Reconsolidation of sucrose instrumental memory in rats: The role of retrieval context

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HIGHLIGHTS

- Conditioned context is essential for an effective instrumental memory retrieval.
- Retrieval inefficacy in a novel context confirms the specificity of our protocol.
- Zif268 and rpS6P protein levels correlate to instrumental memory retrieval in rats.

ABSTRACT

Memory reconsolidation enables the update of a previously consolidated memory trace after its reactivation. Although Pavlovian memory reconsolidation has been widely demonstrated, instrumental memory reconsolidation is still debated. The most critical issue on instrumental memory reconsolidation findings have mainly been linked to the presence of specific boundary conditions for reactivation, for instance contextual parameters. In this study, we investigated the role of the spatial context on molecular markers of sucrose instrumental memory reactivation.

Following withdrawal, rats previously conditioned to sucrose self-administration underwent either instrumental memory retrieval or no-retrieval in the conditioned context (Context A, AA condition) or in a modified version of the conditioned context (Context B, AB condition). Two hours later, the level of GluA1 and GluN2B receptors, Zif268 and phosphorylated-rpS6 (rpS6P) was measured in key brain areas for memory reactivation.

Retrieval in Context A significantly increased GluA1Rs and GluN2Brs in amygdala compared to no-retrieval, indicating that memory successfully reactivated and destabilized. Moreover, Zif268 level was significantly increased after retrieval in Context A in the nucleus accumbens shell, central and basolateral amygdala but not in the hippocampus, while retrieval in Context B significantly increased Zif268 level in all brain areas. On the other hand, rpS6P level was increased in the nucleus accumbens shell and central amygdala, but decreased in the hippocampus, after retrieval in Context A, while retrieval in Context B did not change rpS6P level in brain areas, except for a small but significant decrease in hippocampus.

While the increase of Zif268 level indicated that memory reactivation has been triggered in both the conditions, the lack of change in rpS6P levels after retrieval in Context B – in particular in the central amygdala – suggests that the reconsolidation process could not occur after memory reactivation in a context different from the conditioned one.

1. Introduction

The retrieval of previously consolidated memories may trigger a process of destabilization and lability: re-exposure to unconditioned stimuli, conditioned stimuli and/or context may reactivate and then destabilize emotional memories (Przybyslawski and Sara, 1997; Nader et al., 2000). Amnesic manipulations applied within a limited temporal period of few hours after retrieval (the ‘reconsolidation window’) may...
block the reconsolidation of appetitive memories (Fuchs et al., 2005; Milton and Everitt, 2012).

Although there is strong evidence that Pavlovian memory reconsolidation can be blocked (Lee et al., 2006; Milton and Everitt, 2010; Reichelt and Lee, 2013; Everitt, 2014), it is still debated whether instrumental memory can be reactivated and inhibited (Vousden and Milton, 2017). Early studies showed that instrumental memories did not undergo reconsolidation and therefore could not be disrupted (Hernandez and Kelley, 2004; Brown et al., 2008; Mierzejewski et al., 2009), whereas other authors suggested that these memories are more resistant to destabilization and reconsolidation in comparison to Pavlovian memories (Xue et al., 2012). The modalities under which an instrumental memory reconsolidation is disrupted for sucrose and nicotine instrumental memories occurs have only recently been investigated (Exton-McGuinness et al., 2014; Tedesco et al., 2014a; Exton-McGuinness and Lee, 2015). In our study (Tedesco et al., 2014a), we showed that a limited number of non-reinforced lever pressing on the lever previously associated to nicotine self-administration was able to retrieve and reconsolidate nicotine instrumental memory. This was demonstrated by an increased group response at reinstatement, and by the NMDA channel blocker MK-801 inhibitory effect on the retrieval but not on the no-retrieval group (a critical discussion of these findings are available in Exton-McGuinness et al., 2015).

The main critical issue and discrepancy among studies on instrumental memory reconsolidation are the conditions under which memory is reactivated and reconsolidation occurrence is assessed. It was apparent since early studies that reconsolidation took place only within specific boundary conditions including memory features (e.g., age, strength) (Mileikic and Alberini, 2002; Suzuki et al., 2004) and experimental parameters of retrieval (e.g., conditioned context, time duration, schedule of retrieval) (Auber et al., 2013; Reichelt and Lee, 2013; Dunbar and Taylor, 2017). Finnie and Nader (2012) reviewed the specific molecular events, such as the increase of GluA1-AMPA receptors (GluA1Rs) and of GluN2B-NMDA receptors (GluN2BRs), that are necessary for memory reactivation and for memory destabilization, respectively (Finnie and Nader, 2012). It appears that an experimental evidence of memory reactivation and reconsolidation occurrence might be based on molecular changes. For instance, we have recently reported that sucrose instrumental memory reconsolidation was inhibited by the metaplastic effect of MK-801 as shown by expression changes of transcription factor Zif268 and phosphorylated ribosomal protein S6 (rpS6P) (Piva et al., 2018).

With the present study we aimed to further characterize our model of instrumental memory reconsolidation (Tedesco et al., 2014a; Piva et al., 2018) by assessing the role of spatial context parameters in the process of instrumental memory reactivation. We investigated the expression pattern of Zif268 and rpS6P in the nucleus accumbens shell (NAcS), dorsal hippocampus (Hipp), amygdalar central nucleus (CeA) and basolateral complex (BLA) of rats exposed to instrumental memory retrieval (Piva et al., 2018). Animals trained to sucrose self-administration were re-exposed to a short non-reinforced retrieval session in the conditioned context (AA protocol). In order to test the effect of spatial context features, the same retrieval protocol was performed in a modified experimental box different from the conditioned one (AB protocol). The pattern of expression levels of Zif268 and rpS6P were quantified in the nucleus accumbens shell, dorsal hippocampus and in the amygdalar nuclei after retrieval or no-retrieval conditions for both protocols.

2. Results

The experimental schedule of instrumental memory reconsolidation is reported in Fig. 1. Western blot analysis of total GluA1Rs in the amygdala showed a significantly increased expression in rats exposed to retrieval (Ret) compared to rats exposed to no-retrieval (No-Ret) in Context A (Cx A) (+47% GluA1/tubulin ratio vs. No-Ret; t = 2.1, df = 14; p < 0.05, Student’s t-test), suggesting that our experimental condition induces the retrieval of sucrose instrumental memory in the amygdala (Fig. 2a).

Moreover, the analysis of total GluN2BRs in the same brain area showed a significantly increased expression in rats exposed to Ret compared to rats exposed to No-Ret in Cx A (+43% GluN2B/tubulin ratio vs. No-Ret; t = 3.2, df = 13; p < 0.01, Student’s t-test), suggesting that our memory retrieval is followed by memory destabilization in the amygdala (Fig. 2b).

The marker of memory reactivation Zif268 showed a significantly higher expression in the Ret vs. No-Ret condition in the NAcS, CeA and BLA but not in Hipp, respectively (mean + SEM, expressed as a percentage of No-Ret condition) +28.2 + 6.2% (t = 4.6, df = 10; p < 0.01, Student’s t-test), +30.7 + 5.4% (t = 5.7, df = 10; p < 0.001, Student’s t-test), +68.2 + 7.6% (t = 9.0, df = 10; p < 0.0001, Student’s t-test) and −11.0 + 7.7 (t = 1.4, df = 34; NS, Student’s t-test) (Fig. 3a). To analyze the salience of conditioned context on instrumental memory retrieval in our protocol, we assessed the level of Zif268 in a separate group of rats exposed to memory retrieval in a novel context (i.e. a modified version of the conditioned context, namely Context B – Cx B – provided with the same levers) (Fig. 1). Under this AB protocol, Zif268 expression pattern in the NAcS and in the amygdala was similar to the original AA protocol: +29.0 + 5.2% for NAcS (t = 5.6, df = 10; p < 0.001, Student’s t-test); +72.6 + 9.2% for CeA (t = 7.9, df = 10; p < 0.0001, Student’s t-test) and +49.4 + 7.4% for BLA (t = 6.7, df = 10; p < 0.0001, Student’s t-test). Interestingly, Zif268 was significantly increased in Hipp in Ret vs. No-Ret group, suggesting a context-related effect (+37.7 + 8.0%; p < 0.0001, Student’s t-test) (Fig. 3b).

As a further confirmation of reconsolidation process occurrence, we assessed the phosphorylation level of protein translation marker rpS6 in the same areas after Ret or No-Ret in Cx A or Cx B. Phosphorylated rpS6 was significantly higher after Ret vs. No-Ret in Cx A in NAcS and CeA but not in BLA, respectively +20.7 + 6.9% (t = 3.0, df = 10; p < 0.05, Student’s t-test), +78.5 + 14.4% (t = 5.5, df = 10; p < 0.001, Student’s t-test) and −3.6 + 7.4% (t = 0.5 df = 10; NS, Student’s t-test). In Hipp, rpS6P level was significantly reduced by the Ret exposure (−22.1 + 6.9%; t = 3.2 df = 34; p < 0.01, Student’s t-test) (Fig. 4a). After memory retrieval in Cx B, rpS6P expression level was not changed in the NAcS (−6.2 + 6.0%; t = 1.0, df = 9.9; NS, Student’s t-test), nor in the amygdalar nuclei CeA (4.9 + 10.0%; t = 0.5 df = 7.7; NS, Student’s t-test) and BLA (1.6 + 2.1%; t = 0.7, df = 9.9, NS, Student’s t-test). In Hipp, rpS6P was only slightly decreased (−17.6 + 8.5%; t = 2.1, df = 34; p < 0.05, Student’s t-test) after Ret in Cx B (Fig. 4b).

A summary of the effects of memory retrieval in Cx A or Cx B on Zif268 or rpS6P level is present in Table 1.

Finally, we tested sucrose instrumental memory reconsolidation of rats exposed to Ret or No-Ret in Cx A or Cx B (four subgroups: Ret/Cx A; No-Ret/Cx A; Ret/Cx B; No-Ret/Cx B) with an extinction test in the training context (Cx A; Fig. 5a). Extinction test showed no differences between active lever presses/60 min for No-Ret (87.6 ± 7.1; mean + SEM) vs. Ret (98.1 ± 7.6) condition after memory reactivation in Cx A (t = 1.0, df = 33.4; NS, Student’s t-test). Similarly, extinction test after memory reactivation in Cx B showed no differences between active lever presses/60 min for No-Ret (100.8 ± 14.2) vs. Ret (109.0 ± 11.6) condition (t = 0.4, df = 14.0; NS, Student’s t-test) (Fig. 5b).

3. Discussion

In summary, the reactivation of instrumental memory for sucrose under our AA condition (retrieval in the conditioned context) induces reactivation and destabilization processes (increased GluA1Rs and GluN2BRs respectively) in amygdala. These processes, confirmed by increased Zif268 level in the amygdalar nucleus, allow for sucrose instrumental memory reconsolidation, as demonstrated by the increase of...
Although the control AB condition (retrieval in a modified version of conditioned context) showed a similar increase of Zif268 in the amygdala, the lack of change for rpS6P level in CeA suggested that memory reactivation did not occur after retrieval in a context different from the conditioned one. These findings support our previous data that reconsolidation of appetitive instrumental memory may occur after a non-reinforced retrieval of instrumental responding in the conditioning context. The two-component Zif268/rpS6P assay appears reliable for molecular assessment of instrumental memory reactivation and reconsolidation. In fact, reconsolidation occurrence as an increased behavioural response has rarely been seen (Fuchs et al., 2009; Lasseter et al., 2011; Flavell and Lee, 2013; Tedesco et al., 2014a). The occurrence of reactivation and destabilization was confirmed by increased expression of GluA1 and GluN2B receptors in the amygdala. GluN2B receptors have been shown to play a central role in the molecular processes that allow the destabilization of a memory trace (Finnie and Nader, 2012), whereas GluA1 receptors have been reported to be implicated in memory reactivation (Monfils et al., 2009; Clem and Huganir, 2010).

The transcription factor Zif268 has been widely used as a marker of memory reactivation and reconsolidation (Lee et al., 2004; Lee, 2008; Besnard et al., 2013). In particular, selective inhibition of Zif268 mRNA and protein in BLA is specifically involved in appetitive memory reconsolidation (Lee et al., 2005; Lee et al., 2006; Lee, 2008; Theberge et al., 2010; Besnard et al., 2013; Exton-McGuinness and Lee, 2015). We have demonstrated that high expression levels of Zif268 in amygdala and NAcS (Tedesco et al., 2014b; Piva et al., 2018) were correlated to memory reactivation and reconsolidation. Significant Zif268 increase in CeA but not in dorsal hippocampus further suggest a role of contextual information, without apparently involving a hippocampal-related spatial information component.

Furthermore, it was reported that the mechanistic target of rapamycin complex 1 (mTORC1) inhibition, followed by reduction of downstream rpS6 phosphorylation (Biever et al., 2015) was associated to alcohol-related memory reconsolidation (Barak et al., 2013). This and other reports (Tedesco et al., 2014b; Piva et al., 2018) proposed rpS6P as a molecular marker for memory reconsolidation. The role of CeA-related contextual value is confirmed by an increased level of rpS6P in the CeA. This increase is also a confirmation of the validity of

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### EXPERIMENTAL SCHEDULE

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<td>FORCED ABSTINENCE</td>
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<tr>
<td>RETRIEVAL</td>
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**Fig. 1.** Schematic diagram of the experimental protocol and groups. Boxes represent the different procedures used at the different phases of the study. Arrow represents time progression between consecutive phases. Cx A = sucrose self-administration training context; Cx B = modified version of the training context.

**Fig. 2.** Effect of retrieval on (a) total GluA1 and (b) total GluN2B receptors in amygdala. Upper part of the figure, representative immunoblots for (a) GluA1 (108 kDa, right) and (b) GluN2B (180 kDa, left) receptors level in the amygdala (Amy). In the bottom part of the figure, graphs representing the quantification of the level of total (a) GluA1 and total (b) GluN2B receptors 2 h after No-Retrieval (No-Ret; open column) or Retrieval (Ret; solid column) in the training context (Context A) of sucrose instrumental memory in rats. Data are shown as the mean + SEM and are expressed as a percentage of No-Ret. N = 7–8 rats/group. *p < 0.05; **p < 0.01 vs. No-Ret (unpaired Student’s t-test).
Fig. 3. Immunohistochemistry assessment of Zif268 expressing cells/mm² in NAcS, Hipp, CeA and BLA 2 h after No-Retrieval (No-Ret; open column) or Retrieval (Ret; solid column) in (a) the training context (Context A) or (b) in a modified version of the training context (Context B) of sucrose instrumental memory in rats. On top of both panels are reported representative images of brain areas of interest. Data are shown as mean ± SEM and are expressed as a percentage of No-Ret. Three adjacent sections, both hemispheres, N = 6 rats/group. ***p < 0.001; ****p < 0.0001 vs. No-Ret (unpaired Student’s t-test). Scale bar, 100 μm.
Immunohistochemistry assessment of rpS6P expressing cells/mm² in NAcS, Hipp, CeA and BLA 2 h after No-Retrieval (No-Ret; open column) or Retrieval (Ret; solid column) in (a) the training context (Context A) or (b) in a modified version of the training context (Context B) of sucrose instrumental memory in rats. On top of both panels are reported representative images of brain areas of interest. Data are shown as mean ± SEM and are expressed as a percentage of No-Ret. Three adjacent sections, both hemispheres, N = 4–6 rats/group. *p < 0.05; **p < 0.01; ***p < 0.001 vs. No-Ret (unpaired Student’s t-test). Scale bar, 100 μm.
this marker for memory reconsolidation (Barak et al., 2013; Tedesco et al., 2014b). Moreover, the presence of increased rpS6 phosphorylation in the NAcS in our study reveals the involvement of brain nuclei which are relevant to appetitive memories. In the context-control condition AB, rpS6P level was not different between retrieval and no-retrieval in CeA and NAcS, further confirming the specificity of our protocol of instrumental memory reactivation and reconsolidation under the AA condition. The molecular change in pattern of Zif268 and rpS6P in the areas of interest was different between AA and AB condition, suggesting the occurrence of memory reactivation and reconsolidation only under the former. Currently, however, we cannot explain rpS6P decrease in dorsal hippocampus, although we exclude that this reduction is due to different diurnal oscillation in the retrieval vs. the no-retrieval group (Saraf et al., 2014).

The protocol used in the present study is similar to the one we

Table 1
Summary table of the direction of effects of memory retrieval in sucrose self-administration training context (AA protocol) or in a modified version of the training context (AB protocol) on Zif268 and rpS6P.

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<td>BLA</td>
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NAcS, nucleus accumbens shell; Hipp, dorsal hippocampus; CeA, central nucleus of the amygdala; BLA, basolateral complex of the amygdala. Symbols: ↑ = increase; ↓ = decrease; 0 = no change vs. No-Ret.

Fig. 5. Effect of memory reactivation on sucrose instrumental memory reconsolidation. (a) Schematic diagram of the experimental protocol and groups. Boxes represent the different procedures used at the different phases of the study. Arrow represents time progression between consecutive phases. Cx A = sucrose self-administration training context; Cx B = modified version of the training context (b) Extinction test performance in Context A 24 h after No-Retrieval (No-Ret, open columns) or Retrieval (Ret, solid columns) in Context A (left, AAA) or in Context B (right, ABA). Ordinate represents the number of active lever presses/60 min session of the behavioural test. Data are expressed as mean ± SEM. N = 19 No-Ret AAA; N = 17 Ret AAA; N = 8 No-Ret ABA; N = 9 Ret ABA.
previously used for the reactivation and reconsolidation of nicotine instrumental memory. In that study (Tedesco et al., 2014a), rats were trained to lever press for nicotine reinforcement without association to any discrete cue (e.g., light or tone stimulus). In the present study, we similarly trained rats to respond for sucrose pellets under a limited number of earned reinforcements/session, for a fixed number of daily sessions. After a two-week forced abstinence, retrieval of instrumental memory was induced by 20 non-reinforced lever presses, similarly to the nicotine protocol in Tedesco et al. (2014a).

The reconsolidation of drug-associated memory has been demonstrated in different Pavlovian conditioning paradigms using NMDARs antagonists, such as MK-801, as amnesic agents closely to memory reactivation (Sorg, 2012). However, only recently Exton-McGuinness et al. (2014) reported that destabilization and reconsolidation of well-learned instrumental response for palatable food is possible – as confirmed by inhibited conditioned response for sucrose after MK-801 administration. They showed that instrumental memory reconsolidation occurred when retrieved under the Variable-Ratio (VR20:S′) schedule of reinforcement, but not under the Fixed-Ratio (FR20:S′) or non-reinforced schedules. Their findings were different from the earliest studies reporting no reconsolidation of instrumental memory after both long (Hernandez and Kelley, 2004) and brief (Mierzejewski et al., 2009) training. In their paper, Exton-McGuinness et al. (2014) suggested and demonstrated that a VR schedule may be a more efficient method to destabilize a memory, supporting their hypothesis on the memory destabilization easier.

It could be the case that under our experimental conditions the non-reinforced retrieval induced a prediction error signal greater than the one generated with the non-reinforced session in Exton-McGuinness et al. (2014). In fact, our instrumental training conditions of 10 daily sessions lasting up to 12 delivered sucrose pellets (Exton-McGuinness et al. 2014) used 10 daily sessions lasting up to 60 pellets were obtained or 30 min elapsed) could have created a weaker memory trace, which could be a boundary condition factor making memory destabilization easier.

Finally, the extinction test in the Context A applied 24 h after memory retrieval or No-retrieval in Context A or Context B did not show any difference between the four conditions (Ret/Cx A; No-Ret/Cx A; Ret/Cx B; No-Ret/CxB). However, as previously mentioned, memory reconsolidation has been demonstrated mainly through its inhibition using amnesic agents, protein synthesis inhibitors or NMDARs blockers (Reichelt and Lee, 2013; Haubrich and Nader, 2016). Then, the lack of difference between retrieval and no-retrieval condition is reasonable, considering the absence of a pharmacological treatment closely to memory reactivation process.

4. Conclusions

In conclusion, this molecular approach confirmed that our protocol is able to reactivate instrumental appetitive memory and, possibly, to trigger reconsolidation for sucrose. Together with our previous study on reconsolidation of nicotine and sucrose instrumental responding (Tedesco et al., 2014a; Piva et al., 2018) and those from others (Exton-McGuinness et al., 2014, Exton-McGuiness et al., 2015; Exton-McGuinness and Lee, 2015), we suggest that instrumental appetitive memory could undergo reactivation under context-specific conditions that involve CeA.

5. Experimental procedure

5.1. Animals

Ninety-three male Sprague-Dawley rats (Charles River, Italy) were housed in pairs in temperature and humidity-controlled environment (19–23 °C, 60 ± 20%) on a 12-h light/dark cycle, with light on at 7:30 pm. Rats were food restricted to maintain their body weight in the range of 250 ± 10 g (daily checked), and food (two to four pellets, 10–20 g/day) was made available after each experimental session. Water was available ad libitum, except during experimental sessions. Animals were trained or tested once daily during the dark phase of the light/dark cycle, and all the experimental procedures were carried out in accordance with the European Community Directives (2010/63/EU) and with the Italian Law on Animal Research (D.Lgs. n. 26/2014). All efforts were made to minimize animal suffering and to keep the lowest number of animals used.

5.2. Apparatus

Rats were trained and tested in operant chambers (Coulbourn Instruments, Lehigh Valley, Whitehall, PA, USA) encased in sound-insulated cubicles equipped with ventilation fans (Ugo Basile, Comerio, Italy). Each chamber was equipped with two levers, an active (right) and an inactive lever (left), symmetrically oriented laterally to the food magazine, on the frontal panel. Levers were located 2 cm and food magazine 1 cm above the grid floor. A 2-W white house light located 26 cm above the food magazine provided ambient illumination during the entire session duration of food-shaping, retrieval and reinstatement phases, and for the entire session except for time-out (TO) periods during training phase. Right lever presses produced the delivery of a 45-mg sucrose food pellet (Bilaney Consultants Ltd, UK) with a Fixed-Ratio 1 (FR1) schedule of reinforcement during food shaping and training. Left lever presses did not have consequences. Lever presses and pellet deliveries were recorded, as well as schedule parameters and data acquisition were controlled, by Med-PC software (Med Associates Inc., St Albans, Vermont, USA).

5.3. General procedure

The experimental protocol was designed according to the following phases: Phase I) training to sucrose pellets self-administration (S/A), Phase II) forced abstinence in home cage and, Phase III) memory retrieval (Ret) or no-retrieval (No-Ret). After these three subsequent phases, rats were sacrificed for immunoblotting or immunohistochemical staining. During Phase III, rats were exposed to Ret or No-Ret session in the training context, i.e. Context A (AA protocol) or in a modified version of the training context, i.e. Context B (AB protocol). Separate groups of rats exposed to Ret or No-Ret in Context A or Context B were tested with an extinction test in the Context A (Phase IV) 24 h after Phase III.

5.4. Lever press shaping and training to sucrose self-administration procedures

All rats were initially shaped to associate right lever presses with sucrose pellets as reinforcements. The schedule was FR1:45-mg sucrose pellet, session duration up to 100 reinforcements or 120 min. Once the criterion of 100 reinforcements was reached, rats started training Phase I. During Phase I, right lever pressing corresponded to the delivery of sucrose reinforcements with the schedule: FR1:45-mg sucrose pellet, 60-s inter-reinforcement TO, session duration up to 12 reinforcements or 60 min. During TO right lever presses had no consequences, and the house light switched off. Left lever presses had no consequences for all the experimental sessions. After 10 days of sucrose S/A training, during the forced abstinence phase rats remained in their home cages for
14 days.

5.5. Memory retrieval procedure and molecular analysis

After forced abstinence phase, 2 groups of rats were exposed to Ret or to No-Ret session in the training context. During the Ret session, both levers were presented and rats were allowed to press right active lever up to 20 times, with house light ON. Lever presses had no consequences. During the No-Ret session, no levers were presented and house light was OFF. During Ret or No-Ret session, animals spent a similar amount of time in the training context (181 ± 10 s; mean ± SEM). Two separate groups of rats were exposed to Ret or to No-Ret session in a novel context (Context B; operand conditioning chamber with 5-cm blank striped sheets on the walls and a 1-cm grid on the floor; Auber et al., 2014). Two hours after the first lever pressed during Ret or after the beginning of No-Ret session, all rats were sacrificed for Zif268 and rpS6P immunohistochemistry or GluN2B and GluA1 receptors under western blot investigations.

5.6. Immunohistochemistry

Rats were anesthetized with 350 mg/kg/2 mL i.p. of chloral hydrate (Fluka, Italy), then transcardially perfused with heparin 100 UI/L (Sigma–Aldrich, Milan, Italy) in saline solution and paraformaldehyde (PFA) 4% in phosphate buffered saline solution (PBS). Brains were removed and post-fixed for 2 h at 4 °C into PFA 4% in PBS, then washed 3 times with PBS and left in sucrose 30% in PBS as cryoprotective for 48 h. Free-floating sections (40 µm) containing NAcS (corresponding to a bregma + 1.70 mm section from Paxinos & Watson, 1998), Hipp (bregma + 3.00 mm), CeA (bregma –1.88) and BLA (bregma –3.00 mm) were processed for Zif268 and rpS6P immunoreactivity. After washing in PBS, endogenous peroxidase was neutralized with hydrogen peroxide 0.75% in PBS for 10 min. Sections were blocked with 0.5% Horse Serum (HS; BioWhittaker-Lonza, Basel, Switzerland) + 0.5% Triton X-100 (Sigma-Aldrich, Milan, Italy) in PBS wash solution, and then incubated overnight at 4 °C with anti-Zif268 (1:1000), Santa Cruz, rabbit polyclonal) or anti-PSer235/236-rpS6 antibody (1:1000, Cell Signaling, rabbit polyclonal) or anti-PSer235/236-rpS6 antibody (1:1000). Cell Signaling, rabbit polyclonal) in wash solution. After washes in wash solution, slices were incubated for 2 h at room temperature with anti-rabbit biotinylated antibody (1:1000, AmershamGE Healthcare Europe, Milan, Italy). Following washes in wash solution, and finally in PBS, tissue sections were visualized using VectaStain ABC kit (Vector Laboratories, Rome, Italy) and developed in DAB (3,3-diaminobenzidine) peroxidase substrate (Sigma-Aldrich, Milan, Italy) for 3–4 min. Sections were mounted on gelatin-coated slides, dehydrated and then closed with Entellan (Merck-Millipore, Darmstadt, Germany). The sections were acquired using a light transmission microscope (Axioscope 2 Zeiss, Zeiss). Six images for each region (1 for each hemisphere, 3 sections for each rat, that is 2 × 3 × 6 images/region/rat) were acquired by the connected video camera (Optikam B3) using a 10X objective. Counts of the number of neurons positive to Zif268 and rpS6P were carried out using the NIH software ‘ImageJ’ (www.rsweb.nih.gov) (Caffino et al., 2016).

5.7. Western blot

Rats were anesthetized with 350 mg/kg/2 mL i.p. chloral hydrate (Fluka, Italy), then brains were removed, and 1-mm slices containing amygdala (bregma –3.00 mm) were dissected by using a 1-mm Coronal Brain Matrix (SouthPointe Surgical Supply, Florida, USA). Amygdala were lyzed in a tube containing 1% sodium dodecyl sulfate (SDS). Total protein levels were quantified using the Pierce (Rockford, IL, USA) BCA (bicinchoninic acid) Protein Assay. Forty micrograms of proteins were resolved by electrophoresis on a 8% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes using the transblot TURBO (Bio-Rad, Hercules, CA, USA). Blots were probed overnight at 4°C with the polyclonal rabbit anti-GluA1 or the monoclonal mouse anti-GluN2B antibody (Thermo Scientific, Rockford, IL USA) diluted 1:1000 in 5% milk. Immunodetection was performed with the secondary antibody anti-rabbit or anti-mouse (1:2000) (Amersham Biosciences, UK) conjugated to horseradish peroxidase. The reactive bands were detected using chemiluminescence (ECLplus; Euroclone, Padova, Italy). Quantitative analysis was performed using the QuantityOne analysis software (Bio-Rad, Hercules, CA, USA) (Gerace et al., 2014).

5.8. Extinction test

Twenty-four hours after Ret or No-Ret in Context A or Context B, four separate groups of rats – Ret/CxA; No-Ret/CxA; Ret/CxB; No-Ret/CxB – were tested for sucrose instrumental memory reconsolidation with an extinction test in the Context A. During the 1-h test house light was ON, both levers were presented and rats were allowed to press either the active or the inactive one without any time-out. All lever presses were recorded, but they had no consequences during the test.

5.9. Data analysis

For the immunohistochemistry experiments, intensity threshold, minimum and maximum cell size values were initially determined in an empirical fashion under blind conditions. The dependent variable for the immunohistochemistry experiments was the positive cell count/ mm² for Zif268 or rpS6P. For Hipp, data were pooled from the three subareas: CA1, CA2 and CA3. Immunohistochemistry and western blot data were expressed as mean ± SEM percentage of the No-Ret rats. For the extinction test, the number of active lever presses/60 min for the four groups – Ret/CxA; No-Ret/CxA; Ret/CxB; No-Ret/CxB – was analyzed as dependent variable to assess the effect of retrieval (or no-retrieval) in the conditioning context Cx A (or in a modified version of the context, Cx B). After Grubbs’ test to identify outliers, one subject for Ret/CxA and one for No-Ret/Cx A were excluded from data analysis. All analysis were performed as unpaired Student’s t-test using the GraphPad software package (Prism, version 4; GraphPad, San Diego, California, USA).

Author disclosure

Declarations of interest: none.

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References


