 4I). Among the LiCl 1.0% groups the CR was significant at 4 and 8 but not at 12 h (4 h $P < 0.001$; 8 h $P < 0.01$; 12 h $P > 0.05$) (Fig. 4E) and no significant decay over time was observed ($r^2 = 0.1163$, $P > 0.05$) (Fig. 4F). Finally, the groups of LiCl 0.5% showed a significant CR at all times tested (one-way ANOVA followed by Dunnett's post-hoc test: 4 h $P < 0.001$, 8 h $P < 0.05$, 12 h $P < 0.05$) (Fig. 4B) and no significant decay over time ($r^2 = 0.1274$, $P > 0.05$) (Fig. 4C).

Increased ISI length

Next, we explored the persistence of the CR over time of the prolonged ISIs by testing independent groups at 8 or 12 h after conditioning and tested them again for LTM to see the reactivation effect (we included the results of 4 h in these figures in order to perform a time-dependent analysis).

Among the groups of 5 min a significant CR was found at all times tested (one-way ANOVA followed by Dunnett's post-hoc test: $P < 0.001$ for 4, 8, and 12 h) (Fig. 5A) with no significant decay over time ($r^2 = 0.1152$, $P > 0.05$) (Fig. 5C). Among the 15-min groups, the

CR was significant at 4 and 8 h (one-way ANOVA followed by Dunnett's post-hoc test: $P < 0.001$ and $P < 0.05$, respectively) but not at 12 h ($P > 0.05$) (Fig. 5D) and there was a time-dependent decay ($r^2 = 0.1544$, $P < 0.05$) (Fig. 5F). Among the 30-min groups, the CR was significant at all times tested (one-way ANOVA followed by Dunnett's post-hoc test, $P < 0.001$) (Fig. 5G) but no decay over time ($r^2 = 0.01317$, $P > 0.05$). As for the longest ISI of 60 min, the CR was significant at all times tested (one-way ANOVA followed by Dunnett's post-hoc test: $P < 0.001$ at 4 h; $P < 0.001$ at 8 h; $P < 0.01$ at 12 h) (Fig. 5J), and no significant decay over time ($r^2 = 0.0990$, $P > 0.05$) (Fig. 5L). During the LTM_{Reactivated} tests, the CR of the 5-min groups was again significant at all times tested (one-way ANOVA followed by Dunnett's post-hoc test, $P < 0.001$ for all groups) (Fig. 5B) without significant decay over time ($r^2 = 0.03940$, $P > 0.05$) (Fig. 5C). The 15-min groups showed a significant CR at all times tested (one-way ANOVA followed by Dunnett's post-hoc test: 4 h $P < 0.001$; 8 h $P < 0.001$; 12 h $P > 0.05$) (Fig. 5E) and displayed no significant decay over time ($r^2 = 0.05653$, $P > 0.05$) (Fig. 5F). Among the 30-min groups, the CR was significant at all times tested (one-way ANOVA followed by Dunnett's post-hoc test: 4 h $P < 0.001$; 8 h $P < 0.05$; 12 h $P < 0.01$) (Fig. 5H) and no significant decay over time ($r^2 = 0.03998$, $P > 0.05$) (Fig. 5I). For 60 min, the CR was again significant at all times tested (one-way ANOVA followed by Dunnett's post-hoc test, $P < 0.001$ at 4 h; $P < 0.001$ at 8 h; $P < 0.01$ at 12 h) (Fig. 5K) and no decay over time ($r^2 = 0.02914$, $P > 0.05$) (Fig. 5L).

Discussion

The main finding of this study was that reactivating COA during a STM test strengthens the CR, allowing it to persist up to the LTM test. This effect is different from reconsolidation and extinction since in these two cases the modification of the CR typically occurs in an already consolidated memory. The overall picture that emerges from the 4- to 12-h tests (STM–ITM tests, respectively) suggests that COA does actually take place when reduced US intensities or prolonged ISIs are used during training, but the CR declines progressively if not reactivated at earlier times.

A suboptimal training allows STM but not LTM formation in COA

In COA as well as in conditioned taste aversion (CTA), in which a novel taste (CS) is paired with a gastric malaise-inducing LiCl injection (US), the main variables influencing the magnitude of the CR (rejection to odor or taste, respectively) are ISI length, CS intensity and US intensity (Garcia et al. 1955, 1974; Kalat and Rozin 1973; Andrews and Braveman 1975; Rusiniak et al. 1982; Twining et al. 2016). Of these parameters, ISI has received major attention in CTA and COA because of the apparent violation of the temporal

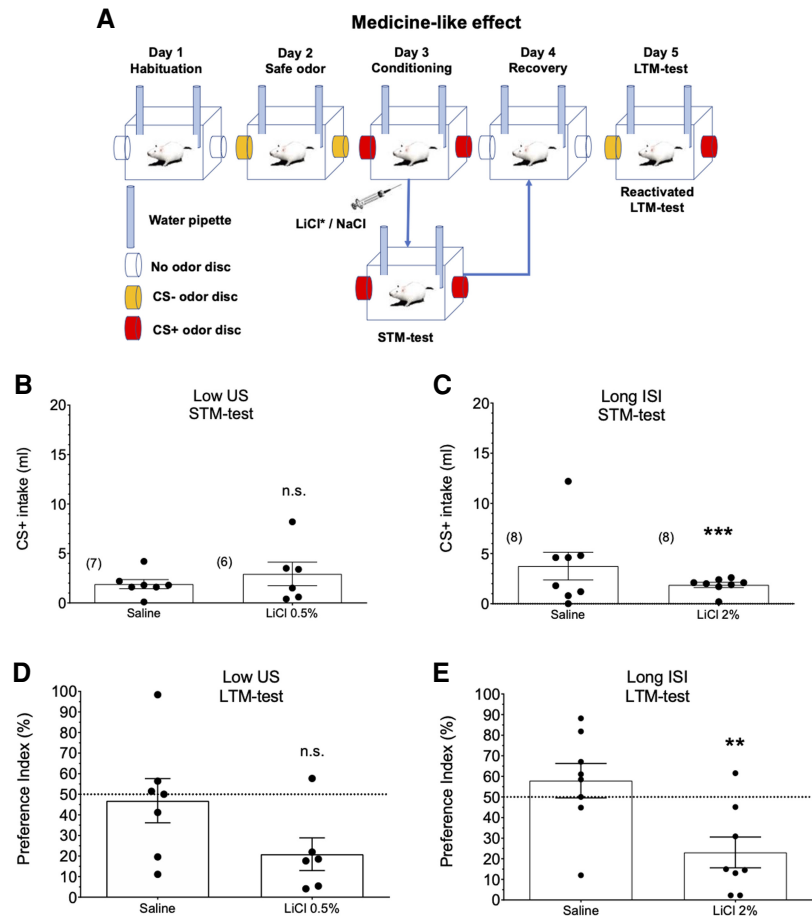


Figure 3. Memory reactivation or medicine-like effect? (A) Experimental protocol; only CS+ was presented during the STM test. (B) STM test of LiCl 0.5% groups. Water intake was similar between saline and LiCl i.p. injected groups (t -test, $P > 0.05$). (C) STM test of long ISI groups. ISI 60-min rats drank significantly less than its respective saline control (t -test, $P < 0.001$). (D,E) P.I. during the LTM test. Only the ISI group reached a statistical significance (t -test, $P < 0.01$).

contiguity rule (Garcia et al. 1966; Kalat and Rozin 1973; Chapuis et al. 2007). Although CTA tolerates ISIs of several hours (Garcia et al. 1966; Andrews and Braveman 1975; Holder and Garcia 1987; Buresh et al. 1998), a number of studies from pioneering descriptions by Garcia's group (Hankins et al. 1973; Rusiniak et al. 1979; Palmerino et al. 1980) to more recent work by others (Ferry et al. 1995, 1996; Ferry and di Scala 1997; Chapuis et al. 2007) have shown that COA cannot be obtained with ISIs > 15 min. In all these instances the CR was tested 48 h after conditioning (LTM test) and the lack of aversion was attributed to the short duration of the odor trace (< 15 min), unavailable by the time illness is induced (Hankins et al. 1973; Palmerino et al. 1980; Ferry et al. 1996; Ferry and di Scala 1997; Chapuis et al. 2007). Under our experimental design similar observations are obtained in which prolonging the ISI impairs LTM. However, our previous (Tovar-Díaz et al. 2011) and current results show a strong aversion during the STM test, even when the ISI is prolonged to 60 min or when the US intensity is lowered to 0.5% BW, suggesting that the CS–US association actually takes place. Furthermore, our ITM results suggest a time-dependent decay where at a given US intensity or ISI length the CR is stronger at earlier testing times, explaining why is evident in the STM test but not in the LTM test. Therefore, a systematic evaluation of STM in COA was lacking.

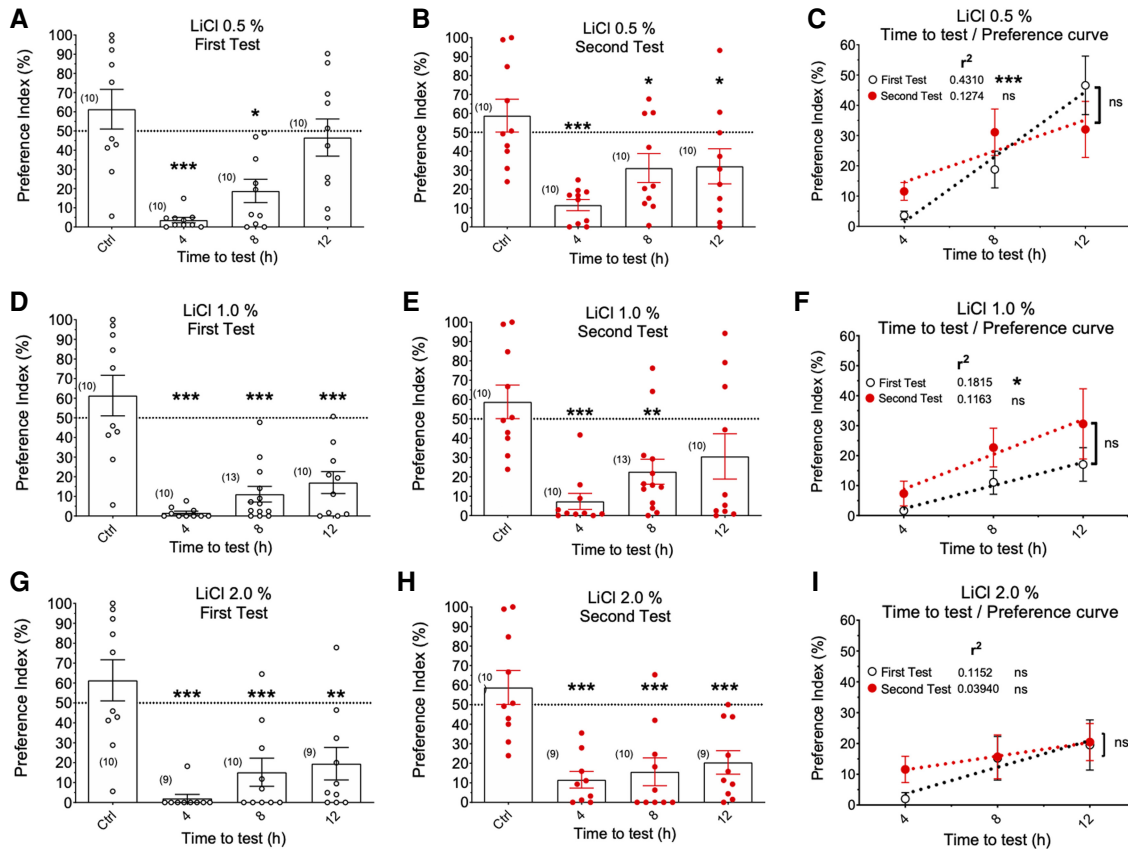


Figure 4. US intensity and time to first test correlation with conditioned response. (A) P.I. for all times tested for 0.5% LiCl, there is a significant reduction at 4 and 8 h but not at 12 h. (B) P.I. of LTM_{Reactivated} test of groups shown in A, there is a significant reduction at all times, and a time-dependent gradient. (C) Linear regression showing a significant time-dependent increase of P.I. at first test ($r^2 = 0.431$, $P < 0.001$) but not at second test ($r^2 = 0.1274$, $P > 0.05$). (D) P.I. for 1.0% LiCl groups, there is a significant reduction at all times tested. (E) P.I. of LTM_{Reactivated} groups shown in D, there is a significant reduction at 4 and 8 h but not at 12 h. (F) Linear regression showing a significant time-dependent increase of P.I. at first test ($r^2 = 0.1815$, $P < 0.05$) but not at second test ($r^2 = 0.1163$, $P > 0.05$). (G) P.I. for 2.0% LiCl groups, there is a significant reduction at all times tested. (H) P.I. of LTM_{Reactivated} groups shown in G, there is a significant reduction at all times tested. (I) Linear regression showing no effect over time for either test ($r^2 = 0.1297$ and 0.0394 , respectively; $P > 0.05$ for both). One-way ANOVA, followed by Dunnett's post-hoc test for all groups. (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$.

Memory reactivation or medicine-like effect?

The long-lasting effects of LiCl and the presence of CS⁻ might complicate the interpretation of early testing, accounting for the scarcity of STM studies. The presence of the CS⁻ by the time of testing could be associated with recovering from sickness, thus increasing its intake (a medicine-like effect) (Green and Garcia 1971; Garcia et al. 1974). In a previous study, a group of rats conditioned with 2% LiCl and 5 min ISI, allowed to choose between odorless water (instead of CS⁻) and CS⁺ during the STM test, drank similar amounts of odorless water than of the amounts of CS⁻ by an independent group tested with both CS⁻ and CS⁺, suggesting that increased preference for the CS⁻ did not contribute to their aversion to the CS⁺ (Tovar-Díaz et al. 2011). In the same reference was also shown that an ISI of 90 min does not induce aversion in the STM test, suggesting that the associability of the US with a CS has a limited time frame and reducing the possibility of a medicine effect at longer times. In the only study we know where COA was tested in STM, anisomycin was infused into the amygdala previous to conditioning and STM was tested (Desgranges et al. 2008). According to the authors, by the time of testing (4 h after conditioning) CS⁺ rejection was not due to the lingering effects of LiCl, since water intake just after CS⁺ was normal. Furthermore, Lamprecht et al. (1997) showed that when testing CTA 4 h after injection of a 0.15 M LiCl solution at 2% BW—corresponding to the

highest US strength used in the present study—lying on the belly and rearing (both signs of LiCl-induced illness) were not detected and there was no difference in the amount of liquid consumed 2–4 h later, suggesting that the aversion observed was not attributable to the lingering effects of LiCl but rather to memory. Moreover, in CTA STM has been evaluated as early as 15 min after training (Haupt and Berlin 1999). Overall, these reports strengthen our interpretation and weaken alternatives like the medicine effect.

STM reactivation induces LTM persistence in COA

Among the groups tested for LTM only, a significant CR was evident only at the highest US (2.0% LiCl) (Fig. 1B) and shorter ISIs (5–30 min) (Fig. 2B), while the CR was significant among all LTM_{Reactivated} groups. We believe that the long-term persistence of the CR in the weakened conditions was due to a reactivation effect induced by the STM test. According to current theories to explain memory persistence over time, reactivating the memory makes it susceptible to modification. New information can interweave with the old, strengthening or weakening the original memory (Lee et al. 2017; Gisquet-Verrier and Riccio 2019). In the classical theory of consolidation, exposure to CS⁺ may induce one of two different processes: extinction or reconsolidation (Bouton and Moody 2004; Dudai and Eisenberg 2004; Stollhoff

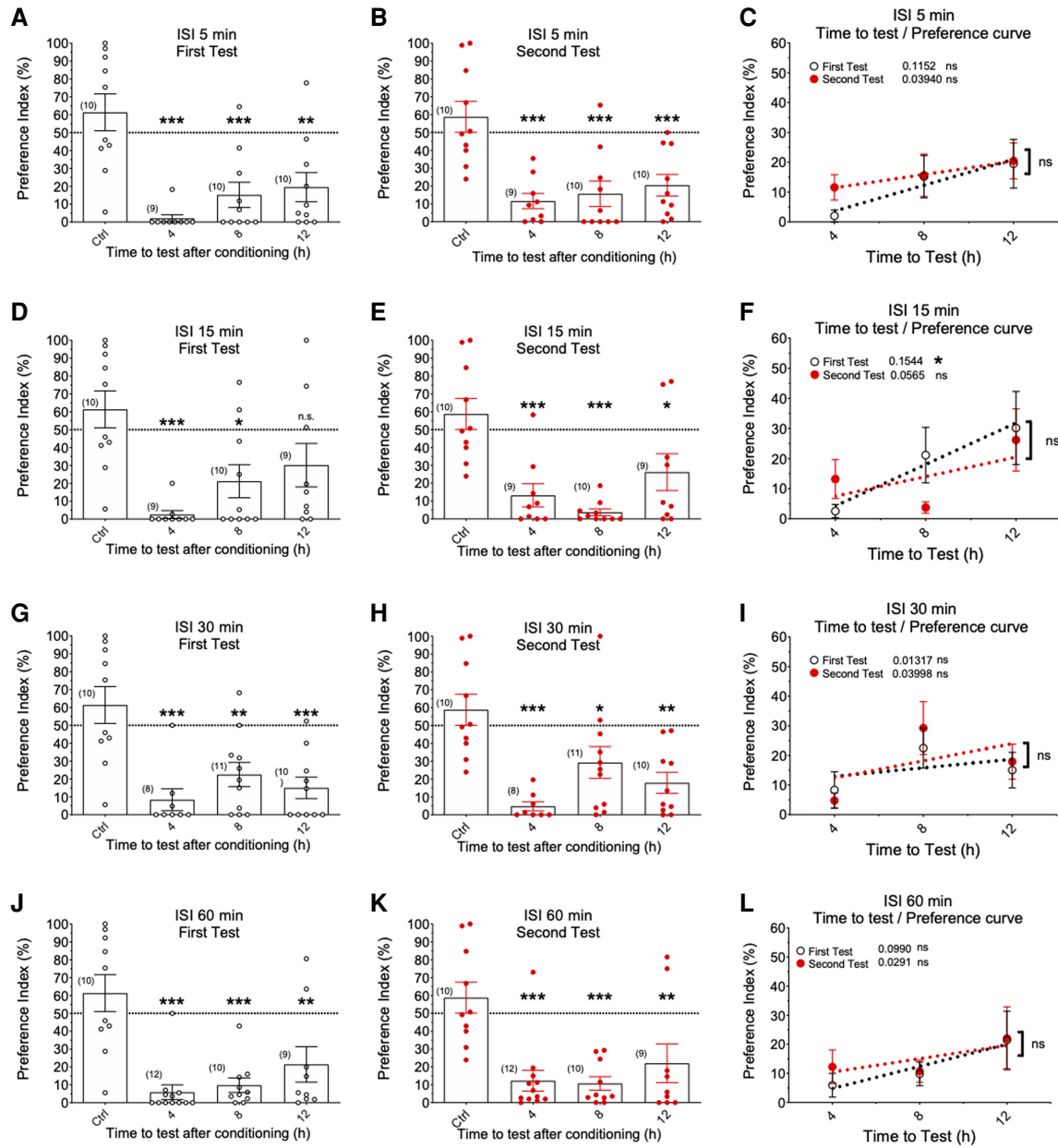


Figure 5. ISI length and time to first test correlation with conditioned response. (A) P.I. for 5-min ISI, there is a significant reduction at all times tested. (B) P.I. of LTM_{Reactivated} groups shown in A; there is a significant reduction at all times tested. (C) Linear regression showing no effect over time on either first or second test ($r^2 = 0.1152$ and $r^2 = 0.03940$, respectively; $P > 0.05$ for both). (D) P.I. for 15-min ISI, there is a significant reduction of P.I. at 4 and 8 but not at 12 h. (E) P.I. of LTM_{Reactivated} groups shown in D, there is a significant reduction at all times tested. (F) Linear regression showing a time effect during the first test ($r^2 = 0.1544$, $P < 0.05$) but not in the second test ($r^2 = 0.0565$, $P > 0.05$). (G) P.I. for 30-min ISI, there is a significant reduction at all times tested. (H) P.I. of LTM_{Reactivated} groups shown in G, there is a significant reduction at all times tested. (I) Linear regression showing no effect over time on either first or second test ($r^2 = 0.01317$ and 0.03998 , respectively; $P > 0.05$ for both). (J) P.I. for 60-min ISI, there is a significant reduction at all times tested. (K) P.I. of LTM_{Reactivated} groups shown in J, there is a significant reduction at all times tested. (L) Linear regression showing no effect of time on P.I. on either first or second test ($r^2 = 0.0990$, $P > 0.05$ and $r^2 = 0.0292$, $P > 0.05$, respectively).

and Eisenhardt 2009; de la Fuente et al. 2011; Lee et al. 2017). Extinction refers to CR loss due to prolonged or repeated CS+ presentation without US reinforcement (Bouton and Moody 2004; Dudai and Eisenberg 2004; Stollhoff and Eisenhardt 2009; de la Fuente et al. 2011). Here we gave one CS+-induced retrieval test instead of repeated or prolonged exposure. However, the STM test was an extinction trial, thus a reduced aversion in the second test would be expected. In contrast, aversion was preserved, if not enhanced. Reconsolidation refers to a process similar to consol-

idation, in which the reactivation of a consolidated memory by brief exposure to CS+ restabilizes it (Dudai and Eisenberg 2004; Stollhoff and Eisenhardt 2009; de la Fuente et al. 2011; Lee et al. 2017). Although pharmacological treatments shortly after reactivation are typically used to induce memory modifications during reconsolidation, behavioral interference may induce similar effects as pharmacological treatments (Lee et al. 2017). Our experimental design based on CS+ exposure reactivation may fit this description, except that we reactivated a STM, that is, not yet consolidated

memory. Thus, the time requirement in consolidation/reconsolidation theory makes the interpretation of our short-term reactivation effect a bit problematic. In this regard, a more parsimonious explanation may be offered by the integration theory, which considers that memory is swiftly established during training and further strengthened by reactivation at any time given the retrieval conditions are similar to the initial event (Gisquet-Verrier and Riccio 2019). However, under the integration theory, it would be expected our reactivation effect to be independent of time to retrieval, which was not the case. Therefore, neither consolidation nor integration seems to adequately explain the STM test reactivation effect described here.

Although the persistence of long-term memories has been widely proposed as dependent on postacquisition processes, little is known about its requirements during induction. According to the consolidation theory, a threshold of activity that filters the traces have been proposed, thereby determining which ones will persist (Dudai 2004; Nadel et al. 2012), or a bottleneck of activity that selects one memory over another (Breton and Robertson 2014). At the behavioral level, salience is considered the most relevant feature of a stimulus that can activate the consolidative processes via induction of selective attention (Salamon 2002; Sarter et al. 2006; Menon and Uddin 2010; Hasselmo and Sarter 2011). The stronger the attention gained by the stimulus, the higher the probability for learning and memory storage. In our experimental design, the low US intensities and the long ISIs could reduce the salience and the consequent level of attention to the learning experience, preventing them from reaching the consolidation threshold. Thus, it is possible that in our experiments, CS+ induced reactivation reengaged the recently recruited neural circuitry overcoming the consolidation threshold. In this regard, our results seem to better fit with the memory integration theory, which foresees that memory reactivation promotes swift consolidation, bypassing the requirement for gradual offline, systems-like consolidation.

Finally, our STM test-induced enhancing effect can be considered the result of a repetition of the learning experience. Intuitively, this appears not to be the case since associative learning requires a new CS-US pairing, while here the CS+ was presented alone during the test. Although we cannot completely discard some lingering effect of the US, we believe our controls lean the interpretation toward a memory reactivation-based effect. This is precisely what is proposed by retrieval-mediated learning, in which reactivating a recently acquired memory by incomplete reminders (in our case, presenting the CS+ only) promotes long-term retention (Antony et al. 2017), through the phenomenon of pattern completion (Hunsaker and Kesner 2013). Accordingly, it has been suggested that under certain circumstances, CS+ presentation can retrieve information about the US, acting as reinforcer (Holland 1990). In CTA, the CR is remarkably similar to the unconditioned response induced by LiCl itself, suggesting that exposure to the CS+ can trigger autonomic responses including nausea or illness (Meachum and Bernstein 1990) and perhaps making its memory persist. Although the CR induced by an odor CS+ does not completely resemble the unconditioned response induced by LiCl (Meachum and Bernstein 1992), it still implies retrieval of the aversive properties of the US. This situation could be considered as a multitrial training, in which early CS+ induced reactivation is required for strengthening the CS-US associative memory. As indicated before, we are aware of one report in which the same subjects were tested for both STM and LTM in COA. In this study, infusing anisomycin into the amygdala before conditioning blocked the LTM but not the STM, suggesting that activity in the amygdala plays an important role for LTM persistence (Desgranges et al. 2008). There is extensive evidence indicating that many drugs and hormones modulate memory, either directly

or indirectly, via noradrenergic activation of the basolateral amygdala whose efferents influence memory processing in many other brain regions (McGaugh and Roozendaal 2009). It is possible to speculate that reactivation during STM could re-engage amygdala activity, thus promoting CR persistence at longer times. Recently, Chen et al. (2018) showed that after CTA training in mice, a flavored CS+ (both its taste and/or its smell) can reactivate a subset of parabrachial nucleus neurons, which initially relayed the US signal, and their inhibition during CTA expression attenuates the CR and accelerates extinction, suggesting that reactivating these neurons can sustain the memory over time.

Overall, the evidence presented in this study suggests that early retrieval by exposure to the CS+ enhances a weak memory of a suboptimal training, allowing it to persist in the long-term. This behavioral phenomenon has not been previously documented in COA and might deserve further analysis.

Materials and Methods

Subjects

Male Wistar rats (250–300 g) obtained from our local breeding colony were used. All animal care and experimental procedures were approved by The Ethics Committee of the Facultad de Medicina, Universidad Nacional Autónoma de México, according to the Mexican Laws for Animal Care and complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council of the National Academies 2011). Rats were kept two per cage in the vivarium of our laboratory under a 12-h/12-h light/dark cycle (lights on at 0800 h), with ad libitum access to food (rodent laboratory chow) and tap water until experiments began.

Apparatus

Experiments were carried out in a plastic box (47 × 32 × 20 cm) equipped with two 30-mL pipettes, graduated to 0.1-mL accuracy, mounted on opposite sides of the box. The tip of each pipette was positioned 10 cm above the floor and 1 cm away from a 1.5-cm circular hole drilled in the wall. A 3-cm filter paper disc was placed inside a plastic cap attached to the hole, on the external side of the wall. This design allows a constant smelling of the odor while the rat is drinking from the tip of the pipette without direct contact with the odorized disc.

Behavioral conditioning

Independent groups of rats were deprived of water for 24 h and submitted to a COA protocol in which they were allowed to drink water for 10 min each day as follows: On day 1, rats were placed in the conditioning box and allowed to drink water only. On day 2, 0.2 mL of vanilla odor extract (unpaired CS, CS-, McCormick) was dropped in the filter disc on both plastic caps and rats drank water in the presence of the odor. On day 3, 0.2 mL of almond odor extract (paired CS, CS+, McCormick) was dropped in new filter discs and new plastic caps and rats drank water in the presence of the odor. After drinking the CS+, an i.p. dose of LiCl was injected according to the specific experiments described below. All control groups were i.p. injected with 0.9% saline, 2.0% BW 5 min after drinking the CS+.

LiCl experiments

On day 3, 5 min after drinking in the presence of the CS+, independent groups of rats received an i.p. injection of 0.15 M LiCl (Sigma-Aldrich Química), dissolved in sterile saline 0.9%, at doses of 0.5%, 1.0%, or 2.0% BW. A first test was performed at 4, 8, or 12 h after CS+ exposure with a double purpose: to test for STM or ITM and to reactivate the memory. Rats were presented with both CS- and CS+ for 10 min. On day 4, all groups drank odorless water for 10 min in the conditioning box, allowing them to recover from manipulations of day 3. On day 5 (48 h after the initial CS+

exposure) the groups tested at 4, 8, or 12 h, were given a second test (LTM_{Reactivated} test) as in day 3. For the LTM groups this was their only test.

ISIs experiments

On day 3, 5, 15, 30, or 60 min after drinking in the presence of the CS+, independent groups received an i.p. injection of 0.15 M LiCl 2% BW. A first test was performed 4, 8, 12, or 48 h after CS+ exposure by presenting both CS- and CS+ for 10 min (thus, the 60 min ISI group was actually tested 1 h earlier relative to LiCl injection than the 5-min group). On day 4, all groups drank odorless water for 10 min in the conditioning box, allowing them to recover from manipulations of day 3. On day 5 (48 h after the initial CS+ exposure) the groups tested at 4, 8, or 12 h were given a second test (LTM_{Reactivated} test) as in day 3. For the LTM groups this was their only test.

Medicine-like effect experiment

On day 1, all groups drank odorless water from both pipettes. On day 2, all groups drank water in the presence of CS- (vanilla) from both pipettes. On day 3, all groups drank water in the presence of CS+ (almond) from both pipettes. For the LiCl groups: LiCl 0.5% or saline 0.5% BW was injected 5 min after CS+ exposure. For ISI groups, LiCl 2% or saline 2% BW was injected 60 min after CS+ exposure. Both sets of groups received their first test 4 h after CS+ exposure on a forced method where both pipettes had CS+. On day 4, all groups drank odorless water from both pipettes. On day 5 a free-choice test (LTM_{Reactivated} test) was performed by presenting both CS- and CS+. In all days, all groups were allowed to drink odorless water for an additional 10-min period in the training box, 4 h after the first drinking session.

Data analysis

Water intake was measured with 0.1 mL accuracy and CS+ preference index (P.I.) was calculated for each rat ($P.I. = [CS+]/[CS-] + [CS+] \times 100$). Data was averaged by group and analyzed with GraphPad Prism 8 statistical software (2019). For all cases we did a one-way ANOVA followed up by a Dunnett's post-hoc, comparing the experimental groups versus the respective control group. A $P < 0.05$ was considered statistically significant. A linear regression analysis was used when indicated. All results are expressed as mean \pm S.E.M.

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