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Research report

Differential effects of spaced vs. massed training in long-term object-identity and object-location recognition memory

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HIGHLIGHTS

- Spaced training promotes more exploration time as compared to massed training.
- Spaced training improves object-identity and not object-location memory.
- Object representation may be improved throughout days of off-line reactivation.
- Spaced training may improve object encoding but also its consolidation.
- The separated streams processing object information respond differently to training.

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ABSTRACT

Here we tested whether the well-known superiority of spaced training over massed training is equally evident in both object identity and object location recognition memory. We trained animals with objects placed in a variable or in a fixed location to produce a location-independent object identity memory or a location-dependent object representation. The training consisted of 5 trials that occurred either on one day (Massed) or over the course of 5 consecutive days (Spaced). The memory test was done in independent groups of animals either 24 h or 7 days after the last training trial. In each test the animals were exposed to either a novel object, when trained with the objects in variable locations, or to a familiar object in a novel location, when trained with objects in fixed locations. The difference in time spent exploring the changed versus the familiar objects was used as a measure of recognition memory. For the object-identity-trained animals, spaced training produced clear evidence of recognition memory after both 24 h and 7 days, but massed-training animals showed it only after 24 h. In contrast, for the object-location-trained animals, recognition memory was evident after both retention intervals and with both training procedures. When objects were placed in variable locations for the two types of training and the test was done with a brand-new location, only the spaced-training animals showed recognition at 24 h, but surprisingly, after 7 days, animals trained using both procedures were able to recognize the change, suggesting a post-training consolidation process. We suggest that the two training procedures trigger different neural mechanisms that may differ in the two segregated streams that process object information and that may consolidate differently.

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1. Introduction

After the characterization of HM's memory impairments [1,2], it was evident that several types of memory exist [3]. One of these is declarative memory, which integrates information that allows us to describe facts and knowledge [4]. Nowadays there is great interest in understanding the cognitive features and underlying neural mechanisms of declarative memory, to ultimately explain memory and design strategies to treat alterations that occur during aging and various pathological conditions in humans [5,6]. For this

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reason, the development of animal models to evaluate declarative memory has been an active endeavor for neuroscientists. In particular the rat model offers the possibility to identify in great detail the neurobiological mechanisms that underlie the expression of this type of memory.

The most widely used rat behavioral model to assess declarative memory is the object recognition memory task, which was originally developed as a one-trial learning task to evaluate working memory [7,8]. This task takes advantage of rats' natural preference for novel objects, such that the time spent in exploring the novel objects compared with the familiar ones is used as a behavioral index to test object recognition memory [9]. The standard behavioral design of this task [8] is to expose a single rat to a particular context, to which the animals may have been habituated [10] and which is different from its home cage. In this context the animal is exposed to two identical copies of an object (sample phase), typically for 3 min, and after a delay of 1 to 15 min (during which the animal is placed back in its home cage), the rat is exposed to a novel object and an identical copy of what is now the familiar object (test phase). Successful recognition is displayed when the rat spends more time exploring the novel object than the familiar object during the test period. The spontaneous recognition of novelty is not simply specific to objects: it is also shown when a familiar object is in a position different from where it was previously encountered [9], indicating that not only the features of the object are represented, but also that the context-specific location of the object is integrated into memory [11]. Furthermore, it appears that object-location and object-identity may involve different neural substrates [11–13].

However, declarative memory in humans allows us to store information for a prolonged period of time and in most cases in the rat model, the object recognition memory task and likewise the memory itself only lasts for a single day [9,10]. By exposing rats repeatedly and briefly to the sample object over a period of a few days, the animals develop a long-term object recognition memory that lasts around 3 weeks [14,15]. This behavioral result suggests that a spaced training procedure that lasts several days is a critical factor for rats in developing a long-term memory representation [16,17] of the experienced object and its location. This is possible since it has long been proposed that:

"*. . .any considerable number of repetitions a suitable distribution of them over a space of time is decidedly more advantageous than the massing of them at a single time*." [18]. This is currently known as the spacing effect (see [19]). It may be that the spacing effect arises because of consolidation processes that take place in the interval between training and testing, such that spaced training allows more time for consolidation [20]. Although the spacing effect and post-training consolidation have been reported a number of times, it is not yet known whether all types of memory show these effects equally, or whether there are system-specific differences that may shed light on the underlying neural mechanisms.

In the present work, we test the effects of both training type (spaced vs. massed trials) and retention interval on memory for object-identity vs. memory for object-location (see Table 1). These variables were compared as follows:

(1) Training type (massed vs spaced training trials)
(2) Retention interval (short (24 h) vs. long (7 days)).
(3) Memory type (fixed vs variable object location during training and test with a new object or a new location with the familiar objects).

We exposed rats to 5 training sessions that lasted 10 min each, either in a spaced training condition that occurred over the course of 5 days or in a massed training condition that occurred on a single day (Fig. 1; Table 1). We also distinguished between training that promotes a representation independent of object location by exposing rats to the objects placed in different locations (Experiments 1 and 3); separate groups of animals were trained with objects placed in the same location to promote an association of the object with that location (Experiment 2). In independent groups of rats, the memory test was done after different retention intervals, i.e., either 24 h or 7 days after the last training trial (In the 3 experiments). In the memory test, the animals were exposed either to a brand-new object when they were trained with the objects placed in switching locations (Experiment 1), or to a brand-new location for the familiar objects when the animals were trained with the objects placed in the same location (Experiment 2), and also when the animals were trained with the objects placed in switching locations (Experiment 3).

We show here that object-identity recognition memory and object-location recognition memory show different responsiveness to spaced training in contrast to massed training, and also found an interesting effect of post-training long-term consolidation. We discuss these results in terms of the different anatomical structures that subserve these different recognition memory types, and the different cellular and molecular events that may underlie the spacing effect.

2. Materials and methods

2.1. Animals

One hundred ninety and two male Wistar rats were used as subjects ($n = 8$ per group). Rats were 5 months old, weighed 400–500 g at the beginning of the experiment, and received free access to food and water throughout the experiment. The colony room was on a 12 h:12 h inverted light-dark cycle with lights on at 20:00, and the temperature was maintained between 22 and 24 °C. Before the experiment was started subjects received two 5-min daily handling sessions for 7 days, the first one in the morning and the second one in the afternoon.

2.2. Apparatus and materials

The apparatus used was an open, acrylic box of 45 cm × 45 cm with walls 45 cm in height, all overlaid with black foam plastic sheets. The floor was covered with wood shavings, which were changed between each trial and each rat, and the box was cleaned with 6% acetic acid. A video camera was positioned over the box, and each trial was videotaped for later analysis.

The test stimuli consisted of six different objects (Supplementary Fig. 2): three light bulbs varying in design were approximately 10 cm in height and 6 cm in width, designated as a, c, and e, & and three glass jars varying in height (9, 12, and 14 cm) and width (4, 7, and 8 cm) were designated as b, d, and f (see Supplementary 2). When subjects were trained and tested, the objects were attached with Velcro to the box floor, at a distance of 10 cm from the back wall and 7 cm from other stimuli. The objects were washed after each trial with a 70% alcohol solution.

2.3. Behavioral procedures

These consisted in four phases: habituation, training, retention interval, and memory test, and all trials were conducted in the same sound-deadening room, with background masking noise.

2.3.1. Habituation

Rats were allowed to free explore the open box, without objects. Each animal was individually placed in it for 3 min per day, for five consecutive days.

2.3.2. Training

Rats were placed into the open box, which contained the 3 sample objects, for five 10-min trials either in a spaced or massed procedure. Animals in the spaced-training group received one training trial per day, while subjects in the massed-training group received five trials in one day, with an inter-trial interval of 10 min. In all groups, the animals remained in their home cage during the inter-trial interval during training. In Experiments 1 and 3, the same series of sample object arrangements was used for all groups (i.e., a-b-c, b-c-a, c-a-b, b-a-c and a-c-b); however, in Experiment 2 the objects were always presented in the same configuration (i.e. a-b-c-).

2.3.3. Retention interval

During the retention interval all subjects remained in their home cages. Independent groups of animals were kept in their home cages for either 24 h or 7 days after last training trial.

Table 1

	Training protocol	Object location during training	Result of training	Consolidation interval	Testing condition	Result of testing
Experiment 1 (effect of massed vs spaced training on recognition of a novel object)						
Group 1	Spaced	Variable	Decrease in exploration through trials, more time exploring objects in total (Fig. 2)	24 h	New object	Object recognition (Fig. 3A)
Group 2	Spaced	Variable		7 days	New object	Object recognition (Fig. 3B)
Group 3	Massed	Variable	Decrease in exploration through trials, less time exploring objects in total (Fig. 2)	24 h	New object	Object recognition (Fig. 3C)
Group 4	Massed	Variable		7 days	New object	No object recognition (Fig. 3D)
Experiment 2 (effect of massed vs spaced training on recognition of a novel object-place association)						
Group 1	Spaced	Fixed	Decrease in exploration through trials, more time exploring objects in total (Fig. 4)	24 h	New location	Location recognition (Fig. 4A)
Group 2	Spaced	Fixed		7 days	New location	Location recognition (Fig. 4B)
Group 3	Massed	Fixed	Decrease in exploration through trials, less time exploring objects in total (Fig. 4)	24 h	New location	Location recognition (Fig. 4C)
Group 4	Massed	Fixed		7 days	New location	Location recognition (Fig. 4D)
Experiment 3 (effect of massed vs spaced training on recognition of a novel object-place association)						
Group 1	Spaced	Variable	Decrease in exploration through trials, more time exploring objects in total (Data not shown)	24 h	New location	Location recognition (Fig. 5A)
Group 2	Spaced	Variable		7 days	New location	Location recognition (Fig. 5B)
Group 1	Massed	Variable	Decrease in exploration through trials, more time exploring objects in total (Data not shown)	24 h	New location	No location recognition (Fig. 5C)
Group 2	Massed	Variable		7 days	New location	Location recognition (Fig. 5D)

2.3.4. Test

During this phase all subjects received 3 test trials following the pre-determined (i.e., 24-h or 7-day) retention interval. In Experiment 1, the subjects were reintroduced for 3 min to the open box containing two familiar objects and one novel object (i.e., glass jar). This trial was repeated two more times (3 test trials total) for each rat, with a different novel object in each memory trial (total of 3 different novel objects) and a new pair of familiar object in each trial, and the position of all objects was changed. Meanwhile, for Experiments 2 and 3, the same objects used during training (a, b, c) were used during the memory test, but a brand-new location for the object was used. During the memory test the location of each familiar object changed in

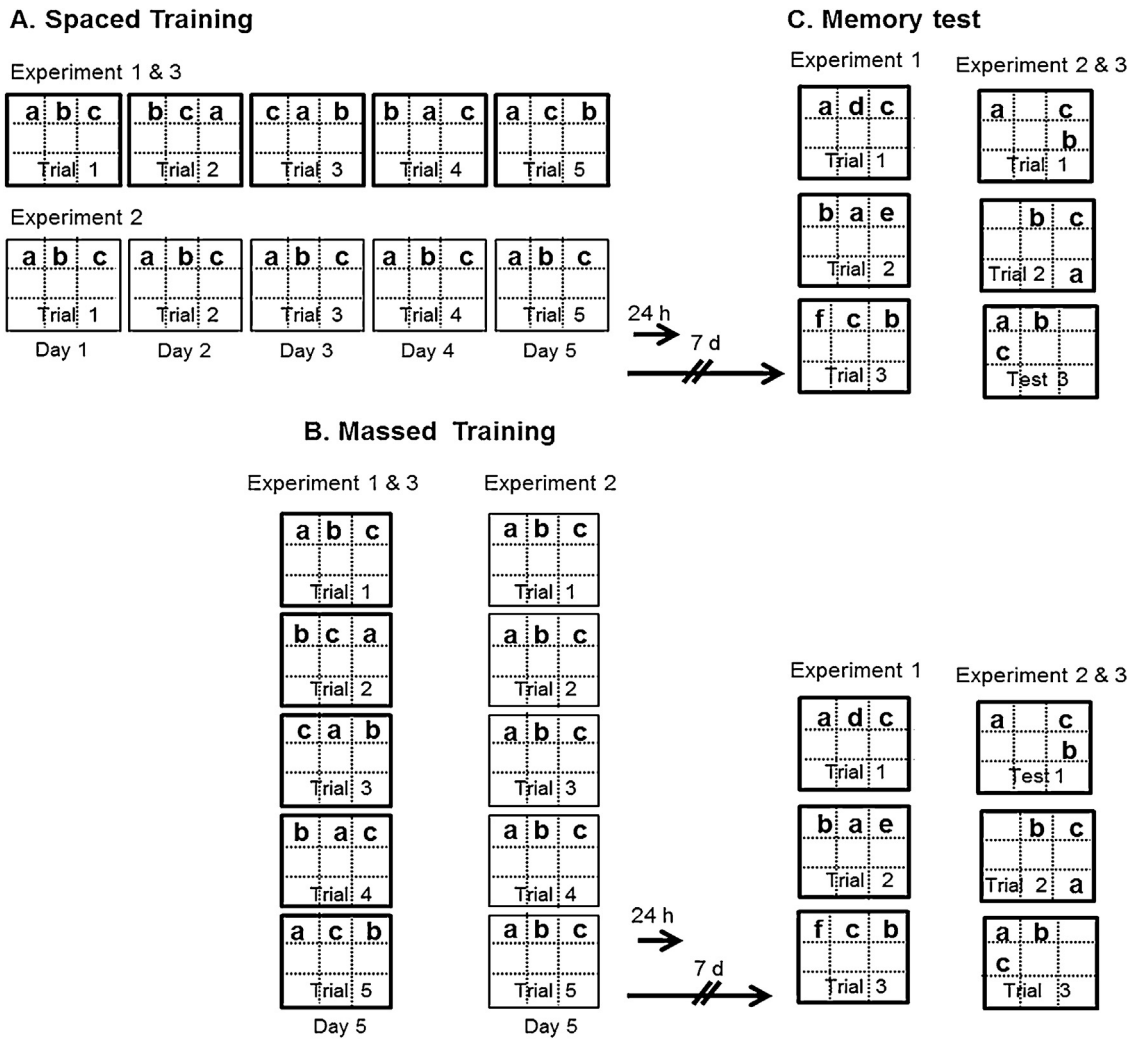

Fig. 1. Schematic representation of the experimental design: Experiment 1 (effect of massed vs spaced training on the recognition of a novel object-identity) Experiment 2 (effect of massed vs spaced training on the recognition of a novel object-location) Experiment 3 (effect of massed vs spaced training on the recognition of a novel object-zone-location). Each square box divided into nine grids represents the context and the location of the objects within it. During training objects that will become familiar (a, b, c) were presented in 5 trials of 10 min each. Note that for Experiments 1 and 3, the objects were placed in different locations, so every trial used a different object configuration; for Experiment 2 the objects were placed in the exact same location for every trial. In the spaced-training condition (A), the inter-trial interval was 24 h, so the five trials occurred on 5 consecutive days, while in the massed-training condition (B) the inter-trial interval was 10 min, so the five trials occurred within a single day (Day 5 of the behavioral procedures). The memory test was performed either 24 h or 7 days after the last training trial. In the memory test (C) of Experiment 1, the animals were exposed to a brand-new object in each of the 3 trials (d, e or f) that appeared in a familiar location replacing one of the familiar objects. It is important to note that the location were each familiar object was placed during test, was used in 2 trials during training. During test each trial lasted 3-min. In Experiments 2 and 3 each familiar object was placed in a brand-new location (note that a location in a different zone of the grid was used) for each trial, such that the 3 familiar objects appeared in 3 different novel locations.

each trial to a brand-new location, i.e., to a location not previously occupied by any of the objects, so 3 different locations were used throughout the memory test (see Fig. 1).

2.4. Behavioral analysis

We decided to use the time exploring the objects as the basic measure for analysis, we consider that by comparing the total time spent in exploration of each of the objects was a more purist way to show whether or not the rats present novel object recognition, since the rationale behind this memory representation is the rats tendency to explore more either new objects and new object locations [9,20]. Exploration of an object was defined as follows: directing the nose at a distance of <1 cm to the object or touching it with the nose. This analysis was done off-line using the videos recorded during behavioral testing by 2 experimenters blind to the experimental conditions; basically, an imaginary boundary line surrounding the object at 1-cm distance was used, and the time during which the animal crossed this boundary with the nose directed to the object was measured.

2.5. Statistical analysis

In order to simplify the presentation of the results, Two-way ANOVAs were used to compare the different conditions by using Statview software.

3. Results

In order to rule out a natural preference for one or some of the objects used in our study (see [20]), or an order of presentation preference, we first evaluated whether object identity or object presentation order affected the exploration time for the objects used in the recognition memory task. An independent group of rats ($n = 6$) underwent a Latin square preference test. This test was conducted under conditions similar to those described in the object recognition training and test procedures. The only difference was that subjects were exposed to the different objects one at a time for a period of 10 min each, and the exploration time was measured. The results revealed similar exploration times averaging ~22 s for all objects (Supplementary Fig. 1A); the ANOVA revealed no significant exploration time difference among the six different objects ($F_{(5,25)} = 0.24$, $p > 0.01$). Moreover, no order preferences were observed ($F_{(5,25)} = 1.769$, $p > 0.01$) (Supplementary Fig. 1B). This indicates that all objects used in the present study were explored

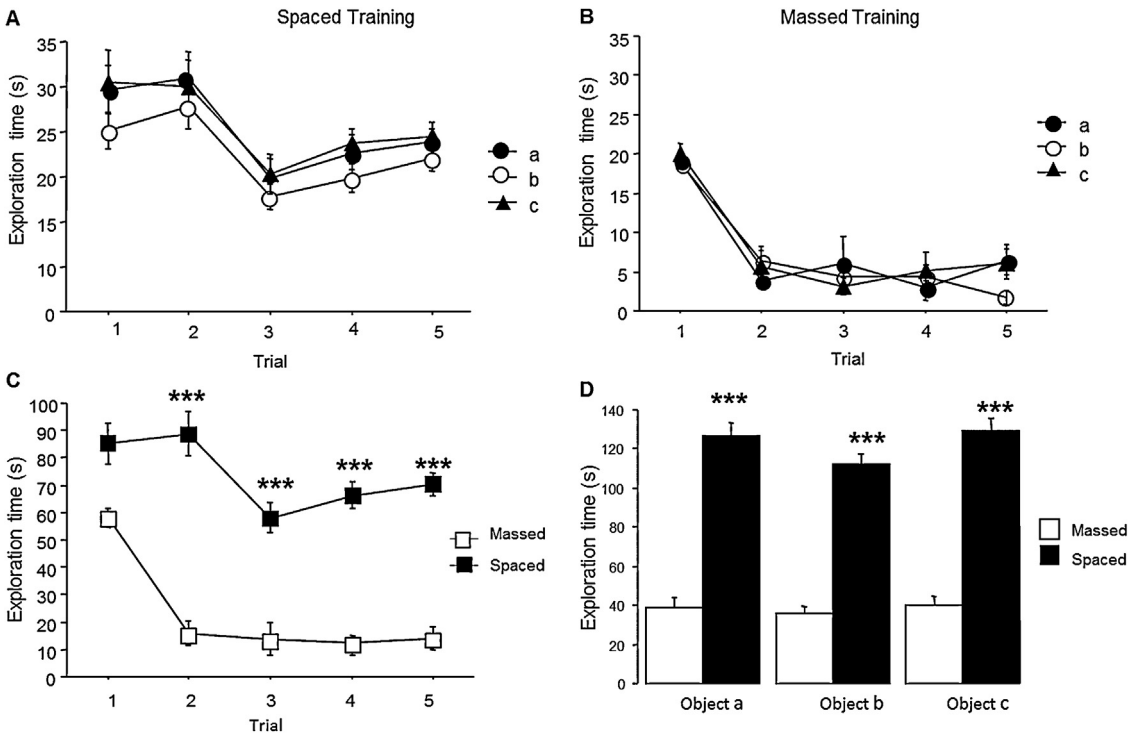

Fig. 2. Training results for object-identity recognition memory (Experiment 1). The total time exploring each of the objects (a, b, c) during the 5 different trials in seconds is shown for the spaced-training condition (A) and for the massed-training condition (B). In both training conditions the exploration time for each object decreased significantly over the 5 trials, with no difference in the exploration time among objects. The average exploration time with all 3 objects was analyzed over the 5 trials (C) for spaced- (black squares) and massed-training animals (white squares); note that a significantly higher exploration time for spaced-training animals was observed in trials 2–5 (***$p < 0.0001$). The average exploration time over the 5 trials is shown for each object (D) in spaced- (black bar) and massed- (white bar) training conditions. Significant differences between training conditions were found in the average exploration time for each object. (***$p < 0.0001$).

for a similar amount of time by the different rats, and that the presentation order does not influence the time spent in exploration.

In all further experiments we compared the effects of spaced vs. massed training conditions. Spaced training consisted of 5 training trials over 5 days, with an inter-trial interval of 24 h, while the massed training consisted of 5 trials conducted on 1 single day with an inter-trial interval of 5 min. Both groups remained in their home cage during the inter-trial interval. In both cases, each trial lasted 5 min, and consisted of exposing the animals to 3 different objects with which to become familiar, named a, b and c. We used three objects to increase the number of items that need to be stored in memory in order to increase the mnemonic challenge, based on previous observations [58]. In each acquisition trial the objects were presented in 1 of the 5 different configurations for Experiments 1 and 3, while for Experiment 2 the objects were present in a single configuration (Fig. 1A and B).

3.1. Experiment 1 (effect of massed vs spaced training on object-identity recognition memory)

Here we used a training strategy in which 3 different objects were placed in locations that switched in each trial, such that the arrangement of objects was different for each trial (Fig. 1A). Importantly, during training with the objects in switching locations, each object was located twice in the same place where they were located during test (see Fig. 1A and C). This training strategy was used to assure that the object arrangement or the object location did not become a relevant part of the object representation. In order to evaluate behavioral performance during object recognition training we used the measure of exploration time spent with each object, in each trial, for the statistical analysis. In the interests of simplicity in the statistical analysis, we first performed a two-way ANOVA with the factor "Object" between and the factor "Trials" in repeated measures, splitting by Training groups (Fig. 2A and B). The main factor Objects did not show significant differences in either the spaced or massed training condition. This is consistent with the Latin square test. However, we obtained significant differences over the course of the trials in both the spaced- ($F_{(4,180)} = 13.63$, $p < 0.0001$) and the massed-training groups ($F_{(4,168)} = 50.97$, $p < 0.0001$). No significant interaction was found in either case. These results indicate that in both training conditions, a significant decrease in the exploration time is observed and is similar for all objects throughout trials.

The decrease in exploration time over the course of the trials can be expected to occur as a consequence of familiarization [20]. However, what is notable is that spaced training animals spent a significantly longer total time exploring the objects as compared to the massed training animals (Fig. 2C). The mean exploration time spent with the 3 objects was compared between the two training conditions, and a two-way ANOVA with the main factors Training and Trials in repeated measures revealed significant differences between Training groups, ($F_{(1,29)} = 144.54$, $p < 0.0001$), and also with the main factor Trials, ($F_{(4,\ 116)} = 16.8$ $p < 0.0001$), with a significant interaction between Training × Trials, ($F_{(4,116)} = 5.63$, $p < 0.01$). These results indicate that massed training produced a more pronounced reduction in the exploration time as compared to spaced training and that there were differences between spaced and massed training groups in how the exploration times changed over the course of the trials. The longer exploration time shown by spaced training animals was also observed when we analyzed the mean exploration for each of the 3 different objects that became

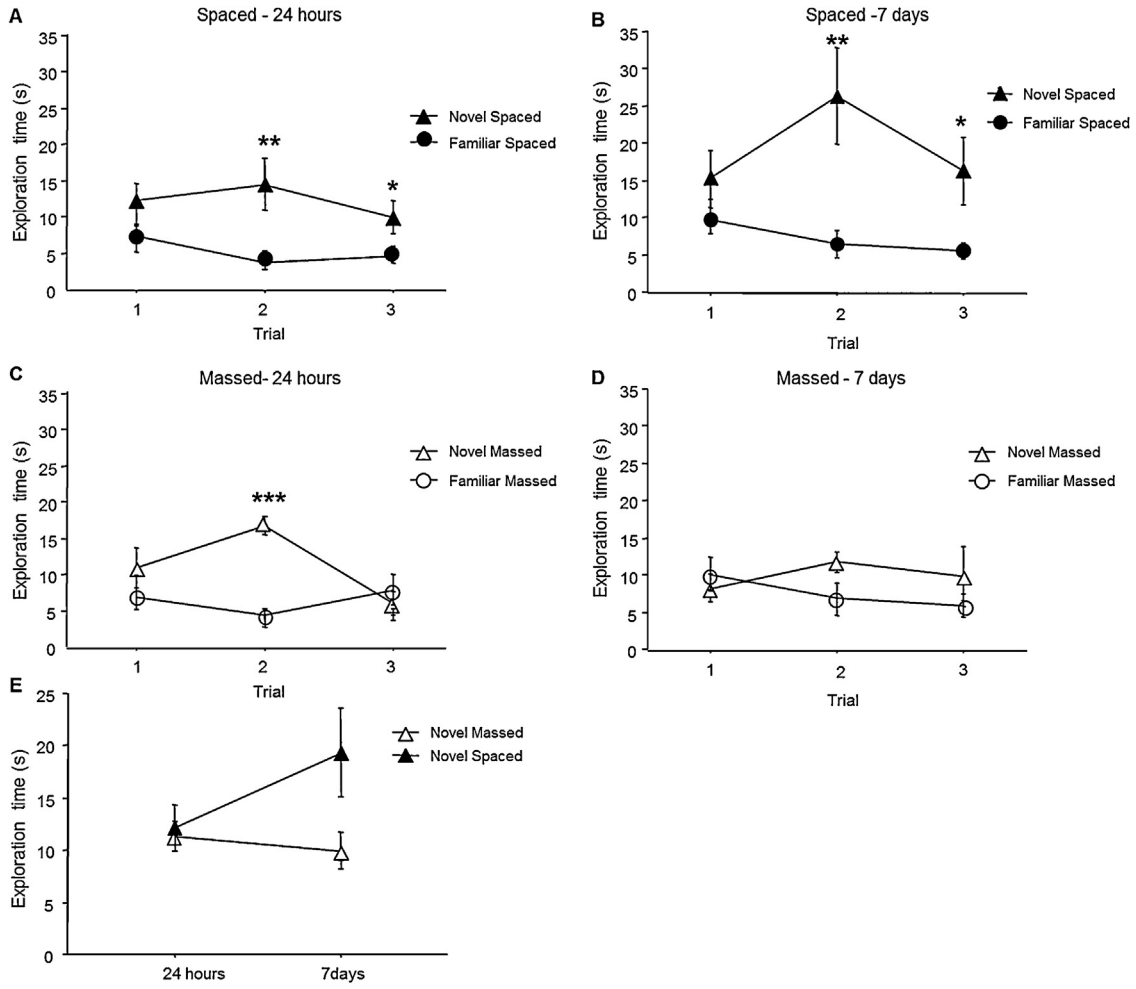

Fig. 3. Memory tests results for object-identity recognition memory (Experiment 1). The exploration time in sec for the novel object (triangle) vs. the average exploration time for the familiar objects (circles: average of 2) in each trial is shown for spaced-training animals in the tests done 24 h (A) and 7 days (B) after training, and for massed-training animals (C and D), respectively. Note that significant differences in the exploration time between novel and familiar objects ($^{*}p < 0.01$, $^{**}p < 0.001$, $^{***}p < 0.0001$) are found in both training conditions (Spaced and Massed) in the 24-h test but only for spaced-training animals in the 7-day memory test. The average exploration time in all 3 trials for the novel object revealed no significant differences between spaced- and massed-training animals or between the two retention intervals (E).

familiar in all trials (Fig. 2D). A two-way ANOVA for the mean exploration time, with one factor Training condition and the second factor Objects, revealed significant differences for the main factors Training ($F_{(1,29)} = 144.54$, $p < 0.0001$) and Objects ($F_{(2,58)} = 7.53$, $p < 0.05$), with no significant interaction. Post hoc Fisher's analysis showed that the mean exploration time for each object was significantly longer for the animals trained in the spaced condition and that the differences between objects were observed between objects *a* and *c* relative to *b* ($p < 0.015$). However, these differences between objects were only observed for the spaced-training animals, no differences were found in the exploration time between objects for the massed-training animals.

Memory performance was evaluated at two retention intervals, either 24 h or 7 days after the last training trial. We decided to use independent groups of animals for each retention interval in all the experiments, in order to avoid confounding effects of reconsolidation that may modify the behavioral performance of the animals [21,22]. The memory test consisted of placing the animals into the context and exposing them to 2 familiar objects and 1 brand-new novel object, replacing 1 familiar object (see Fig. 1 and Supplementary Fig. 2). A total of 3 trials were conducted, presenting 3 different brand-new objects that each replaced one of the familiar ones, and these familiar objects were placed in a different location in each trial (see Fig. 1C).

We analyzed the memory test results as follows. First, we used the exploration time with each object in each trial as the dependent variable for ANOVA in order to directly test whether or not the animals spent more time exploring the novel object and consequently showed evidence of object recognition memory. With this rationale in mind, we measured the average exploration time spent with the 2 familiar objects and compared it statistically with the exploration time spent with the novel object (Fig. 3).

In the test done 24 h after training, both training conditions showed evidence of object recognition memory (Fig. 3A and C). We used a two-way ANOVA splitting by training groups with the between-groups factor Objects (Novel vs Familiar) and repeated-measures factor Trials. The results of the analysis in the spaced-training group revealed that the exploration time between objects was significantly different ($F_{(1,14)} = 8.82$, $p < 0.01$). No significant differences were found between trials and there was no significant interaction. Post hoc Fisher's analysis revealed that the exploration time differed between the novel and familiar objects in trial 2 ($p < 0.01$) and trial 3 ($p < 0.05$) but not in trial 1. In the massed-training group, the two-way ANOVA analysis revealed significant differences between objects ($F_{(1,14)} = 6.55$, $p < 0.05$) and significant differences between trials ($F_{(1,14)} = 57.93$, $p < 0.0001$), with no interaction. Post hoc Fisher's analysis revealed that the exploration time differed between the novel and familiar objects only in trial 2

($p < 0.0001$). These results indicate that both training conditions produced an object memory representation that was evident 24 h after training; nevertheless, spaced-training animals showed more consistent object recognition that was evident in two trials.

In rats tested seven days after the last training trial, more differences between spaced and massed training were found in object recognition memory (Fig. 3B and D). Splitting by training group, a two-way ANOVA with the between-groups factor Objects (novel vs familiar) and Trials in repeated measures, revealed that the spaced-training animals spent a significantly longer time exploring the novel object than the familiar ones, ($F_{(1,14)} = 7.74$, $p = 0.015$). No significant differences were found between trials and there was no significant interaction. Post hoc Fisher analysis indicated that the differences in the exploration time between the novel and familiar objects were observed in trial 2 ($p < 0.01$) and trial 3 ($p < 0.05$). In contrast, the massed-training animals showed no significant differences in the exploration time spent between the novel and the familiar objects. This indicates that no object recognition memory was observed at 7 days after training in the massed-training animals (Fig. 3D), while the memory representation in spaced-training animals was well preserved (Fig. 3B).

It is apparent from Fig. 3A and B that spaced-training animals spend more time exploring the novel object in the test done 7 days after the last training trial as compared to the one done at 24 h, and that this time may differ between training conditions. For this reason, we obtained the average exploration time for the novel objects in all test trials for either the test done 24 h or 7 days after training and used it for statistical analysis (Fig. 3E). A two-way ANOVA between Training (spaced vs massed) and the memory test retention interval (24 h vs 7 days) revealed no significant differences between groups, ($F_{(1,1)} = 3.56$, $p = 0.07$), with no significant interaction and no significant differences between retention intervals. The high variability of exploration time for the novel object found at 7 days may explain these results. Nevertheless, this does not conflict with the fact that only space training animals exhibited a significantly higher exploration time for the novel than for familiar objects for both retention intervals, while the massed-training animals only showed evidence of object recognition memory in the 24-h memory test.

3.2. Experiment 2 (effect of massed vs spaced training on novel object-location recognition memory)

To evaluate whether spaced training can also improve the recognition of a new object location, we performed a training procedure in which the objects were placed in the same location throughout all 5 trials, to ensure that the object location became a relevant part of the memory representation; in the memory tests of this second experiment, we evaluated the recognition of a brand-new location for the familiar objects (see Fig. 1).

The results obtained during training showed no differences in the exploration time between objects, similar to the results obtained in the first experiment (Supplementary Fig. 3). We obtained the average exploration time with the 3 objects for each trial and compare spaced- and massed-training conditions (Fig. 4A). The two-way repeated measures ANOVA revealed significant differences between the Training procedures, ($F_{(1,30)} = 25.13$, $p < 0.0001$), and also significant differences between Trials, ($F_{(1,4)} = 22.17$, $p < 0.0001$), with no significant interaction. These results indicate a significant decrease in the exploration time over the course of the acquisition trials, with the spaced-training animals again showing a longer exploration time. The average exploration time in all trials for the 3 objects was compared with a two-way ANOVA with the factor Training and Objects in between. We found a significant difference for the main factor Training condition, ($F_{(1,30)} = 25.13$, $p < 0.0001$), and also for the main factor Objects, ($F_{(2,\ 60)} = 9.39$, $p < 0.01$), with no significant interaction. A Post hoc Fisher's analysis showed that the mean exploration time for each object was significantly longer for objects "a" and "c" as compared to "b" ($p < 0.05$), and no differences were found between objects "a" and "c" (Fig. 4B). These differences between objects were observed for both training conditions when the analysis was done by splitting the training groups ($p < 0.05$).

The results for the memory test done 24 h after training in the spaced-training condition revealed significant differences in the time spent exploring the brand-new object location when compared with the average exploration time with the familiar Object locations, ($F_{(1,14)} = 23.53$, $p < 0.01$), with significant differences between Trials, ($F_{(2,28)} = 14.62$, $p < 0.0001$), and a significant interaction between Trials and Object-location, ($F_{(2,28)} = 5.18$, $p < 0.05$). The Post hoc analysis revealed differences in trial 1 ($p < 0.05$) and trial 2 ($p < 0.01$) but not in trial 3 (Fig. 4C). These results indicate that spaced-training animals present a clear object-location recognition memory after the 24-h retention interval. The massed-training animals also spent a significantly longer time exploring the object in novel location as compared to the familiar one in the test done 24 h after training, ($F_{(1,14)} = 10.47$, $p < 0.01$) (Fig. 4E). No significant differences between the 3 test trials and no significant interaction were found; the Post hoc analysis revealed that the differences between the time exploring the object in the brand-new vs. the familiar location were present only in trial 2 ($p < 0.01$). This indicates that also the massed trained animals showed object-location recognition memory in the test done 24-h after training, but their performance suggest that this recognition is less consistent.

In the memory test done 7 days after the last training trial for the spaced-training animals (Fig. 4D), exploration time for the object in the brand-new location was longer than that for the objects in the familiar location, ($F_{(1,14)} = 15.60$, $p < 0.01$), with significant differences between Trials, ($F_{(2,28)} = 22.91$, $p < 0.0001$), and no significant interaction. The post hoc analysis revealed that the differences in the exploration time between object locations were found in trial 1 ($p < 0.01$) and trial 2 ($p < 0.05$). For the massed-training animals tested 7 days after training (Fig. 4F), the object in the brand-new location was explored for a longer time than the object in the familiar one, ($F_{(1,14)} = 7.63$, $p < 0.05$), with no significant differences between trials and no significant interaction. The post hoc analysis revealed that the differences in the time spent exploring the object in the novel location were found only in trial 2 ($p < 0.01$). The average time spent exploring the object in the brand-new location did not differ between the two training conditions (Fig. 4G). It is important to acknowledge that objects "a" and "c" were displaced 2 quadrants (Test Trial 2 and 3 see Fig. 1C), while object "b" was displaced only 1 quadrant (Trial 1), which may represent a more difficult challenge for the animals. However, the memory test results revealed that spaced trained animals did recognize this displacement (see Trial 1 in Fig. 4C and D), making this factor as non-crucial to explain our results. Overall, these results indicate that both massed and spaced trained animals present a clear long-term object-location recognition memory.

These results contrast with those obtained in the first experiment in which we evaluate object-identity recognition memory, since both training groups showed evidence of object-location recognition memory at both retention intervals (at 24 h and 7 days), whereas in the object-identity test, the massed-training group tested at the long retention interval were impaired (Fig. 3D). However, there is a suggestion that the spaced-training animals showed a more consistent recognition, since the difference between the time exploring the object in the novel location compared to that in the familiar location, was found in 2 trials, while in the massed-training animals, this difference was found only in 1 trial for both retention intervals.

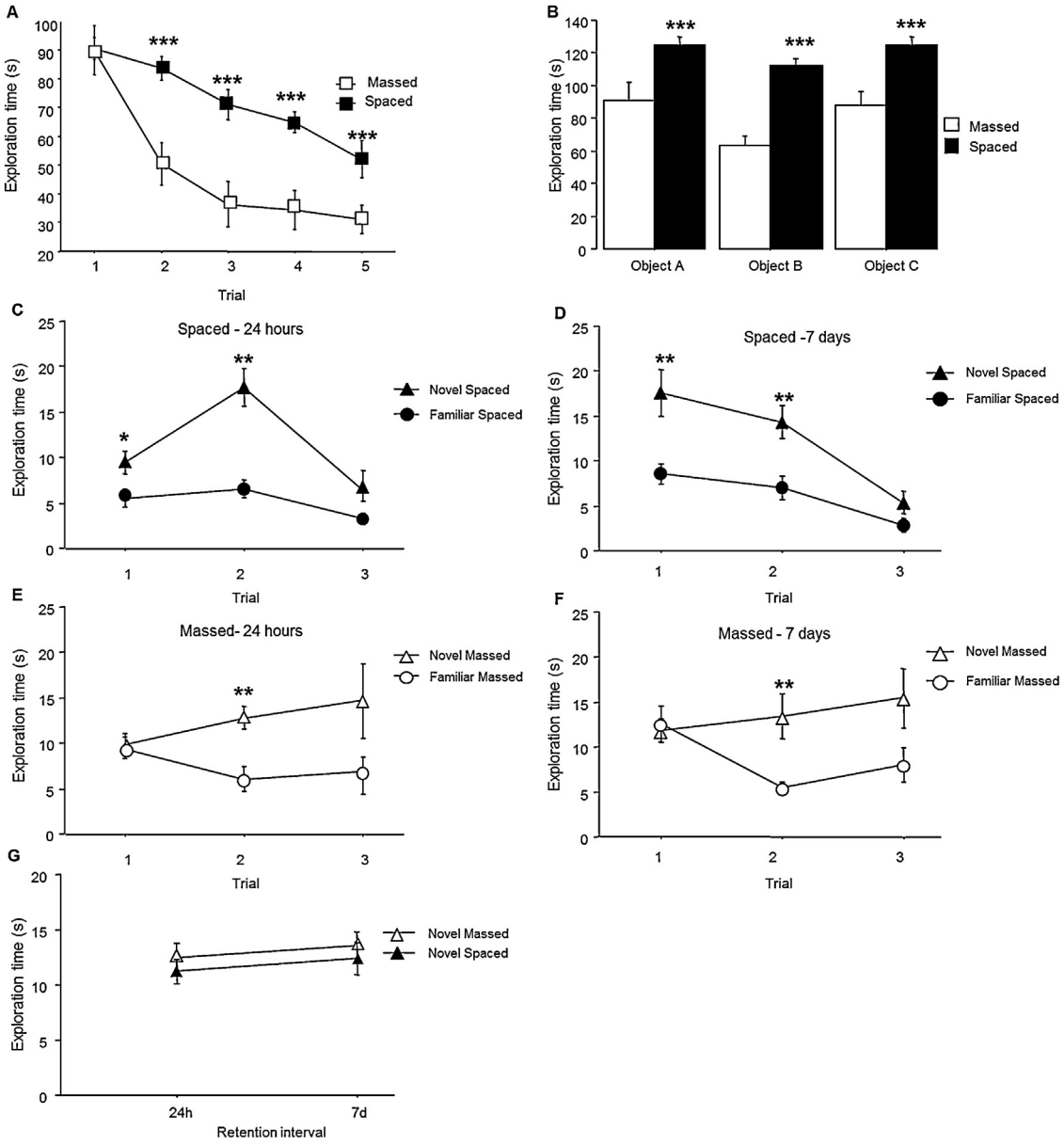

Fig. 4. Training and memory test results for object-location recognition memory (Experiment 2). The average exploration time (sec) spent with the 3 objects over the 5 training trials were compared for the spaced- (black squares) vs. the massed-training condition (white squares) (A). Note that a significantly longer exploration time is evident in the spaced-training animals (***$p < 0.0001$). The exploration time was averaged over the 5 training trials for each object (B) in spaced- (black bar) and massed- (white bar) training conditions (*** $p < 0.0001$). In the memory test, the time spent exploring the object in the novel (triangle) and familiar locations (circles are the average of the 2) is shown for spaced- (C and D) and massed-training animals (E and F) in the tests done 24 h and 7 days after training. Note that rats in both training conditions showed a significantly greater exploration time for the object in the novel location (*$p < 0.01$, **$p < 0.001$). No significant differences between training conditions or retention intervals were found in the 3-trial average of the time spent exploring the object in the novel location (G).

3.3. Experiment 3 (effect of massed vs spaced training on recognition of a novel object-place association)

In order to test whether the long-term object-location recognition memory, which we observed in both spaced- and massed-training conditions, is dependent on the association of each object with its specific location, we performed a final experiment in which we switched the object arrangement over the course of the training, as in the first experiment (Fig. 1), assuming that the precise location of each object was not associated with the object during encoding; the animals were then tested with the familiar objects in a brand-new location, as in the second experiment.

During acquisition we found similar results to those obtained in the first experiment (Suplementary Fig. 4), observing a significant decrease in the exploration time throughout trials, and that this decrease was more dramatic in massed trained animals, being spaced trained animals the ones that explored the objects more time. The memory test results of the time spent exploring the objects after the 24-h retention interval were analyzed for each training group with a two-way ANOVA with the factors Objects (novel vs familiar) in between and Trials in repeated measures. The analysis revealed that the spaced-training animals spent significantly more time exploring the object in the brand-new location than in the familiar one, ($F_{(1,12)} = 13.53$, $p < 0.01$); no significant differences were found between trials and there was no significant interaction (Fig. 5A). The post hoc Fisher analysis indicated that differences in the exploration time between the novel and the familiar objects were observed in trial 1 ($p < 0.001$), trial 2 ($p < 0.001$), and

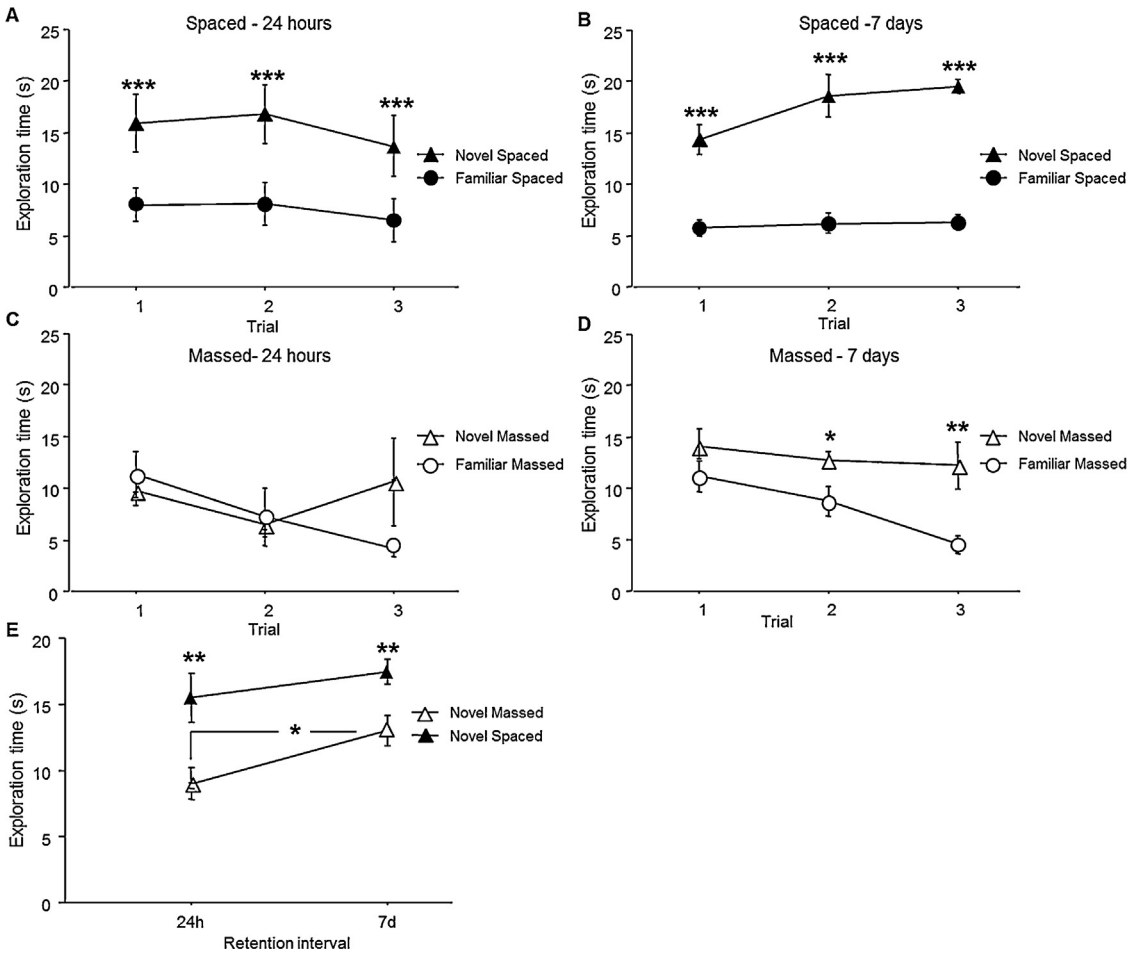

Fig. 5. Memory test results for object-zone-location recognition memory (Experiment 3). Exploration time of novel (triangles) and familiar objects (circles) in the 3 test trials for spaced- (A and B) and massed- (C and D) training animals. Note that spaced-training animals show a significantly longer exploration time for the novel object after both retention intervals, while massed-training animals show this increase only 7 days after training. Comparison of the 3-trial average exploration time for the novel object for both retention intervals and both training conditions (E) showed that spaced-training animals spent more time exploring the novel object than the massed-training animals after both retention intervals, but massed-training animals spent significantly more time exploring the novel object in the test done 7 days after training than in the one done at 24 h after training ($^{*}p < 0.01$, $^{**}p < 0.001$, $^{***}p < 0.0001$).

trial 3 ($p < 0.001$). This indicates that spaced-training animals developed an object-zone-location recognition memory, allowing them to recognize that a brand-new location is being occupied by familiar objects. In the massed-training group no differences were found in the exploration times between the novel and the familiar objects in the 24-h retention interval, suggesting that this object-zone-location recognition memory was not developed after massed training (Fig. 5C).

The results obtained in the memory test done at 7 days after the last training trial were more striking. Splitting by Training conditions, a two-way ANOVA with the between-groups factor Objects (novel vs familiar) and with Trials in repeated measures showed that the spaced-training animals spent significantly more time exploring the object in the brand-new location when compared to the objects in the familiar location ($F_{(1,14)} = 105.95$, $p < 0.0001$), with significant differences between trials ($F_{(2,28)} = 3.54$, $p < 0.05$), and no significant interaction. The post hoc Fisher analysis indicated that differences in the exploration time between the novel and familiar objects were observed in all three trials ($p < 0.001$ in each trial). This indicated that the object-zone-location recognition memory, developed after spaced training, is preserved for at least 7 days (Fig. 5B). Surprisingly, when we evaluated the performance of the massed-training animals 7 days after the last training trial, the two-way ANOVA revealed that they spent significantly more time exploring the object in the brand-new location than in the familiar one, ($F_{(1,14)} = 11.35$, $p < 0.01$), with significant differences between trials, ($F_{(2,28)} = 3.95$, $p < 0.05$), and no significant interaction. The post hoc Fisher analysis indicated that these differences between objects were found in trials 2 ($p < 0.05$) and trial 3 ($p < 0.01$). This is a very consistent object-zone-location recognition memory shown only after the 7-day retention interval, suggesting that the zone representation for the familiar objects was developed after 24 h in the massed-trained animals (Fig. 5D). Here again, it is apparent that spaced-training animals spent more time exploring the object in the novel location than the massed-trained animals, and that the exploration time was longer when tested 7 days than only 24 h after training. When the average exploration time for the object in the brand-new location in all trials was compared, between spaced and massed-training animals, and between the two retention intervals, 24 h and 7 days (Fig. 5E), we found significant differences between spaced- and massed-training animals, ($F_{(1,1)} = 17.97$, $p < 0.01$), and also between the two retention intervals, ($F_{(1,1)} = 5.38$, $p < 0.05$), with no significant interaction. This indicates that the average time that the spaced-training animals spent exploring the object in the novel location was longer than that spent by the massed-training animals after both retention intervals (post hoc p's < 0.01). Moreover, the exploration time for the object in the novel location significantly increased in the massed-training animals when tested

after 7 days as compared to the test done 24 h after the last training trial ($F_{(1,14)} = 5.94$, $p < 0.05$). This suggests that massed-training animals had changed their behavior toward the object in the novel zone-location after 7 days. It is notable that by comparison, in the independent group of animals tested 24 h after massed-training did not show evidence of object-zone-location recognition memory. This suggests a consolidation process operating in the interval between 1 and 7 days.

It is to be noted that both Experiments 2 and 3 evaluate object location recognition memory; however, in this 3rd experiment the animals developed object-zone location recognition, since during training the objects were placed in variable locations. Given the different results obtained it is possible to argue that different computational resources are used to encode each type of objet-location representation.

4. Discussion

The main finding of these experiments is that spaced vs massed training produced a difference in both the learning and consolidation of object-location memory. Furthermore, this difference was, in itself, different for the learning of new objects vs the learning of new locations. The implication is that different types of plasticity underlie object- vs place-learning, both in new learning and in consolidation.

4.1. The spacing effect on training

In our experiments we replicated the well known superiority of spaced over massed training in improving memory performance. There are two possible reasons for this superiority. First, it may relate to the amount of exploration time during training. Clearly, in both the spaced- and massed-training groups the exploration time decreased over the course of the trials, but spaced-training animals spent more total time exploring the objects during acquisition (Fig. 2). This was observed in both training procedures, either when switching the object location or when it remained fixed. This greater time expended in exploring the objects may be a key element in explaining the spacing effect [18] in the object recognition memory task. Similar results were obtained by Commins et al. [16] when they compared massed- vs. spaced-training animals in an object-displacement task. It is interesting to speculate about the mechanistic and adaptive reasons for this difference in exploration time. Mechanistically, it may be due to the operation of an across-trial habituation process that recovers between days in the space-trained animals. Adaptively, it may be that it benefits animals to check, after an absence, that some relevant change has not occurred to an object. The result is that the spaced-trained animals spent more time with the objects, which may imply more attention and hence improved encoding with more opportunity to form an enduring memory of those objects.

The second reason for the superiority of spaced training is that encoding may be improved through the consolidation process occurring between days in the course of training, which could not occur in the massed trained animals because training was completed all in one day. This is an interesting possibility since here we used 24 h long inter-trial intervals during spaced training, and similarly, in humans, it has been shown that spaced training with 24 h inter-trial intervals, in contrast to massed training, slows down the rate of forgetting without enhancing the initial memory performance, suggesting that the spacing effect may depends on improved memory consolidation [20]. Further research needs to be done to further characterize the role of memory consolidation in the spacing effect in animals and its neurobiological substrates.

4.2. Spaced training improves long-term object-identity-recognition memory

In the memory test done 24 h after training, both training conditions (massed and spaced) produced an object-identity recognition memory response, perhaps a little more consistently in the spaced-training animals. However, in the Test done 7 days after training, the massed-training animals showed no evidence of object-identity recognition memory, while the representation was fully preserved in the spaced training animals (Fig. 3B vs D). This is direct evidence that spaced training promotes a long-term object-identity recognition memory, as opposed to massed training, in which this representation lasted only around 24 h. Although this effect was first described in the 19th century and a large psychological literature about it is available [23,24], the neurobiological mechanisms underlying this effect have only recently become a focus of attention [25,26]. Molecules such as MAPK, CREB, PKA, and PKC are involved in the spacing effect for short inter-trial intervals in *Aplysia* and *Drosophila* [26], and some are also important in rodents [26–29]. It may be that the replenishment of molecules underlies the spacing effect, but the evidence suggests that it is not that simple; for example, in spaced stimulation the persistent activity of PKA is known to be required for long-term synaptic facilitation in *Aplysia* [30,31], whereas massed stimulation leads to activation of both PKA and PKC [30,32]; PKC is known to inhibit PKA-mediated function [33], so the suppression of PKC activity and the disinhibition of PKA could be the mechanism through which spaced training leads to long-term memory formation [32,33]. For longer time intervals the picture is less clear, but there is evidence showing that factors such as the immediate early gene *Arc* are expressed in a consistent number of hippocampal neural units (about 40% of the CA1 neuronal population) after spaced spatial exploration, which included 9 consecutive 5-min trials with inter-trial intervals of 24 h; on the other hand, after massed spatial exploration including 9 consecutive 5-min trial with inter-trial intervals of several minutes, the number of Arc-expressing neurons decreased nearly to cage control levels (about 10% of the CA1 neural population). Since the same number of neural units showed an electrophysiological response to spatial exploration (about 40%) in both spaced and massed conditions, this was interpreted as an electro-transcription decoupling that occurred only after massed spatial exposure [34]. This interesting result suggest that for long time intervals, spaced training increases the likelihood that *Arc* can accomplish its role during synaptic plasticity [35], leading to optimal memory consolidation [36,37], but further research is required to elucidate many other molecular and cellular mechanisms underlying the spacing effect for long time intervals in mammals. The current behavioral results may well serve as a model for future designs to address these issues.

4.3. Spaced training differentially affects object-identity and object-location recognition memory

Interestingly, contrasting results were obtained in an object-in-place task, when we trained the animals with the objects fixed in a particular location and then tested the animals with familiar objects in a brand-new location (Experiment 2). In these conditions we found that both training conditions (massed and spaced) resulted in object-location recognition memory after both retention intervals. Our results contrast with previous findings using the object displacement task, in which 4 objects were fixed in a particular configuration during acquisition (either spaced or massed) and during the memory test done 7 days later, one object was displaced to a new location [16]. Their results indicate that the number of contacts with the displaced object increased significantly between the last acquisition trial and the test, but only in the spaced- and not

in the massed-training condition. However, neither the massed- nor the spaced-training animals had more numerous nose contacts with the displaced object than with the objects that remained in the fixed location [16]; thus, there was no clear evidence of object-location memory [9,10]. In the present work both training procedures (spaced and massed) produced evident object-location recognition memory that remained stable after both retention intervals (Fig. 4); however, since spaced-training animals showed significantly more time exploring the novel vs. the familiar objects in 2 trials and massed-training animals only in 1, perhaps it can be argued that the object-location recognition is more consistent in spaced-training as compared to massed-training animals. Nevertheless, the results clearly contrast with the effect observed in the object-identity recognition memory, where object recognition memory was not observed in massed-training animals 7 days after training.

This difference in the spacing effect observed between object-identity and object-location recognition memory may be explained by the "two streams hypothesis" of visual information processing, which was originally developed for primates [38] which remains a valid notion [39] that also applies to rodents, in which evidence indicates that temporal cortex lesions affect object recognition [40], whereas parietal cortex lesions affect spatial location [41]. Furthermore, recent evidence revealed that in mice, the projections arising from the medial/anterior extra-striate visual areas strongly connect to parietal, motor, and limbic regions, whereas the lateral extra-striate areas preferentially connect with the temporal and para-hippocampal regions [42], indicating that in mice these two anatomically segregated streams resemble those in primates; these streams may have subtle anatomical differences between primates and rats [43], but they can be functionally similar [44]. Thus, it is possible to argue that the molecular and cellular events underlying the spacing effect may differ between the two information-processing streams, a finding that could well be the focus of future research.

Finally, when we performed the training with the objects in variable locations, and the memory test was done by displacing a familiar object to a brand-new location, we found that in the test done 24 h after training, spaced-training animals were able to consistently recognize a change in the zone in which the objects had been placed 24 h earlier, while massed-training animals did not. Surprisingly, in the test done 7 days after the last training trial, both the spaced- and massed-training animals showed clear evidence of object-zone-location recognition memory. This intriguing result suggests that in the massed-training animals, the representation is not strong enough at 24 h to yield an evident behavioral outcome, but 7 days after training the representation has strengthened sufficiently to produce behavioral evidence of object-zone-location recognition memory, but this apparent long-term consolidation effect may not be exclusive of massed training (see Suplementary Fig. 5).

5. Conclusion

Memory is a dynamic process in which the record of experience is ever evolving [45]. Memory consolidation is a process during which the acquired representations are stabilized by a series of molecular and cellular events that modify synaptic efficiency [46,47]. Further changes are promoted at the systems level, integrating different neural networks in one particular multi-trace representation [48]. However, consolidation is not a process that occurs as a single, post-acquisition event; when the animal is re-exposed to the same task or context where learning occurred, the information is reactivated on-line (in the presence of the stimulus), and a new round of consolidation is triggered such that memories again become labile, a phenomenon known as reconsolidation [49]. Reconsolidation may be subject to several "boundary conditions" [22], which are limits beyond which reactivated memories remain resistant to disruption. Particularly interesting is the finding that reconsolidation may occur only when memory is updated [50], so we can argue that after reconsolidation, object recognition memory evolves [51] perhaps into a more complex and/or stronger representation. It is also noticeable that reactivation of information occurs off-line [52–54] and may be spontaneously triggered several days after learning occurred [55] either during sleep or awake resting states [52,56]. It has been proposed that during spontaneous 'off-line' reactivation, memories are redistributed in the absence of interfering external inputs, leading to a reorganization of neuronal representations and to qualitative changes of the memory content [56]. Moreover, off-line memory reactivations may also be accompanied by a transient destabilization of memory traces [57], but unlike the on-line reactivation, in which memories are updated with respect to current perceptual input, off-line reactivation that could occur over several days may allow a gradual adaptation of the newly acquired information to pre-existing long-term memories, possibly by integrating multiple memory traces into anatomically separated neural networks [48], that may transform the information into a memory trace that is stronger and easier to retrieve. Together these considerations could explain why massed-trained animals, and possibly also spaced-trained animals (see Supplementary Fig. 5), do not express a good object-location memory representation at 24 h after training but do so efficiently at 7 days.

Overall the behavioral results presented here have important implications for the neural mechanisms underlying the spacing effect, and will serve as a model for future research that can bring insights into the neural mechanisms underlying it.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbr.2013.04.047.

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