TITLE: Recovering spatial information through reactivation: brain oxidative metabolism involvement in males and females.

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Abstract

Memory involves a complex network system of interconnected brain areas in which labile trace memories are transformed into enduring ones and reorganized in a time-dependant manner. Although it has been observed that remote memories are less prone to destabilizing, they can become fragile and lead to behavioural decline. We explored the behavioural outcomes of male and female rats in response to the reactivation of a previously acquired allocentric spatial reference memory, under conditions in which animals have shown a retrieval decay. In addition, we assessed their brain metabolic activity through cytochrome c oxidase histochemistry. Our results show that a spatial memory amnesia-like behaviour with a time interval of 45 days can be recovered after re-exposure to the environmental configuration with the reinforced contingencies. Moreover, we observed that, following reactivation, male rats reveal a decrease in metabolic activity in septal nuclei and thalamic structures, whereas female rats add a metabolic reduction in the hippocampus, amygdala, mPFC, and retrosplenial and parietal cortices, suggesting that they efficiently employ these brain areas when reactivation a memory that has suffered a decay with time. Finally, although male and female rats perform the behavioural task equally, we found sex differences at the brain metabolism level, revealing the differential contribution of brain limbic system energy demands by sex, even when their performance is similar. In conclusion, our work provides behavioural and brain data about remote spatial retrieval and memory reactivation processes.

Keywords: cytochrome c oxidase; forgetting; reactivation; retrieval; spatial memory.

Introduction

Memory formation involves a complex network system of interconnected brain areas that encode, consolidate, and store information, in which labile trace memories are transformed into enduring ones (Lechner et al., 2018; Larry R. Squire et al., 2015). The purpose is to retrieve stored items, when necessary, in order to guide our behaviour according to our own experiences and the challenging environmental demands (Frankland et al., 2019; Spear, 1973). The traditional view of memory processes argued that, following consolidation, a memory becomes stable and fixed, with the subsequent phases interpreted as passive processes (McGaugh, 1966). However, today we know that all the entities related to memory are active from a neurobiological point of view, including the maintenance of memories over time, retrieval, forgetting, or extinction (Haubrich & Nader, 2018; Mendez et al., 2018). Previously consolidated memories, when reactivated, can re-enter a labile state where they are capable of being modified, leading subsequently to reconsolidation (Misanin et al., 1968). Thus, they could be altered or even erased, even when successfully consolidated (Misanin et al., 1968).

It is not clear how memories are destabilized and under what circumstances it occurs (Haubrich & Nader, 2018). In fact, it has been suggested that the possibility of a trace memory entering a fragile state following reactivation depends on multiple conditions such as the time elapsed since learning consolidation, proposing the idea that older memories will be in a state protected from potential interferences following reactivation (Bustos et al., 2009; Eisenberg & Dudai, 2004; Suzuki et al., 2004; Winters, Boyer et al., 2009). Nevertheless, it is important to mention that other authors have observed the opposite: although memories were consolidated a long time ago, they can also be destabilized in a time-dependent manner (Diergaarde et al., 2006; Robinson & Franklin, 2010; Wang et al., 2009).

Spatial cognition has been widely explored from multiple approaches in human and animal models, due to its marked relevance for survival (Epstein et al., 2017; Vorhees & Williams, 2014). Although there is some consensus about the dynamic nature of the brain networks involved in spatial learning and memory (Solari & Hangya, 2018), relatively few studies have addressed allocentric spatial approaches –that is, a navigation strategy that integrates and establishes associations between multiple information sources present in the environment (Epstein et al., 2017; Tolman, 1948)– during retrieval with long time

intervals after learning acquisition. We previously observed (Zorzo et al., 2020) that healthy male and female rats successfully remember allocentric spatial information up to 30 days, but they show a retrieval deficit after this period of time, particularly when 45 and 60 days have elapsed since learning acquisition. We suggested that, as time goes by, the cognitive process of recovering the necessary spatial information to successfully reach a particular location could become increasingly difficult, leading to behavioral forgetting. There are two main theories related to the underlying brain regions involved in remote memories. The standard model supports the idea that memories encoded in the hippocampus are later stored in the neocortex (Albo & Gräff, 2018; L R Squire & Alvarez, 1995), and the multiple trace theory argues that in addition to the neocortex, the hippocampus is always needed during retrieval (Nadel & Moscovitch, 1997; Wartman et al., 2014). In this regard, some authors have revealed the functional role of the prefrontal cortex (Barry et al., 2016; Teixeira et al., 2006), the parietal cortex (Barry et al., 2016; Gusev & Gubin, 2010), the retrosplenial cortex (Barry et al., 2016; Gusev & Gubin, 2010) and the hippocampus (Barry et al., 2016; Broadbent, 2006; Martin et al., 2005). Other cortical regions that have also been linked to remote retrieval are the perirhinal cortex (Barry et al., 2016) and the entorhinal cortex (Barry et al., 2016; Hales et al., 2018), in addition to subcortical structures such as the thalamus (Lopez et al., 2009; Loureiro et al., 2012). Furthermore, the septum and amygdala have been related to spatial memory function (Arias et al., 2015; Conejo et al., 2010), but have not yet been portrayed in remote spatial retrieval.

The assessment of the contribution of different brain areas during cognitive processes, including spatial memory, can be explored by labelling cytochrome c oxidase (CCO) activity given that it represents a measure of the energy consumption after behavioral tasks (Gonzalez-Lima & Cada, 1994; González-Pardo et al., 2012). CCO is a mitochondrial enzyme involved in ATP production, thus, it is an endogenous marker of oxidative energy consumption in cellular respiration (Gonzalez-Lima & Cada, 1994; Wong-Riley, 1989). Hence, the quantitative CCO histochemisty represents an index of mitochondrial competence (Wong-Riley, 1989), which is commonly used in order to study cognitive responses (Rubio et al., 2012; Wong-Riley, 1989).

In the present study, we aimed to investigate the behavioral consequences of memory reactivation under conditions in which we previously observed a retrieval deficit (Zorzo et al., 2020), with the same time-interval from learning acquisition. To do this, we trained

male and female rats on an allocentric spatial reference protocol performed in the Morris Water Maze (MWM) on five consecutive days, and we evaluated remote memory and memory reactivation 45 days post-learning acquisition. We aimed to study the CCO activity in response to the behavioural outcome to decipher the underlying brain metabolic demands, in addition to the potential differences between male and female rats. In particular, we addressed brain areas linked to remote spatial memories, such as the neocortex (prefrontal, parietal, retrosplenial), hippocampus and related cortices (entorhinal and perirhinal) and thalamus, but also the septum and amygdala due to its functional contribution on spatial learning and memory processes.

Material and methods

Animals

A total of 17 young male Wistar rats (*Rattus norvegicus)* (255.28 ± 13.99 g. at the beginning of the experiment) and 17 young female Wistar rats (198.39 ± 5.53 g. at the beginning of the experiment), all of them aged 12-13 weeks, were employed. All the animals were housed in plastic cages (34 x 20 x 21 cm) (Tecniplast-Leica, *Madrid*) in a room with a constant temperature (20 ± 2 ºC), relative humidity (65-70 %), a 12-h artificial light-dark cycle (08:00-20:00 / 20:00-08:00 h), and *ad libitum* access to **food** and tap water. The animals were divided into four groups: male retrieval 45 days post-learning acquisition (R45M), n=9; female retrieval 45 days post-learning acquisition (R45F), n=9; male reactivation 45 days post-learning acquisition (RA45M), n=8; and female reactivation 45 days post-learning acquisition (RA45F), n=8. Prior to conducting the behavioural procedures, all the animals were handled daily for seven days in order to reduce the stress generated by contact with the experimenter. The behaviour tests were performed between 8:00 h and 14:00 h.

The procedures and manipulations of the animals employed in the present study were carried out according to the European Communities Council Directive 2010/63/UE and Royal decree Nº 53/2013 of the Ministry of the Presidency related to the protection of animals used for experimentation and other scientific purposes, and the study was approved by the local committee for animal studies of the Agriculture Council of the Principality of Asturias.

Behavioural procedure

Apparatus

Spatial reference learning and memory, remote memory, and memory reactivation were evaluated in the pool designed by Morris (Morris, 1984). It consists of a black circular fiberglass tank measuring 150 cm in diameter and 49 cm in height, placed 35 cm above the floor. The pool was filled with tap water with a temperature of 22 ± 2°C, and the water level was 39 cm. We used the Atlantis platform (Ugo Atlantis platform, *Noldus Information Technologies, Wageningen, The Netherlands*) (Spooner et al., 1994), which consists of an adjustable escape platform measuring 10 cm in diameter, 37 cm in height when accessible, and 27 cm in height when inaccessible. When we wanted to make it accessible, we hid it two cm below the surface of the water, whereas when we wanted to make it inaccessible, we hid it 12 cm below the surface of the water. The pool was divided into four imaginary quadrants (A, B, C, and D) to locate the start positions, and the escape platform was located in the centre of quadrant D. The MWM was located in the centre of a room measuring 16 m^2, illuminated by an indirect light of 4000 lx from two lamps facing the walls of the room. The pool was surrounded by black panels, located 30 cm away from the maze, on which allocentric cues were placed. Specifically, they consisted of five geometric visual cues with different volumes and colour patterns. We recorded *(*V88E, *Sony, Spain*) the behaviour of the experimental groups, and we used a computerized video-tracking system (Ethovision XT 14.0, *Noldus Information Technologies, Wageningen, The Netherlands*) to analyze the path.

Habituation

The first day of the protocol was devoted to habituation to the testing contingencies of the MWM. Therefore, R45M, R45F, RA45M, and RA45F performed four trials in which they had to reach a visible platform located in the centre of the maze that protruded four cm from the water. On each trial, the rats were released from each quadrant (A, B, C, or D) facing the pool wall, following a pseudo-randomised sequence. Once the habitation session had ended, the animals were carefully dried and returned to their home cage.

Spatial reference learning and memory task

On the following five days after habituation, R45M, R45F, RA45M, and RA45F rats were required to locate a hidden platform placed in the centre of quadrant D (target quadrant)

in relation to the external visual cues located in the black panels. Training was performed in blocks of six trials per day: four acquisition trials, one learning probe trial, and one additional trial to avoid possible extinction of learning (Figure 1). The training trials ended when the animal had found the platform or, if not, when 60 s had elapsed. To begin each trial, the rats were placed in the water facing the maze wall in one of four quadrants, and the daily order of entry into these quadrants was pseudo-randomized. Once the rats had found the platform, they remained in the reinforced place for 15 s. If the animals failed to reach the platform after 60 s, they were gently guided to the platform. The inter-trial interval consisted of 30 s, during which the animals were placed in a black bucket. Following these four acquisition trials, a learning probe trial was carried out. On this test, the escape platform was removed, and the animals were introduced into the pool for 60 s in the quadrant opposite to where the platform had been located in previous trials (in this study, quadrant C), in order to check whether the animals remembered the platform location. Then, the rats were moved to the black bucket for 30 s, and, finally, they received an additional trial with the escape platform located in the usual position to avoid extinction. Once the daily learning session had ended, the animals were dried and returned to their home cage. We recorded latencies to reach the platform during the first four trials of the spatial reference learning and memory task, and the permanencies in each quadrant during the learning probe trial. We also recorded speed and cumulative distance.

Spatial reference remote retention task

R45M and R45F rats were submitted to a memory retention task 45 days post-learning acquisition in a single 60 s remote retention probe trial (Figure 1). For this purpose, the platform was removed from the pool, and the animals were released from the opposite quadrant (quadrant C) under the same conditions as those described in the learning probe trial performed during the spatial reference learning and memory task. Once the daily remote retention task had ended, the animals were dried and returned to their home cage. We recorded permanencies in each quadrant during the remote retention trial and speed.

Spatial reference memory reactivation task

RA45M and RA45F rats were submitted to a memory reactivation task 45 days post-learning acquisition (Figure 1). It consisted of seven consecutive trials using the Atlantis Platform (Ugo Atlantis platform, *Noldus Information Technologies, Wageningen, The Netherlands*) (Spooner et al., 1994), which allows the evaluation of remote memory and

avoidance of extinction because the platform can remain inaccessible for a predetermined time and appear again after a selected delay, due to the fact that it is already accessible, but hidden under the water level (Broadbent, 2006; Morris et al., 2006). The first trial (remote retention probe trial) was performed with the aim of verifying the absence of spatial remote retrieval in order to study reactivation processes. The evaluation consisted of a single trial lasting 60 s with the escape platform located 12 cm below the water level, thus remaining inaccessible. The rats were introduced into the pool for 60 s in the quadrant opposite to where the platform had been located during the spatial reference learning and memory task. Once 60 s had elapsed, the escape platform became accessible but was hidden two cm below the surface of the water in a second trial (extinction avoidance (EA) trial), without removing the animal from the pool, thus allowing it to serve as a reinforced memory test. If the animals failed to reach the platform after 60 s, they were gently guided to the platform. Then, an inter-trial interval of 30 s took place, during which the animals were placed in a black bucket. After the inter-trial interval after EA, four reinforced reactivation (RA) trials were conducted, with the platform accessible but hidden two cm below the surface of the water. In these trials, the rats were placed in the water facing the maze wall in one of four quadrants, pseudo-randomized. Once the animals had found the platform, they remained in the reinforced place for 15 s. If the animals failed to reach the platform after 60 s, they were gently guided to the platform. After each trial, an inter-trial interval of 30 s took place, during which the rats were placed in a black bucket. Finally, in the last 60 s, a reactivation probe trial was carried out in order to assess whether the animals remembered the location of the platform after the spatial reactivation task. For this purpose, the escape platform was located 12 cm below the water level, thus remaining inaccessible, and the rats were introduced into the pool in the quadrant opposite to where the platform had been located during the spatial reference learning and memory task and the spatial reference memory reactivation task. Once the spatial memory reactivation task had ended, the animals were dried and returned to their home cage. We recorded the permanencies in each quadrant during the remote retention and reactivation probe trials when the platform was inaccessible. We also recorded the latencies to reach the platform during the RA trials, speed, and cumulative distance.

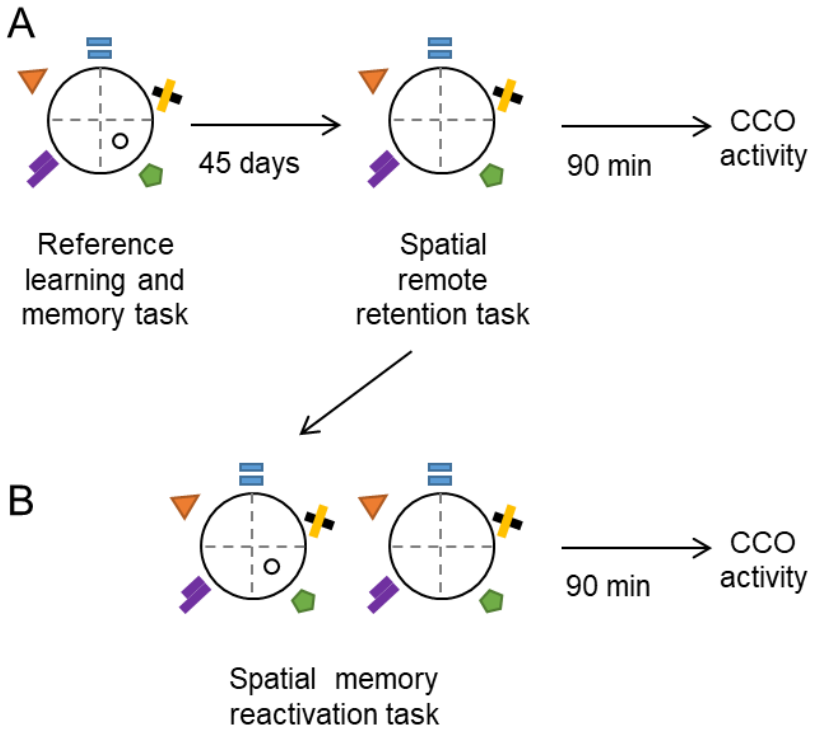

Figure 1.

Sacrifice and brain processing

R45M and R45F rats were decapitated 90 minutes after the remote retention probe trial. RA45M and RA45F rats were decapitated 90 minutes after the last reactivation probe trial. The brains were immediately removed, frozen in isopentane (*Sigma-Aldrich, Germany*), and stored at -40 °C to make coronal sections with a thickness of 30 µm in a cryostat at -20 ° C (*Leica CM1900, Germany*). For CCO histochemistry, we mounted the sections on non-gelatinized slides. We anatomically defined the regions of interest according to Paxinos and Watson's atlas (Paxinos and Watson, 2005). The regions of interest and their distances in mm counted from bregma were: +3.24 mm for the cingulate (CG), infralimbic (IL), and prelimbic cortex (PL); +0.72 mm for the septum (medial septum (MS) and lateral septum (LS)); -1.44 mm for thalamus (anterodorsal (ADT), anteroventral (AVT) and mediodorsal (MDT)); -2.28 mm for the amygdala (central (CeA), basolateral (BLA) and lateral (LaA)); -3.48 mm for the CA1 and CA3 subfields of the dorsal hippocampus, dentate gyrus (DG), granular retrosplenial (RSG), agranular retrosplenial (RSA), and parietal cortex (PAR); and −4.68 mm for the entorhinal (ENT) and perirhinal (PHr) cortices (Figure 2).

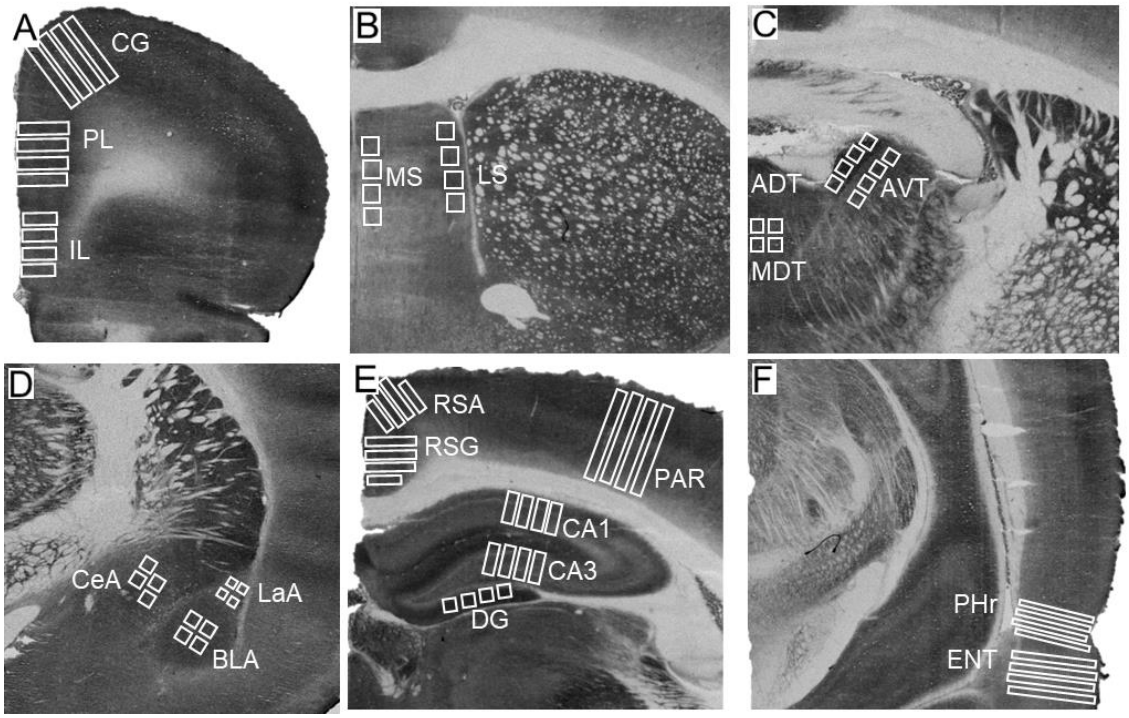

Figure 2.

Cytochrome c oxidase histochemistry

Brain tissue was processed with quantitative CCO histochemistry, as described by Gonzalez-Lima & Cada (1994) and following the protocol previously described by González-Pardo et al. (2019). Briefly: sets of tissue homogenate standards from control Wistar rat brains were cut at thicknesses of 10, 30, 50 and 70 µm and included with each bath of brain tissue slides. Then, standards and sections were incubated in 0.1 phosphate buffer (PB) with 10% (w/v) sucrose and 0.5 (v/v) glutaraldehyde. Later, three baths of 0.1 PB with 10% (w/v) sucrose were used, following a bath in 0.05M Tris buffer, with 275 mg/l cobalt chloride, sucrose, and 0.5 (v/v) dimethylsulfoxide. Then, sections and standards were incubated in a solution containing 0.0075 % cytochrome-c (w/v), 0.002 % catalase (w/v), 5 % sucrose (w/v), 0.25 % dimethylsulfoxide (v/v), and 0.05% diaminobenzidine tetrahydrochloride (*Sigma-Aldrich, Spain*). The reaction was stopped in buffered 4% (v/v) formalin. Finally, the slides were dehydrated, cleared with xylene, and cover-slipped with Entellan (*Merck, Germany*).

CCO optical density quantification

The CCO histochemical staining intensity was carried out with optical densitometry using a computer-assisted image analysis workstation (*MCID, Interfocus Imaging Ltd., Linton, England*). It consists of a high precision illuminator, a digital camera, and a computer

with specific image analysis software. The mean optical density of each region was measured employing four non-overlapping readings in each section across three consecutive sections, thus obtaining a total of 12 readings per area and subject. The mean optical density values were converted into CCO activity units (µmol of cytochrome c oxidized/min/g tissue wet weight), determined by the enzymatic activity of the standards obtained with spectrophotometry (Gonzalez-Lima & Cada, 1994).

Data analysis

All the data were analyzed with the SigmaStat 12.5 program (Systat, Richmond, USA) and expressed as means ± SEM. The normality assumption was evaluated using the Shapiro-Wilk test ($P > .05$), and the homoscedasticity of variances was evaluated by employing the Levene test ($P > .05$). In the repeated measures analysis of variance (ANOVA), sphericity was tested through Mauchly's W ($P > .05$). If the assumption was violated, the F value was corrected with the Greenhouse-Geisser procedure. Statistical significance was set at the .05 level. For graphic representation, the SigmaPlot 12.5 program (Systat, Richmond, USA) was employed.

Behavioural data

The permanencies in each of the four quadrants during the learning, remote retention and reactivation probe trial were analyzed comparing the reinforced quadrant time with the average time in the other three non-reinforced quadrants for each group. First, normality and homoscedasticity assumptions were tested and then, a two-way repeated-measures ANOVA was performed (Factor A: quadrant, Factor B: day; Factor of repetition: permanencies). Then, post hoc multiple comparison analyses were carried out using the Holm-Sidak method. A statistically significant difference between quadrants was considered a learning criterion for the reference learning and memory task, a retrieval criterion for the reference remote retention task, and a reactivation criterion for memory on the memory reactivation task. The time spent in the reinforced quadrant across R45M, R45F, RA45M, RA45F groups on remote retention probe trial was also compared with a one-way ANOVA.

To analyze the escape latencies on the spatial reference learning and memory task (days one to five), in addition to the memory reactivation task (EA and RA trials on day 45 post-learning acquisition) for each group, a one-way repeated-measures ANOVA was

performed when normality and homoscedasticity assumption tests were passed, but a Friedman repeated-measures ANOVA on ranks was performed when they failed. Then, post hoc multiple comparison analyses were carried out using the Holm-Sidak method for parametric samples and Tukey's test for non-parametric samples. In the spatial reference learning and memory training protocol, latencies for the four trials were averaged per day. In the case of the memory reactivation task, latencies on the EA trial were recorded, as well as the average of the following four RA trials.

Moreover, in order to analyze differences between latencies from the first reinforced trial of the first day of the spatial reference learning and memory task and latencies from the first reinforced trial of the spatial reference memory reactivation task, a paired t-test or the Wilcoxon Signed Rank Test was performed within the RA45M and RA45F groups. The same analysis was performed to analyze the cumulative distance to reach the platform.

Finally, the speed during the learning, retention, and reactivation probe trials was evaluated with a one-way repeated-measures ANOVA, as normality and homoscedasticity assumption tests were passed.

CCO activity

In order to compare group differences in CCO activity taking into account the training, sex, and their interaction, a two-way ANOVA ((training (retrieval, reactivation) x sex (male, female)) was performed in each brain region. Then, post hoc multiple comparison analyses were carried out using the Holm-Sidak-method.

Results

Behavioural results

The R45M analysis regarding quadrant permanencies across days showed an interaction effect between both factors ($F_{5,40}$= 23.559, P< .001), revealing that rats spent more time in the target quadrant in comparison with the non-target ones on day one (P< .001), two (P< .001), three (P< .001), four (P< .001) and five (P< .001) but there was not difference on day 45 (P= .878). Thus, the R45M group achieved the learning criteria from day one and showed an impaired retrieval on day 45 (Figure 3A). The R45F group also showed an interaction effect between quadrants and days ($F_{5,30}$= 6.722, P< .001) and post-hoc

analyses revealed that rats spent more time in the target quadrant on day two ($P< .001$), three ($P< .001$), four ($P< .001$), and five ($P< .001$) but that there were no differences on day one ($P= .367$) and 45 ($P= .929$) (Figure 3C). These results indicated that the R45F group achieved the learning criteria from day two, and showed an impaired remote retention. Regarding the reactivation groups, the RA45M group revealed an interaction effect ($F_{6,42}= 11.540$, $P< .001$), displaying a preference for the target quadrant on day one ($P< .001$), two ($P< .001$), three ($P< .001$), four ($P< .001$), five ($P< .001$) and RC ($P< .001$) but there was not difference on day 45 ($P= .878$) (Figure 4A). This reveals that RA45M rats achieved the learning criteria from day one and showed a remote impairment which was rescued after the reactivation task. Similar results were obtained in the RA45F group as there was an interaction effect ($F_{6,42}= 13.106$, $P< .001$), and rats spent more time in the reinforced quadrant on day one ($P< .001$), two ($P< .001$), three ($P< .001$), four ($P< .001$), five ($P< .001$), and RC ($P< .001$) but there was no difference on day 45 ($P= .170$) (Figure 4C). Furthermore, analysis of time spent in the reinforced quadrant on the remote retention probe trial revealed no differences between R45M, R45F, RA45M and R45F groups ($F_{3,30}= .889$, $P= .458$).

Analysis of the escape latencies showed a statistically significant reduction across learning days in the R45M, R45F, and RA45F groups, whereas the RA45M group did not show a statistically significant latency decrease. In the R45M group, there was a significant reduction in the escape latencies from days one to four ($F_{4,32=}$ 6.326, $P= .007$), one to five ($F_{4,32=}$ 6.326, $P= .001$), and two to five ($F_{4,32=}$ 6.326, $P= .029$) (Figure 3B). In addition, the R45F group showed a significant reduction in the escape latencies across days of training, specifically, from days one to three ($F_{4,3=}$ 6.447, $P= .031$), one to four ($F_{4,3=}$ 6.447, $P= .008$), one to five ($F_{4,32=}$ 6.447, $P< .001$), and two to five ($F_{4,32=}$ 6.447, $P= .038$) (Figure 3D). The RA45M group did not show a statistically significant reduction in the escape latencies during learning ($F_{6,42=}$ 5.223, $P> .05$) (Figure 4B), whereas the RA45F group showed a statistically significant reduction between days one and five ($X^2_{6=}$ 24, $P< .05$) (Figure 4D).

Moreover, analysis of escape latencies taking into account the learning and memory task, in addition to the reactivation process, showed that the RA45M group reduced the escape latency from EA trial with respect to days four ($F_{6,42=}$ 5.223, $P= .025$) and five of training ($F_{6,42=}$ 5.223, $P= .005$), and also in comparison with the RA trials ($F_{6,42=}$ 5.223, $P= .002$) (Figure 4B). In the case of the RA45F group, the animals decreased their escape latency

from EA trial with respect to day five ($X^2_{6=}$ 24, P< .05), in addition to reducing the latency on the reactivation day compared to day one ($X^2_{6=}$ 24, P< .05) (Figure 4D).

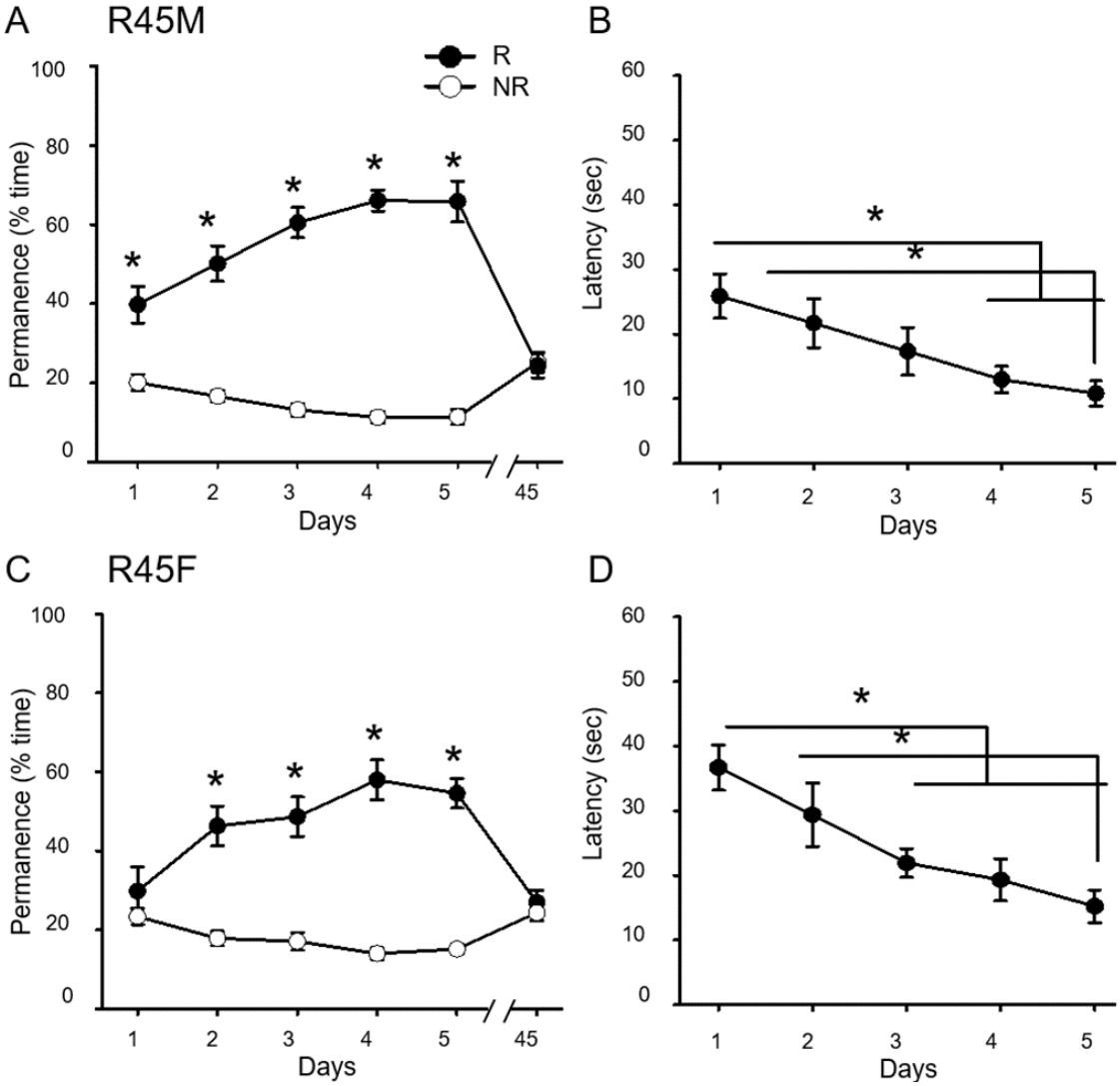

Figure 3.

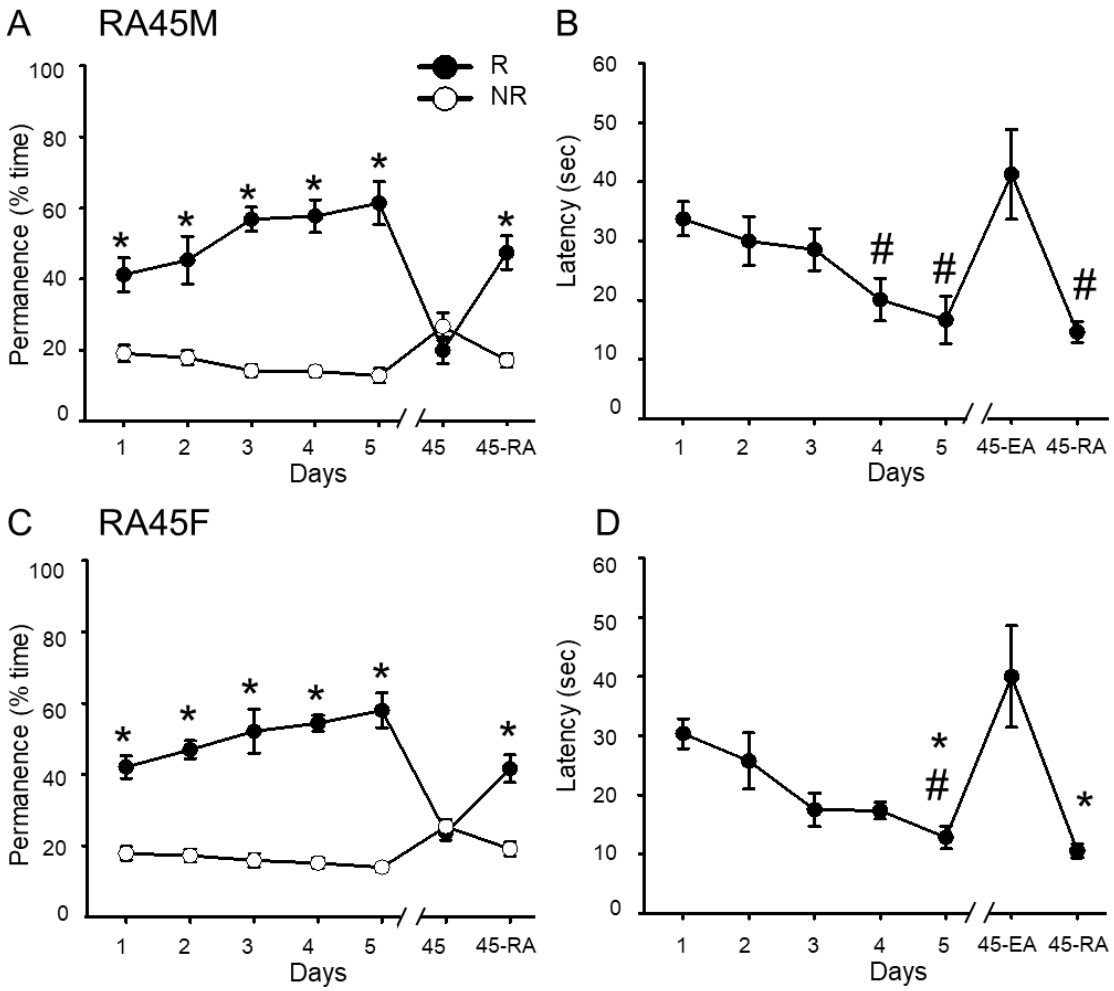

Figure 4.

Analysis comparing escape latencies of the first reinforced trials of the learning and memory task and memory reactivation task revealed that the RA45M group decreased the time to reach the platform in the first trial of the memory reactivation task with respect to the learning and memory task (t_7= 7.121, P< .001) (Figure 5A). Similar results were obtained in the RA45F group (t_7= 4.592, P< .001) (Figure 5B). Finally, the cumulative distance to reach the platform was also reduced in the first trial of the memory reactivation task with regard to the first trial of the learning and memory task both in males (Z_7= -2.521, P= .008) (Figure 5C) and females (Z_7= -2.380, P= .016) (Figure 5D).

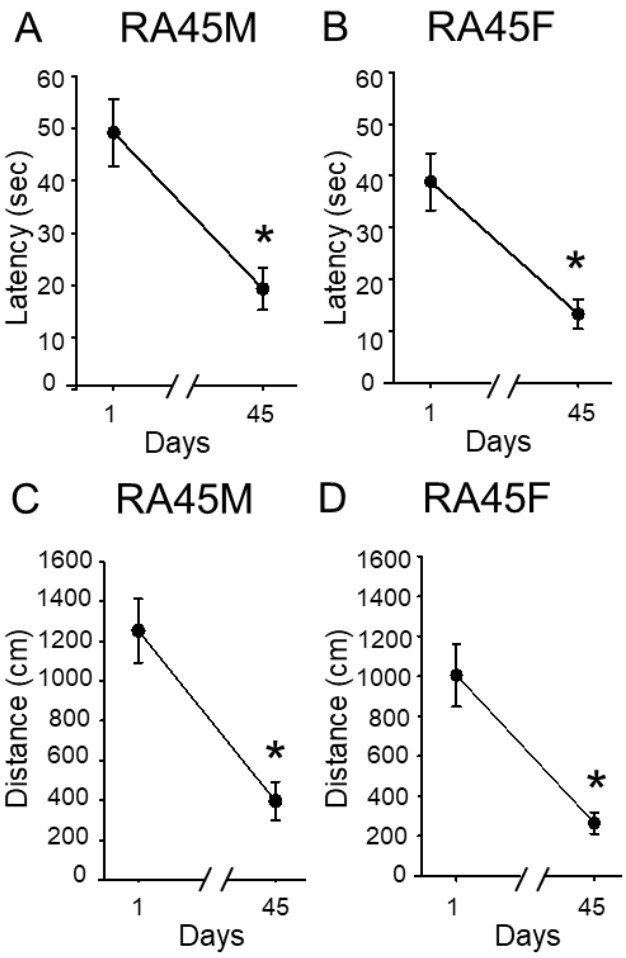

Figure 5.

Swimming speed results

Analysis regarding swimming speed revealed there were not differences across probe trials in males ($F_{6,42=}$ 1.823, P= .118) (Figure 6A) or females ($F_{6,42=}$ 1.772, P= .128) (Figure 6B).

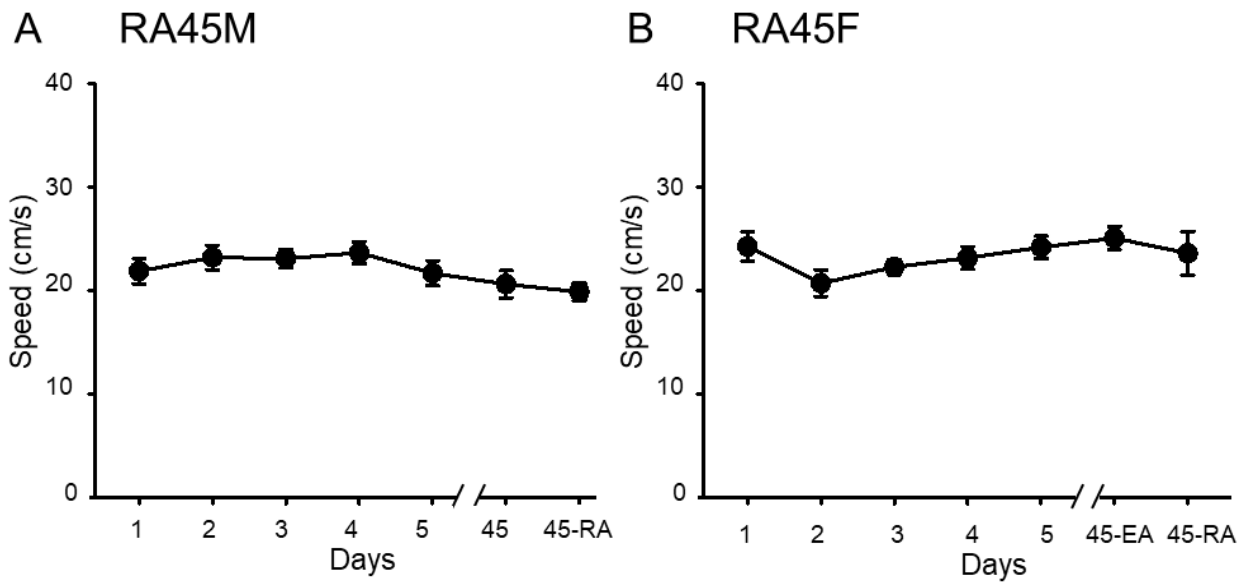

Figure 6.

CCO activity results

Analysis of CCO activity revealed statistically significant differences between groups. Brain metabolic activity in the medial prefrontal cortex (mPFC) showed a training effect (CG: $F_{1, 30}$= 17.784, *P*< .001; PL: $F_{1, 30}$= 26.161, *P*< .001; IL: $F_{1, 30}$= 17.108, *P*< .001) and a training x sex effect (CG: $F_{1, 30}$= 9.424, *P*= .005; PL: $F_{1, 30}$= 11.938, *P*= .002; IL: $F_{1, 30}$= 9.181, *P*= .005). Holm-Sidak test revealed that the R45F group presented higher metabolic activity than the R45M group (CG: *P*= .021; PL: *P*= .004; IL: *P*= .002) and in comparison with the RA45F group (CG, IL, PL: *P*< .001). As for the septum measurements, CCO activity reflected the effect of sex (MS: $F_{1, 30}$= 4.516, *P*= .042; LS: $F_{1, 30}$= 8.359, *P*= .007), training (MS: $F_{1, 30}$= 15.140, *P*< .001; LS: $F_{1, 30}$= 39.272, *P*< .001), and the interaction in LS ($F_{1, 30}$= 7.7, *P*= .009). Post-hoc analysis showed that the R45F group presented higher metabolic activity than the R45M group (MS: *P*= .011; LS: *P*< .001), and in comparison with the RA45F group (MS, LS: *P*< .001). Moreover, the R45M group presented higher metabolic activity than the RA45M group in LS (*P*= .02). As for the thalamus results, an effect of training (ADT: $F_{1, 30}$ = 18.995, *P*< .001; AVT: $F_{1, 30}$= 28.596, *P*< .001; MDT: $F_{1, 30}$= 13.353, *P*= .001) and training x sex (ADT: $F_{1, 30}$= 8.412, *P*= .007; ADV: $F_{1, 30}$= 4.343, *P*= .046; MDT: $F_{1, 30}$= 5.316, *P*= .029) was found. Specifically, the R45F group showed higher metabolic activity than the R45M group in MDT (*P*= .017), and compared to the RA45F group (ADT, AVT, MDT: *P*< .001). Moreover, the R45M group showed higher metabolic activity than the RA45M group in AVT (*P*= .035), and higher COO activity was found in ADT in the RA45M group,

compared to the RA45F group (P= .031). In terms of the amygdala, a training (CeA: $F_{1, 30}$= 4.641, P= .04; BLA: $F_{1, 30}$= 5.235, P= .03) and training x sex effect was found (CeA: $F_{1, 30}$= 8.8193, P= .008; LaA: $F_{1, 30}$= 6.979, P= .013; BLA: $F_{1, 30}$= 11.97, P= .002). Post-hoc analysis showed that the R45F group presented higher metabolic activity than the R45M group (CeA: P= .001; LaA: P= .004; BLA: P= .001), and in comparison with the RA45F group (CeA: P= .001; LaA: P= .011; BLA: P< .001). In the case of hippocampal CCO activity, an effect of training (CA1: $F_{1, 30}$= 27.094, P< .001; CA3: $F_{1, 30}$= 11.074, P= .02; DG: $F_{1, 30}$= 14.714, P< .001), sex (CA1:$F_{1, 30}$= 10.485, P= .003; CA3: $F_{(1, 30)}$ = 12.027, P= .02; DG: $F_{(1, 30)}$ = 6.896, P= .013), and the interaction (CA1: $F_{1, 30}$= 11.55, P= .002; DG: $F_{1, 30}$= = 8.404, P= .007) was found. Holm-Sidak method determined that the R45F group presented higher metabolic activity than the R45M group (CA1, CA3, DG: P< .001) and compared to the RA45F group (CA1, DG: P< .001; CA3: P= .001). When analysing retrosplenial cortices, training (RSG: $F_{1, 30}$= 5.951, P= .021), sex (RSG: $F_{1, 30}$= 4.952, P= .034; RSA: $F_{1, 30}$= 4.952, P= .034), and training x sex (RSG: $F_{1, 30}$= 5.881, P= .022) effects were also found, revealing that the R45F group had higher CCO activity than the R45M group (RSG: P= .002; RSA: P= .006) and the RA45 group (RSG: P= .002). As for PAR, ANOVA analysis showed effects of training ($F_{1, 30}$= 14.397, P< .001), sex ($F_{1, 30}$= 4.961, P= .034), and the interaction ($F_{1, 30}$= 6.704, P= .015), with the R45F group showing higher COO activity than the R45M group (P= .002) and the RA45F group (P< .001). Finally, rhinal cortex metabolic activity showed an effect of training (PHr: $F_{(1, 30)}$ = 6.631, P= .015) and training x sex (ENT: $F_{1, 30}$= 4.274, P= .048; PHr: $F_{1, 30}$= 4.794, P= .037). Post-hoc analysis revealed that R45F rats presented higher metabolic activity than RA45F animals (ENT: P= .017; PHr: P= .003) (Table 1).

Table 1.

CCO activity values

Brain area	**R45M**	**R45F**	**RA45M**	**RA45F**
Cg	20.48 ± 1.26	24.30 ± 1.34 *	19.17 ± 0.61	15.99 ± 1.08 &
PL	20.39 ± 1.02	25.20 ± 1.55 *	18.51 ± 0.58	15.50 ± 1.03 &
IL	18.14 ± 0.81	23.50 ± 1.61 *	16.89 ± 0.71	15.43 ± 1.03 &

MS	17.75 ± 0.95	21.53 ± 1.21 *	15.27 ± 0.69	15.84 ± 1.10 &
LS	23.52 ± 0.84	30.31 ± 1.68 *	19.33 ± 0.80 $	19.47 ± 1.16 &
ADT	34.63 ± 1.32	38.41 ± 1.72	32.33 ± 1.88	26.92 ± 1.35 # &
AVT	27.74 ± 0.71	31.15 ± 1.92	23.58 ± 0.87 $	21.69 ± 0.71 &
MDT	21.22 ± 0.66	25.31 ± 1.63 *	19.58 ± 1.57	18.05 ± 0.69 &
CeA	18.62 ± 1.08	24.29 ± 2.19 *	19.69 ± 0.84	16.66 ± 0.95 &
LaA	12.83 ± 0.59	18.50 ± 1.61 *	14.64 ± 1.18	13.43 ± 1.48 &
BLA	18.27 ± 0.85	25.39 ± 2.11 *	19.92 ± 0.63	17.29 ± 1.16 &
CA1-D	13.93 ± 0.37	18.36 ± 0.74 *	12.73 ± 0.63	12.62 ± 0.87 &
CA3-D	13.92 ± 0.46	18.69 ± 0.93 *	12.41 ± 0.50	14.05 ± 1.49 &
DG-D	24.54 ± 0.68	31.51 ± 1.80 *	23.35 ± 0.86	23.01 ± 1.31 &
RSG	20.10 ± 0.57	24.12 ± 1.14 *	20.09 ± 0.95	19.92 ± 0.61 &
RSD	19.14 ± 0.50	22.55 ± 1.10 *	19.63 ± 0.81	20.53 ± 0.84
PAR	17.57 ± 0.50	21.86 ± 1.07 *	16.50 ± 0.77	16.18 ± 1.10 &
PHR	16.84 ± 0.94	18.98 ± 0.80	16.51 ± 0.69	14.90 ± 0.92
ENT	14.65 ± 0.59	16.80 ± 1.25	15.13 ± 0.69	13.36 ± 1.15

Table 1 shows the CCO activity values (mean ± SEM) in R45M, R45F, RA45M and RA45F groups for all the areas studied. * R45M in comparison with R45F; # RA45M in comparison with RA45F; $ R45M in comparison with RA45M; & R45F in comparison with RA45F ($P < .05$). Cingulate cortex= CG, Infralimbic cortex = IL, Prelimbic cortex

= PL, MS= Medial septum, LS= Lateral septum, ADT= Anterodorsal thalamus, AVT= Anteroventral thalamus, MDT= Mediodorsal thalamus, CeA= Central amygdala, LaA= Lateral amygdala, BLA= Basolateral amygdala, Gyrus = DG, Granular retrosplenial cortex= RSG, Agranular retrosplenial cortex= RSA, Parietal cortex= PAR, Perirhinal cortex= PRh Entorhinal cortex = ENT.

Discussion

In this study, we explored the behavioural outcomes of male and female rats, in addition to the assessment of the limbic brain metabolic activity, in response to reactivation of a previously acquired allocentric spatial reference memory. In particular, the assessment was performed under conditions in which animals showed a retrieval deficit as a result of a long time period without exposure to the spatial contingencies that made it possible to establish a consolidated spatial memory. We have found that 45 days after memory acquisition, the allocentric spatial retrieval can be recovered by a brief re-exposure to the reinforced contingencies linked to memory trace reactivation. Furthermore, we have shown that male reactivated rats display a septal and thalamic CCO decrease in comparison to non-reactivated ones, whereas female reactivated rats exhibit a reduced energetic metabolism across a wider number of brain regions, including prefrontal, retrosplenial and parietal cortices, in addition to septal, thalamic, amygdalar and hippocampal areas, when compared to the non-reactivated females. These brain metabolic results suggest that a reduction in CCO activity is required to stabilize a preceding consolidated spatial cognitive mapping. Moreover, male and female rats displayed CCO activity differences, suggesting the differential contribution of brain limbic system energy demands in response to sex.

The accurate transmission of information through time, which leads to remembering, requires a modification in certain brain networks, in addition to a reactivation of the neural ensembles that were present in the encoding phase of learning (Richards & Frankland, 2017; Tanaka et al., 2014). Nevertheless, a destabilization of the aforementioned neural connectivity can trigger the opposite process, that is, forgetting, which results in a loss of memory events by making them latent or decaying (Awasthi et al., 2019; De Hoz et al., 2004). Our behavioural data reveal that when 45 days have elapsed since an allocentric spatial learning task occurred, there is a retrieval deficit, with

the animals not being successful in remembering a spatial location through the integration of several visual distal cues that allowed them, a long time before, to consolidate a particular spatial learning. Therefore, the R45M and R45F groups showed increased difficulty in achieving the retrieval criteria, defined as significant time spent in the target quadrant during the remote retention probe trial, with no sex differences. We previously observed (Zorzo et al., 2020) that spatial retrieval in healthy male and female rats is preserved seven, 15, and 30 days post-training, but not after longer time intervals, specifically, after 45 and 60 days. Nevertheless, we can assume that the behavioural forgetting that animals seem to display is not due to a failure during encoding, storage, or fragile learning because the animals achieved the learning criteria from day one or two (depending on the experimental groups) and maintained it over the five consecutive days of the task. Moreover, regarding latencies to reach the platform, the R45M and R45F groups showed a significant reduction on the last training days. From our point of view, both permanency and latency results indicate that animals have reached asymptotic levels of training when there is no need for new encoding (García-DeLaTorre et al., 2009). Our results agree with spatial memory cognition in humans, which, through the exploration of the retention of spatial knowledge over time, revealed that memory can suffer a decay, represented as a negatively accelerated forgetting curve (Ishikawa, 2013)

Memory age can display an impact on the possibility that a memory trace will re-enter a fragile state susceptible to interference (Milekic & Alberini, 2002; Nader et al., 2000; Ribeiro et al., 2013; Tian et al., 2011; Tronson et al., 2006). It has been consistently observed that remote memories seem to be less prone to destabilization following reactivation sessions (Bustos et al., 2009; Eisenberg & Dudai, 2004; Suzuki et al., 2004; Winters, Boyer et al., 2009). However, this does not rule out that certain older memories can also trigger this labile state (Diergaarde et al., 2006; Inda et al., 2011; Robinson & Franklin, 2010; Wang et al., 2009), and also, if destabilized, after providing reminders or reactivation sessions, we can hypothesize memory could be more stable. Our behavioural results show that the RA45M and RA45F groups, when submitted to the reinforced contingencies that allowed them previously to solve the task, rapidly reach the reactivation criteria, spending significantly more time in the target quadrant during the reactivation probe trial, but not during the remote retention trial. As for the latencies recorded, we can observe that the RA45M and RA45F groups showed an increase in latencies in locating the platform in the EA trial, compared to the last learning trials

performed in training, in addition to RA trials. It is important to mention that the EA trial is performed after the remote retention probe test, and it is the first reinforced trial of the 45-day old spatial memory. Thus, the EA trials are expected to reveal a non-successful behavioural outcome, consistent with the remote retention probe test. After carrying out a re-exposition to the environment in which the reinforced contingencies are available, there is not prior remembering, but after four trials of retraining, animals display a successful spatial retrieval that can indicate a reactivation of previous memory. However, if we examine the latencies of RA45M and RA45F groups in the first trial of the first day in comparison with the first trial of the reactivation day –that is, 45 days later–, there is a decrease in the time to reach the platform in both male and female groups. Thus, we do not consider animas are performing a new learning, but rather it is happening a facilitation of a previously learned task due to reactivation sessions. Together, these data strengthen the hypothesis that a failure on the remote retention probe test is not due to weak learning acquisition or an impairment during encoding and/or memory storage, and that the spatial remote retrieval impairment can be prevented by submitting animals to reinforced reactivation sessions, leading to a behavioural facilitation. Similar results have been obtained in other behavioural paradigms. For example, it has been shown that the retrieval of an inhibitory avoidance memory task decays over weeks, with this retrieval depletion being rescued after performing three reactivation sessions (Inda et al., 2011). With regard to spatial cognition studies, long non-reinforced reactivation trials (16 consecutive 60-second memory probe trials without the hidden platform) result in memory extinction (Rossato et al., 2006), but a single retention non-reinforced test triggers positive behavioural results in the MWM (Rossato et al., 2015).

Considering both the retention and reactivation sessions described above, our results support the idea that, although stronger memories, or those that reach asymptotic levels of learning, have characteristics that prepare them to be resistant, they can enter a fragile state after prolonged disuse, as proposed previously (Robinson & Franklin, 2010). It is important to note that forgetting is as necessary as remembering in being flexible and adapting our behaviour according to the challenging environmental demands (M. C. Anderson, 2003). Thus, it was recently proposed that the combination of remembering and forgetting is the key to achieving flexible behaviours (Richards & Frankland, 2017), probably because some goal-relevant memory traces can benefit from suppressing competing or distracting memories (M. C. Anderson, 2003). The physiological role of

memory suggests that there is a plastic period following reactivation that offers the opportunity to enhance weak memories or incorporate new information into an existing stored memory (Lee et al., 2017; Nader, 2015; Nader et al., 2000) when a suitable cue reminder of the original memory is available (Bustos et al., 2009). Hence, here we explore the neurobehavioral consequences of a re-exposure of a certain spatial location when animals suffer a memory decay, probably due to extended disuse. We consider that the spatial memory has become labile due to the passage of time, something that we consistently showed on the remote retention probe tests. The animals sequentially recovered the spatial knowledge after re-exposure to the environmental configuration, with one non-reinforced 60-second duration trial followed by five consecutive reinforced trials, suggesting that the information directly linked to a stored memory would be better coded by reactivation-mediated updating (Haubrich & Nader, 2018). It is important to note that, in addition to permanencies' results, the EA trial offers evidence that supports that it is not only the re-exposure to the environment which allows to recover a spatial information previously acquire, but there is also a need to carry out a re-training with the reinforced stimulus. Moreover, after deepening in the behavioural data, we show that the mentioned spatial memory recovery do to require the same training as for establishing a new learning as it occurs in day one of the learning task, when four consecutive reinforced trials are needed, but latencies and cumulative distance to reach the platform are grossly decreased with one single trial. This suggests that one single trial is effective in order to recover a successful behavior. It is worth noting that we have not found any differences in regard to swimming speed, therefore, we can state that the reduced latency and cumulative distance are not a result of rats having increased their procedural capacity of swimming across days.

There is cumulative research focused on deciphering the brain regions involved in spatial navigation, which enables us to elucidate the role of complex networks that involve both hippocampal and extrahippocampal substrates (for review see Hunsaker & Kesner. (2018)). However, despite the large number of animal and human spatial memory studies, the brain areas engaged in the cognitive phases that follow learning acquisition, such as maintenance, forgetting, or reactivation, are still being debated.

With the purpose of studying the differences in brain metabolism demands of the R45M, R45F, RA45M and RA45F groups, we employed CCO histochemistry. This technique, by measuring the mitochondrial enzymes that catalyse oxygen consumption during cellular respiration, is a reliable marker for ATP synthesis (Gonzalez-Lima & Cada, 1994) and highly useful in a wide variety of spatial learning paradigms (González-Pardo et al., 2019; Gutiérrez-Menéndez et al., 2019; Méndez-López et al., 2013; Zorzo et al., 2019) because it reveals sustained changes in brain metabolism (Gonzalez-Lima & Cada, 1994). We observed that male rats subjected to the spatial reinforced contingencies of the reference memory task, *i.e.*, with the hidden platform available, in comparison with males that displayed a retrieval deficit 45 days after memory acquisition, assessed on a single non-reinforced trial, showed a reduction in brain metabolic activity in the LS and AVT areas, suggesting that less septal and thalamic metabolic activity is required to stabilize a previously consolidated trace memory. It is known that the hippocampus, the main area involved in spatial cognition (Eichenbaum, 2017), projects to the septum (Okada & Okaichi, 2010) and, therefore, is relevant in spatial navigation. Indeed, it has been shown that bidirectional hippocampal-neocortical interactions may be coordinated by it and result in a spatial memory retrieval significance (Mei et al., 2018). Furthermore, in the case of the thalamus, several studies have unveiled its critical role in spatial cognition, particularly to enable spatial remote memory, suggesting its functional contribution within hippocampal connectivity (Klein et al., 2019; Mendez-Couz et al., 2015). Regarding female brain metabolism, there was a decrease in the CCO activity of the RA45F group, compared to the R45F rats, in the mPFC (CG, IL, PL), septum (MS, LS), thalamic (ADT, AVT, MDT), amygdalar (CeA, LaA, BLA), dorsal hippocampus (CA1-D, CA3-D, DG-D), RSG, and PAR brain structures, which shows that in order to re-enter a consolidating state, the decrease in metabolic activity involves a higher number of brain areas. Under conditions where a memory suffers a decay and, consequently, needs new memory encoding, the pivotal role of the hippocampus has been proposed, suggesting that protein synthesis within this area needs to occur in order to stabilize a spatial memory labile trace (Artinian et al., 2007, 2008; Da Silva et al., 2008; De Jaeger et al., 2014; Morris et al., 2006). Additionally, the role of the mPFC is being clarified, specifically during remote retrieval (Frankland & Bontempi, 2005), but also during the reconsolidation of a spatial memory trace (Rossato et al., 2015). Moreover, the extended network system involved in spatial navigation comprises other cortical regions, such as the retrosplenial cortex and PAR, which gain importance in long-term memory

representations (Kesner, 2009; Milczarek et al., 2018), in addition to the subcortical structures mentioned –hippocampus, septum, thalamus–, or even, amygdaloid nuclei (Aggleton, 2012).

Taking into account brain metabolism expenditure in response to spatial cognitive processes, some stress models have shown an increase in the CCO activity across several brain areas, suggesting an elevated energy cost in male and female stressed animals after performing a spatial reference memory task. The female study revealed, particularly, that higher energy demands can result in less effective performance (Banqueri et al., 2017, 2018). Indeed, it has been postulated that higher brain metabolic activity can be found in several brain regions of animals that exhibit depressive and anxiety-like behaviours, or those that are more susceptible to stress (Harro et al., 2014; McCoy et al., 2019). In line with this, increased CCO activity has been linked to anxious states during spatial memory performance (Sampedro-Piquero et al., 2013), whereas protective conditions, such as environmental enrichment, can trigger an enhancement of brain metabolic efficiency in order to better solve a spatial memory task (González-Pardo et al., 2019; Sampedro-Piquero et al., 2013). Thus, all of this evidence shows that a faster acquisition of the spatial reference memory could lead to a reduction in brain metabolism when the task is learned (Banqueri et al., 2017). Similarly, in humans, reduced neuronal resources were achieved with the use of cognitive enhancers, subsequently leading to improvements in a specific cognitive task, and this occurs only when the performance is not optimal (Volkow et al., 2008). Therefore, here we hypothesize that the lower energy metabolism across different brain regions employed by the RA45M and RA45F groups in comparison with their sex-equivalent retrieval groups, which displayed a spatial amnesia-like behaviour, can reflect an accurate behavioural response after a re-exposure to the reinforced contingencies and environmental context, assessed by permanencies and latencies recorded in the MWM. In addition, we have noted differences at the beginning of the training, taking into account a single trial, supporting that the behaviour performed 45 days later is more effective. Specifically, our brain metabolic results revealed that male rats employ septal nuclei and thalamic structures in an efficient manner in order to reactivate a memory that has suffered a decay due to the passage of time, whereas female rats add the metabolic costless use of the hippocampus, amygdala, mPFC, retrosplenial, and parietal cortices.

Sex differences in the spatial domain have been found in human and rodent studies, showing a better performance of men and male animals (Fernandez-Baizan et al., 2019; Monfort et al., 2015). However, these differences can be confined to the initial phases of training (E. M. Anderson et al., 2013; Woolley et al., 2010) and seem to disappear in posterior stages, such as retrieval (Piber et al., 2018), or during reactivation (Flint et al., 2007). Thus, we found similar behavioural results for male and female rats, but a different contribution of brain area specific metabolic demands when animals suffer a spatial amnesia-like behaviour of a previously acquired allocentric memory and also, after re-training. In particular, the R45F group revealed an enhancement of their CCO activity, in comparison with the R45M group, in mPFC (CG, IL, PL), septum (MS, LS), thalamus (MDT), amygdalar (CeA, LaA, BLA), dorsal hippocampus (CA1-D, CA3-D, DG-D), retrosplenial and PAR brain areas, showing a higher metabolic demand in response to a failure to locate a target zone. In the spatial memory reactivation process, male rats needed higher metabolic activity than their analogous female group in the thalamus, particularly in the ADT. Together, these findings suggest the differential contribution of brain limbic system energy demands in response to sex, although they were similar at a behavioural level.

Finally, as it has been shown that there is a distinct pattern of brain energy consumption at different time points after learning (Méndez-López et al., 2013), exploring the distinctions across immediate and remote memory brain metabolic activity can be of great interest.

In conclusion, our study indicates that 45 days is a long enough time interval to generate an amnesia-like behaviour in an allocentric spatial remote memory that can be recovered after re-exposure to the environmental configuration with the reinforced contingencies that made it possible to acquire the task, as a result of memory reactivation, in both male and female rats. Moreover, we observed a different brain metabolism pattern that revealed a behavioural and sex effect, determined by CCO histochemistry.

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