# Long-term associative memory in rats: effects of familiarization period in object-place-context recognition test

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**Abstract**

Spontaneous recognition tests take advantage of innate exploratory behaviors toward a novel object in rodents. In these paradigm, not only simple non-associative recognition memory but also more complexed associative memory can be evaluated without the use of appetitive or aversive reinforcement, or learning of task rules. In the present study, we investigated whether the length of the object familiarization period improves subsequent novelty discrimination in the spontaneous object, place, and object-place-context (OPC) recognition tests in rats. In the OPC test, rats showed a significant novelty preference only when the familiarization period was 30 min, but not when it was 5 min or 15 min. On the other hand, rats showed successful discrimination behaviors under 15 min and 30 min familiarization period conditions in the place recognition test, and under all conditions of 5, 15 and 30 min in the object recognition test. Our results suggest that the extension of the familiarization period improves discrimination performance in the spontaneous recognition paradigms, and a longer familiarization period is necessary for long-term associative recognition memory compared to non-associative memory.

**Keywords**

recognition memory, long-term memory, object-place-context test, rats

## Introduction

Recognition memory is necessary to discriminate novel information from that is already known. Since animals have an innate tendency to respond to or explore toward novel stimuli, the habituation-dishabituation paradigm has been regarded as a useful behavioral test to assess recognition memory in various animal species including Aplysia (Pinsker, Kupfermann, Castellucci, and Kandel, 1970), rodents (Ennaceur and Delacour, 1988), monkeys (Mishkin and Delacour, 1975), and humans (Fantz, 1964). In particular, researchers have evaluated rodents' recognition memory using several types of spontaneous recognition tests.

Spontaneous recognition tests have been used to evaluate not only simple non-associative recognition memory (e.g. object recognition test, Ennaceur and Delacour, 1988; place recognition test, Ennaceur and Meliani, 1992) but also more complexed associative recognition memory (e.g. object-context recognition test, Dix and Aggleton, 1999; object-place-context recognition (OPC) test, Eacott and Norman, 2004). Non-associative recognition memory is typically composed of single information, such as objects or locations. On the other hand, associative recognition memory is necessary to recognize objects using combined information on multiple stimuli which typically include the context where animals encountered the objects, in addition to the information on objects and locations themselves.

A standard object recognition test consists of a sample phase and a test phase, with a delay period inserted between the two phases. In the sample phase, a rat is allowed to explore an open-field arena, in which a pair of two identical objects are placed, or a few minutes for familiarization. After the delay period, the rat is returned to the arena where one of the objects is replaced with a novel object (test phase). A preferential exploration toward the novel object is defined as a successful discrimination, and the rat is considered to display great ability of recognition memory. In general, performance in the test depends on the length of the delay period, and performance in the tests with a shorter (< 24 hours) and a longer (≧24 hours) delay period reflect short-term and long-term recognition memory, respectively (Ennaceur & Delacour, 1988). In addition, performance in spontaneous recognition memory test could also be affected by the length of familiarization periods (sample phase). Previous studies systematically examined how the extension of familiarization periods improved performance in non-associative object or place recognition tests. For example, in an object recognition test, animals showed novel object preference in a 5 min familiarization with both short (15 min) and long (24 hours) delay periods (Gaskin et al., 2010). Likewise, in a place recognition test with a long (24 hours) delay period, a 20 min, but not 5 min, familiarization was sufficient for rats to

discriminate a replaced object from a placed one (Ozawa et al., 2011).

In associative recognition memory tests, such as OPC test, on the other hand, rats would make the association between objects, places, and contexts. Typically, the OPC test consists of two sample phases, a test phase, and a delay inserted between the second sample and test phases. In the first sample phase, rats are familiarized with a pair of two different objects in a context. In the second sample phase, the rats are moved to another context in which the same pair of objects are placed in a swapped position. In the test phase, then, the rats are placed in one of the contexts, and allowed to explore a pair of one of the objects that the rats encountered in either the first or the second contexts. In the OPC test, rats are expected to explore the replaced object longer than the stayed one in each context. Several studies indicated that short-term recognition memory has been evident in the OPC test which consisted of 2-5 min familiarization and 2-15 min delay period (Davis et al., 2013; Eacott and Norman, 2004; Easton et al., 2011; McLean et al., 2018; Ramsaran et al., 2016; Langston and Wood, 2010; Cozannet et al., 2010; Lee et al., 2014; Wilson et al., 2013), while long-term recognition memory (≧24 hours) has never been tested.

In the present study, we hypothesized that (1) the extension of the familiarization period in the sample phase facilities associative recognition memory, and enables animals to show long-term associative recognition memory in the OPC test, (2) a longer familiarization period is necessary for the formation of long-term associative recognition memory compared to that of non-associative memory. Here, we systematically investigated the relationship between the lengths of familiarization periods (5, 15, or 30 min) and subsequent novelty discrimination performance in the object, place or OPC recognition test in rats.

## Materials and methods

### Subjects

Thirty-two Long-Evans male rats (10-11 weeks old; weighing 346.05 ± 11.61 g at the beginning of behavioral experiments: Institute for Animal Reproduction, Ibaraki, Japan) were used. They were housed individually and kept on a 12-h light/dark cycle (lights on at 8:00 a.m.) free access to food and water throughout the experiments. All experimental tests were conducted during the light phase. All experiments were approved by the University of Tsukuba committee on Animal Research.

### Apparatus

Two open-field arenas (900 × 900 × 450 mm) made of black polyvinyl chloride or white acrylic plexiglass were used in order to test in two different contexts. The black context consisted of the gray floor and the black walls. On one of the walls, a white–black vertically striped pattern was attached as a spatial cue. The white context consisted of the white floor and walls. A white–black checkered pattern was attached on one of the walls. The illumination of the center of each arena was 60 lx. An overhead camera was used to record the movement of the rat for the analysis. Background white noise (50 dB) was continuously present during all experimental tests to mask any extraneous noise. The stimulus objects were copies of 10 different objects made of glass, metal, or plastic and varied in height between 7 and 15 cm. All the objects were heavy enough or fixed on the heavy metal plate so that the rat could not move them.

**Habituation**

Habituation sessions were conducted for 3 consecutive days. On each day, rats were received 5 min of handling by the experimenter, and then placed in each of the black and white contexts without any objects for 30 min (with at least 60 min interval). The order of the exposure to each context was counterbalanced.

After the habituation, rats were divided into three groups in which the length of the familiarization periods were different (5min, $n$ = 11; 15min, $n$ = 11; 30min, $n$ = 10, see below for details).

**Object-place-context test**

The OPR test consisted of two sample phases and a test phase (Fig.1A). A 24 hours delay period was inserted between the second sample and test phases. In the first sample phase (sample 1), rats were allowed to explore the black context where a pair of two different objects were presented diagonally placed, at 22.5 cm from adjacent two walls (e.g., object A on the top left and object B on the bottom right). In the second sample phase (sample 2), the rats were placed at the other white context in which the same pair of objects were placed in a swapped position relative to the first sample phase (e.g., object B on the left and object A on the right). Each rat was allowed to explore freely these objects for 5, 15, or 30 min at each sample phase. In the 5-min test phase, rats explored a pair of one of the sample objects (e.g. object A-A) in one of the contexts (e.g. black context). If rats had associative recognition memory on objects, places, and contexts, they would show a preferential exploratory behavior towards the object placed in the novel place-context combination (the dashed arrow in Fig. 1A). The positions of the novel objects (e.g. top left or bottom right) in the test phase were counterbalanced. After each

phase, the floor of the arena was cleaned using a wet cloth containing sodium hypochlorite solution and the objects were wiped with 70% ethanol to eliminate odor.

**Place recognition test**

3-7 days after the OPC test, rats were tested on a place recognition test. All rats were subjected to re-habituation to the black context for 15 min without any objects. Two identical objects (object C) and the black context were used in this test (Fig. 1B). Animals were allowed to explore two objects for 5, 15, or 30 min in the sample phase. After 24 hours delay interval, animals were returned to the context in which one of the objects were moved to a novel location, and allowed to freely explore for 5 min in the test phase. Note that for place recognition test, one of the objects was presented in the same location as familiar, whereas the other was moved to a different location (dashed arrow in Fig. 1B), which was 30 cm away from the familiar object and 22.5 cm from a sidewall (two locations were possible).

**Object recognition test**

3-7 days after the place recognition test, rats were tested in an object recognition test (Fig. 1C). All rats were subjected to re-habituation in the black context without the spatial cue for 15 min. In the object recognition test, animals were allowed to explore two identical objects (object D) for 5, 15, or 30min in the sample phase. After the delay interval, animals were returned to the same open-field arena where one of the objects are replaced with a novel object (object E) in the 5 min test phase. The positions of the novel objects in the test phase were counterbalanced.

**Data analysis**

The ANY-maze video tracking software (Stoelting Co., Illinois, USA) was used to analyze the rats' performance and locomotor activity in each test. In each phase, we manually counted the time rats spent exploring the objects. Exploration was defined as the rat sniffing, pawing, and directing its nose toward to the objects within a distance of 2 cm, except standing or climbing the objects. As a measure of discrimination behavior in the test phase, discrimination index (DI) was calculated by dividing the difference in the time spent exploring the novel and familiar by the total amount of exploration for both objects [DI = $(T_{novel} - T_{familiar})$ / $(T_{novel} + T_{familiar})$]. A value of zero indicates no preference, while a positive value indicates more exploration of the novel object and a negative value indicates to the preferential exploration to the familiar object. Exploration time in the OPC, place recognition, object recognition test at each phase was analyzed

using a paired *t*-test (two-tailed) or two-way (object × trial) analysis of variance (ANOVA) followed by a post-hoc Scheffe test. DIs were compared to the theoretical chance level (0%) using a one-sample *t*-test (two-tailed). The exclusion criteria of a statistical outlier defined that DI exceeded ± 2 standard deviations from the mean of all rats in each test. If subject meet the criteria, the data in sample and test phases in was excluded. All values are expressed as mean ± standard error of the mean (*SEM*). Statistical significance was set at $\alpha = 0.05$.

## Results

### Object-place-context test

In 5 min condition, one subject was excluded from the analyses according to the criteria of a statistical outlier. In the sample phase 1, the mean ± *SEM* exploration times of familiar object (5 min = 38.66 ± 4.96, 15 min = 61.43 ± 8.19, 30 min = 67.49 ± 9.93) and novel object (5 min = 36.27 ± 2.98, 15 min = 60.53 ± 7.86, 30 min = 83.38 ± 12.99) indicated similar all condition. In the sample phase 2, the mean ± *SEM* exploration times of familiar object (5 min = 29.05 ± 3.63, 15 min = 41.80 ± 3.48, 30 min = 59.51 ± 8.20) and novel object (5 min = 26.57 ± 3.19, 15 min = 35.16 ± 3.56, 30 min = 55.33 ± 7.37) were also similar all conditions. Note that 'familiar' means the object that will be the same, and 'novel' means the object that will be replaced by the novel object in the test phase. Data in the sample phases ware analyzed using a repeated measures two-way ANOVA, Object (familiar vs. novel) as the within-subjects variable × Trial (sample 1 vs. sample 2) as the between-subjects variable, which showed only a significant main effect of Trial in 30 min condition [$F(1, 9) = 7.91$, $p = .020$], 15 min condition [$F(1, 10) = 26.97$, $p = .004$], and but not in 5 min condition. A main effect of Object and an interaction were not significant each condition. In the test phase, Fig. 1D shows the mean exploration time of the familiar and novel objects in each familiarization. Paired t-tests revealed, only in 30 min condition, rats explored the novel object significantly more than familiar one ($t(9) = 3.14$, $p = .011$). The mean DIs of three familiarization period conditions are shown in Fig. 1H. One-sample t-tests revealed DI was significantly higher than chance level only in the 30 min condition ($t(9) = 2.85$, $p = .018$).

### Place recognition test

In 5 min and 30 min condition, one subject from each condition was excluded from the analyses for according to the criteria of a statistical outlier. The mean ± *SEM* exploration times of familiar object (5 min = 17.38 ± 2.53, 15 min = 50.73 ± 4.48, 30 min

= 68.21 ± 9.45) and novel object (5 min = 22.73 ± 4.20, 15 min = 44.51 ± 3.38, 30 min = 58.57 ± 10.41) were similar, and a paired *t*-test revealed that there was no significant difference of exploration time. In the test phase, the mean exploration time of each object (Fig. 1E) and DIs under three different familiarization period conditions (Fig. 1H) were compared. Paired *t*-test revealed that rats explored the novel object significantly more than familiar one when the sample phases were 15 min ($t(10) = 5.73$, $p < .001$) and 30 min ($t(8) = 2.57$, $p = .032$). Furthermore, one-sample *t*-tests revealed that DIs were significantly higher than chance level in the 15 min ($t(10) = 7.66$, $p < .001$) and 30 min ($t(8) = 2.83$, $p = .022$).

**Object recognition test**

In 15 min and 30 min conditions, one subject from each condition was excluded from the analyses for accoding to the criteria of a statistical outlier. The mean ± *SEM* exploration times of familiar object (5 min = 20.09 ± 2.83, 15 min = 67.05 ± 21.70, 30 min = 65.24 ± 13.78) and novel object (5 min = 17.47 ± 2.13, 15 min = 58.46 ± 7.85, 30 min = 60.69 ± 15.01) were similar, and a paired *t*-test revealed that there was no significant differences of exploration time. In the test phase, the mean exploration time of each object (Fig. 1F) and and DIs under three different familiarization period conditions (Fig. 1I) were compared. Paired *t*-test revealed that rats explored the novel object significantly more than familiar one in all groups (5 min, $t(10) = 6.74$, $p < .001$); 15 min, $t(9) = 3.32$, $p = .008$); 30 min, $t(8) = 3.48$, $p = .008$)). One-sample *t*-tests also revealed that DIs were significantly higher than chance level in all groups (5 min, $t(10) = 9.19$, $p < .001$); 15 min, $t(9) = 4.39$, $p = .001$); 30 min, $t(8) = 3.87$, $p = .004$)).

**Discussion**

In the present study, we investigated the relationship between the length of familiarization at the sample phase (5, 15, 30 min) and subsequent novelty discrimination performance at the test phase in the object, place and OPC recognition tests with a 24-hour delay period. In the OPC test, rats showed a significant novelty preference when the familiarization period was 30 min, but not when it was 5 min or 15 min. In contrast, rats showed successful discrimination even under the shorter familiarization conditions such as 15 min in the place recognition and 5 min in the object recognition, respectively. These results demonstrated that the long-term (24 hours) associative recognition memory can be evident by extending the familiarization period to 30 min in the OPC test. Furthermore, it is also suggested that the formation of the long-term complexed associative memory

requires longer familiarization periods compared to the simple non-associative memory.

The findings that successful recognition memory was evident in 5-min familiarization in the object recognition test, but not in the place recognition test, are consistent with our previous study showing that rats needed longer familiarization in the place recognition test than the object recognition test (Ozawa et al., 2011). Since rats are required to process the information on both of objects and locations in the place recognition, it is reasonable that rats have to process more complexed information in the place recognition test than in the object recognition test. Thus, our results showing the relationship between the performance of recognition memory tests and the length of familiarization periods are likely to reflect the differences in difficulty among the object, place and OPC recognition tests. Previous studies reported that primates and humans spent more time to gaze at a novel image than a familiar one in the habituation-dishabituation paradigm, and the longer familiarization period is required when the stimulus is more complex (Fagan, 1964; Lasky, 1980; Bachevalier, Brickson, Hagger, 1993). Although Gaskin et al. (2010) demonstrated that longer exploration to the objects in the familiarization period did not improve non-associative memory performance in the object recognition test in rodents (Gaskin et al., 2010), our findings showing that the longer the familiarization period was, the more time rats spent in exploration to the objects demonstrated that the formation of associative recognition memory needs much longer exploration of the objects. These results suggest that a sufficient exploring the environment, as well as objects and/or locations, can lead animals to make the associations between each information, such as objects, locations and contexts.

The associative recognition memory has been regarded as an episodic-like memory, which is typified as the comprehensive information of “what”, “where” and “when” acquired from a single experience (Eacott and Norman, 2004). Indeed, although several tests have been developed to measure episodic-like memory in rodents, some of them are thought to be inappropriate for episodic-like memory tests. For example, a food reinforcement-based test (Veyrac et al., 2015) that needs multiple trainings is not supported by the definition of episodic-like memory described above. In addition, it is thought to be impossible to compare directly between associative and non-associative memories even in reinforcement-free spontaneous recognition tests due to differences in the procedures of the tests. The associative memory tests (episodic-like memory test; Dere et al., 2005; Kart-Teke et al., 2006) used more objects (e.g. 4 objects) compared to those used in the non-associative memory tests in which two objects are usually used. In the OPC test, on the other hand, only two objects are used and multiple trainings are not required. Thus, the OPC test can be the most appropriate paradigm as a rodents’ episodic-

like memory test, and it can systematically investigate the cognitive and neural mechanisms in both associative and non-associative memory by combining with object recognition and place recognition tests.

In conclusion, our results showed that the long-term associative recognition memory is evident when the familiarization period was extended to 30 min in the OPC test. The findings suggested that longer familiarization periods are necessary for the recognition of the complexed associative memory compared to simple non-associative memory. We propose that a spontaneous recognition paradigm is a useful tool for the systematical assessment of long-term associative and non-associative recognition memory in rats.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** All experiments were approved by the University of Tsukuba committee on Animal Research.

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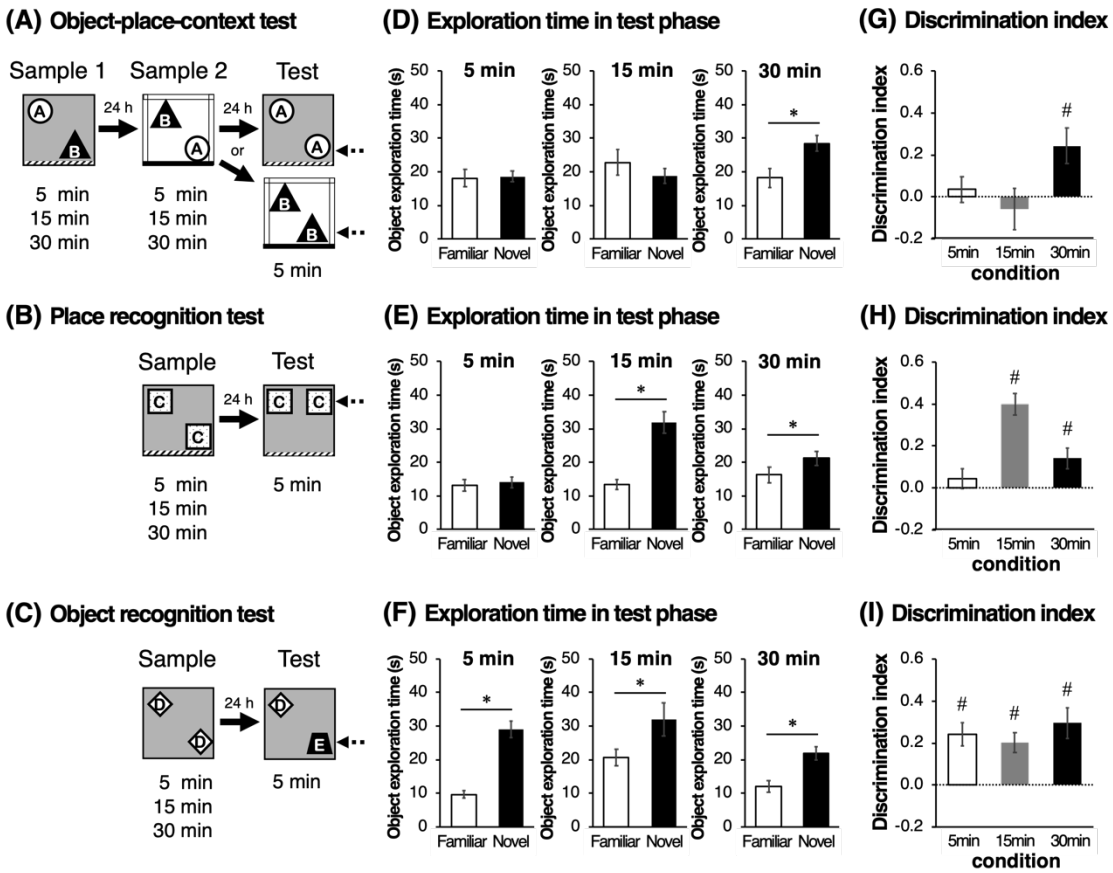


**Fig.1** Effect of familiarization period on discriminatory behavior in associative and non-associative memory in spontaneous recognition paradigm. **(A, B, C)** Schematic illustration of object-place-context test **(A)**, place recognition test **(B)** and object recognition test **(C)**. Each test consists of the sample phase (5, 15 or 30 min), the delay interval (24 hours) and the test phase (5 min). Successful discrimination behavior was defined as the preferential exploration to the 'object in novel context-place combination', 'the object in novel place' or 'the novel object' which is indicated by dashed arrow. In object-place-context test **(A)**, animals encountered two different objects (object A and B), placed at left and right position respectively, in the 'black context' (sample phase 1). After 24 hours, they were put in the 'white context' where the same pair of objects were placed at the swapped position (sample phase 2). In the test phase, animals were allowed to explore two of object A in the 'white context' or that of object B in the 'black context'. In the place recognition test **(B)**, two identical objects (object C) were used through a trial. Animals were allowed to explore two objects in the sample phase. After the delay interval, animals were returned to the same open-field arena where one of the objects were moved to a novel position (test phase). In the object recognition test **(C)**, animals were allowed to explore two identical objects (object D) in the sample phase. After the delay interval,

animals were returned to the same open-field arena where one of the objects are replaced by novel object (object E) in the test phase. **(D, E, F)** Time spent for the exploration of each object in test phase. **(G, H, I)** Mean discrimination index of each test in the test phase. Data are presented as mean ± standard error of the mean (*SEM)*. The horizontal dotted line indicates the chance level (0%). *$p$<.05. #$p$ < .05 vs. chance level (0%) in one-sample $t$-test.