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A Within-Subject Experimental Design Using an Object Location Task in Rats

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TITLE:

A Within-Subject Experimental Design Using an Object Location Task in Rats

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KEYWORDS:

Object location task, spatial memory, recognition memory, rat, within-subject design

SUMMARY:

This protocol provides detailed steps for an object location task with four repetitions using the same cohort of rats. Weak and strong encoding can produce short- and long-term memories. The flexibility of the protocol with repetition can be beneficial for studies involving surgical operations by saving time and labor.

ABSTRACT:

Object place recognition is a prominent method used to investigate spatial memory in rodents. This object place recognition memory forms the basis of the object location task. This paper provides an extensive protocol to guide the establishment of an object location task with the option of up to four repetitions using the same cohort of rats. Both weak and strong encoding protocols can be used to study short- and long-term spatial memories of varying strength and to enable the implementation of relevant memory-inhibiting or -enhancing manipulations. In addition, repetition of the test with the counterbalancing presented here allows the combination of results from two or more tests for within-subject comparison to reduce variability between

rats. This method helps to increase statistical power and is strongly recommended, particularly when running experiments that produce high variation in individual behavior. Finally, implementation of the repeated object location task increases the efficiency of studies that involve surgical procedures by saving time and labor.

INTRODUCTION:

Spontaneous recognition tasks (*e.g.,* object recognition, object place recognition) have been utilized to a great extent in the investigation of memory in rodents. These tests are unlike the variety of tests used for assessing memory that are based on either fear conditioning or reward motivation, in that spontaneous recognition tasks are based solely on spontaneous exploratory behavior towards new stimuli. This behavior, referred to as 'neotic preference'[1], is inherent in rodents as well as in other mammalian species and some non-mammalians such as birds and fish[2]. Object place recognition, which depends on spatial memory, can be observed using the object location task (also known as spatial object recognition task)[3]. Lesion studies have shown that object place recognition requires an intact hippocampus[4,5]. Because of the relatively simple training protocol and the absence of any reinforcement, this task is preferable in many studies. The absence of both positive and negative reinforcement minimizes the additional parameters and brain regions that might drive the behavior. Hence, behavior here is neutral and is based on curiosity and spatial memory, allowing the investigation of mechanisms that are involved in encoding, consolidation, and retrieval of the spatial memory.

The protocol for object location task typically consists of habituation sessions followed by a single session of encoding and test trials, separated by a delay period, which varies from several minutes to hours. It is strongly recommended that rats are handled beforehand to minimize the stress level of the animals, and hence, behavior that could affect recognition memory, such as aversion towards novelty. Similarly, a well-designed habituation protocol plays an essential role in preventing stress that might hinder the natural behavior of the rat during the task. However, the extent of handling and habituation varies largely between laboratories and experimenters, which may contribute to low replicability[6-8]. In the encoding trial, the rat is given time to explore an arena with two identical objects located in two designated corners. In the test trial, which is delayed by a period, the rat is given time to explore the arena with the same pair of objects, but this time one of them has been moved to a novel location. The spontaneous preference exhibited by the rats and the resulting increase in time spent exploring the object at the novel location are indicative of spatial recognition and the memory of the object locations[3]. Modification of the encoding trial (duration and number of repetitions) influences the strength of the memory.

Depending on the aim of the study, the length of the delay between encoding and test trials can be modified to model protein-synthesis-independent short-term memory or protein-synthesis-dependent long-term memory. Hence, the object location task can be used for a wide variety of studies by adapting the protocol as needed. Further, implementation of experimental manipulations, such as pharmacological and optogenetic interventions, are also possible between these trials, as is *in vivo* imaging. There are several studies[9,10] that report repeated iterations of the object location task within the same rat cohort. This contrasts the traditional use in which one animal has one session with no repetitions. However, the effectiveness of these

paradigms has not been thoroughly investigated, nor are there any method papers describing these. To the best of our knowledge, this is the first reported description of a protocol that describes in detail an object location task with up to four repetitions using the same rat cohort, which also systematically compares the results from each repetition. Repetitions can be used to counterbalance experimental conditions to allow within-subject comparison with reduced variability between tests. The reliable repetition of the task allows data to be pooled, meaning that sufficiently large amount of data can be generated using a relatively small number of rats. Finally, repetitions using the same rat can be beneficial in experiments involving surgical operations and implantations by lowering the number of rats required that, consequently, saves time and labor costs.

This study presents an extensive protocol detailing how to perform an object location task in adult rats using strong and weak encoding trials followed by test trials with 1-h and 24-h delays. The strong encoding protocol produces statistically significant recognition memory when tested with 1-h and 24-h delays and can thus be used to study both short-term and long-term memories upon implementation of manipulations to inhibit these memories[11]. In contrast, the weak encoding protocol only produces significant short-term memory when tested with a 1-h delay. The absence of long-term memory can be used to study manipulations for enhancing the retention of memory[11,12]. This protocol also includes detailed handling and habituation sessions, which aim to increase the replicability of the object location task. This paper also demonstrates repetition of the task in four distinct contexts with the same cohort of rats using the weak encoding protocol, which is confirmed to produce replicable and consistent results each time.

PROTOCOL:

All methods described here have been approved by the Danish National Authorities (License number: 2018-15-0201-01405) in accordance with Danish and EU animal welfare legislations.

1. Experimental setup and preparation of distinct contexts

1.1. Object location arena with context

NOTE: The setup below is demonstrated in an enclosed soundproof box (**Figure 1B**) with the light source located along the edges of the ceiling and the camera located at the center of the ceiling of the box. The arena, 60 cm x 60 cm with walls that are 100-cm high (**Figure 1B**), is placed inside the box and is fully isolated from the surrounding room. All spatial cues are inside the arena. This simplifies the process of creating distinct contexts. A similar level of isolation from the surrounding room can be achieved by enclosing a normal open-field arena with a uniform curtain around the walls.

1.1.1. Obtain a square arena made of opaque, non-porous hard plastic with a minimum 60-cm width and a minimum of 50-cm height. Choose a color for the floor contrasting with the color of the rat for successful recording of rat movements by the automated software (if applicable). Place the arena either inside a box (**Figure 1B**) or on a platform that is enclosed by a curtain.

1.1.2. To create a context, obtain a second layer of insertable walls (*e.g.,* wall covering made of the same material as the arena, or plastic wallpaper that can be easily cleaned) in different colors and/or patterns (*e.g.,* black, white, stripes, or dots). Insert the second layer of walls in the arena such that they are distinct from each other.

1.1.3. Obtain three-dimensional (3D) spatial cues (1–2 per context) with dimensions varying between 10 cm x 10 cm x 5 cm and 20 cm x 15 cm x 15 cm (width x length x height) and have (i) distinct geometric shapes and (ii) colors that contrast the wall color. Hang them on the walls high enough so that rats cannot reach these cues.

1.1.4. Obtain different pairs of objects (as many as the context number) that are non-porous, non-chewable, and easy to clean. Aim to have distinct geometric shapes and textures for each new object. Choose objects that are between 5 to 15 cm in width and height (avoid any higher objects). See **Figure 1D** for examples of four distinct objects (cones, footballs, rectangular prisms, and triangular prisms).

NOTE: Each object should be of similar interest to rats, so that the total exploration times for all objects are comparable.

1.1.5. Find the best solution for attaching the objects on the floor of the arena (*e.g.,* using sticky mats, double-sided tape, attaching a metal plate under the object and a pairing magnet underneath the arena *etc.*).

1.1.6. When creating another context, re-create the walls such that they contrast the distribution of color and pattern of the walls from the previous context(s). Use new 3D spatial cues that are different from, and contrasting with, all previous cues. See **Figure 1C** for examples of four distinct contexts.

1.1.7. Obtain a light source that will ensure a diffused and equal illumination within the arena and that has a dimmer option. Adjust the light intensity to approximately 100–120 lux at the corners of the arena after creating each context. Obtain a camera and place it at the center of the ceiling of the box.

NOTE: The light intensity can be adjusted to a lower level if automated scoring software is not being used.

1.2. Object bucket

1.2.1. Obtain a bucket (>50 cm in diameter). Do not choose a square shape to avoid any resemblance to the experimental arena. Fill it with bedding material.

1.2.2. Obtain 5–10 objects of different shapes and sizes (different from the objects that are going to be used in the experiment) and randomly place them all in the bucket (**Figure 1A**).

177
178 [Place **Figure 1** here]
179
180 **1.3.** Camera and tracking software (optional)
181
182 1.3.1. Obtain software that can be used to remotely control the camera recorder and that can
183 track rat noses. Make the software adjustments for each specific context and rat strain before
184 each experiment.
185
186 **1.4.** Counterbalancing of object locations and experimental groups
187
188 1.4.1. Prepare possible combinations of object locations for encoding and test trials, and name
189 these as counters. Create the combinations such that they cover all corners as object locations,
190 and object movement from adjacent to diagonal corners and *vice versa* (**Figure 2A**).
191
192 1.4.2. Prepare a schedule for the specific experiment, matching each rat in one experimental
193 group with a counter. Use each pair of the two paired counters (**Figure 2A**) within one group, if
194 there are enough rats. Use the same set of counters for both experimental groups in a single
195 encoding/test session (**Figure 2B**). Reassign counters for the following sessions (*i.e.,* each new
196 context).
197
198 NOTE: Run the rats in a mixed order during encoding/test sessions (*e.g*., do not run all rats in one
199 cage one after another; instead, rotate cages to ensure a calm environment within a cage of more
200 than one rat).
201
202 1.4.3. When using two or more contexts to counterbalance the experimental groups (*e.g.,* 1-h
203 memory versus 24-h memory groups), assign rats to each group, and change the groups in the
204 following contexts (**Figure 2B**).
205
206 [Place **Figure 2** here]
207
208 NOTE: All handling, habituation, and encoding/test sessions in this protocol were optimized
209 during the light phase of a 12-h light/dark cycle, and hence, it is recommended that experiments
210 be performed during the light phase.
211
212 **2. Handling and habituation**
213
214 **2.1.** Start handling rats beginning either from weaning (if the rats are bred in the home facility)
215 or 2–3 weeks before the beginning of experiments (in the case that the rats are ordered from an
216 external facility, after allowing them to acclimate for one week after arrival).
217
218 **2.2.** Spend at least 10–15 min on each cage of 4 rats for 2 or 3 days a week until the rats are
219 comfortable being touched and picked up by the experimenter. Adjust the time allocated per
220 cage depending on the number of rats in a cage.

NOTE: It is important that all experimenters expecting to work with the rats are present during handling.

2.3. In cases where handling begins at weaning, reduce handling to a minimum (optional) once this level is reached. If beginning 2–3 weeks before the experiments, continue handling until the beginning of habituation sessions.

2.4. Bring the rats in their cages to the experiment room to habituate the rats to the transport as well as to the experimental room. Allow rats to sit for at least 30 min to give them time to calm down and habituate. After this time, return the rats/cages to the housing room.

NOTE: Step 2.4 can be combined with handling and repeated as many times as needed. Additional habituation can be implemented at this step if the protocol includes any further manipulations (*e.g.,* handling for the procedure of injections etc.).

2.5. Perform object habituation to habituate rats to interacting with objects and to reduce general stress levels stemming from the experience of new environments.

2.5.1. For session 1, bring all home cages to the experiment room, and let the rats habituate to the room and settle for at least 30 min. Put rats (2–4 rats) from the same cage together in the bucket for 20 min. Clean the bucket by removing any fecal matter between each group of rats. Repeat the procedure for all cages. Put all rats in their home cages and return to the housing room.

2.5.2. For session 2, bring all cages to the experiment room and leave for at least 30 min. Put each rat individually in the bucket for 10 min. Place the rat back into the home cage and clean the bucket after each rat. Return all cages to the housing room.

2.5.3. For session 3, repeat step 2.5.2 on a separate day.

2.6. If the experimental apparatus is an enclosed box (**Figure 1B**), opt to perform empty box habituation to habituate the rats to the new experimental apparatus. In session 4, bring all cages to the experiment room and leave for at least 30 min. Place rats from the same cage together (2–4 rats) in the empty arena with no context or spatial cues (**Figure 1B**, middle) for 20 min. Place all rats back into the home cage, and wipe the arena with 70% ethanol after each group of rats.

NOTE: Steps 2.5 and 2.6 should performed in a single week, preceding the context habituation week (step 2.7). A break for a couple of days during these steps is acceptable. However, after starting step 2.7, each step should be performed on consecutive days as specified, until the end of test trial (step 2.9).

2.7. Perform context habituation to habituate rats to the context and 3D cues, to reduce general stress levels and to support the spatial learning of the environment (**Figure 3**).

2.7.1. Modify the empty arena to create the first context as described in section 1.1, but do not put the objects in the arena. Prepare the recording equipment.

2.7.2. For session 1, bring all cages to the experiment room and leave for at least 30 min. Start the recorder if doing this manually. Place the first rat in the center of the arena, and allow the rat to explore the arena for 10 min. Then, stop the recorder (if manual),and place the rat back into the home cage. Wipe the arena thoroughly with 70% ethanol after each rat, and return all cages to the housing room when finished.

2.7.3. For sessions 2 and 3, repeat step 2.7.2 for each rat over two consecutive days such that there are 3 sessions of context habituation per rat in total.

NOTE: Consider shuffling the order in which rats go into the arena, especially when dealing with a large group. This avoids running specific rats repeatedly at the same time of the day.

[Place **Figure 3** here]

2.8. Encoding trial (Session 4)

NOTE: In the case of pharmacological manipulations, a reasonable time to administer an agent can be either before or immediately after the encoding trial(s) and/or before the test trial depending on the nature of the pharmacological agent.

2.8.1. Bring all cages to the experiment room and leave for at least 30 min. Using the schedule prepared beforehand (**Figure 2B**), place the first identical pair of objects in the designated locations (at 2 corners and a distance of >10 cm from each respective wall; an L-shaped piece of cardboard can be used to maintain the same distance each time) by using sticky mats or double-sided tape.

2.8.2. Start the recorder (if manual). Place the first rat in the arena facing a wall or a corner that is not occupied by any object (equal distance to each object).

NOTE: Follow the steps below for either weak or strong encoding.

2.8.3. **For weak encoding (1 trial)**, allow the rat to explore the arena and objects for 20 min. Then, stop the recorder (if manual), and place the rat back into the home cage. Remove the objects, and wipe both the objects and the arena thoroughly with 70% ethanol.

2.8.4. Repeat step 2.8.3 for all rats so that each rat receives 1 encoding trial of 20 min.

2.8.5. **For strong encoding (3 trials)**, allow the rat to explore the arena and objects for 5 min. Then, stop the recorder (if manual), and place the rat back into the home cage. Do not remove the objects. Wipe the arena and objects with 70% ethanol.

2.8.6. Repeat step 2.8.5 two more times with the same rat such that there are 3 trials in total. Place the rat back into the home cage when the time is up. Remove the objects for thorough cleaning, and wipe the objects and the arena with 70% ethanol.

NOTE: Inter-trial interval for a rat should be approximately 1–2 min.

2.8.7. Repeat steps 2.8.5–2.8.6 for each rat.

2.8.8. If the delay time is shorter than 24 h, keep the cages in the experiment room until the test trial. If not, return all cages to the housing room when completed.

2.9. Test trial (Session 4)

NOTE: The delay period should be counted from the beginning of the encoding trial.

2.9.1. In case of a 24-h delay (or any delay that requires the test trial to be performed the following day), bring all cages to the experiment room, leaving sufficient time ahead of the first test so that the rats can be left for at least 30 min. According to the schedule, place the objects in the designated locations (one of the objects at a new location).

2.9.2. When it is time, start the recorder (if manual). Place the first rat in the arena facing a wall or a corner that is not occupied by any object (equal distance to each object).

2.9.3. Allow the rat to explore the arena and objects for 5 min. Then, stop the recorder (if manual). Place the rat back into the home cage. Remove the objects, and wipe both the objects and the arena thoroughly with 70% ethanol.

2.9.4. Repeat the steps 2.9.2–2.9.3 for each rat. Return all cages back to the housing room.

NOTE: In each following encoding/test session, start the habituation protocol from step 2.7 (context habituation) after an interval of at least 48 h and up to 1 week.

3. Data analysis

3.1. For each rat, score the exploration time for each object in both the encoding and test trials by using software designed for this purpose or using a manual setup. Score encoding trials for the whole duration. Score test trials for 2 min for best discrimination performance[3]. If using automated online software scoring, export the scoring data from the software.

3.2. Count exploration time when the rat is in contact with the object, sniffing the object or facing the object at a distance less than 2 cm. Include climbing and sitting on the object as exploration unless the attention of the rat appears to be somewhere other than the object (*e.g.,* looking away from the object).

3.3. Calculate the total exploration time for both objects for each rat. Consider excluding any rat that has a total exploration time of less than 10 s in the test trial (for 2 min scoring) from this test, as it may reflect unreliable exploration.

3.4. Calculate the percentage of exploration for each object (equation 1)or the discrimination index (DI) for each rat (equation 2), and calculate mean values for the groups.

$$\% \text{ exploration of object 1 (novel location)} = \frac{\text{Time spent exploring object 1 (novel loc.)}}{\text{Total exploration time}} \times 100\% \quad (1)$$

$$\text{DI} = \frac{\text{Time spent exploring object 1 (novel loc.)} - \text{Time spent exploring object 2 (stable)}}{\text{Total exploration time}} \quad (2)$$

NOTE: If % exploration is 50% or DI is 0, it means that the performance is at the chance level, and the rat has no preference for either object. The mean percentage exploration and DI during encoding trials should be ~50% or 0, respectively. Any rat showing a preference higher than [mean ± (2 × SD)] for either object in the encoding trial should be excluded from the analysis of the respective test. This allows for reliable interpretation of preference in the test trial as the memory of the stable object location. This value can be calculated for an individual test or for combined encoding data from several tests.

3.5. Analyze the data by the method best fitting the experimental setup. Use a one-sample *t*-test for detecting a significant preference above the chance level.

3.6. While using more than one context with counterbalancing, combine the results of the same experimental condition across contexts.

NOTE: This will result in groups that consist of the same rats, enabling within-subject comparison using a paired *t*-test for two groups and using repeated measures analysis of variance (ANOVA) for more than two groups.

REPRESENTATIVE RESULTS:

Shown here are the representative results for both the strong and weak encoding protocols described using tyrosine hydroxylase (Th)-Cre transgenic rats[13] with Long-Evans strain backcrossed four times to Lister Hooded strain and wild-type Lister Hooded rats. Th-Cre transgenic rats were used as this rat line will be used in future studies involving optogenetics. Using each protocol, memory was tested with delays of 1 and 24 h. Tests at 1 h demonstrate short-term memory, while 24-h tests demonstrate long-term memory. The exclusion value for encoding preference was calculated as described in the protocol, using the combined data from five tests (strong and weak encoding protocols) as [50.8% ± (2×10.8%)]. Rats that had an encoding preference above and below these values were excluded from the analyses of the respective tests.

During the strong encoding trials (3 × 5 min encoding; **Figure 4A**), there was no significant preference for either object (52.0 ± 1.9%, n = 16, t_{15} = 1.1, p = 0.29; one-sample *t*-test versus chance level). This strong encoding protocol led to preference for the object at the novel location, as shown in terms of mean percentage exploration, which was significantly higher than the chance level (50%) in tests with both 1-h and 24-h delays (1-h memory, 77.9 ± 2.4%, n = 8, t_7 = 11.8, p < 0.001; 24-h memory, 65.2 ± 5.3%, n = 8, t_7 = 2.8, p = 0.025; one-sample *t*-test versus chance level). There was no significant difference between 1-h and 24-h memory (p = 0.056; unpaired Welch's *t*-test).

During the weak encoding trials (20 min encoding; results pooled from four contexts; **Figure 4B**), there was no significant preference for either object (51.1 ± 1.0%, n = 66, t_{65} = 1.2, p = 0.24; one-sample *t*-test versus chance level). This weak encoding protocol produced a significant increase in the preference for the object at the novel location compared to chance level in tests with a 1-h delay, but not 24-h delay (combined data from all four contexts; 1-h memory, 66.7 ± 2.0%, n = 32, t_{31} = 8.2, p < 0.001; 24-h memory, 49.6 ± 2.6%, n = 34, t_{33} = 0.16, p = 0.87; one-sample *t*-test versus chance level). There was a significant difference between the performance in tests with 1-h and 24-h delays (1-h memory: n = 32, 24-h memory: n = 34, $t_{61.5}$ = 5.2, p < 0.001; unpaired Welch's *t*-test).

Memory at the group level was not observed in the 24-h delay test as indexed by chance-level performance, but showed individual variations. This higher variation for weak to no-memory conditions (*e.g.*, 24-h test) was commonly observed due to more random exploration of the objects. Hence, it is important not to interpret the performance of rats individually. Instead, distribution of individual data points can be used along with the group average as the reliable outcome of the test. The stronger the encoding, the more uniform the behavior of the rats becomes, and the fewer the number of rats needed for reaching statistical significance, as can be observed in **Figure 4A** for the strong encoding protocol. In contrast, larger groups are needed for obtaining reliable results for weak conditions (**Figure 4B**).

[Place **Figure 4** here]

A significant advantage of this established protocol is that it can be performed four times using four distinct contexts (**Figure 1C**) with the same cohort of rats. The results shown in **Figure 5** demonstrate one possible way of using counterbalancing with two experimental groups (1-h and 24-h memory groups). The two groups were counterbalanced over two contexts (contexts 1 and 2), and this was repeated in two additional contexts (contexts 3 and 4; **Figure 5A**). The results from the four contexts are presented individually in **Figure 5B,D**, where the memory for each experimental group was assessed by comparing the preference to chance level in each context (1-h memory: Context 1, 69.9 ± 3.6%, n = 9, t_8 = 5.5, p < 0.001; Context 2, 65.6 ± 3.9%, n = 9, t_8 = 4.0, p = 0.004; Context 3, 65.2 ± 3.8%, n = 7, t_6 = 4.0, p = 0.007; Context 4, 65.3 ± 5.6%, n = 7, t_6 = 2.7, p = 0.035; 24-h memory: Context 1, 45.1 ± 6.4%, n = 9, t_8 = 0.77, p = 0.46; Context 2, 49.1 ± 4.9%, n = 9, t_8 = 0.18, p = 0.86; Context 3, 57.2 ± 4.1%, n = 8, t_7 = 1.7, p = 0.12; Context 4, 47.6 ± 4.7%, n = 8, t_7 = 0.52, p = 0.62; one-sample *t*-test versus chance level).

In contexts 1, 2, and 4, between-subject comparison of the groups revealed significant differences between 1-h and 24-h memory (1-h memory versus 24-h memory: Context 1, $t_{12.7}$ = 3.4, p = 0.005; Context 2, $t_{15.2}$ = 2.6, p = 0.019; Context 3, $t_{13.0}$ = 1.4, p = 0.17; Context 4, $t_{12.2}$ = 2.4, p = 0.032; unpaired Welch's *t*-test). For a better representation and within-subject comparison of the data, the results from two counterbalanced contexts were combined (**Figure 5C,E**). The combined experimental groups were compared to chance level individually again (Contexts 1 and 2 combined: 1-h memory, 67.8 $\pm$ 2.6%, n = 18, t_{17} = 6.7, p < 0.001; 24-h memory, 47.1 $\pm$ 3.9%, n = 18, t_{17} = 0.74, p = 0.47; Contexts 3 and 4 combined: 1-h memory, 65.3 $\pm$ 3.3%, n = 14, t_{13} = 4.7, p < 0.001; 24-h memory, 52.4 $\pm$ 3.2%, n = 16, t_{15} = 0.73, p = 0.48; one-sample *t*-test versus chance level). Then, the experimental groups were compared to each other.

In both context pairs, there were significant differences between groups as revealed by within-subject comparisons (1-h memory versus 24-h memory: Contexts 1 and 2 combined, t_{16} = 3.5, p = 0.003; Contexts 3 and 4 combined, t_{13} = 2.4, p = 0.032; paired *t*-test). Comparable results were obtained with wild-type Lister Hooded rats, too, in the weak encoding protocol using contexts 1 and 4 for the two counterbalanced sessions (data not shown). The replicability and reliability of the results were validated by comparing each data set using one-way ANOVA. No significant difference was detected among the four contexts (1-h memory: $F_{3,28}$ = 0.31, p = 0.81; 24-h memory: $F_{3,30}$ = 0.99, p = 0.41). Therefore, the object location test can be repeated reliably with minimum influence of repetitions, given that the instructions in this protocol are followed.

[Place **Figure 5** here]

FIGURE AND TABLE LEGENDS:

Figure 1: The experimental setup, including four distinct contexts and objects. (**A**) The object bucket for object habituation. (**B**) The experiment apparatus (left), enclosing the object location arena, the camera, and the light source. The experimental box and arena before context setup (middle) and arena with context setup (right). (**C**) Four contexts (1–4) with distinct wall colors and patterns, as well as three-dimensional spatial cues. (**D**) Four objects that are used in contexts 1–4, respectively.

Figure 2: Representative counterbalancing methods. (**A**) Possible orientations of objects in the arena at encoding and test trials are named as counters. Object 1 is always the moving object. Every two counters are counterbalanced such that the location of the moving object changes. Each corner is occupied twice, and object 1 is moved from diagonal to adjacent and *vice versa* for an equal number of times. (**B**) Example of encoding/test schedule for two counterbalanced sessions (*e.g.,* contexts 1 and 2). Rats are assigned to experimental conditions in context 1 (session X, left). A set of counter pairs (*i.e.,* 1-2, 3-4, 5-6, and 7-8) are selected and assigned to each rat in one experimental group. The same set of counters is assigned to rats in both experimental groups. In the following session in context 2 (session X+1; right), the rats in the experimental groups are changed for counterbalancing, and a new set of counter pairs are assigned. The time at the beginning of encoding and test trials should be noted.

Figure 3: The design of the behavioral experiment including handling, habituation, and object location task protocols. Rats should be handled regularly starting from few weeks prior to the habituation week. In week 0, object and experimental box habituations are carried out over 4 sessions with at least 24-h intervals in between. In week 1, context habituation is carried out over 3 consecutive sessions with 24-h intervals in between, followed by encoding and test trials. There should be a minimum of 48 h and up to 1 week interval before proceeding with the following session (*e.g.,* begin habituation to the next context in week 2 or 3). Abbreviation: Hab., habituation.

Figure 4: Memory performance after strong and weak encoding. (**A**) The strong encoding trial (3 × 5 min encoding) followed by either 1-h or 24-h test trials. There was no significant preference for either object during encoding trials (n = 16). The strong encoding produced significantly increased preference for the object at the novel location in the tests with both 1-h and 24-h delays compared to chance level (1-h and 24-h memory: n = 8 in each group). There was no significant difference between groups. (**B**) The weak encoding trial (20 min encoding) followed by either 1-h or 24-h test trials. There was no significant preference for either object as a group during encoding trials (n = 66). The weak encoding produced significantly increased preference for the object at the novel location in the test with a 1-h, but not 24-h delay, compared to chance level (1-h memory: n = 32; 24-h memory: n = 34). There was a significant difference between the performance in tests with 1-h and 24-h delays. The results were pooled from four contexts. Individual data points are presented as dots. All bars show the percentage of exploration of the object at novel location as mean ± SEM. *$p < 0.05$, ***$p < 0.001$; one-sample *t*-test versus chance level (50%, dashed line). $^{###}p < 0.001$; ns, not significant; unpaired Welch's *t*-test.

Figure 5: Different ways of presenting and analyzing the results of the weak encoding protocol with two experimental groups counterbalanced over two sessions. (**A**) The experimental design for counterbalancing with two experimental groups (1-h and 24-h memory groups) over two sessions (contexts 1 and 2). The counterbalancing was repeated in two additional sessions (contexts 3 and 4). (**B** and **D**) The results from each context and the experimental groups were individually compared to chance level and to each other. In all four contexts, the preference for the object at the novel location in tests with a 1-h delay was significantly increased compared to chance level [Context 1 and 2: n = 9 per group (**B**); Context 3 and 4: n = 7 per group (**D**)]. In 24-h delay tests, the preference for the object at the novel location did not differ from chance (Context 1 and 2: n = 9 per group; Context 3 and 4: n = 8 per group). There was a significant difference between the preferences of experimental groups in contexts 1, 2, and 4, but not context 3, as revealed by between-subject comparison. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; one-sample *t*-test versus chance level (50%, dashed line). $^{#}p < 0.05$; $^{##}p < 0.01$; ns, not significant; unpaired Welch's *t*-test. (**C** and **E**) The results are presented after combining the experimental groups from the two counterbalanced contexts [Contexts 1 and 2 combined, n = 17 per group (**C**); Contexts 3 and 4 combined, n = 14 per group (**E**)]. The preference for the object at the novel location was significantly increased compared to chance level in tests with a 1-h, but not 24-h delay, in both context pairs. The within-subject comparison of the experimental groups revealed significant

differences between the preferences for the object at the novel location in tests with 1-h and 24-h delays in both context pairs. ***$p < 0.001$; one-sample *t*-test versus chance level (50%, dashed line). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$; paired *t*-test. Individual data points are presented as dots. All bars show the percentage of exploration of the object at the novel location as mean ± SEM.

DISCUSSION:

The object location task can be used in a variety of studies to investigate spatial memory as described earlier. The flexibility of the setup enables the modeling of short-term and long-term memory of different strengths, and it can be easily implemented at a low cost. However, as there are many parameters in the protocol that can influence results, and different studies vary slightly in these parameters[6], one might face difficulties successfully implementing the task for the first time. The above protocol is intended to guide readers through this process smoothly. Further crucial steps that might be significant in the successful implementation of the task with high replicability will be discussed below.

Although the encoding/test session often is the focus when running object location experiments, handling and habituation protocols have a profound effect on the outcome of these kind of behavioral tests where the outcome depends on undisturbed natural rat behavior[14,15]. As such, the steps preceding the encoding/test session should be designed with caution, as they can influence rat behavior and memory and consequently, influence the end results. A good level of handling and habituation such that rats become familiar with the experimenter and the task will minimize the effect of stress factors whilst increasing the likelihood of exhibiting natural behavior[8]. As mentioned in the protocol, handling can begin as early as weaning of pups if the rat strain is maintained in the home facility. Based on previous experience (data not shown) and from that of several previous studies[16,17], this early handling results in low anxiety and enhanced curiosity in the months that follow.

As the object location task depends solely on the intrinsic exploratory drive of the rats, expected behavior can be easily hindered if rats are not eager to explore or reluctant to approach novelty, which is referred as 'neophobic behavior'[1]. As such, it is highly recommended to include a thorough handling and habituation protocol according to the specific needs of the study. This protocol can be used as a minimum requirement guide, and further steps can be implemented (*e.g.*, if the study is to include injections at a later stage, habituation to injection procedures and specific holding position are required). The strain and the age of the experimental rats are two other influential factors and should be considered before planning an experiment to avoid suboptimal results. Different rat strains can have different behaviors and baseline anxiety levels[18-20] and therefore, specific adjustment to the protocol may be required depending on strain used.

This protocol is confirmed to work well with Th-Cre transgenic rats with Long-Evans strain backcrossed four times to Lister Hooded strain and wild-type Lister Hooded rats. A logically ideal starting age for rats in behavioral experiments is around 12 weeks[20], but inter-strain variability and the specific requirements of the task should be accounted for. It could also be possible to use developing rats if it is of interest for the study, though adjustments to the protocol may be

required and are not covered here. However, it is important to consider whether the rat at a given age has developed the cognitive functions required to successfully perform this task. A study[21] investigating this has reported that only the adolescent rats at postnatal day 38 and not before, showed allocentric spatial memory reflected in preference for object at the novel location, as observed in adult rats. The protocol presented here was successful using rats that were 15–16 weeks old at the beginning of the first encoding/test session. Previously, the same strong encoding protocol produced suboptimal to negative results when using 23-week-old rats that had not reached the optimal level of habituation due to lack of handling and habituating at a young enough age. These rats either failed to perform differently from chance level or in fact, exhibited aversion towards novelty as observed in terms of preference for the stable objects instead of the displaced objects (data not shown). These results provide evidence that the age and the timing of the handling habituation can have an impact on the effectiveness of habituation and as a result, contribute to the observation of anxious and neophobic behavior in the tests.

Here, two different protocols are outlined, ensuring strong or weak encoding in the object location task. During the establishment of these protocols, it was observed that interest in the objects declined after 5–10 min of exploration during single long trials (*e.g.,* 20 min encoding), and rats eventually stopped exploring. This results in weaker memory of the object locations. An encoding protocol that interleaves encoding trials with short rest periods (*e.g.,* 3 x 5 min encoding) overcomes this and leads to high exploration throughout the trials. Thus, the active exploration time and the different layout of these two encoding protocols influences the strength of the memory, which is stronger after 3 x 5 min encoding than after 20-min encoding protocols. Similar results can also be achieved using slightly different durations with single trial versus interleaved trial protocols, and adjustments can be made to suit the needs of the study and the rat strain.

As opposed to protocols using a plain white open field with only external cues in the room, the protocol presented here uses an arena with distinct contexts and intra-maze cues that likely requires more time to learn. Hence, the addition of a context habituation step in the protocol prior to the encoding trial is recommended. This will allow rats to form a spatial map of each context during habituation and decrease the duration of the following encoding trial, as the rats will only need to encode the locations of the objects in relation to this map. Furthermore, context habituation will allow rats to habituate to any possible distractor within each context, such as the 3D spatial cues, minimizing behaviors other than object exploration in the encoding/test session to follow. With the implementation of a thorough counterbalancing method consisting of several levels (*i.e.,* a wide range of object location combinations (counters) and direction of the object displacement), unwanted preferences that may rise due to variations in light intensity and wall colors/patterns at the corners of the arena are minimized.

Several factors should be considered when repeating the task to increase the replicability between encoding/test sessions and minimize the influence of repetition. First, distinct contexts (as many as the number of repetitions of encoding/test sessions) need to be designed to avoid the accumulation of spatial memory that might be caused by performing the repeated sessions using the same context. To achieve this, an apparatus with replaceable walls of different colors

and patterns was used (**Figure 1B,C**). The distinct walls and 3D objects (such as toys or small everyday items of distinct colors and shapes, see protocol and **Figure 1C**) hung on the walls are the spatial cues and landmarks that the rats potentially use to learn object locations in relation to their contexts. In the case that a test fails to produce preference for the moved object, changing these parameters of the context (wall design and spatial cues) can be considered. Alternatively, a rectangular- or circular-shaped arena can be used for object location tasks instead of a square arena as in this protocol. Circular arenas are reported to eliminate corner preferences[22] that is often observed in arenas with corners, and hence, it can be beneficial when dealing with a particularly high-anxiety rat or mouse strain. While the requirements of creating four distinct contexts in this protocol works most optimally with a quadrangular shape, a circular arena can also be made functional following some adjustments.

Second, the intervals between each encoding/test session should be determined such that rats retain the same level of interest each time, while avoiding the risk of cumulative learning resulting from a dense schedule of repetitions. Usually, an interval of at least double the length of the delay time between encoding and test trials is sufficient, with longer intervals being more favorable for more than two repetitions. This means, while a minimum of 48-h interval after a 24-h test is sufficient for one or two repetitions, using a 1-week interval is recommended for four repetitions. As the results in **Figure 5** and the comparison using ANOVA show, the task can be successfully repeated four times. Based on this, the established protocol can be used to counterbalance up to four experimental conditions. The number of experimental groups determines the number of repetitions of encoding/trial sessions in distinct contexts. The results in **Figure 5** represent one possible way of using the protocol with two experimental groups. The groups were counterbalanced in two sessions, and the same conditions were repeated in two additional sessions (for validation purposes). The second set of counterbalanced sessions could also be used to counterbalance new conditions. Similarly, three or four experimental conditions can be compared using three or four counterbalanced sessions, respectively.

In these cases, the contexts should be designed to accommodate contrasting characteristics described in the protocol. It is noteworthy that the counterbalanced design may not be suitable for experiments in which additional manipulations, such as a pharmacological intervention that might leave a long-lasting effect or damage, are to be used. To maintain the effectiveness and replicability of the tests, the experiment should be designed accordingly. The data from repeated tests can be presented and analyzed in several ways, as demonstrated in **Figure 5**. For an initial analysis, the experimental groups in each context can be individually compared to chance level using one-sample *t*-test to determine any significant preference (**Figure 5B,D**). This can be helpful to get a quick understanding of the data, but it ensures only an indirect comparison of the groups. So, for comparing two or more groups, the data should be analyzed using two-sample *t*-tests (paired or unpaired) or ANOVA, respectively. This can be in the form of between-subject comparison of the groups within a single encoding/test session (**Figure 4A** and **Figure 5B,D**) or within-subject comparison of the groups from two (or more) counterbalanced contexts (**Figure 5C,E**). The latter method is strongly recommended, especially when dealing with weak memory conditions, which, as explained previously, results in high variance due to randomness in behavior.

Combining the counterbalanced contexts leads to larger groups that are required to reliably visualize the behavior of the group with minimal variation. Using a protocol with repetitions in counterbalanced sessions, one can expect a decrease in the number of rats to around one third of the number that would be required using a single test with the same statistical power. Usually, sample sizes in a range of 7 to 15 rats (total) for counterbalanced sessions and in a range of 20 to 50 rats (10 to 25 per group) for a single session with an effect size and power both larger than 0.8 are sufficient. It is important at this step to keep in mind that random rat behavior, which is not accompanied with a strong memory, may result in individual strong preferences both below and above chance, but the group average should yield a preference not significantly different from chance. Individual data should be interpreted carefully. The distribution of individual data points within a group can also be informative for interpreting results. As seen in **Figure 4** and **Figure 5**, the distribution changes depending on the strength of the memory. Overall, the protocol presented here can be followed easily to implement the object location task with repetitions to model short-term and/or long-term spatial memory. The simple and flexible training protocol and the possibility of implementing further manipulations make this task a popular choice. These modifications to the protocol enable the investigation of particular steps such as memory acquisition, consolidation, and recall.

ACKNOWLEDGMENTS:

We would like to thank Antonios Asiminas, Dorothy Tse, Kiichi O'Hara, and David Bett for insightful comments and suggestions. This study was supported by Erasmus+ (to G.B. and L.N.); the Graduate School of Health, Aarhus University (to K.H.); Novo Nordisk Foundation Young Investigator Award 2017 (NNF17OC0026774), Lundbeckfonden (DANDRITE-R248-2016-2518) and PROMEMO – Center for Proteins in Memory, a Center of Excellence funded by the Danish National Research Foundation (DNRF133) (to T.T.).

DISCLOSURES:

The authors have nothing to disclose.

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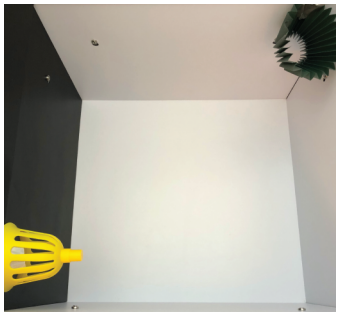
Figure

Click here to access/download;Figure;Figure_1.pdf

A Object bucket

B Object location box

C Context 1

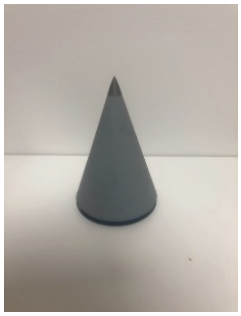


Context 2

Context 3

Context 4

D Object 1



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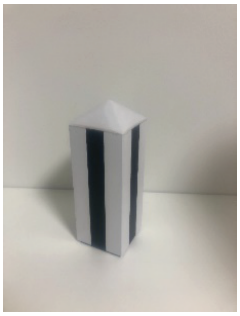

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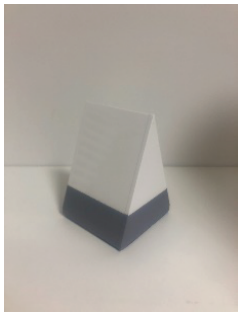
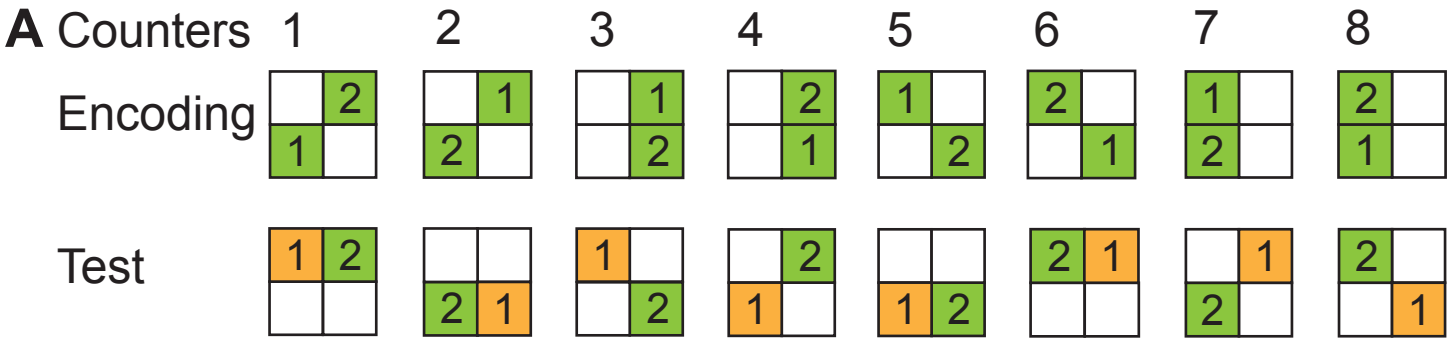


A



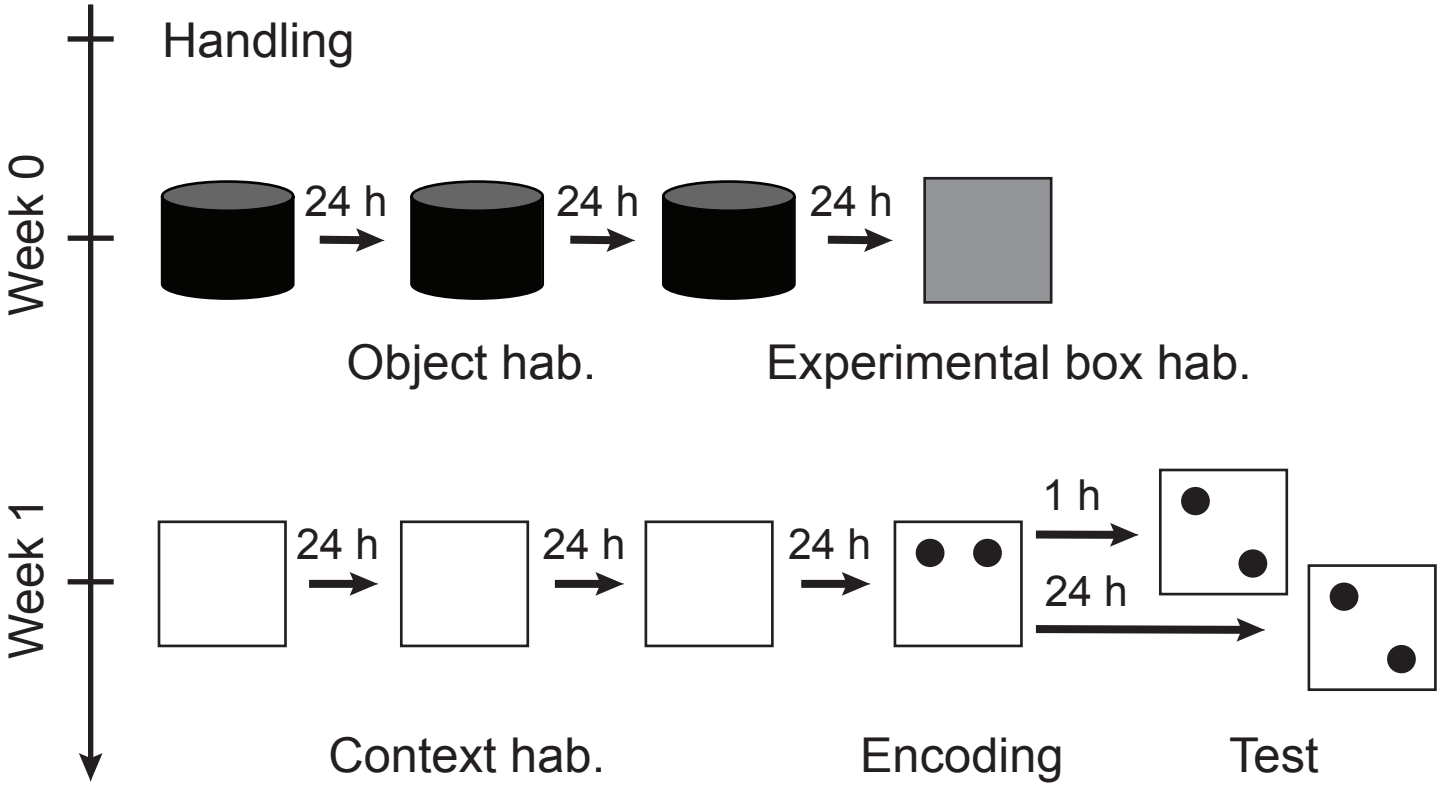
B

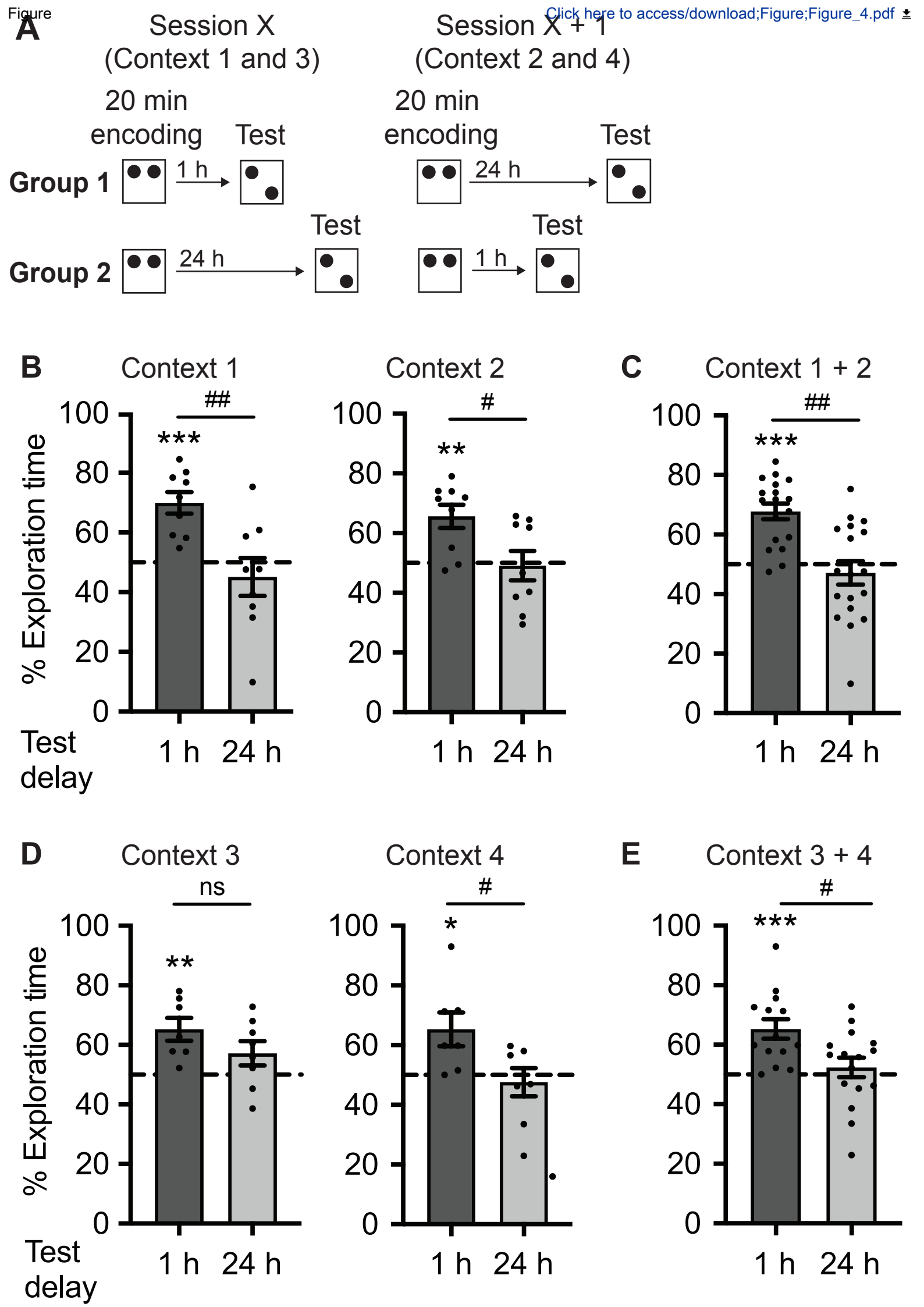
Session X

Context	Rat ID	Cage	Condition	Counter	Encoding time	Test time
1	1	1	24 h	1		
1	2	1	1 h	7		
1	3	1	24 h	3		
1	4	1	1 h	4		
1	5	2	24 h	4		
1	6	2	1 h	1		
1	7	2	24 h	8		
1	8	2	1 h	3		
1	9	3	24 h	7		
1	10	3	1 h	2		
1	11	3	24 h	2		
1	12	3	1 h	8		

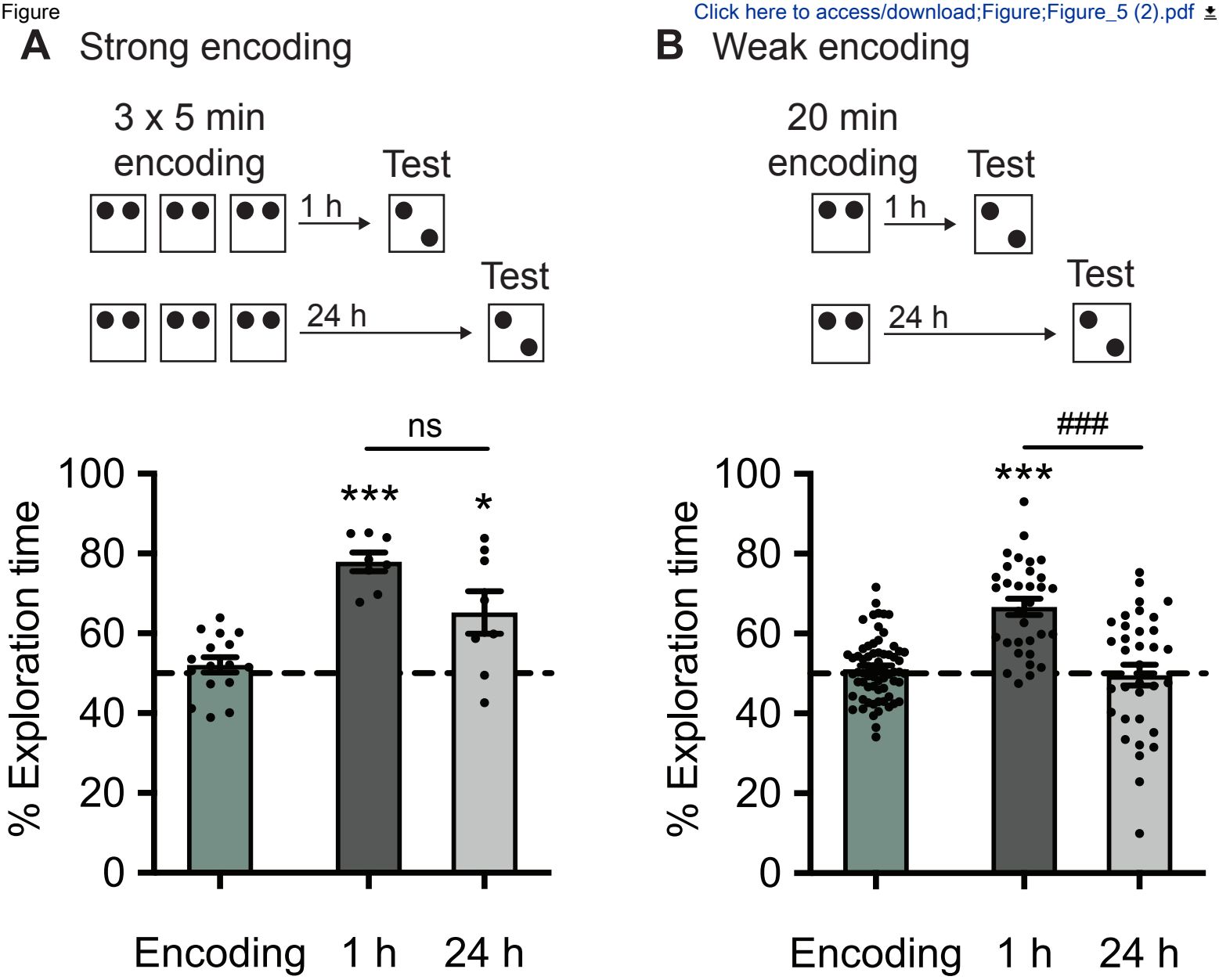
Session X+1

Context	Rat ID	Cage	Condition	Counter	Encoding time	Test time
2	1	1	1 h	2		
2	2	1	24 h	5		
2	3	1	1 h	8		
2	4	1	24 h	2		
2	5	2	1 h	5		
2	6	2	24 h	7		
2	7	2	1 h	6		
2	8	2	24 h	1		
2	9	3	1 h	1		
2	10	3	24 h	8		
2	11	3	1 h	7		
2	12	3	24 h	6		





Figure



Name of Material/Equipment	Company	Catalog Number	Comments/Description
Open-field/experimental box	O'Hara & Co (Japan)	OF-3001	Open-field box for the object location task
Object 1: cones	O'Hara & Co (Japan)	ORO-RR	
Object 2: footballs	O'Hara & Co (Japan)	ORO-RB	
Object 3: rectangular blocks	O'Hara & Co (Japan)	ORO-RC	Rectangular blocks were modified after purchase
Object location task apparatus	O'Hara & Co (Japan)	SPP-4501	Sound attenuating box that contains the open-field box fo
Tracking software	O'Hara & Co (Japan)	TimeSSI	For movement tracking and automated camera functions
Wild-type Lister Hooded rats	Charles River	603	

ɹr the object location task

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response:
Thank you. The manuscript was proofread again.

2. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Response:
We have made the change accordingly (line 124).

3. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Response:
We have made these corrections.

4. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

Response:
We have moved these parts to the discussion section.

5. Line 141-143: Please specify the dimensions of the 3D spatial cues.

Response:
We have added this information (lines 150-151).

6. Line 207: Please use standard abbreviations for time units preceded by a numeral. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks

Response:
We corrected this (line 230) and checked for others throughout the manuscript.

7. Please title case and italicize journal titles and book titles. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.

Response:
We corrected our referencing style accordingly.

Response to Reviewers

Reviewer #1

Manuscript Summary:

This manuscript by Bayraktar et al provides a detailed protocol for the use of a spontaneous exploration, object-place spatial memory task. The protocol is presented well, the schematics help understand complicated aspects (such as counterbalancing conditions) and the authors provide convincing evidence of the utility of this assay as a way to study memory persistence. There are no major issue with the manuscript. The manuscript will benefit from some rewording/editing to aid clarity. Moreover, I have a series of comments, suggestions and questions and the authors may wish to address.

Major Concerns:

None

Comment 1:

Line 68 since this behaviour is not only innate in rodent (pigs, birds etc) maybe here you can rephrase to emphasize that preference for novelty (in some ways reflecting curiosity) is extremely conserved across species.

Response:

Thank you for your comment. We updated the phrasing (line 68).

Comment 2:

Line 69: setting, maybe use a different word, since the setting is not natural. The behaviour is spontaneous and naturalistic but the setting is highly artificial.

Response:

We agree on this point and we removed this phrasing from line 70, instead we updated the phrasing in line 74-75 to convey this message accordingly.

Comment 3:

Line 75: This sentence does not follow logically from the previous. This object-location task requires the hippocampus as well as perirhinal cortex (lesion studies cite). I would suggest refraining from using semi-quantitate words such as 'mainly' since there is no evidence on the relative contribution of different neural systems in object-location memory.

Response:

Thank you -we rephrased the part starting with line 70. However, lesion studies have shown show that the perirhinal cortex is required for object recognition but not for spatial components, so it appears that it is not required for object location recognition task.

Please see page 4 in *Aggleton & Nelson, Brain Neurosci Adv., 2020*: 'Consistent with this prediction, hippocampal lesions are associated with object-location deficits (Barker and Warburton, 2011b; Mumby et al., 2002; Save et al., 1992), while perirhinal cortex lesions spare performance (Barker et al., 2007).'

Comment 4:

Line 80: Can you substantiate this claim? Are there any studies suggesting that not well handled animals are more neophobic?

Response:
We do not know of any study that directly shows handling minimizes neophobia. Combining the pieces of evidence and experiences, however, these two seem likely to influence one another. There are studies reporting that anxiety/fear state can cause neophobia if it overcomes the exploratory instinct of the animal (*Ennaceur, Michalikova, Bradford, & Ahmed, Behav Brain Res, 2005*; *Hughes, Neurosci. Biobehav. Rev., 2007*). Other studies report that not well-handled animals tend to exhibit higher anxiety in tasks such as open-field test and elevated plus maze (*Schmitt and Hiemke, Pharmacol. Biochem. Behav., 1998*; *Hurst and West, Nat. Methods, 2010; Costa et al., J Am Assoc Lab Anim Sci, 2012; Gouveja and Hust, Sci Rep, 2017*). Hence, these suggest that a not-well-handled animal may experience high anxiety due to handling during the encoding/test trials, which may result in loss of attention in the task and hindered natural behavior that is driven by curiosity and is what the task at hand is largely dependent on, and finally cause reluctance to approach novelty.

Comment 5:
Lines 95-96: I think the authors should consider citing two papers: Migues et al 2016 J Neurosci and Maingret et al 2015 Nat Neurosci. In both these manuscripts the authors used the object location task multiple times (within animal design) to study memory decay (Migues et al) and the importance of HPC-PFC coordination during sleep for the consolidation of spatial memories (Maingret et al). Moreover, many other studies have reported repeated object exploration tasks in the same animals either for adults or during development (to name a few; Langston and Wood 2010, Barker and Warburton 2017, Asiminas et al 2019).

Response:
Thank you for referring to these studies. We rephrased our statement and referred to some of these papers in the text (line 97 and on).

Comment 6:
Line 130: Can the authors comment on the importance of the shape of the box. Is square essential? Could a cylindrical box work as well since it does not have any corners which are known to serve as strong spatial cues to rodents?

Response:
Thank you for addressing this. Indeed, there are studies in the literature that reports the use of either square, rectangular or cylindrical arenas/open fields for object location tasks and similar kinds. So, it would be possible to use another shape for the arena in this protocol too. However, some considerations should be given before deciding for a change of arena shape. One of the main characteristics of this protocol is the use of four distinct contexts. For this we largely make use of different patterns/colors of second layer of each wall and 3D cues, which altogether serve as spatial cues. We think that while this can still be easily achieved using a rectangular arena, the use of cylindrical arena poses a bigger challenge for this purpose. However, it still can be made possible with adjustments and given that these conditions are provided, we think that a cylindrical arena would also ensure the same results.

We have now added a part about this in the discussion (line 623 and on).

Comment 7:
Line 141: Can the authors comment on the significance of 3D cues instead of 2D?

Response:
Our colleagues have previously experienced that rats need salient 3D cues for allosteric spatial learning in the Morris water maze task. Based on that experience, we expect that 3D cues in the open field represent spatial 'landmarks' for rats and promote allocentric learning strategies in the object location task better than 2D cues. So, based on these, we use and recommend the use of distinct 3D cues for this task.

Comment 8:
Line 211: Can the authors comment on the significance of this? Why reduce handling to a minimum?

Response:
This was intended to be an option, so if the animals reach a point where they are comfortable being handled, we think that maintaining a dense handling protocol is not necessary. The frequency can be reduced or kept the same, depending on the wish of the experimenter. We changed the sentence such that it is now clear that it is 'optional' (line 237).

Comment 9:
Line 217: Can the authors comment on the reason behind the 30min idle time in the experimental room?

Response:
This is a common practice whenever the animals are transported from the housing room to another room (e.g., the experimental room). The time (not strictly 30 min) is given such that before beginning any handling, habituation or experiment, the animals can calm down and habituate to the room and the presence of the experimenters. Reaching this state before proceeding with any process is important as high activity or anxiety arising due to the change can agitate the animals, reduce the efficiency of the habituation or reduce the attention of the animals on the task. In this particular step, no additional process follows. So basically, this time is given for a general habituation before returning the cages back to the housing room.

Comment 10:
Line 348: Is that for only higher or lower as well? If a rat is deemed an outlier in one of the four trials, will it be excluded from all trials? What is the biological basis of that exclusion?

Response:
It is in the case of both higher and lower preferences than mean ± (2 × SD). We tried to implicate this by saying higher preference for either object, as a high preference for one object means low preference for the other (line 384).

The exclusion is based on the encoding trial of each session and the rat is excluded from the analysis of only that particular session (e.g., only in context 1).

The reliable interpretation of a change in the preference for exploring the object at the novel location in test trials depends on the absence of any initial preferences for either object in encoding trials. On this basis, we decided that rats that have stronger preference than mean ± (2 × SD) should be excluded. In a test trial this value and above is typically interpreted as a preference that indicates a strong memory of the object location. Thus, we do not accept the presence of such a strong preference during encoding.

Comment 11:
Line 351: Can you please clarify this? Preference against what? Since the objects are identical why would there be a preference in objects? Also if an animal has a preference for a given corner, is this take into account in the interpretation of the test phase results?

Response:
Thank you for pointing out this remark. We intended to say that there might be differences in the corners hosting the objects that may rise from the differences in the surrounding context.

We removed this from the text, but we discuss how our counterbalancing method overcomes this problem in the discussion, also addressing your second question (line 609 and on).

Our extensive counterbalancing method is meant to minimize the systematic recurrence of this kind of preferences and hence its effect on the interpretation of the test results. So unless the animal has a preference for a corner hosting an object and this appears as a strong preference for either object in encoding trials, we do not take any action to exclude or disregard this animal. Only if there is a strong preference for either object in the encoding trial as explained in the protocol, we exclude the rat from further analysis of the respective test.

Comment 12:
Line 375: 'preference' Do you mean memory? Or preference for novelty?

Response:
The meaning of ‘preference’ was explained in the following part of the sentence. So we mean the preference for exploring the object at the novel location, and this represents both the memory for stable object location and the preference for novelty. We hope this explanation helps. We also made a slight change in the phrasing of the sentence now in line 413.

Comment 13:
Line 507: Can you please clarify what do you mean by interference?

Response:
We removed this from the text as we decided that it does not reflect what we aimed to tell. Thank you for addressing it.

Reviewer #2

Manuscript Summary:

The authors applied a well-described protocol of the object place preference test, a commonly applied paradigm in the measurement of object recognition memory in rodents, and experimental results resulted from the protocol. They provided a stepby-step illustration of the protocol that can be used to run the same cohort of animals several times by introducing animals into distinct contexts. The protocol is also applicable and convenient in the investigation of weaker or stronger traces of memory as controlling times of learning. The description of the protocol is direct, straightforward and in detail, images of the materials, testing environments and objects used are concrete, and the experimental results are genuine. Below I have some points that do not compromise the main structure of the manuscript.

Major Concerns:

Comment 1:

1. Please add connected sentences about why each step is required to conduct. For instance, why habituate animals into the object bucket? To reduce neophobia towards objects or general stress (or help the animal to learn in the following contexts as in the discussion)? Why different times of learning were used to create weak or strong memory? Cite articles if possible. A review paper (Chao et al., 2020 Neuroscience and Biobehavioral Reviews) can be referenced.

Response:

We added this connecting information to sections where it was missing (lines 250, 266 and 277). We hope that it is clearer now - thanks.

Changing the encoding duration affects the strength of the memory and hence, whether it will be behaviorally observed as a short-term and/or long-term memory in test trials. We explain our reason behind demonstrating strong and weak memories in this protocol, that is, these memories of varying strength can either be used for studies of memory enhancement or impairment (lines 113-116). We now refer to this review paper that you recommended in the text (lines 113 and 116).

Comment 2:

2. The weak memory results from a single trial of learning (20 min exposure to object explorations), while the strong memory comes from 3 times of object exposure of learning (5 min x3). Apparently, the total amount of exploration time is longer in the weak condition (20 min) than the strong one (5min x3), which is counter-intuitive the general concept and empirical data of the longer the encoding, the stronger the memory. Although the experimental results support the protocol, the reason to apply this design is lacking. Why not applying a single 10 min encoding versus a single 15 min encoding in the weak and strong memory conditions, respectively (control times of learning trial)? Or 15 min encoding in the weak condition versus 5 min x3 encoding in the strong condition (control the total amount of object exploration)? Is it more beneficial to use the design of the protocol?

Response:

Thank you for addressing this. We understand that it is not straightforward to see the rationale behind these protocols. We added a section explaining this in the discussion (line 590 and on).

As we explain now in the text, the main difference and the critical point here is that one protocol utilizes a single long trial whereas the other a protocol with trials interleaved with short rest periods. The former results in rats losing interest in objects after 5-10 min of exploration, the latter results in higher attention and exploration of the objects. Thus, they ensure weak and strong encoding, respectively. We observed these as we started out trying 20 min encoding initially, then switched to 3 x 5 min encoding. These protocols were functional, and the results were good enough for our purposes and that's why we did not try to further optimize the durations. However, it certainly is possible to use a slightly different combination of encoding durations as you suggest that would still give the same results.

Comment 3:
3. In 3. Data analysis, lack of statistical methods of applying ANOVA in the comparison of different groups or conditions. Please specify.

Response:
Thank you for reminding us of this. We added the option of using ANOVA for comparison of more than two groups in data analysis section 3.6 (line 395).

Minor Concerns:

Comment 4:
1. In 1.1.7. Please specify the adjustment of light intensity in the corners of open fields as different light intensity might be affecting the animals' exploratory behaviors.

Response:
We made changes in the text to clarify it (line 174). The mentioned range (100-120 lux) is reflecting the values measured at the corners of the arena. There is a variation rising from the differences in wall colors and patterns, but this falls within the range. As the difference is not very large, we do not observe strong tendencies in animals' preferences for corners. Additionally, any potential preference will not be able to accumulate as we implement a complex counterbalancing method that eliminates or minimizes these effects. We also discuss this now in the discussion (line 610).

Comment 5:
2. In 2.8.1. Please specify whether identical or different objects were used in the protocol.

Response:
We clarified it by adding 'identical' (line 302).

Comment 6:
3. If one likes to treat animals with pharmacological agents or others, at which step is appropriate to administrate? And what details should be noted? Please add it.

Response:

We added possible options for such a scenario in the protocol, first in the handling section after 2.4 (line 246) and then in the encoding section after 2.8 (line 297). However, precisely when and how these should be done would depend on the nature of the manipulation.

Reviewer #3

Manuscript Summary:

This article provide a set of standardized procedures to assess hippocampal-dependent spatial memory in rats using an object location task. They included an extensive protocol to perform repetitions using the same cohort of rats. Interestingly, the authors added two separate protocols to provide weak and strong encoding for forming short and long-term memories. I believe this protocol represents a contribution to the behavioral neurosciences and I will definitively support its publication. I have only one major comment and some minor comments which the authors may address to optimize their article.

Major Concerns:

Comment 1:

The authors claim that the object place recognition task is a hippocampus-dependent type of memory; however, to my understanding according to the protocol the experimental setup and preparation of the distinct contexts include the preferential use of proximal cues (i.e., spatial cues presented only within the arena). In my view this is an important matter in any behavioral study. Whether the test used is valid i.e., to what extent a test accurately measures what it is supposed to measure, is at utmost importance. The validity of the test will support the correct interpretation of the data obtained. Accordingly, the authors should clarify to what extent a spatial task which only contains proximal cues can be solved by an allocentric spatial strategy (and therefore claim as being hippocampal-dependent). It has been suggested that allocentric, contrary to egocentric navigation, is characterized by the ability to navigate using distal cues that is cues or landmarks located outside and at some distance from the organism (Vorhees and Williams, 2014_ILAR Journal). The authors should comment on this issue and make clear which spatial strategy (i.e., egocentric or allocentric) is used to solved this protocol and confirm its hippocampal-dependency.

Response:

Thank you for raising this important remark. We agree that it is important to make sure that a test measures what is intended to. Object place recognition tasks have been shown to require intact hippocampus. There are variations in the object place recognition apparatus used in these reported studies and particularly in the use of spatial cues. In contrast to studies using the Morris water maze, the features and the localization of these cues are often not clear (e.g., whether it is a proximal or distal location) in studies using object place recognition tasks, while some report explicitly that they are located distally, as you suggest. Similarly, the discussion of the extent of allocentric versus egocentric strategies utilized in object place recognition tasks are sparingly found in these studies. Regardless, the results largely overlap with indications of requiring intact hippocampus for a successful discrimination of object locations.

In our setup, the whole context and spatial cues are contained within the enclosed box. Altogether these cues (wall patterns and 3D cues) form a complex environment that the rats need to understand in order to successfully acquire the object locations in the encoding trial before exhibiting a preference for the displaced object in the test trial. Even though these cues are proximal, we believe that they, especially the 3D cues, still represent spatial 'landmarks' similar to distal ones. The rats would potentially need to use these 3D cues to disambiguate the features of the distinct walls and the locations of the objects, which calls for more dominantly allocentric strategies. The rat needs to use the relative information of these three factors, the walls, the 3D cues and the object locations, to be able to correctly discriminate the object at a novel location.

This is also supported by the fact that we use a thorough counterbalancing method and a large group of object location combinations (counters). Due to this counterbalancing, we do not think it very likely that the rats always use one single cue in isolation to learn object locations and discriminate subsequent changes in object location (from encoding to test), although perhaps this does happen on rare occasions.

The task itself does not contain any real navigation, serial turns or choice points that promote egocentric strategies. Thus, we do not think that there is a big egocentric strategy component to this task and setup. Additionally, the start location and orientation of the rats (i.e., the wall or corner that the rat faces when put in the box) is different in encoding and test trials as a result of one of the objects moving to a new location. This should also promote a more allocentric strategy.

There are studies indicating that proximal cues can also influence place cell firing which is associated with allocentric spatial mapping and work together with distal cues to create a spatial map (see a review; *Knierim and Hamilton, Physiol. Rev., 2011*). *Cressant et al., J Neurosci., 1997* shows that addition of a single white cue card on the wall of a cylindrical arena containing 3D objects placed at the center results in control over the angular position of hippocampal place cell firing fields, in contrast to the scenario without the cue card on the wall, in line with the previous report from *Muller and Kubie, J Neurosci., 1987*. Furthermore, a similar control over place-cell firing was observed by Cressant et al. when the 3D objects were placed next to the walls instead of the center of the arena, indicating that intramaze cues that are at the periphery (i.e., a more distal location) can contribute to place cell activity and spatial mapping of an arena.

Using a similar setup with 3D intramaze cues, but in the Morris water maze, two studies (*Sakamoto and Okaichi, Psychobio., 1998; Save and Poucet, Behav. Brain Res., 2000*) reported comparable results to the previously described place cell studies, that is, hippocampal lesioned rats had impaired performance to an extent and impaired use of a 'place strategy' in learning the platform location in this kind of setup, indicating the need of the hippocampus for spatial learning based on proximal cues in the Morris water maze.

Altogether, the setups that were reported in these mentioned studies resemble our setup with slight differences: our cues are 3D and hung on the walls instead of the floor of the arena/hung over the pool, making them inaccessible to the rats and potentially representing more stable and distal landmarks to guide spatial mapping.

Finally, *Tintorelli et al., Sci. Rep., 2020* uses a very similar setup as ours, not enclosed but with only proximal cues being reported. They describe a rectangular box with white floor, two

opposite walls that are white and with visual cues hung on these walls, one transparent front wall and one back wall that is hatched. They show that inhibition of neuronal activity in the dorsal hippocampus by muscimol injections immediately after encoding trial in this described box for spatial object recognition task impairs the memory in the test trial whereas an increased preference for moved object in the control (vehicle injected) group is reported. Similar to this, other manipulations in the hippocampus interferes with the memory in this study. These supports that this task in the described setup, which shows high resemblance to ours, still largely depends on hippocampal function, as the disruption of hippocampal neural activity impaired the object place recognition.

So, we are aware that our setup withholds some variations, but we believe that the essence of the task is still comparable to the originally reported versions of object place recognition task. Based on the points described above, we believe that this task using this specific setup should still largely use allocentric strategies and spatial memory that requires hippocampal function.

Minor Concerns:

Comment 1:
1. Would be useful when the authors point out at the beginning of the article that this protocol is meant to be used only for adult rats. In my view, before to claim that can be used for pup rats several modifications should be done.

Response:
We added that we use adult rats in this protocol in the introduction (line 110). We also added a part in the discussion (line 575 and on) that discusses the possibility of using younger rats under certain circumstances and after required considerations and adjustments. We hope that these have addressed your concerns correctly.

Comment 2:
2. The authors should mention at the Introduction section (not only at the end in the Discussion) that one of the advantages (very important one) of the recognition tasks based in spontaneous preference is that the correct handling and habituation will reduce significantly the level of "stress".

Response:
Thank you very much for your valuable advice. We emphasized this further in line 81-82.

Comment 3:
3. Strictly, the tasks based in spontaneous recognition include not just the object recognition and object place recognition but several others. L.64

Response:
Thank you for the correction. We changed the text accordingly (line 63).

Comment 4:

4. I am not sure if the concepts of positive and negative reinforcement are correctly used in the Introduction section. Negative reinforcement is not directly comparable with fear as the authors mentioned. L74-75.

Response:
We made changes to the text to clarify it (lines 74-75). We intended to say that these reinforcements introduce further parameters and brain regions as responsible for the task and hence, making it less straightforward to study spatial memory.

Comment 5:
5. Within the field, a more standard name for "box" is "open field" or "arena".

Response:
We made changes such that now we refer to the enclosed experimental apparatus as ‘box’ and the object location task box as ‘arena’.

Comment 6:
6. The proper use of the object needs a prerequisite which involved testing whether they are equally comparable in terms of the unconditioned preferences.

Response:
Thank you for raising this issue. We added a sentence emphasizing this in our protocol (line 161).

For our case, all four objects result in comparable total exploration time and we do not observe a significant correlation between the total exploration time in the encoding trial and the respective performance in the test trial.

Comment 7:
7. Would be appropriate if the authors describe the position of the light within the setup. In our experience the best location for the lights is from below and never above the open field.

Response:
The position of the light source is described in the protocol in the note below 1.1 (line 132) and we further give information on the features of the light source at 1.1.7 (line 173).

In our setup, the LED light is located along the edges of the ceiling and it creates a more diffused light. This works well in our setup, but alternatives of light position should be functional as well. We also added a note about the option of changing the light intensity (line 178).

Comment 8:
8. I could not find the ITI (inter trial interval) between the task´s repetitions. Please the authors should present a clear timeline, which should include the total time need it to apply the four repetitions. To my knowledge the minimum of the ITI should be twice long the retention

interval used i.e., if the retention interval lasted 24 hours, then before to repeat the task an interval of 48 hours should be applied.

Response:
Thank you for your suggestion. We clarified the description regarding the intervals between sessions (repetitions). Now the reader can find the information in the note (line 356) under 2.9.4 in the protocol and in the legend of Figure 3 (line 500-501).

We also talk about it in the discussion starting at line 632.

Comment 9:
9. It is not clear enough how many times the steps should be done in 2.8.3 until 2.8.6 Are those steps including the repetitions? If yes, please make it clear.

Response:
We made some changes to the text to clarify these.

Comment 10:
10. One easy way to assure that the objects are not going to be moved is making them heavy enough. In this way one can avoid the use of tape or any other material which make the cleaning even more difficult.

Response:
Thank you for the suggestion. We agree that this could be a reasonable option for mice but with rats, they are typically able to move objects no matter how heavy they are (bearing in mind size constraints) unless they are somehow stuck down. Climbing behavior also makes it risky to not use any attaching method. We also think that using too-heavy objects poses practical challenges.

Comment 11:
11. Could the authors specify why they choose to present data by using transgenic rats?

Response:
This transgenic rat line will be used in our future experiments that involves optogenetics. That's why we use these rats. As mentioned in the results and discussion section, the results produced using this transgenic rat line and wildtype rats were comparable. We also added a sentence explaining the reason in the results section (line 403).

Comment 12:
12. Would be important to mention what is the number of animals expected to be used in order to get enough statistical power in both conditions: one single trial or with repetitions.

Response:
Thank you for addressing this. We now added a part explaining this in the discussion (line 663 and on) based on our power and sample size calculations.

Comment 13:
13. I find a bit strict the criteria of minimum object exploration established by the author in 10 s, could the authors support this statement.

Response:
It should of course depend on the evaluation of the behavior of the population and the characteristics of each specific setup. In our case, we almost never experience such low total exploration time, the average is around 33 seconds for test trials. Thus, a rat with below 10 seconds of total exploration represents an outlier in our population using this protocol.

Comment 14:
14. Why do the authors name the axis Y as "% exploration time" and in the text they name it "Discrimination ratio". Would be better to keep consistent.

Response:
We use the term 'discrimination index' only in the protocol 3.4 (lines 380 and 382), to show the reader that there are two common ways of calculating the percentage or discrimination for exploration of an object (i.e., 1. percentage of exploration, and 2. discrimination ratio). In our calculations we use the first way, that is, the percentage of exploration for each object. For the rest of the text and results we use the percentage of exploration for an object, also referring to it as 'preference' at times. We hope this does not any confusion for the reader.

Comment 15:
15. When exactly should be done the habituation to the different contexts when using the protocol for repetitions? Considering all the habituations, how many days are required to apply the four repetitions?

Response:
We hope to clarify this with the addition of the information about the interval between repetitions in line 356 and in the legend of Figure 3.

Each repetition including the respective context habituation over 3 days and encoding/test session over 1 or 2 days depending on the delay period, takes around 4-5 days. We recommend giving a break of 48 hours to 1 week between each repetition.

So in the case of 48-hours breaks, meaning repetitions are performed in consecutive weeks, it would require 5 weeks (1 week for initial object bucket and box habituation followed by 4 weeks for repetitions). In the case of following 1-week breaks, it would take roughly 8 weeks (1+7 weeks).

Comment 16:
16. It caught my attention that the weak encoding last longer than the strong encoding (20 min vs. 15 min). Could the authors explain this effect?

Response:

We understand that it is not straightforward to see the rationale behind these protocols. We added a section to explain this in the discussion (line 590).

As we explain now in the text, the main difference and the critical point here is that one protocol consists of a single long trial whereas the other consists of trials interleaved with rest periods. The former results in rats losing interest and sitting in a corner after 5-10 min of exploration, the latter results in higher attention and exploration of the objects. Thus, they ensure weak and strong encoding, respectively.

Comment 17:
17. Please specify the time of the day that the protocol should be done.

Response:
We added the information on when we have performed our experiments (Line 220).

Comment 18:
18. In the Discussion section it is mentioned that the rats could present "aversion toward novelty". What would be the rational and empirical foundation for such a statement?

Response:
We do not know of any study that directly shows this, but there are studies that indicate in this direction.

Some studies report that anxiety/fear state can cause neophobia if it overcomes the exploratory instinct of the animal (*Ennaceur, Michalikova, Bradford, & Ahmed, Behav Brain Res, 2005*; *Hughes, Neurosci. Biobehav. Rev., 2007*). As novelty has an inverted U-shape effect, with maximal effect when the novelty is medium level, weak novelty can go unnoticed and too strong novelty can cause anxiety or fear that stops the rat from interacting with it.

Other studies report that not-well-handled animals tend to exhibit higher anxiety in tasks such as open-field test and elevated plus maze (*Schmitt and Hiemke, Pharmacol. Biochem. Behav., 1998*; *Hurst and West, Nat. Methods, 2010; Costa et al., J Am Assoc Lab Anim Sci, 2012; Gouveja and Hust, Sci Rep, 2017*).

A combination of these suggest that a not-well-handled or not-well-habituated animal may experience high anxiety due to handling and experiencing new environments during the encoding/test trials, which may result in loss of attention in the task and hindered natural behavior that is driven by curiosity and is what the task at hand is largely dependent on, and finally cause reluctancy to approach novelty.

In the referred case (line 582 and on), with some rats we observed an increased preference for exploring the stable object (at the familiar location) instead of the object that was at the novel location. Based on the combination of the preceding sub-optimal handling these rats had received due to starting at a late age, their general anxious behavior and the preference for the familiar location instead of the novel, we speculated that the rats were perhaps not sufficiently habituated to handling, which also resulted in general high anxiety in the experimental setup and hence, were not comfortable in approaching the ‘novelty’ that is the object at the novel

location. This is of course a speculation, but based on the evidence in the literature, it is a likely scenario, with at least partial responsibility of the observed behavior.

Comment 19:
19. There are two typo mistake from an old ms version, probably. L422 and L504.

Response:
Thank you. We hope that they are corrected now.