Elsevier Editorial System(tm) for
Neurobiology of Learning and Memory
Manuscript Draft

Manuscript Number: NLM-20-58R1

Title: Retrieval of allocentric spatial memories is preserved up to thirty days and does not require higher brain metabolic demands.

Article Type: Research paper

Keywords: cytochrome c oxidase; spatial retrieval; recent; remote; allocentric; reference memory.

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Abstract: Spatial orientation is a cognitive ability that is indispensable for survival. Several visual distal cues present in the context can be integrated, establishing a cognitive map. Although there is cumulative evidence about the neural substrate involved in spatial memory acquisition, the brain networks mediating the processes involved in the retrieval of allocentric spatial memories have been studied less. Here, we aimed to explore the role of neuronal oxidative metabolism in the retrieval of allocentric spatial memories through cytochrome c oxidase (CCO) histochemistry seven, 15, 30, 45, and 60 days after task acquisition. Our behavioural results show that spatial memory retrieval in male and female rats is preserved seven, 15, and 30 days post-acquisition, but there is forgetfulness after this time, with subjects not being able to remember the position of the hidden platform after 45 and 60 days. Regarding the study of male brain metabolism, we observed reduced CCO activity in the medial prefrontal cortex, the parietal, retrosplenial, rhinal cortex, and the hippocampal regions in all the groups that failed to solve the task. Similar results were found for female brain oxidative metabolism, in addition to certain differences between successful-retrieval female groups. In conclusion, our work adds information about the behavioural retrieval of an allocentric spatial reference task, suggesting that recovering spatial information seven, 15, and 30 days after acquisition is a simple task that does not require a high metabolic demand, in both male and female rats.

Abstract

Spatial orientation is a cognitive ability that is indispensable for survival. Several visual distal cues present in the context can be integrated, establishing a cognitive map. Although there is cumulative evidence about the neural substrate involved in spatial memory acquisition, the brain networks mediating the processes involved in the retrieval of allocentric spatial memories have been studied less. Here, we aimed to explore the role of neuronal oxidative metabolism in the retrieval of allocentric spatial memories through cytochrome c oxidase (CCO) histochemistry seven, 15, 30, 45, and 60 days after task acquisition. Our behavioural results show that spatial memory retrieval in male and female rats is preserved seven, 15, and 30 days post-acquisition, but there is forgetfulness after this time, with subjects not being able to remember the position of the hidden platform after 45 and 60 dfearays. Regarding the study of male brain metabolism, we observed reduced CCO activity in the medial prefrontal cortex, the parietal, retrosplenial, rhinal cortex, and the hippocampal regions in all the groups that failed to solve the task. Similar results were found for female brain oxidative metabolism, in addition to certain differences between succefearssful-retrieval female groups. In conclusion, our work adds information about the behavioural retrieval of an allocentric spatial reference task, suggesting that recovering spatial information seven, 15, and 30 days after acquisition is a simple task that does not require a high metabolic demand, in both male and female rats.

Keywords: cytochrome c oxidase; spatial retrieval; recent; remote; allocentric; reference memory.

1. Introduction

Spatial orientation is a cognitive ability that is preserved across most species, and it is indispensable for survival in animal groups such as insects (Zars, 2009), fish (Davis et al., 2014), birds (Sherry et al., 2017), and mammals, including humans (Brandt et al., 2017; Etienne & Jeffery, 2004), because we all need to reach a goal location and/or to return safely to a place as efficiently as possible without getting lost (Vorhees & Williams, 2014).

To do this, it is necessary to recognize the surrounding environment, in addition to our location, and integrate it in a reference system that allows the flexible mapping of different environments (Bellmund et al., 2018). Thus, this cognitive function requires handling a wide variety of information in order to learn, memorize, and remember locations and routes for everyday functioning (Chersi & Burgess, 2015; Wolbers & Hegarty, 2010). For this purpose, two distinct strategies –egocentric and allocentric– can be employed. The egocentric strategy is characterized by the ability to navigate using internal cues, taking our own body and its feedback about directions, distances, and turns as a reference (Vorhees & Williams, 2014). The allocentric strategy draws on several visual distal cues present in the context and integrates them, establishing a complex representation of multiple associations, *i.e.* a cognitive map (Epstein et al., 2017; Tolman, 1948).

Once a spatial memory is encoded, a consolidation process takes place in which the newly formed memory gradually begins to become stabilized (Albo & Gräff, 2018; Müller & Pilzecker, 1900). Therefore, the learning does not produce an instantaneous permanent memory, but rather it needs a period of time to become fixed, leading to a transformation from an initially labile state to a more permanent one (Frankland & Bontempi, 2005; Lechner et al., 2018). However, consolidation can be understood in two different phases, at a synaptic level or a systemic level. Synaptic consolidation is considered fast, lasting a few hours after learning, and it involves the stabilization of changes in synaptic connectivity. By contrast, systemic consolidation is a slower and more extended process of reorganization of brain networks, supporting memory retrieval in a time-dependent manner (Frankland & Bontempi, 2005). According to the standard model of consolidation, it has been suggested that, once learning occurs, the knowledge is stored in the hippocampus to later become consolidated mainly in the neocortex (Frankland & Bontempi, 2005; Larry R Squire & Alvarez, 1995), but the time scale of the hippocampal-cortical dialogue is not clear (Barry et al., 2016). In contrast, multiple trace theory supports the idea that the hippocampus is necessary for retrieval, regardless of the memory's age (Nadel & Moscovitch, 1997).

Furthermore, considering the passage of time, there is a dissociation between recent and remote memories. It was first noted by Ribot in cases of brain damage (Ribot, 1882), and then further identified as temporally graded retrograde amnesia following hippocampal complex damage (Scoville & Milner, 1957; L. R. Squire et al., 1975). However, currently, there is no clearly defined classification for the specific period of time that has to elapse to consider a memory recent or remote, with recent memory ranging from short intervals, such as one month, to longer ones, such as five years (Irish

et al., 2014). A similar controversy is observed in rodents. Therefore, in this study, we consider remote memory traces to be superior to two weeks, based on previous research (Albo & Gräff, 2018). Moreover, the majority of studies have been performed in males, with an underrepresentation of studies on spatial cognition in females (Beery & Zucker, 2011).

Although there is cumulative evidence about the neural substrates involved in spatial memory acquisition, revealing the role of the hippocampus and related brain structures (Buzsáki & Moser, 2013; Conejo et al., 2010; Epstein et al., 2017; Moscovitch et al., 2016; O'Keefe & Nadel, 1978; L. R. Squire et al., 2004), the substrates mediating the processes involved in the retrieval of allocentric spatial memories have been studied less. Studies have proposed the critical role of the prefrontal (Barry et al., 2016; Teixeira et al., 2006), entorhinal (Hales et al., 2018), parietal (Kesner, 2009) and retrosplenial (Milczarek et al., 2018; Todd & Bucci, 2015) cortices, and also hippocampal formation (Barry et al., 2016; Broadbent et al., 2006; Conejo et al., 2013), suggesting a functional network connectivity required for memory retrieval (Holschneider et al., 2019). However, these studies have reached up to 30 days post-acquisition, with a lack of information about the behavioural outcomes and brain regions involved after this time.

Considering the above, in the present study we aimed to explore the brain metabolism involved in the retrieval of allocentric spatial memories in both male and female rats using different degrees of delay after spatial reference memory acquisition. With the objective of addressing the brain oxidative metabolism underlying these processes, we employed cytochrome c oxidase (CCO) histochemistry seven, 15, 30, 45, and 60 days after the behavioural task. CCO is a mitochondrial enzyme involved in the oxidative phosphorylation process and a reliable marker of brain energy demands (Gonzalez-Lima & Cada, 1994; Wong-Riley, 1989). It can be used to detect brain regional differences in the metabolic capacity in response to cognitive processes, such as spatial memory retrieval (Mendez-Couz et al., 2015).

2. Material and methods

2.1. Animals

A total of 54 male young Wistar rats (271.5 ± 4.8 g. at the beginning of the experiment) and 56 female Wistar rats (210.8 ± 3.3 g. at the beginning of the experiment), all of them aged between 12 and 15 weeks old, were used. All the animals were housed with a constant room temperature (20 ± 2 ºC), relative humidity (65-70%), and an artificial light-dark cycle of 12 h (08:00-20:00/20:00-08:00 h). The animals had *ad libitum* access to food and tap water. The procedures and manipulation of the animals used in this study were carried out according to the European Communities Council Directive 2010/63/UE and the Spanish legislation on the care and use of animals for experimentation (RD 53/2013), and the study was approved by the local committee for animal studies (University of Oviedo).

Prior to conducting the behavioural procedures, all the animals were handled daily for seven days to reduce the stress generated by contact with the experimenter. All the behaviour tests were performed between 8:00 h and 14:00 h.

2.2. Experimental procedure

We trained five groups of male rats and five groups of female rats on a spatial reference memory task. Then, we studied their memory retention with different degrees of delay. Each group per sex received a retention test seven days (male: R7M, *n = 8*; female: R7F, *n=8*), 15 days (male: R15M, *n = 10*; female: R15F, *n=10*), 30 days (male: R30M, *n = 8*; female: R30F, *n=9*), 45 days (male: R45M, *n = 10*; female: R45F, *n=10*), and 60 days (male: R60M, *n = 9*; female: R60F, *n=10*) after the end of the acquisition. Additionally, a swim control group was added for each sex (male: SCM, *n = 9*; female: SCF, *n=9*) (Figure 1).

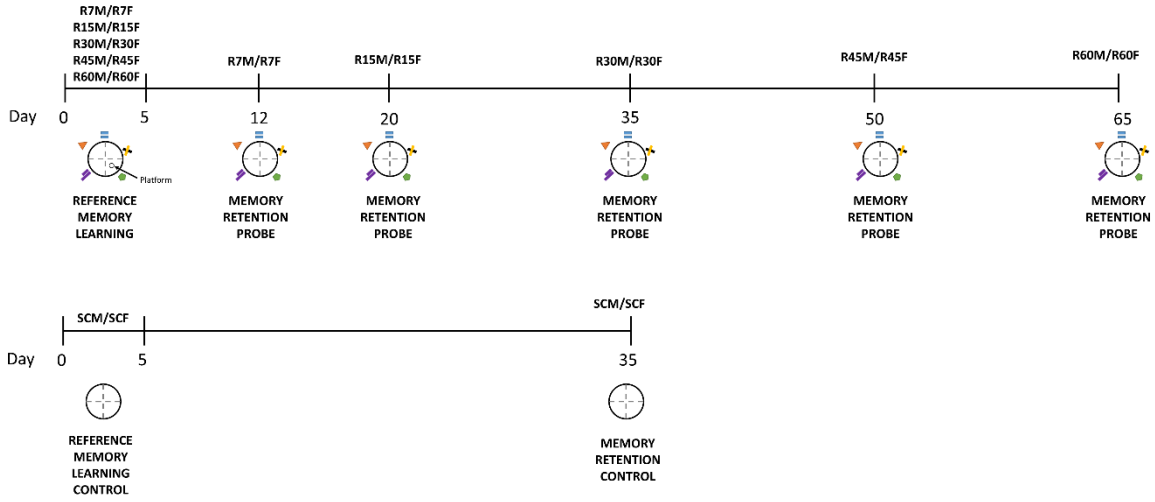

Figure 1. Overview and timeline of the behavioural test. The rats were divided into twelve groups (six male, six female). Five groups per sex were trained on a spatial reference memory task on five consecutive days, and their memory retention was tested after seven (male: R7M, n = 8; female: R7F, n=8), 15 (male: R15M, n = 10; female: R15F, n=10), 30 (male: R30M, n = 8; female: R30F, n=9), 45 (male: R45M, n = 10; female: R45F, n=10), and 60 (male: R60M, n = 9; female: R60F, n=10) days of delay. One group per sex was employed as a swim control group (male: SCM, n = 9; female: SCF, n=9).

2.3. Behavioural procedure

2.3.1. Apparatus

Spatial reference memory and memory retention were evaluated in the Morris Water Maze (MWM) (Morris, 1984). It consists of a black circular fibreglass tank measuring 150 cm in diameter and 75 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 30 cm. An escape platform was hidden beneath the surface of the water. It consists of a 10 cm diameter cylinder measuring 28 cm in height, located 2 cm below the surface of the water. The MWM was located in the centre of a 16 m^2 room 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 from the maze, on which five geometric visual cues with different volumes and colour patterns were placed, acting as allocentric cues. 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 animal's behaviour was recorded *(*V88E, *Sony*, *Spain)*, and its path was analysed using a

computerized video-tracking system (Ethovision XT 14.0, *Noldus Information Technologies, Wageningen, The Netherlands*).

2.3.2. Habituation

Animals were habituated to the testing contingencies of the MWM. For this purpose, R7M, R7F, R15M, R15F, R30M, R30F, R45M, R45F, R60M, and R60F performed four trials in which they had to reach a visible platform that protruded 4 cm from the water and was located in the centre of the pool. In each session, animals were released from each quadrant facing the pool wall following a pseudo-randomised sequence. The SCM and SCF groups swam in the maze for an equivalent average time as their male or female reference memory retention groups, but without the visible platform and the visual cues. Once the habitation session had ended, the animals were carefully dried and returned to their home cage.

2.3.3. Reference memory task

On the following five days, the animals were required to locate a hidden platform placed in the centre of quadrant D. Therefore, they had to use the external visual cues to perform the task. The subjects received four acquisition trials per day. Once the animal had found the platform, it remained in the reinforced place for 15 s. If the animal failed to reach the platform after 60 s, it was placed on it for 15 s. The inter-trial interval lasted 30 s, during which the animals were placed in a black bucket. For each trial, the animals 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. After completing the four daily training trials, a test (or transfer test) was carried out, in which the escape platform was removed, and the rat was introduced from the opposite quadrant to where the platform had been located in previous trials (in our experiment, quadrant C), in order to find out whether the animal remembered the location of the hidden platform. This trial lasted 60 s. Then, the animal was moved to the black bucket for 30 s and, finally, received an additional trial with the hidden platform located in the usual position to avoid the possible extinction of learning. The SCM and SCF groups swam in the MWM for an average time on the same number of daily trials and training days as the male and female reference memory retention groups, but without the presence of the hidden platform and the visual cues located on the panels around the maze. Once the daily learning session had ended, the animals were dried and returned to their home cage. We recorded the time of permanence in each quadrant during the transfer test.

2.3.4. Retention test

Rats underwent a memory retention test in a single 60 s probe trial. 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 transfer test. The SCM and SCF groups swam in the maze for the same amount of time, but without the visual cues located on the panels around the pool. Once the retention test was over, the animals were picked up from the maze, dried, and returned to their home cage. Memory retention was evaluated by measuring the amount of time spent in each quadrant during the probe trial seven, 15, 30, 45, and 60 days after the last acquisition trial in the different groups, also segregated by sex.

2.4. Sacrifice and brain processing

Animals were decapitated 90 minutes after the retention test. The brains were immediately removed, frozen in isopentane (*Sigma-Aldrich, Germany*), and stored at -40 ° C to make coronal sections 30 µm thick in a cryostat at -20 ° C (*Leica CM1900, Germany*). We mounted the sections on non-gelatinized slides for CCO histochemistry. We anatomically defined the regions of interest according to Paxinos and Watson's atlas (Paxinos & Watson, 2005). The regions of interest and their distances in mm counted from bregma were: +3.24 mm for the cingulate (CG), infralimbic (IL), prelimbic cortex (PL) and primary motor cortex (M1); -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), perirhinal (PHr) and primary auditory cortices (Au1) and dorsal lateral geniculate nucleus (DLG) (Figure 2).

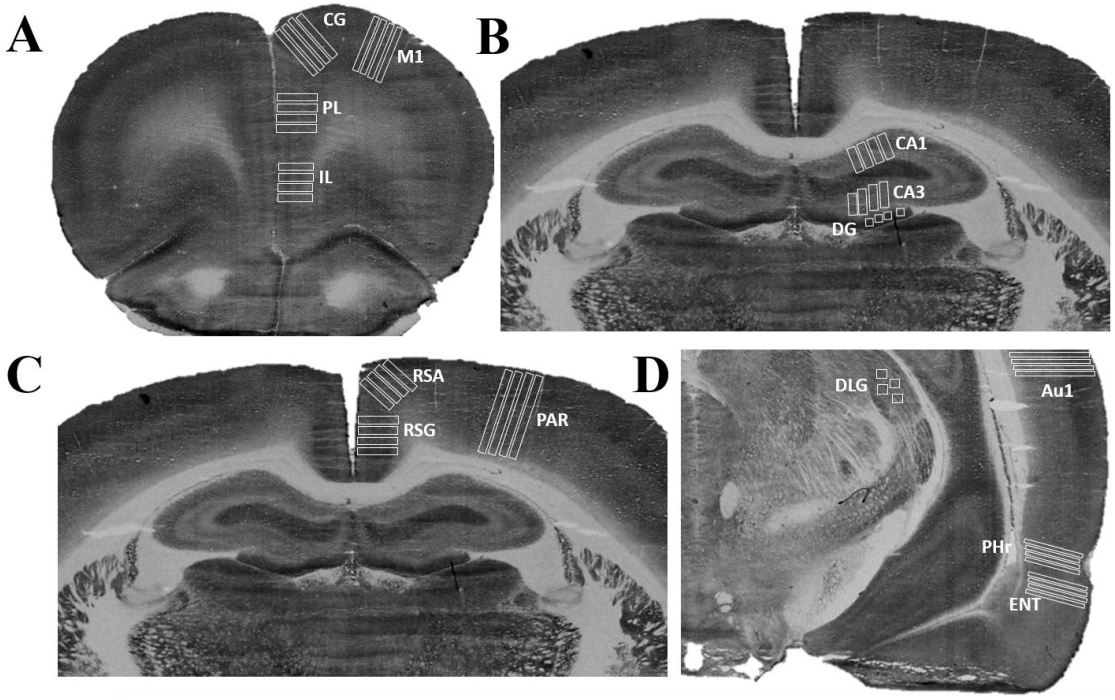

Figure 2. Sampling frames of CCO histochemistry in the regions of interest. Au1= Primary auditoy cortex, CG= Cingulate cortex, DG= Dentate Gyrus, DLG= Dorsal lateral geniculate nucleus, ENT= Entorhinal cortex, IL=Infralimbic cortex, M1= Primary motor cortex, PAR= Parietal cortex, PRh= Perirhinal cortex, PL= Prelimbic cortex, RSA= Agranular retrosplenial cortex, RSG= Granular retrosplenial cortex.

2.5. Cytochrome c oxidase histochemistry

We processed the section slides with quantitative CCO histochemistry, as described by Gonzalez-Lima & Cada (1994) and following the protocol previously described by Zorzo, Méndez-López, Méndez, & Arias (2019). Briefly: sets of tissue homogenate standards from Wistar rat brains were cut at different thicknesses and included with each bath of slides. Sections and standards were incubated for 5 min in 0.1 phosphate buffer (PB) with 10% (w/v) sucrose and 0.5 (v/v) glutaraldehyde, followed by three 5 min baths of 0.1 PB with 10% (w/v) sucrose after 8 min in 0.05M Tris buffer, with 275

mg/l cobalt chloride, sucrose, and 0.5 (v/v) dimethylsulfoxide. Then, for 1 h at 37°C, 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 for 30 min. Finally, the slides were dehydrated, cleared with xylene, and cover-slipped with Entellan (*Merck, Germany*).

2.6. CCO optical density quantification

Quantification of CCO activity was performed by optical densitometry using a computer-assisted image analysis workstation (*MCID, Interfocus Imaging Ltd., Linton, England*) made up of a high precision illuminator, a digital camera, and a computer with specific image analysis software. Measurements of standards in each of the incubation baths were taken. The average optical density of each region was measured on the right hemisphere, using four non overlapping readings in each section across three consecutive sections. Consequently, a total of 12 measurements per area and subject were collected, where the square-shaped sampling window was adjusted for each region size. The average optical density values were transformed into CCO activity units, determined by the enzymatic activity of the standards that were taken by spectrophotometry (Gonzalez-Lima & Cada, 1994).

2.7. Data analysis

We employed the SigmaStat 12.5 program (*Systat, Richmond, USA*) for data analysis. We used the Shaphiro-Wilk test to evaluate the normality assumption, and the Levene test to assess the equality of variances for ANOVA analysis. When the data had a normal distribution ($P > .05$) and variances were equally distributed ($P > .05$), we used a parametric test. Otherwise, we used non-parametric tests. Statistical significance was set at the .05 level. Finally, for the graphic representation, we employed the SigmaPlot 12.5 program (*Systat, Richmond, USA*). We presented the data as mean ± SEM.

2.7.1. Behavioural data

Time spent in each of the four quadrants of the MWM during the transfer test on the five consecutive days of the reference memory task and during the retention probe was analysed using a *t-test* per group and day, separately. We compared the permanence in the reinforced quadrant (quadrant D) with average permanence in the non-reinforced quadrants (quadrants A, B, and C). When data did not fit the normality assumption, we used *Mann-Whitney's U* statistic.

2.7.2. CCO activity

Group differences in CCO activity were assessed by *one-way ANOVA* analysis in each brain region when the data had normal distribution and variance homoscedasticity. When one of these assumptions failed, we employed the *Kruskal–Wallis one-way ANOVA of Ranks* (*H*). The *Holm-Sidak* post-hoc method was applied with parametric tests, whereas *Dunn's* method was applied with non-parametric tests.

2.7.3. Correlations

The analysis of correlations between brain metabolic activity across different brain regions and the rate of retrieval, regardless the passage of time was analysed by

calculating Pearson product-moment correlations. For that, we have pool all the experimental groups excluding the swim controls.

3. Results

3.1. Behavioural results

We analysed the permanence in the reinforced quadrant with average permanence in the non-reinforced quadrants. A statistically significant difference between them was considered a learning criterion for the reference memory task and a retrieval criterion on the retention probe test.

3.1.1. Reference memory task

With regard to male quadrant permanencies during reference memory training, all the groups that performed the reference memory task achieved the learning criteria from day one to the end of the training days. Specifically, the R7M group showed a preference for the reinforced quadrant on the five consecutive days of the task (D1: t= -4.566 , P< .001; D2: U= 12, P< .001; D3: t= -6.055 , P< .001; D4: t= -7.731 , P< .001; D5: t= -14.557 , P< .001) (Figure 3B), just as the R15M group did (D1: U= 0, P< .001; D2: U= 17, P< .001; D3: t= -7.498, P< .001; D4: U= 10, P< .001; D5: U= 0, P< .001) (Figure 3C), the R30M group (D1: t= -4.736 , P< .001; D2: t= -2.301, P= .028; D3: U= 20.5, P= .001; D4: t= -7.61, P< .001; D5: U= 0, P< .001) (Figure 3D), the R45M group (D1: t= -4.536 , P< .001; D2: t= -10.677, P< .001; D3: t= -14.112, P< .001; D4: t= -20.838, P< .001; D5: t= -13.695, P< .001) (Figure 3E), and the R60M group (D1: U= 7, P< .001; D2: U= 6, P< .001; D3: t= -16.66, P= .028; D4: U= 16, P< .001; D5: t= -15.067, P< .001) (Figure 3F). However, the SCM group did not show a preference for the rewarded quadrant across the task days (D1: U= 115.5, P= .841; D2: U= 84.5, P= .182; D3: U= 118, P= .913; D4: U= 100.5, P= .453; D5: U= 119, P= .942) (Figure 3A).

With regard to female quadrant permanencies during reference memory training, all the groups that performed the reference memory task achieved the learning criteria before the end of the training days. The R7F group spent significantly more time in the reinforced quadrant, compared to the non-reinforced quadrants, from day 2 (D1: U= 54, P= .071; D2: t= -8.269, P< .001; D3: t= -6.135, P< .001; D4: t= -5.752, P< .001; D5: t= -7.524, P< .001) (Figure 4B), similar to R15F group (D1: t= -.957, P= .345; D2: t= -6.515, P< .001; D3: U= 0, P< .001; D4: U= 32, P< .001; D5: t= -11.72, P< .001) (Figure 4C). The R30F group achieved the learning criteria from day 1 (D1: U= 33, P= .001; D2: t= -6.489, P< .001; D3: t= -12.656, P< .001; D4: t= -15.221, P< .001; D5: U= 0, P< .001) (Figure 4D), R45F from day 2 (D1: U= 111, P= .229; D2: t= -7.553, P< .001; D3: U= 13, P< .001; D4: U= 1.5, P< .001; D5: U= 0, P< .001) (Figure 4E), and R60F from day 1 (D1: t= -2.033, P= .0491; D2: t= -8.125, P< .001; D3: t= -7.895, P< .001; D4: t= -14.673, P< .001; D5: U=3, P< .001) (Figure 4F). Moreover, as expected, the SCF group did not spend significantly more time in the rewarded quadrant across the task days (D1: U= 98.5, P= .410; D2: U= 93, P= .306; D3: U= 76, P= .1; D4: t= -1.058, P= .298; D5: t= -.0114, P= .991) (Figure 4A).

3.1.2. Retention probe

Regarding male permanencies, the R7M, R15M, and R30M groups achieved the retrieval criteria. The R7M group spent more time in the rewarded quadrant (t= -2.955, P= .006) (Figure 3B), similar to the R15M group (U= 52, P= .002) (Figure 3C) and

R30M group (*t*= -2.082, *P*= .0459) (Figure 3D). However, the R45M and R60M groups did not achieve the retrieval criteria. The R45M group did not show differences between the reinforced quadrant and the non-reinforced ones (*U*= 141, *P*= .791) (Figure 3E), similar to the R60M group (*t*= -1.865, *P*= .0708) (Figure 3F). Moreover, the SCM group did not show differences between the reinforced quadrant and the non-reinforced ones (*U*= 113.5, = .522) (Figure 3A).

As for female permanencies, the R7F, R15F, and R30F groups achieved the retrieval criteria similarly to the male groups. The R7F group swam more time in the reinforced quadrant (*t*= -8.269, *P*< .001) (Figure 4B), just as the R15F group (*U*= 84, *P*= .041) (Figure 4C) and the R30F group (*t*= -3.494, *P*= .001) (Figure 4D) did. However, the R45F and R60F groups did not achieve the retrieval criteria. The R45F group did not show differences between quadrants (*t*= -1.313, *P*= .197) (Figure 4E); nor did the R60M group (*t*= -.704, *P*= .486) (Figure 4F). Furthermore, the SCF group did not show differences between the rewarded quadrant and the non-rewarded ones (*t*= -1.315, *P*= .197) (Figure 4A).

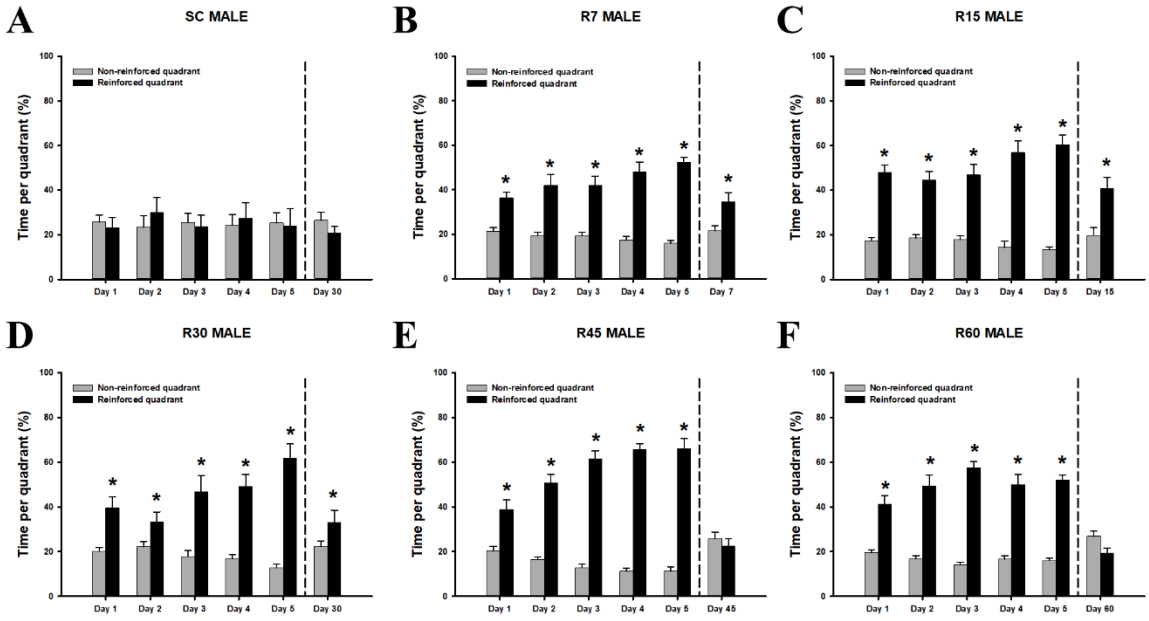

Figure 3. Morris water maze male results. Time spent in reinforced and non-reinforced quadrants during the transfer test on the five consecutive days of the reference memory task and during the retention probe. The x-axis shows the days. (A) Permanencies in the SCM group. They did not show differences between quadrants (*P*> .05). (B) Permanencies in the R7M group. They reached the learning criteria on the first day, and they remembered the location of the platform seven days post-acquisition. (C) Permanencies in the R15M group. They reached the learning criteria on the first day, and they remembered the location of the platform 15 days post-acquisition. (D) Permanencies in the R30M group. They reached the learning criteria on the first day, and they remembered the location of the platform 30 days post-acquisition. (E) Permanencies in the R45M group. They reached the learning criteria on the first day, but they did not remember the location of the platform 45 days post-acquisition. (F) Permanencies in the R60M group. They reached the learning criteria on the first day, but they did not remember the location of the platform 60 days post-acquisition.

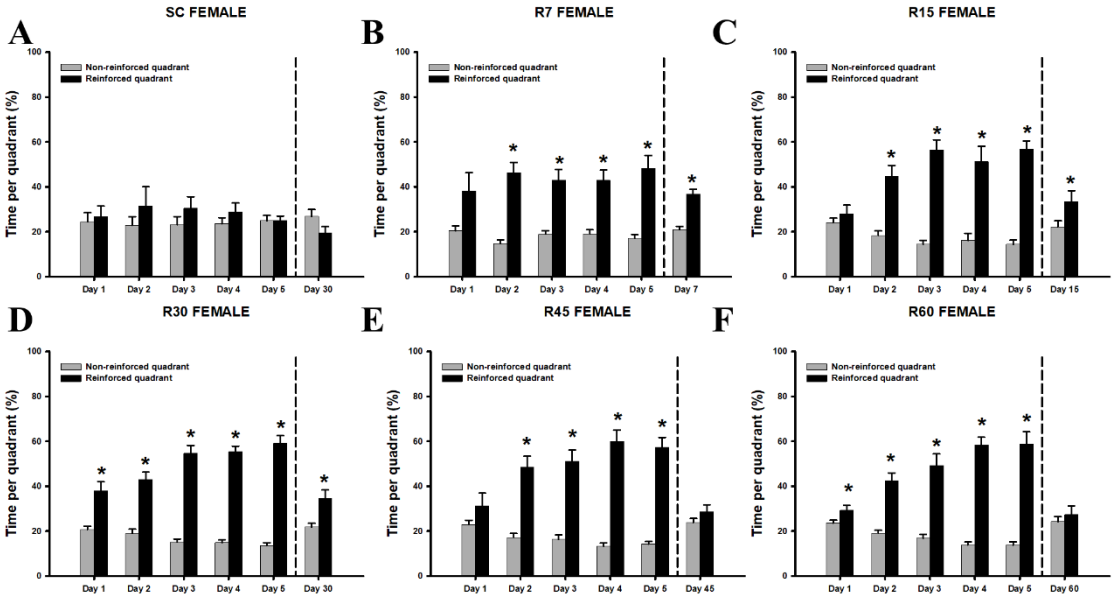

Figure 4. Morris water maze female results. Time spent in reinforced and non-reinforced quadrants during the transfer test on the five consecutive days of the reference memory task and during the retention probe. The x-axis shows the days. (A) Permanencies in the SCF group. They did not show differences between quadrants (*P*> .05). (B) Permanencies in the R7F group. They reached the learning criteria on the second day, and they remembered the location of the platform seven days post-acquisition. (C) Permanencies in the R15F group. They reached the learning criteria on the second day, and they remembered the location of the platform 15 days post-acquisition. (D) Permanencies in the R30F group. They reached the learning criteria on the first day, and they remembered the location of the platform 30 days post-acquisition. (E) Permanencies in the R45F group. They reached the learning criteria on the second day, but they did not remember the location of the platform 45 days post-acquisition. (F) Permanencies in the R60F group. They reached the learning criteria on the first day, but they did not remember the location of the platform 60 days post-acquisition.

3.2. CCO activity

Male densitometric analysis of CCO activity revealed group differences in all the areas measured. Detailed CCO activity in the medial prefrontal cortex (mPFC) showed statistically significant group differences (CG: F= 17.078, P< .001; PL: F= 21.283, P< .001; IL: F= 24.796, P< .001). Holm-Sidak's method revealed that differences in CG were between SCM, R7M, R15M, and R30M compared to R45M (P< .001) and R60M (P< .001), as in PL (P< .001) and IL (P< .001). In reference to M1, we have found significant group differences (F= 11.699, P< .001). Holm-Sidak's method revealed that differences in M1 were between SCM, R7M and R30M compared to R45M (P< .001) and R60M (P< .001), and R15M compared to R45M (P= .010). As for the hippocampus, we found group statistically significant differences (CA1: H= 37.942, P< .001; CA3: H= 39.351, P< .001; DG: F= 15.377, P< .001). Post-hoc analysis indicated that in CA1, group differences were between SCM, R7M, R15M, and R30M compared to R45M (P< .005), and between SCM and R30M compared to R60M (P< .005). As for CA3, group differences were found between SCM, R15M, and R30M compared to R45M (P< .005), and between SCM and R30M compared to R60M (P< .005). Finally,

DG results showed different CCO activity between SCM, R7M, R15M, and R30M compared to R45M (P< .001) and R60M (SCM: P= .021; R7M: P= .001; R15M: P= .012; R30M: P= .025). Moreover, group differences between R45M and R60M (P= .049) were found. Regarding the retrosplenial cortices, we found significant group differences in both cortices (RSG: H= 35.963, P< .001; RSA: H= 33.927, P< .001). Dunn's method showed that differences in RSG were between SCM, R7M, R15M, and R30M compared to R45M (P< .005), and between R7M and R15M, compared to R60M (P< .005). As for the PAR, we found statistically significant group differences (F= 23.996, P< .001), with Holm-Sidak's method showing differences between SCM, R7M, R15M, and R30M compared to R45M (P< .001), and between the same groups and R60M (SCM, R7M, R30M: P< .001; R15M: P= .011). For the parahippocampal cortices, we also found statistically significant group differences (PRh: F= 23.377, P< .001; ENT: F= 24.144, P< .001). Post-hoc analysis revealed that in both cortices, differences were found between SCM, R7M, R15M, and R30M compared to R45M (P< .001) and R60M (P< .001). Au1 results showed significant group differences (F= 18.010, P< .001), and post-hoc analysis revealed that differences in Au1 were between SCM, R7M and R30M compared to R45M (P< .001) and R60M (P< .001), and R15M compared to R7M (P= .019), R45M (P= .005) and SCM (P= .004). Finally, as for DLG (F= 14.716, P< .001), differences were obtained between SCM, R7M and R30M compared to R45M (P< .001) and R60M (P< .001), and R15M compared to R7M (P= .023) and SCM (P= .003) (Table 1).

Table 1.
CCO male values

Structures	SCM	R7M	R15M	R30M	R45M	R60M
CG	32.11 ± 1.19 *^	32.84 ± 1.06 *^	28.55 ± 1.56 *^	31.20 ± 2.36 *^	20.31 ± 1.14	19.74 ± 0.77
PL	32.30 ± 0.92 *^	32.67 ± 1.10 *^	29.46 ± 1.70 *^	30.67 ± 1.70 *^	20.28 ± 0.91	19.87 ± 1.11
IL	31.59 ± 0.94 *^	31.09 ± 1.26 *^	28.89 ± 1.81 *^	31.31 ± 1.75 *^	18.06 ± 0.73	18.22 ± 1.15
M1	33.78 ± 0.88 *^	33.89 ± 1.52 *^	29.11 ± 2.04 *	30.74 ± 1.31 *^	22.53 ± 0.88	23.74 ± 1.37
CA1	26.90 ± 0.65 *^	23.47 ± 0.96 *	22.06 ± 1.45 *	25.73 ± 1.92 *^	14.00 ± 0.34	14.57 ± 1.32
CA3	27.93 ± 0.61 *^	22.97 ± 1.06 *	22.94 ± 1.22	26.96 ± 1.73 *^	13.93 ± 0.41	15.89 ± 1.35
DG	40.57 ± 1.02 *^	44.08 ± 1.74 *^	40.98 ± 2.23 *^	40.55 ± 3.20 *^	24.40 ± 0.62	31.84 ± 2.02
RSG	34.48 ± 0.82 *	35.25 ± 0.96 *^	34.79 ± 1.69 *^	35.17 ± 2.27 *	20.19 ± 0.52	24.07 ± 1.16
RSA	33.76 ± 0.96 *	32.67 ± 1.35 *	32.89 ± 1.42 *	34.91 ± 2.49 *	19.36 ± 0.50	25.92 ± 1.12
PAR	31.20 ± 0.72 *^	30.88 ± 1.16 *^	27.69 ± 1.72 *^	31.95 ± 1.77 *^	17.60 ± 0.47	21.88 ± 1.19
PRh	30.55 ± 0.85 *^	25.69 ± 1.30 *^	27.45 ± 1.61 *^	28.57 ± 1.31 *^	16.49 ± 0.96	17.23 ± 1.37
ENT	27.71 ±	23.72 ±	25.02 ±	26.26 ±	14.38 ±	15.25 ±

	0.50 *^	1.30 *^	1.61 *^	1.42 *^	0.63	1.29
Au1	33.22 ± 1.05 *^&	33.71 ± 1.79 *^&	27.05 ± 1.25 *	33.21 ± 1.28 *^	21.13 ± 0.60	23.25 ± 1.39
DLG	31.51 ± 1.37 *^&	30.71 ± 1.56 *^&	24.95 ± 1.20	29.60 ± 1.16 *^	21.19 ± 0.94	21.42 ± 1.01

Table 1 shows the male CCO values (mean ± SEM) in the SCM, R7M, R15M, R30M, R45M, and R60M groups for all the structures studied. Sampling frames of CCO histochemistry in the regions of interest. Au1= Primary auditoy cortex, CG= Cingulate cortex, DG= Dentate Gyrus, DLG= Dorsal lateral geniculate nucleus, ENT= Entorhinal cortex, IL=Infralimbic cortex, M1= Primary motor cortex, PAR= Parietal cortex, PRh= Perirhinal cortex, PL= Prelimbic cortex, RSA= Agranular retrosplenial cortex, RSG= Granular retrosplenial cortex. * Differences compared to R45M ($P < .05$). ^ Differences compared to R60M ($P < .05$). & Differences compared to R15M ($P < .05$).

In terms of female densitometric analysis of CCO activity, we also found differences between groups in all the regions measured. Specifically, brain metabolic activity in mPFC showed statistically significant group differences (CG: $H= 32.889$, $P< .001$; PL: $H= 24.483$, $P< .001$; IL: $H= 24.931$, $P< .001$). Dunn's method revealed that in CG, differences were found between the SCF and the R15F, R45F, and R60F groups ($P< .005$), between the R7F and R60F groups ($P< .005$), between the R15F and R30F groups ($P< .005$), and between R30F compared to the R45F and R60F groups ($P< .005$). In PL, differences were found between SCF, R7F, and R30F compared to the R60F group ($P< .005$), and in IL, differences were found between the SCF and R30F compared to the R45F and R60F groups ($P< .005$). As for M1, we found as well significant group differences ($H= 23.623$, $P< .001$). Dunn's method showed that differences in M1 were between R15F, R45F and R60F compared to SCF ($P< .005$). Regarding the hippocampus, statistically significant differences between groups were also found (CA1: $F= 13.532$, $P< .001$; CA3: $F= 9.585$, $P< .001$; DG: $F= 5.461$, $P< .001$). Post-hoc analysis revealed that in CA1, group differences were found between SCF, R7F, and R30F compared to R45F (SCF, R30F: $P< .001$; R7F: $P= .017$), between SCF, R7F, R15F, and R30F compared to R60F (SCF, R7F, R30F: $P< .001$; R15F: $P= .006$), and between R15M and R30M ($P= .041$). In CA3, group differences were found between SCF compared to R15F ($P= .025$), R45F ($P< .001$), and R60F ($P< .001$), between R7F compared to R45F ($P= .021$) and R60F ($P= .002$), and, finally, between R30F compared to R45F ($P= .003$) and R60F ($P< .001$). As for the DG results, different CCO activity was found between SCF compared to R45F ($P= .029$) and R60F ($P= .002$), between R7F compared to R60F ($P= .023$), and between R30F compared to R45F ($P= .01$) and R60F ($P< .029$). Regarding the retrosplenial cortices, statistically significant group differences were also found (RSG: $F= 11.029$, $P< .001$; RSA: DG: $F= 12.045$, $P< .001$). Holm-Sidak's method revealed RSG differences between SCF, R7F, and R30F compared to R45F ($P< .001$), but also between SCF, R7F, R15F, and R30F compared to R60F (SCF, R7F, R30F: $P< .001$; R15F: $P= .045$). The differences in RSA were found between SCF, R7F, R15F, and R30F compared to R45F (SCF, R7F, R30F: $P< .001$; R15F: $P= .032$), and between SCF, R7F, and R30F compared to R60F (SCF, R30F: $P< .001$; R7F: $P= .002$). In the case of the PAR cortex, statistically significant group differences were found ($F= 11.186$, $P< .001$) between SCF, R7F, and R30F, compared to R45F (SCF: $P< .001$, R7F: $P= .004$; R30F: $P= .001$) and R60F ($P< .001$).

Parahippocampal cortices showed statistically significant differences between groups (PRh: F= 17.26, P< .001; ENT: F= 16.112, P< .001). Holm-Sidak's method revealed that differences were found between SCF, R7F, and R30F compared to R45F (SCF, R30F: P< .001; R7F: P= .008), between SCF, R7F, R15F, and R30F compared to R60F (SCF, R7F, R30F: P< .001; R15F: P= .006) and between R7F and R15F compared to R30F (R7F: P= .028; R15F: P= .002). In relation to ENT, differences were found between SCF and R30F compared to R45F (SCF: P= .002; R30F: P< .001), between SCF, R7F, R15F, and R30F compared to R60F (SCF, R30F: P< .001; R7F: P= .002; R15F: P= .015), and between R7F and R15F compared to R30F (R7F: P= .01; R15F P< .001). In reference to auditory cortex, group differences were found, in addition to the observed in DLG (Au1: H= 33.535, P< .001; DLG: H= 31.035, P< .001). As for Au1, differences were found between R15F, R45F and R60F compared to SCF (P< .005), in addition to R7F differences when compared with R45F and R60F (P< .005). Finally, as for DLG, we observed group differences between R15F, R45F and R60F compared to SCF (P< .005) and between R7F and R30F with R60F (P< .005) (Table 2).

Table 2.
CCO female values

Structures	SCF	R7F	R15F	R30F	R45F	R60F
CG	36.93 ± 3.17 *^&	32.26 ± 1.71 ^	25.98 ± 1.34 +	33.92 ± 1.57 *^	24.88 ± 1.33	21.07 ± 1.81
PL	36.27 ± 3.37 ^	31.14 ± 1.41 ^	26.19 ± 1.47	33.80 ± 1.58 ^	25.88 ± 1.55	20.77 ± 1.06
IL	34.81 ± 3.50 *^	29.00 ± 0.99	25.49 ± 1.43	32.57 ± 1.59 *^	24.09 ± 1.55	20.64 ± 1.36
M1	40.40 ± 3.13 *^&	31.75 ± 1.34	25.59 ± 0.88	31.82 ± 1.70	25.41 ± 2.04	27.96 ± 1.02
CA1	27.70 ± 2.28 *^	25.53 ± 1.00 *^	22.64 ± 1.20 ^+	28.21 ± 1.64 *^	19.09 ± 0.98	15.83 ± 0.68
CA3	27.55 ± 2.04 *^&	25.60 ± 1.30 *^	21.63 ± 1.08	26.75 ± 1.72 *^	19.28 ± 1.02	17.69 ± 0.81
DG	40.50 ± 2.72 *^	38.74 ± 1.20 ^	35.33 ± 2.08	41.63 ± 1.82 *^	32.19 ± 1.75	29.86 ± 1.26
RSG	36.012 ± 2.42 *^	34.61 ± 1.11 *^	30.99 ± 1.55 ^	35.45 ± 1.50 *^	24.97 ± 1.33	24.72 ± 1.28
RSA	35.83 ± 2.60 *^	33.85 ± 0.97 *^	30.45 ± 1.76 *	36.73 ± 1.34 *^	23.39 ± 1.30	24.41 ± 1.53
PAR	31.83 ± 2.44 *^	30.99 ± 1.20 *^	25.69 ± 1.59	31.54 ± 1.69 *^	22.40 ± 1.10	19.82 ± 1.16
PRh	29.31 ± 1.94 *^	26.69 ± 1.26 *^+	24.90 ± 1.18 ^+	32.86 ± 2.18 *^	19.50 ± 0.83	17.90 ± 0.85
ENT	25.20 ± 1.79	22.68 ± 1.30 ^+	20.95 ± 1.12 ^+	29.39 ± 1.51 *^	17.44 ± 1.22	14.93 ± 0.94
Au1	34.45 ± 2.38 *^&	30.68 ± 1.36 *^	23.87 ± 0.78	28.79 ± 1.37	22.44 ± 1.30	22.93 ± 0.65
DLG	33.73 ± 2.21 *^&	28.28 ± 1.22 ^	24.89 ± 0.61	28.07 ± 1.37 ^	22.06 ± 1.54	21.74 ± 0.48

Table 2 shows the female CCO values (mean ± SEM) in the SCF, R7F, R15F, R30F, R45F, and R60F groups for all the structures studied. Au1= Primary auditoy cortex, CG= Cingulate cortex, DG= Dentate Gyrus, DLG= Dorsal lateral geniculate nucleus, ENT= Entorhinal cortex, IL=Infralimbic cortex, M1= Primary motor cortex, PAR= Parietal cortex, PRh= Perirhinal cortex, PL= Prelimbic cortex, RSA= Agranular retrosplenial cortex, RSG= Granular retrosplenial cortex. * Differences compared to R45F ($P < .05$). ^ Differences compared to R60F ($P < .05$). + Differences compared to R30F ($P < .05$). & Differences compared to R15F ($P < .05$).

3.3. Correlations

Interregional correlations of CCO activity and rate of retrieval expressed as percentage of time are present in Table 3, both in male and female groups.

Table 3
Correlations between COO activity and rate of retrieval

Structures	Rate of retrieval
CG	*.393 (P < .001*) n=88*
PL	.363 (*P* < .001*) n=90
IL	.375 (*P* < .001*) n=90
M1	.257 (*P*= .0221*) n=86
CA1	.338 (*P* < .001*) n=92
CA3	.334 (*P*= .0252*) n=92
DG	.279 (*P*= .0315*) n=91
RSG	.419 (*P*= .0037*) n=92
RSA	.306 (*P*= .0638) n=91
PAR	.335 (*P*= .0342*) n=90
PRh	.322 (*P*= .0206*) n=89
ENT	.319 (*P*= .0206*) n=89
Au1	.126 (*P*= .224) n=88
DLG	.257 (*P*= .0825) n=89

Table 3 shows the Pearson correlations between different brain regions COO activity and the rate of retrieval. Each table cell shows the calculated Pearson's correlation r value, the *P* level for the calculated correlation coefficient and the number of samples (n). Au1= Primary auditoy cortex, CG= Cingulate cortex, DG= Dentate Gyrus, DLG= Dorsal lateral geniculate nucleus, ENT= Entorhinal cortex, IL=Infralimbic cortex, M1= Primary motor cortex, PAR= Parietal cortex, PRh= Perirhinal cortex, PL= Prelimbic cortex, RSA= Agranular retrosplenial cortex, RSG= Granular retrosplenial cortex. P-value was considered significant when < .05.

4. Discussion

The purpose of the present study was to explore, both behaviourally and at a brain metabolism level, the retrieval of allocentric spatial memories with different degrees of delay after a reference memory task assessed in the MWM, taking into account male and female animals. We cover a wide range of time periods after learning formation, and we distinguish between recent memories –those evaluated when seven days have

elapsed since the memory task– and remote ones with diverse time intervals, *i.e.* 15, 30, 45, and 60 days after task acquisition, studying the underlying brain functioning through CCO activity.

The study of memory retention in rodents has helped us to understand the neurobiology of memory storage and, thus, delve more deeply into the molecular and cellular mechanisms responsible for the transformation of newly formed memories into enduring ones (Frankland & Bontempi, 2005). To our knowledge, memory retrieval has mainly been assessed shortly after the task (Khakpour-Taleghani et al., 2009; Kraeuter et al., 2019; Mendez-Couz et al., 2015; Timić et al., 2013), using different behavioural paradigms such as the Y-maze, the radial arm maze (RAM), and the MWM. In addition, some researchers have focused on the study of both behaviour performance and brain-related activity up to one week after spatial learning acquisition (Barry et al., 2016; Milczarek et al., 2018; Moosavi et al., 2012; Teixeira et al., 2006), whereas fewer have evaluated retention with higher time lags, such as from one week to one month. Conversely, the behavioural outcome and the brain networks involved in the long-term retrieval of spatial information with time delays that exceed a month have not been described yet.

Our behavioural results showed that spatial memory retrieval in male and female rats is preserved seven, 15, and 30 days post-acquisition, but there is forgetfulness after this time, with subjects not being able to remember the position of the hidden platform when 45 and 60 days have elapsed. In reference to our retrieval results, they are consistent with previous literature, as other authors have shown successful retrieval assessed in the MWM after one (Barry et al., 2016; Guskjolen et al., 2017; Harati et al., 2013; Roozendaal et al., 2003; Teixeira et al., 2006) and seven days (Barry et al., 2016; Mendez-Couz et al., 2015; Ramíarez-Amaya et al., 2001). Similar results have also been found using other mazes, revealing a conserved memory six days after the last acquisition trial, although a slight decline (represented by an increase in the percentage of errors in the RAM) was found (Milczarek et al., 2018). Regarding spatial remote memories up to one month, the opposite results are present in the literature. Some of the research in the field shows similar performance to those presented here, showing, therefore, conserved retrieval with higher time delays, such as 14 and 30 days post-acquisition (Barry et al., 2016; Guskjolen et al., 2017; Ramíarez-Amaya et al., 2001; Teixeira et al., 2006). However, others have observed an enhancement of errors compared to the last training session (Milczarek et al., 2018), or even an absence of memory retrieval under standard conditions (Harati et al., 2013). Finally, we have provided information about spatial remote retrieval failure with greater time delays than one month. Because all the male groups and two of the five female groups achieved the learning criteria on the first day of the task, and the other three female groups achieved it on the second day, we suggest that the forgetting found is not a consequence of weaker encoding or impaired storage, but rather it is specific to a deficit in the competence of remembering allocentric information that was consolidated so long ago. Nevertheless, some authors have focused on aversive remote memories through contextual fear conditioning, identifying a strengthening memory with intervals superior to one month (Cox et al., 2013; Quinn et al., 2008), even reaching retention almost seven months post-training (Quinn et al., 2008). This difference may be due to the nature of the learning because fear conditioning causes a strongly emotional aversive

label (Bocchio et al., 2016), and, therefore, it could be easier to remember a potential threat that is ultimately linked to adaptation and survival.

CCO histochemistry reflects changes in the brain metabolic capacity and therefore, is a reliable marker of mitochondrial metabolic competence (Gonzalez-Lima & Cada, 1994; Wong-Riley, 1989). By measuring the mitochondrial enzymes that catalyse oxygen consumption during cellular respiration involved in ATP production, the CCO technique reveals energy demands of neurons underlying prolonged stimulation or training on behavioural tasks (Arias et al., 2015; Hescham et al., 2014; Zorzo, Higarza, et al., 2019). Thus, it has been previously employed to evaluate metabolic changes related to spatial recovery processes (Conejo et al., 2013; Mendez-Couz et al., 2015).

Regarding the study of male brain metabolism, our results showed that, when animals perform the task successfully, that is, the R7M, R15M, and R30M groups, they do not differ from controls or each other. This suggests that recovering spatial information with these time lags is a simple task that does not make a higher metabolic demand. Animals remember the spatial location of the hidden platform by using distal cue information, without requiring a statistically significant energy expenditure (Gonzalez-Lima & Cada, 1994). In contrast, when male animals fail to successfully perform the task, *i.e.* R45M and R60M groups, there is a general decrease in CCO activity compared to controls and the successful-retrieval groups. This result could suggest that failing to remember previously acquired spatial learning leads to an alteration in several brain areas' metabolic activity. Specifically, mPFC neuronal metabolic activity at CG, PL, and IL was reduced in the R45M and R60M groups, suggesting that downregulation of this region leads to an impairment in retrieving spatial information. Accordingly, numerous studies have described the role of mPFC in recent and remote spatial retrieval (Barry et al., 2016; Lopez et al., 2012; Maviel et al., 2004; Teixeira et al., 2006), proposing that memories later become reorganised within the neocortex (Larry R Squire & Alvarez, 1995) and that a reduction in neuronal activity can be linked to a dysfunctional behavioural outcome (Bosch et al., 2013; Cholvin et al., 2016; Rojas et al., 2012). Moreover, patients who suffer from autobiographical–episodic memory loss show a reduction in PFC glucose metabolism, suggesting a functional correlate of memory leakage (Brand et al., 2009; Tomadesso et al., 2015). According the M1 results, there is a decrease of CCO activity in groups that failed to solve the task, suggesting that the motor activity when driven to a certain location that was learned time ago, executes an active role in the performance of the retention task. Furthermore, the R45M and R60M groups showed a CCO decrease in the CA1, CA3, and DG subfields of the dorsal hippocampus, in addition to PAR, PRh, and ENT. The hippocampal formation has also been pointed out as a brain region involved in both recent and remote retrieval (Barry et al., 2016; Bosch et al., 2013; Broadbent et al., 2006; Conejo et al., 2013), as well as in PAR in human mild cognitive impairment patients (Bosch et al., 2013; Nishi et al., 2010). Moreover, previous studies have found no differences in the hippocampus and PRh and ENT compared to control groups, whereas an involvement in a functional network responsible for correctly remembering a cognitive map and the driven behaviour to reach the platform was found in the CA1 and CA3 subfields of the dorsal hippocampus, in addition to a connection with mPFC (Mendez-Couz et al., 2015). In particular, lesions in the ENT lead to an impairment in the retrieval of recent and remote spatial memories (Hales et al., 2018). As for the retrosplenial cortex results, we found

that the R45M group showed reduced neuronal metabolic activity compared to successful-retrieval male groups and controls, both in RSC and RSA. In addition, a reduction in CCO activity was found when comparing the R60M group to the R7M and R15M groups in RSC. Similar results were found in mnemonic retrieval human deficits, suggesting that retrosplenial hypometabolism could be a potential contributor to memory alterations (Milczarek et al., 2018; Nestor et al., 2003; Todd & Bucci, 2015), and that successful reference memory retention involves the stability of retrosplenial cortex engrams (Milczarek et al., 2018). Accordingly, other studies have revealed no changes in retrosplenial CCO activity after spatial retrieval, whereas animals showed successful recent memory (Mendez-Couz et al., 2015). Finally, although the non-successfully retrieval groups showed a decreased metabolic activity in Au1 and DLG, the result is not specific to the behavioural outcome, as other groups that remember the task, had also lower activity, suggesting –and discussed later– that the CCO activity of Au1 and DLG is not related to the retrieval rate.

As for female CCO activity, comparable results can be described. The R45F and R60F groups showed reduced neuronal metabolism in all the measured areas of the limbic system compared to controls, with the exception of the R45F group in PL. Therefore, the mPFC, hippocampal, retrosplenial, PAR, PRh, and ENT areas are essential in memory recall (Barry et al., 2016; Bosch et al., 2013; Mendez-Couz et al., 2015; Todd & Bucci, 2015). Furthermore, the R15F group showed a reduced metabolism, compared to controls, in the CG and CA3 subfields of the dorsal hippocampus, suggesting that inhibitory activity in these areas may be taking place in order to solve the task (Ma et al., 2014). Moreover, the R45F group showed reduced activity compared to successful retrieval groups, *i.e.* R7F, R15F, and R30F groups. As for the recent memory group, differences were found in CA1, CA3, RSG, RSA, PAR, and PRh, compared to the R15F group in the RSA and the R30F group in the CG, IL, hippocampus, retrosplenial, PAR, PRh, and ENT. As for the long-lasting spatial retrieval female group, similar results were found, revealing decreases in CCO activity compared to the R7F group in the CG, PL, hippocampus, retrosplenial, PAR, PRh, and ENT; compared to the R15F group, differences were found in CA1, RSG, PRh, and ENT; and compared to R30F, in all the areas measured. Finally, the R30F group showed an enhancement of CCO activity compared to R15F in CG, CA1, PRh, and ENT, and compared to R7F in PRh and ENT. Similar to what happens in some male brain areas, lower metabolism was found in the non-successfully retrieval groups across M1, Au1 and DLG. This difference was no specific to the behavioural outcome, showing other groups that perform adequately the task, also a decreased in CCO activity, proposing that the brain metabolism of these areas is not related to the percentage of retrieval. The existing differences in male animals between the groups that do not remember the location of the hidden platform and those that have great performance were not systematically found in female rats. However, the pattern of CCO activity shows more differences between groups, regardless of a positive *versus* negative behavioural outcome. On balance, these results could suggest greater heterogeneity in the neuronal metabolism of female animals, probably because ovarian hormones influence both brain activity and different memory systems (Yagi et al., 2017).

Overall, although both male and female animals showed similar behavioural performance, it is important to note that CCO activity seems to be more related to

behavioural success or failure than to the time elapsed since spatial acquisition in male rats, but in female animals, there is also an effect of time intervals that modifies brain metabolic activity. Moreover, we have to highlight that we are examining neuronal metabolic activity in response to performance on a behavioural task. When learning takes place, reduced CCO activity has been linked to a positive impact on spatial behavioural performance (Méndez-López et al., 2009; Sampedro-Piquero, Zancada-Menendez, Begega, Mendez, et al., 2013; Sampedro-Piquero, Zancada-Menendez, Begega, Rubio, et al., 2013), as well as to acquired learning criteria during acquisition (Conejo et al., 2010). With regard to the neuronal metabolic results in the non-successful retrieval groups, we can also propose that the CCO reduction in the majority of the limbic brain regions could be due to the fact that the animals are trying to learn new visual distal relationships in order to solve the task, that is, to find the hidden platform. It is important to note that the contingencies the animals are subjected to during the task are known, and although they do not remember the exact position of the platform, the R45 and R60 groups probably remember that there was a driven behaviour that led to escaping from an aversive event, such as the water. However, these changes are not present in the control groups or good recall groups because the latter do not need to modify their neuronal metabolic activity in order to reach the platform, due to previously well-consolidated learning. Conversely, the study of correlations between CCO activity across different brain areas, and the rate of retrieval, regardless the time that has been elapsed since memory acquisition, has revealed a positive correlation between the time spent in the reinforced quadrant and the CCO activity in the CG, PL, IL, M1, CA1, CA3, DG, RSG, RSA, PAR, PRh and ENT of male and female rats. It is clear that all of them, excluding M1, take part from brain limbic system, and that mPFC, hippocampus, retrosplenial, parietal and rhinal cortices contributes in the process of remote spatial retrieval, as previously described (Barry et al., 2016; Broadbent et al., 2006; Hales et al., 2018; Nishi et al., 2010; Todd & Bucci, 2015). Moreover, the M1 CCO activity, lower in the groups that failed to remember the hidden platform, can be useful during retrieval, as animals need to perform a motor activity and deliberate movements in order to address to the target quadrant. However, no significant correlations were found in Au1 and DLG, suggesting that these areas are not specifically linked to the percentage of retrieval that animals showed in the probe test.

In general, neocortical, hippocampal, and parahippocampal brain regions appear to be essential to achieve retention criteria, with these data supported by both the standard consolidation theory, which proposes a reorganization of memories over time into cortical structures (Larry R Squire & Alvarez, 1995), and the multiple trace theory, which highlights the role of the hippocampus in retrieval (Nadel & Moscovitch, 1997). These theories suggest that retrieving memories that were previously acquired and dependent on hippocampal and neocortical circuits involves the reactivation of neuronal engrams established during learning, and they shed light on the present results because decreased metabolic activity could be linked to spatial retrieval failure (Tayler et al., 2013).

Finally, gender differences in spatial learning and memory have been reported in humans, determining that men usually obtain better performance than women (Fernandez-Baizan et al., 2019; León et al., 2016; Sneider et al., 2015). Thus, there is growing evidence suggesting the role of various influential variables, such as the

strategy employed (Pletzer et al., 2019), with females showing a preference for using landmark information, whereas males favour cardinal directions (Andersen et al., 2012), experience or familiarity with the environment (De Goede & Postma, 2015), task demands (Chamizo et al., 2011), or sex-hormone impacts (Scheuringer & Pletzer, 2017). In rodents, sex differences favouring males have also been depicted in hippocampal-dependent learning and memory (Jonasson, 2005), which have been linked, among others, to different strategy choices displayed to solve the task (Duarte-Guterman et al., 2015; Grissom et al., 2013) and close attention to the specific features of landmarks (Chamizo et al., 2014). Moreover, it has been revealed that pre-training sessions can lead to an improvement in female performance, suggesting that these differences may be ubiquitous to the initial phases of learning (Anderson et al., 2013; Woolley et al., 2010).

However, little is known about the role of sex in spatial memory retrieval because only a few studies have addressed this question (Qi et al., 2016; Sebastian et al., 2013). To our knowledge, this is the first study to provide information about what is occurring behaviourally and at a brain metabolism level in healthy males and females in the retention of recent and remote allocentric memories covering a broad time scale and assessed in the MWM. We have shown that male and female rats behave similarly, obtaining successful retrieval seven, 15, and 30 days after training, but an impaired retrieval after 45 or 60 days. Our results agree with recent human studies that employed the virtual version of the MWM and showed that, although sex differences were found during the learning phase, there were no differences in spatial memory recovery (Piber et al., 2018). In contrast, it has been reported that males outperformed females at seven and 28 days post-acquisition (Qi et al., 2016). However, it is important to note that the subjects' ages differ because the aforementioned study was carried out during adolescence, not adulthood, suggesting that the ability to solve the task can also be dependent on the age of the animals when tested. Additionally, other authors, using a different spatial procedure, *i.e.* the RAM, found better performance by females one day after training, whereas male outperformed them 30 days after learning (Sebastian et al., 2013). Furthermore, regarding gonadal hormones, females in proestrous, compared to estrous, have shown worse performance on spatial learning, suggesting that one of the reasons can be stress novelty, which is more likely to interfere during the proestorus stage (Duarte-Guterman et al., 2015).

5. Conclusions

In conclusion, our work adds information about the behavioural retrieval of an allocentric spatial reference task, showing successful performance seven, 15, and 30 days after learning acquisition, but impaired performance after 45 and 60 days, in both male and female rats. Moreover, we observed reduced neuronal metabolic activity in the medial prefrontal cortex, the parietal, retrosplenial, rhinal cortex and hippocampal regions, measured through CCO histochemistry, in all the groups that failed to solve the task, in addition to certain differences between the successful-retrieval female groups.

Credit Author Statment

Candela Zorzo: investigation, formal analysis, Writing - Original Draft, visualization.

Jorge L. Arias: conceptualization, methodology, Writing - Review & Editing, supervision, project administration, resources, funding acquisition.

Marta Méndez: conceptualization, methodology, Writing - Review & Editing, supervision, project administration, resources, funding acquisition.

Acknowledgements

This study was funded by Project grants of Secretaría De Estado De Investigación, Desarrollo E Innovación Del Gobierno de España (PSI2017-83893-R and PSI2017-90806-REDT) and Programa "Severo Ochoa" de Ayudas Predoctorales de la Consejería De Cultura Y Deporte del Principado de Asturias (PA-18-PF-BP17-011) to C.Z. We thank AINDACE Foundation (Ayuda a la Investigación del Daño y Enfermedades Cerebrales).

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Figure1

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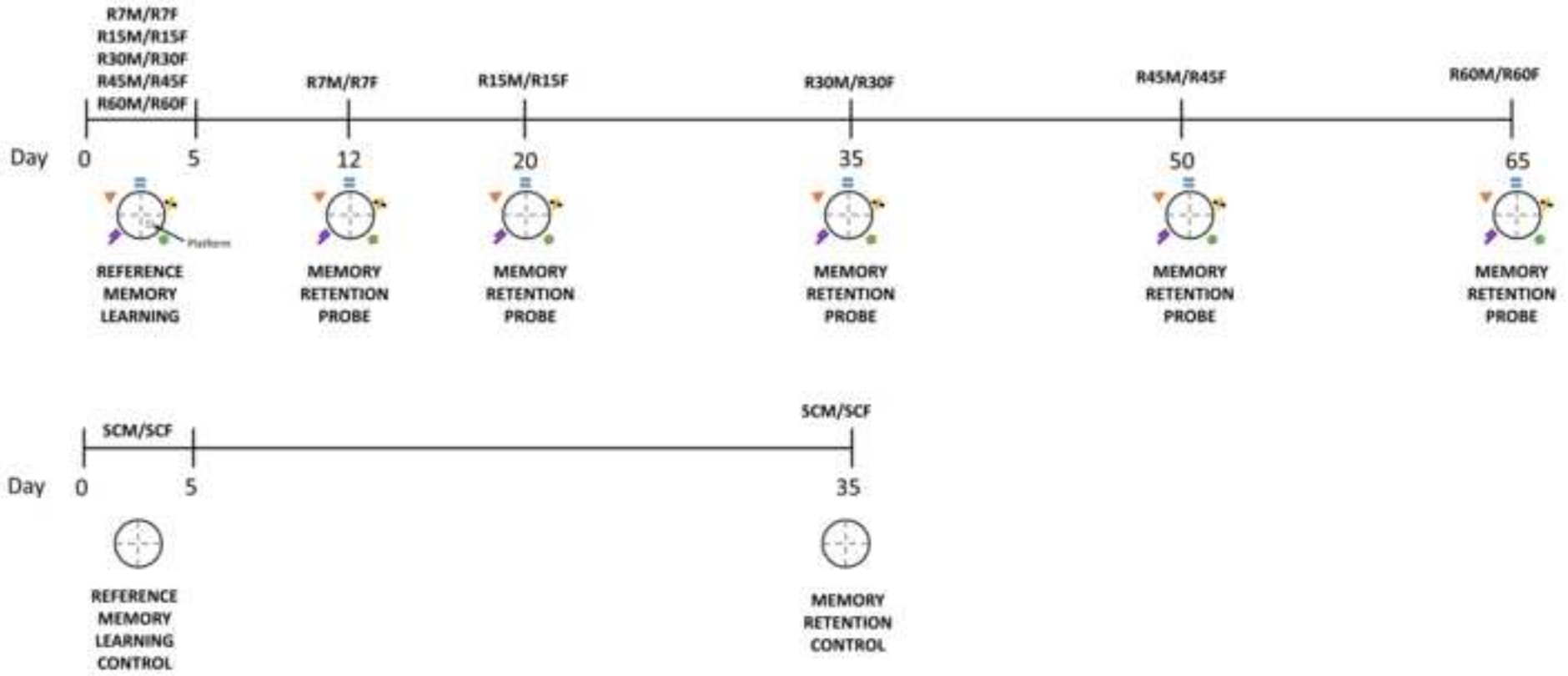

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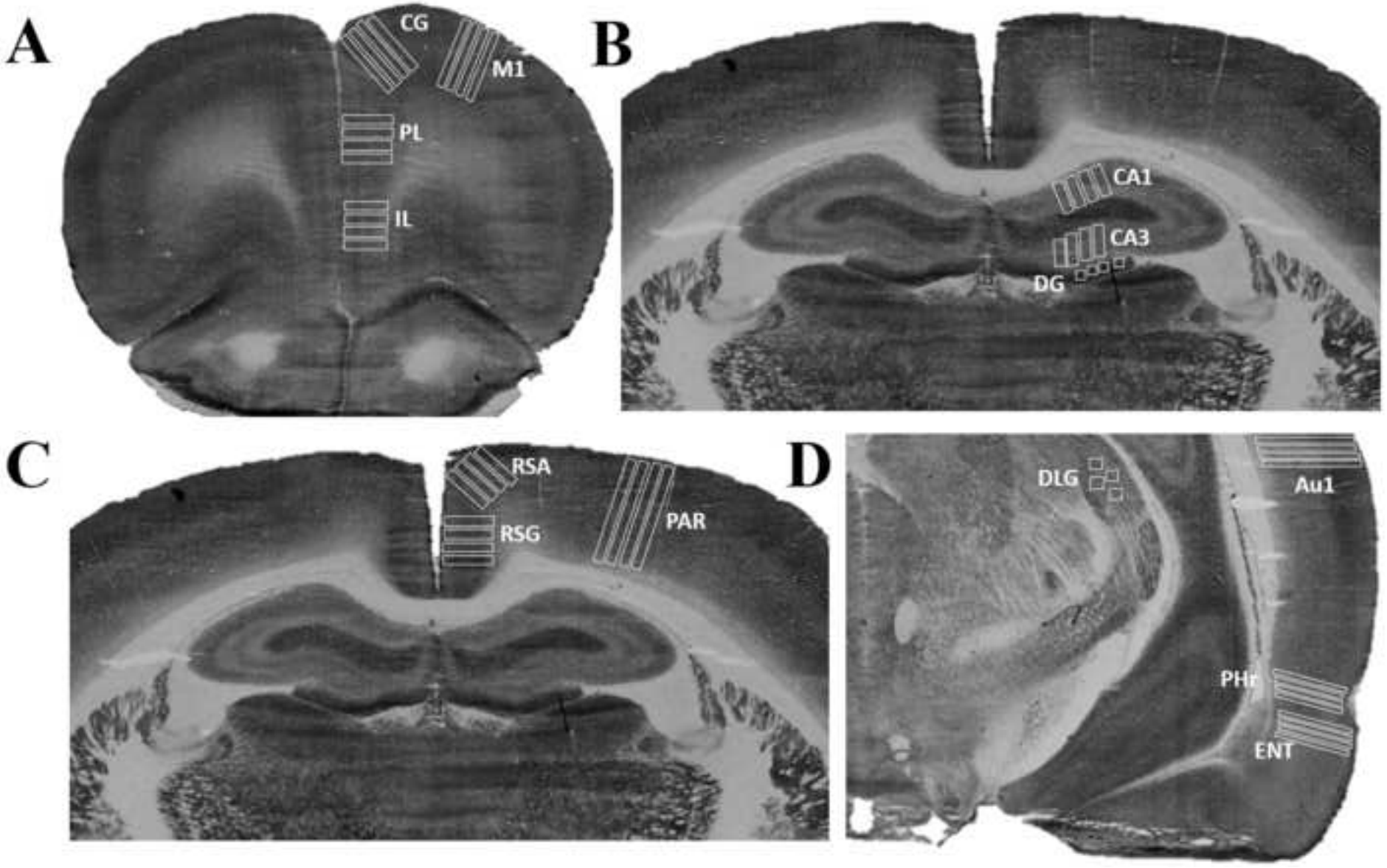

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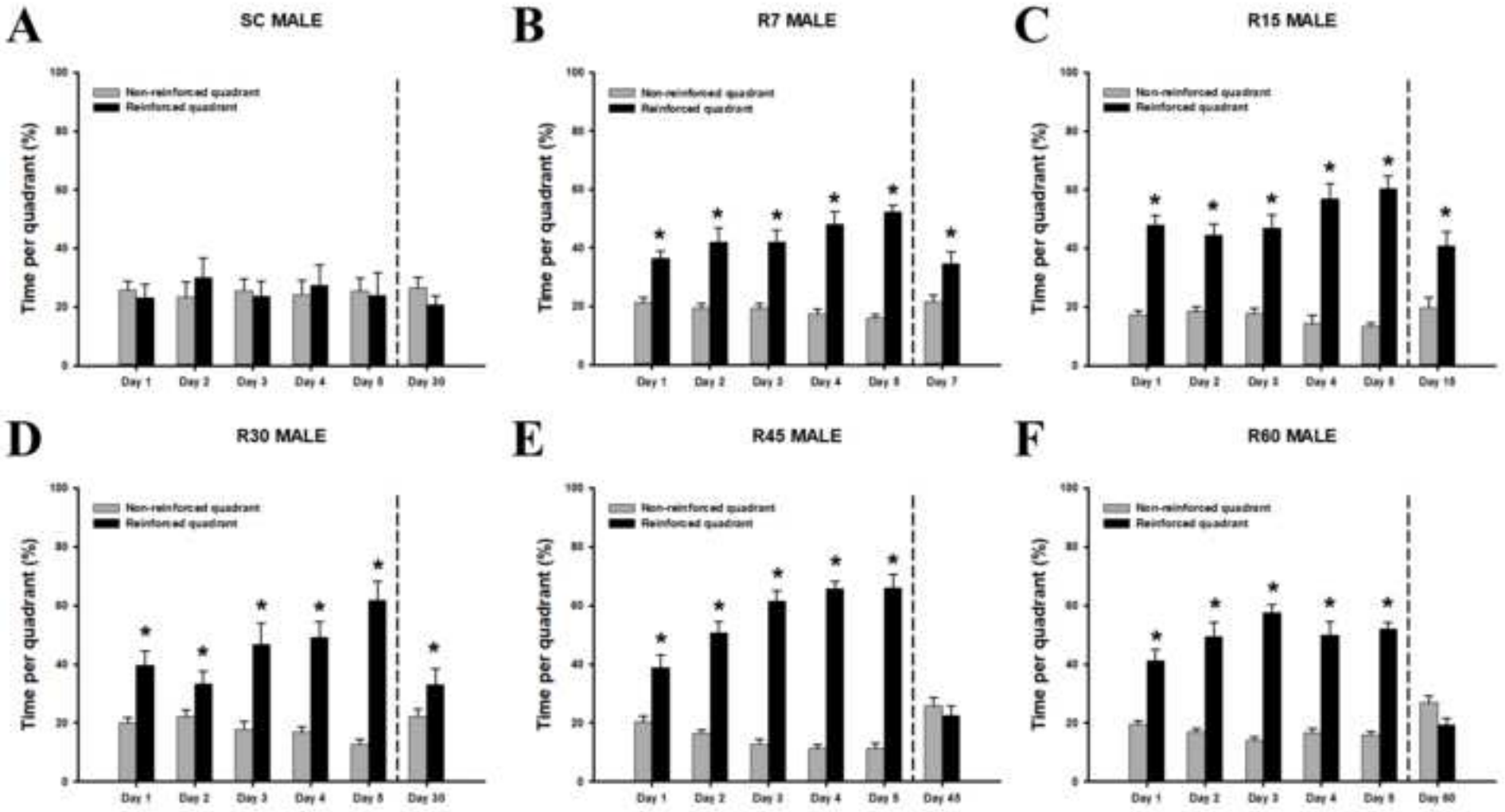

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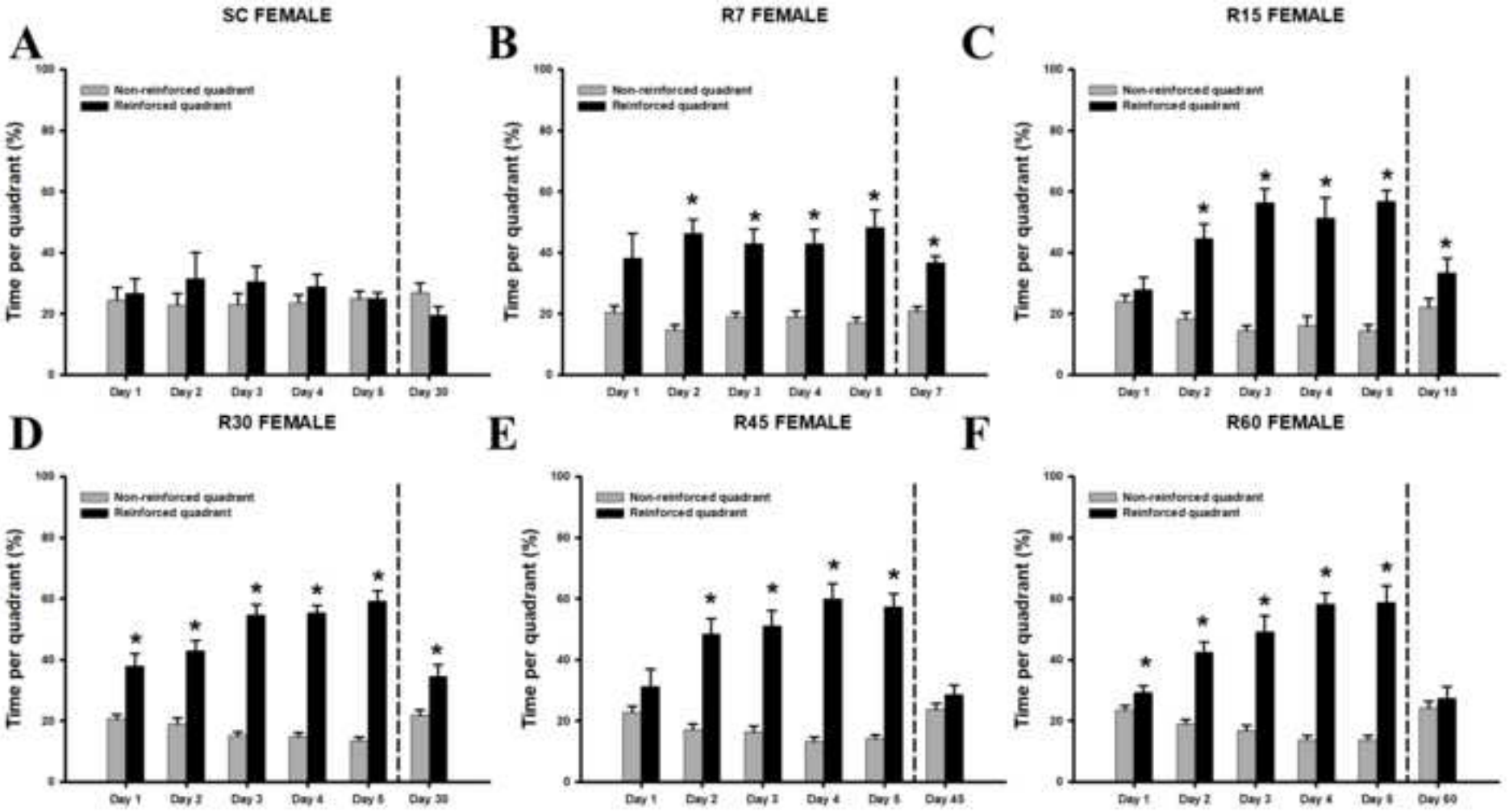

Table 1.

CCO male values

Structures	SCM	R7M	R15M	R30M	R45M	R60M
CG	32.11 ± 1.19 *^	32.84 ± 1.06 *^	28.55 ± 1.56 *^	31.20 ± 2.36 *^	20.31 ± 1.14	19.74 ± 0.77
PL	32.30 ± 0.92 *^	32.67 ± 1.10 *^	29.46 ± 1.70 *^	30.67 ± 1.70 *^	20.28 ± 0.91	19.87 ± 1.11
IL	31.59 ± 0.94 *^	31.09 ± 1.26 *^	28.89 ± 1.81 *^	31.31 ± 1.75 *^	18.06 ± 0.73	18.22 ± 1.15
CA1	26.90 ± 0.65 *^	23.47 ± 0.96 *	22.06 ± 1.45 *	25.73 ± 1.92 *^	14.00 ± 0.34	14.57 ± 1.32
CA3	27.93 ± 0.61 *^	22.97 ± 1.06 *	22.94 ± 1.22	26.96 ± 1.73 *^	13.93 ± 0.41	15.89 ± 1.35
DG	40.57 ± 1.02 *^	44.08 ± 1.74 *^	40.98 ± 2.23 *^	40.55 ± 3.20 *^	24.40 ± 0.62	31.84 ± 2.02
RSG	34.48 ± 0.82 *	35.25 ± 0.96 *^	34.79 ± 1.69 *^	35.17 ± 2.27 *	20.19 ± 0.52	24.07 ± 1.16
RSA	33.76 ± 0.96 *	32.67 ± 1.35 *	32.89 ± 1.42 *	34.91 ± 2.49 *	19.36 ± 0.50	25.92 ± 1.12
PAR	31.20 ± 0.72 *^	30.88 ± 1.16 *^	27.69 ± 1.72 *^	31.95 ± 1.77 *^	17.60 ± 0.47	21.88 ± 1.19
PRh	30.55 ± 0.85 *^	25.69 ± 1.30 *^	27.45 ± 1.61 *^	28.57 ± 1.31 *^	16.49 ± 0.96	17.23 ± 1.37
ENT	27.71 ± 0.50 *^	23.72 ± 1.30 *^	25.02 ± 1.61 *^	26.26 ± 1.42 *^	14.38 ± 0.63	15.25 ± 1.29

Table 1 shows the male CCO values (mean ± SEM) in the SCM, R7M, R15M, R30M, R45M, and R60M groups for all the structures studied. Au1= Primary auditoy cortex, CG= Cingulate cortex, DG= Dentate Gyrus, DLG= Dorsal lateral geniculate nucleus, ENT= Entorhinal cortex, IL=Infralimbic cortex, M1= Primary motor cortex, PAR= Parietal cortex, PRh= Perirhinal cortex, PL= Prelimbic cortex, RSA= Agranular retrosplenial cortex, RSG= Granular retrosplenial cortex.* Differences compared to R45M ($P < .005$). ^ Differences compared to R60M ($P < .005$).

Table 2.
CCO female values

Structures	SCF	R7F	R15F	R30F	R45F	R60F
CG	36,93 ± 3,17 *^&	32,26 ± 1,71 ^	25,98 ± 1,34 +	33,92 ± 1,57 *^	24,88 ± 1,33	21,07 ± 1,81
PL	36,27 ± 3,37 ^	31,14 ± 1,41 ^	26,19 ± 1,47	33,80 ± 1,58 ^	25,88 ± 1,55	20,77 ± 1,06
IL	34,81 ± 3,50 *^	29,00 ± 0,99	25,49 ± 1,43	32,57 ± 1,59 *^	24,09 ± 1,55	20,64 ± 1,36
CA1	27,70 ± 2,28 *^	25,53 ± 1,00 *^	22,64 ± 1,20 ^+	28,21 ± 1,64 *^	19,09 ± 0,98	15,83 ± 0,68
CA3	27,55 ± 2,04 *^&	25,60 ± 1,30 *^	21,63 ± 1,08	26,75 ± 1,72 *^	19,28 ± 1,02	17,69 ± 0,81
DG	40,50 ± 2,72 *^	38,74 ± 1,20 ^	35,33 ± 2,08	41,63 ± 1,82 *^	32,19 ± 1,75	29,86 ± 1,26
RSG	36,012 ± 2,42 *^	34,61 ± 1,11 *^	30,99 ± 1,55 ^	35,45 ± 1,50 *^	24,97 ± 1,33	24,72 ± 1,28
RSA	35,83 ± 2,60 *^	33,85 ± 0,97 *^	30,45 ± 1,76 *	36,73 ± 1,34 *^	23,39 ± 1,30	24,41 ± 1,53
PAR	31,83 ± 2,44 *^	30,99 ± 1,20 *^	25,69 ± 1,59	31,54 ± 1,69 *^	22,40 ± 1,10	19,82 ± 1,16
PRh	29,31 ± 1,94 *^	26,69 ± 1,26 *^+	24,90 ± 1,18 ^+	32,86 ± 2,18 *^	19,50 ± 0,83	17,90 ± 0,85
ENT	25,20 ± 1,79	22,68 ± 1,30 ^+	20,95 ± 1,12 ^+	29,39 ± 1,51 *^	17,44 ± 1,22	14,93 ± 0,94

Table 2 shows the female CCO values (mean ± SEM) in the SCF, R7F, R15F, R30F, R45F, and R60F groups for all the structures studied. Au1= Primary auditoy cortex, CG= Cingulate cortex, DG= Dentate Gyrus, DLG= Dorsal lateral geniculate nucleus, ENT= Entorhinal cortex, IL=Infralimbic cortex, M1= Primary motor cortex, PAR= Parietal cortex, PRh= Perirhinal cortex, PL= Prelimbic cortex, RSA= Agranular retrosplenial cortex, RSG= Granular retrosplenial cortex. * Differences compared to R45F ($P < .005$). ^ Differences compared to R60F ($P < .005$). + Differences compared to R30F ($P < .005$). & Differences compared to R15F ($P < .005$).

Credit Author Statment

Candela Zorzo: investigation, formal analysis, Writing - Original Draft, visualization.

Jorge L. Arias: conceptualization, methodology, Writing - Review & Editing, supervision, project administration, resources, funding acquisition.

Marta Méndez: conceptualization, methodology, Writing - Review & Editing, supervision, project administration, resources, funding acquisition.