Object recognition testing: Methodological considerations on exploration and discrimination measures

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Abstract

The object recognition task (ORT) is a popular one-trial learning test for animals. In the current study, we investigated several methodological issues concerning the task. Data was pooled from 28 ORT studies, containing 731 male Wistar rats. We investigated the relationship between 3 common absolute- and relative discrimination measures, as well as their relation to exploratory activity. In this context, the effects of pre-experimental habituation, object familiarity, trial duration, retention interval and the amnesic drugs MK-801 and scopolamine were investigated. Our analyses showed that the ORT is very sensitive, capable of detecting subtle differences in memory (discrimination) and exploratory performance. As a consequence, it is susceptible to potential biases due to (injection) stress and side effects of drugs. Our data indicated that a minimum amount of exploration is required in the sample and test trial for a significant discrimination performance. However, there was no relationship between the level of exploration in the sample trial and discrimination performance. In addition, the level of exploration in the test trial was positively related to the absolute discrimination measures, whereas this was not the case for relative discrimination measures, which correct for exploratory differences, making them more resistant to exploration biases. Animals appeared to remember object information over multiple test sessions. Therefore, when animals have encountered both objects in prior test sessions, the object preference observed in the test trial of 1 h retention intervals is probably due to a relative difference in familiarity between the objects in the test trial, rather than true novelty per se. Taken together, our findings suggest to take into consideration pre-experimental exposure (familiarization) to objects, habituation to treatment procedures, and the use of relative discrimination measures when using the ORT.

Keywords

Object Recognition Task
Methods
Memory
Exploration
Discrimination Measures

List of abbreviations

d1	discrimination index 1, difference score
d2	discrimination index 2, relative difference score
d3	discrimination index 3, investigation ratio
e1	total object exploration in T1
e2	total object exploration in T2
i.p.	intra peritoneal
NOP	Novel Object Preference task
NOR	Novel Object Recognition task
ORT	Object Recognition Task
OLT	Object Location Task
p.o.	per os, oral injection
T1	sample- or learning trial
T2	test trial

1. Introduction

Twenty-three years ago, Ennaceur and Delacour [1] introduced a new one-trial learning test for neurobiological studies. Since its introduction it has been referred to as Novel Object Preference (NOP) test [2], Novel Object Recognition (NOR) task [3] and Object Recognition Task (ORT) [4]. In the present paper we use the ORT abbreviation to refer to object recognition testing. The ORT is based on the underlying principle that, in a familiar environment, laboratory rodents show an instinctive attraction towards novelty, or neotic preference [5]. In general, animals are allowed to freely explore two identical sample objects during a sample- or learning trial. After a certain delay, the animals perform a test trial, in which they are confronted with one of the sample objects and a novel one. If rodents show more interaction with the novel object during the test trial, it can be presumed that they have remembered the sample object. This novel object preference is used as an indication of memory.

Because it utilizes the natural behaviour of the animals, the ORT is relatively free of stress if properly administered. Furthermore the property of one-trial learning makes it suitable for the examination of temporal aspects of memory. Over time, it has become a widely used tool for the assessment of memory functions in combination with pharmacological treatments [6-10] and brain lesions [11-14]. The ORT is mainly used for rodent experiments, most research is done with rats and mice but the task is also suitable for testing other rodent species [15]. It has also been successfully applied using larger animal species like dogs [16], pigs [17, 18] and horses [19], making it a versatile tool suitable for inter-species comparisons.

The most essential brain structures implicated in object recognition memory of rodents, are the hippocampus and rhinal cortices, perirhinal and in particular the postrhinal cortex [20]. Of note, the human and primate counterpart of the postrhinal cortex is the parahippocampal cortex. Although there is much evidence that the hippocampus is implicated in object recognition testing [21-23], its role in processing of the actual object information is more an integrative one, i.e. combining object and contextual information into integral episodic memories [20, 24]. Concerning object information itself, the perirhinal cortex is considered to be the most crucial structure whereas the postrhinal cortex is implicated in contextual information processing [24, 25].

Many variations on the original task have been developed over time, making it difficult to compare results between different laboratories. Differences exist in object and arena features like the size, shape and materials. Also, differences in animal strain, age, gender, and housing conditions have been reported to influence object recognition [15, 26-29]. Furthermore, different test protocols are being used. For example, some studies use flexible sample trial durations, allowing all animals to reach a specific amount of object interaction [12, 30-32]. Other experiments have fixed sample trial durations and each individual animal differs in the amount of sample object exposure [1, 11, 25, 33]. Before testing starts, the animals are often familiarized or habituated and protocols differ greatly between laboratories. In some laboratories the animals are allowed to familiarize with only the apparatus [2, 11, 12, 23], others also introduce objects in the pre-experimental phase [25] or even let the animals undergo the full testing procedure, including injections [34, 35]. Even more fundamental, there is no consensus about the definition of object investigation. In the original test, object investigation was defined as directing the nose towards

the object at a distance of 2 cm or less [1]. However, also minimal proximities of 1 cm [11] and 4 cm [2] have also been reported. Generally, climbing and leaning on objects is not considered to be object examination [36], although some do in fact consider this to be relevant object-directed behaviour [2, 37, 38].

It is also important to note that novel object preference is being analyzed and reported in various ways. Some authors calculate the absolute difference in exploration times between the novel and the sample object, resulting a difference score (DS). In others, object discrimination can also be reported in terms of the relative interaction with each object in the test trial, resulting in a percentage or investigation ratio (IR). Lastly, results of the ORT have also been reported as the relative difference score (RDS), which divides the DS by the total time spent exploring both the objects in the test trial. Theoretically, random exploration would result in a (R)DS of 0 or an IR of 0.5, a higher score indicates that an animal directed the majority of exploration towards the novel object and thereby implicating that it has remembered the sample object. Since studies generally report only one of the different discrimination measures it is difficult to directly compare them. To our knowledge, it has not yet been fully investigated in one and the same study how the different discrimination indices are related.

The aim of our study is to shed more light on the effects of familiarization and habituation to the procedures on the measures of exploration and discrimination in the ORT. Additionally, we want to deepen our understanding of the relationships between the different discrimination and exploration measures in the sample- and test trial. This could be helpful in determining the minimum amount of exploration required for reliable object discrimination, by this we mean the amount of exploration that is representative of normal exploratory behavior and allows for a meaningful statistical interpretation. We have included historical data from 28 ORT studies performed in our lab to test the potential of several drugs to reverse natural or drug-induced forgetting. To investigate the latter, one hour retention intervals were used in combination with amnesic drugs, the non-competitive N-methyl-d-aspartate (NMDA) receptor antagonist MK-801 or the muscarinic receptor antagonist scopolamine. Both drugs have proven reliably to attenuate the novel object preference in a 1 h retention delay, likely due to drug induced memory impairment [6, 39, 40]. 24 h retention intervals were used to investigate drug effects on natural

forgetting, as our male Wistar rats normally do not discriminate anymore between the novel and the familiar object after such an interval [1, 8, 33-35].

2. Methods

2.1. Animals

All experiments were performed using male Wistar rats ordered from Harlan (the Netherlands). The animals (n = 731) were ordered at an age of 2 months and tested between 3 and 5 months of age. After arrival, animals were individually housed in standard Makrolon™ Type III cages with sawdust bedding. A cardboard tunnel with a diameter of 10 cm, length of 12.5 cm and a 0.5 cm thick wall was provided as enrichment together with a piece of wood. Food and water were provided at libitum, water was acidified (pH~3) to prevent bacterial infections and refreshed every week. Testing was done in the same room as where the animals were housed. During the first two weeks the rats were allowed to get accustomed to a reversed 12 h day/night cycle (lights were on from 19:00 till 07:00). After this they were habituated to handling and familiarized with the testing procedures. All testing was done during their active phase, i.e. during dark phase at daytime. During the dark phase red fluorescent tubes provided illumination for which the rat-retina is relatively insensitive. A radio played softly, providing background noise 24 h a day, also during testing. The room temperature was kept constant at 20 °C and the humidity of the air was kept within a range of 60-80%.

2.2. Apparatus

The test setup that was used is a slightly adapted version of the object recognition task as described by Ennaceur and Delacour [1]. Instead of a square arena of 65 x 45 x 45 cm, as used by Ennaceur and Delacour, a circular arena, with a diameter of 83 cm was used in our studies. The floor plate and back-half of the arena wall were made of gray (RAL 7035) polyvinyl chloride. The frontal half of the 40 cm high arena wall was made of transparent polyvinyl chloride to enable direct observation of the animals. Light intensity on the apparatus floor was about 20 lux. Illumination was provided by red fluorescent tubes in the ceiling and a 40 watt light bulb, which was switched on during behavioural observations, in the corner of the room covered by a lamp-shade.

Two objects were placed at symmetrical positions on the left and right side of the arena, the center of the object was positioned 24 cm from the arena wall. Four different sets of objects were used. Each set consisted of 3 identical copies. The different objects were; 1) a standard 1L transparent glass bottle (diameter 10 cm, height 22 cm) filled with water, 2) a metal cube (5 x 7.5 x 10 cm) with two tunnels (diameter 1.5 cm) drilled across, 3) a cone (height 16 cm, diameter at base 18 cm) consisting of a gray polyvinyl chloride base with a collar on top made of aluminum (diameter 5.0 cm) and 4) a solid, 'bullet-shaped' aluminum cube with a tapering top (8 x 8 x 13 cm) and a groove near the bottom. The four sets of objects were rotated for over the test sessions, using the novel object from the previous session as familiar object in the subsequent session. Because we only used 4 sets of objects the objects were re-used after the 4th test session. So from the 4th test session on, the novel object was always the same object as the familiar object from the 3st test session before. Rats were unable to displace the objects, a picture of each object is shown in Figure 1.

2.3. Experimental procedure and treatment conditions

An ORT session consisted of a sample (T1) and a test trial (T2), of 3 min each, separated by a retention interval of 1 h or 24 h. At the start of each trial animals were placed in front of the objects, facing the wall. During T1 animals were confronted with two identical sample objects, a_1 and a_2. In T2 these were replaced by a 'fresh' sample object, a_3, and a novel object, object b. The objects were cleansed before each trial with a damp cloth containing a 70% ethanol solution. During a trial, animals could freely explore the arena and objects, the time spent interacting with each individual object during T1 and T2 was recorded live on a personal computer. Six experienced observers contributed to the current dataset. We have previously shown that experienced observers achieve very high levels of concordance on both exploration and discrimination parameters of the ORT [41].

Before experimental testing, the animals need to be habituated to the apparatus and the testing procedures. Although familiarization with the apparatus is known to affect object interaction and possibly discrimination [38], there is no apparent consensus about what should be involved in ORT familiarization. In our lab two familiarization protocols are used, depending on the type of retention delay which will be used in the subsequent experiment. We use a '1 h familiarization protocol' to prepare animals for 1 h retention interval testing in combination with drug-induced

forgetting. However, when animals are prepared for testing of natural forgetting during a 24 h retention interval we use a '24 h familiarization protocol', which is a slightly adapted version of the '1 h familiarzation protocol'.

In both protocols, familiarization starts with a 3 min habituation trial in the empty arena on 2 consecutive days, in order to let the animals get accustomed to the test environment. After this we start with the 1st familiarization session, a test session with a 1 h retention interval in which the animals are not subjected to any injections. This is followed by the 2nd familiarization session, a 24 h retention interval test, also without injecting the animals. These first 2 sessions combined with the first 2 habituation trials give the animals sufficient opportunity to get accustomed to handling and ORT test procedures. On a subsequent day, animals are familiarized to the administration procedure via saline injections, without submitting the animals to ORT testing. These can be one or more injections, intra peritoneal (i.p.) and/or oral (p.o.), depending on the demands of the subsequent experiment. Usually, we observe that the animals still discriminate between the novel and old object in the 2nd familiarization session. Therefore, in '24 h familiarization protocol', we continue with 24 h retention intervals, until the animals show no discrimination anymore. This is different from the '1 h familiarization protocol', which is completed by subjecting the animals to a final 1 h retention interval in the 3rd familiarization session. In both protocols, training beyond the 2nd session always includes saline injections at the same time point, volume and via the same route as will be used during the experiment. This is done to check whether the animals still perform properly when the ORT procedures are applied in combination with the injections. Usually, the familiarization data are not reported in articles because no scientific hypotheses were tested. However, we have included the familiarization data included in the dataset, to investigate the effects of training on exploration and discrimination.

A supplementary experiment was performed to investigate whether the object discrimination we observed in the 2nd familiarization sessions is due to the absolute novelty of the novel object, i.e. animals seeing the novel object for the first time in their life. Data from this experiment was not included in the other analyses in this article, because different object sets were used. 24 fully familiarized animals were subjected to a 24 h retention interval in the usual test setup that has been described above. However, we used 2 sets of objects that were completely novel to the animals as novel and familiar object (Figure 2). The objects in the sets were red tin cans

(diameter 9 cm, height 11.5 cm) and white porcelain cups (diameter 7.3 cm, height 9 cm). Animals were tested twice and in the 2nd test session, 72 h after the 1st test session, the animals received the exact same objects in the same locations as in the 1st test session.

The dataset contained 3771 individual ORT sessions divided over 6 conditions which were firstly based on the retention interval used (1 h or 24 h) and pharmacological treatment and secondly, on whether the data was collected during or after familiarization, which will be referred to as 'non-familiarized' and 'familiarized' animals, respectively.

Four different treatment groups with a 1 h retention delay were included in the dataset. First, animals that did not receive any kind of treatment were labeled the '1 h untreated' group. Second, the '1 h saline' group contained animals that received 1 ml/kg saline i.p., 30 min before T1. In addition, these animals received an injection with the vehicle of the experimental drug. Because the dataset was composed of animals from different experiments, these additional vehicle injections vary in composition (saline, 1-20% pluronic solution with tylose 0.5-3%, saline with 2% tween 80, tylose solutions of 0.5-5% with 2% tween 80, and 1% tylose with 5% mannitol), volume (1-10 ml/kg), timing (2 h or 0.5 h before T1, or 3 h after T1), and administration route (i.p. or p.o.). Third, the 'scopolamine' group was treated identical to the 'saline' group, only now the 1 ml/kg saline injection contained scopolamine, dissolved in a concentration of 0.1 mg/ml. Finally, the 'MK-801' group received the same treatment as the 'scopolamine' group, but now a concentration of 0.125 mg/ml MK-801 was dissolved in the 1 ml/kg saline injection. So, the 1 h untreated group did not receive any injections. All other 1 h groups received a vehicle injection in addition to a saline, scopolamine, or MK-801 injection.

In the remaining experiments, pharmacological agents that are considered to facilitate memory were administered to subjects in order to assess their ability to counteract the natural decline of novel object preference that is seen in untreated subjects over a 24 h retention delay. From these experiments, only the conditions in which the animals received no pharmacological treatment, i.e. untreated and vehicle treated animals were included in the dataset. Together with the familiarization data from the 24 h retention experiments these sessions will be referred to as the '24 h' group. The vehicles used varied between experiments, for details, see the vehicles described for 1 h retention experiments. Administration route was either i.p. or p.o. and injections were always given after T1 (0 min - 3 h). Compositions are amongst the vehicles

described for the 1 h retention studies. To summarize, animals in 24 h experiments either received a single vehicle injection after T1, or did not receive any injection.

2.4. Statistical analysis

The basic output measures in the ORT are the times spent by rats in exploring each object during T1 and T2. From these basic output measures, several variables can be calculated (see Table 2). e1 and e2 are measures of the total time spent investigating both objects during T1 and T2 respectively. The d1 index depicts the absolute difference between the sample and the novel object. The d2 index is a relative measure of discrimination corrected for the level of exploration in the test-trial (e2) and the d3 index shows the proportion of e2 devoted to the novel object.

One-sample *t*-statistics were performed to assess whether the d1 and d2 indices were different from zero, since random exploration in T2 would result in equal exploration of both objects. The d3 index was compared to 0.5 because if both objects in T2 are equally explored, the proportion of exploration directed towards the novel objects would be 50%. Between group effects on e1, e2 and the d measures were also assessed using one-way ANOVA. In case of significant differences, pair-wise comparisons were performed using Bonferroni correction. The relation between the different output measures was investigated by calculating their Pearson correlation coefficient.

3. Results

3.1. Familiarization effects

3.1.1. 1 h familiarization protocol

The '1 h familiarization protocol' involves 3 sessions. The '1st session' (n = 443), which is a 1 h retention interval. The '2nd session' (n = 444), a 24 h retention interval. Familiarization is completed after the '3rd session', a 1 h retention interval (n = 336) with saline injection(s). The '4th session' was also included separately (n = 96). All subsequent 1 h saline test sessions were combined and will be referred to as the '≥5th session' (n = 901). These test sessions only included data from vehicle treated animals.

The development of e1 and e2 is depicted in Figure 3. One-way ANOVA showed that e1 and e2 were significantly different over the course of familiarization, $F_{(4,2215)} = 101.78$, $p < 0.001$ and $F_{(4,2215)} = 11.55$, $p < 0.001$ respectively. Individual sessions were compared via post hoc analyses with Bonferroni correction. This revealed that, in the 1st session, e1 was higher than in all subsequent sessions ($p < 0.001$). Furthermore, e1 in the 2nd session was significantly higher, compared to the ≥5th session ($p < 0.001$). e2 proved to be not significantly different in the 1st, 2nd and ≥5th sessions ($p > 0.05$). In the 3rd session, e2 was higher than in all other sessions ($p < 0.05$). The 4th session showed a lower e2 compared to all other sessions ($p < 0.01$), except for the ≥5th session, which had a statistically equal e2 ($p > 0.05$).

Paired-samples *t*-tests were performed on e1 and e2 within each session, the results of which are displayed in Figure 3.

The d1 values of the successive sessions are shown in Figure 4A. Using one-sample *t*-tests, the d1 was found to be significantly higher than zero in all of the sessions. ANOVA revealed that the d1 values of the familiarization sessions were significantly different $F_{(4,2215)} = 54.39$, $p < 0.001$. Post hoc comparison revealed no significant differences between the 1st, 4rd and ≥5th session ($p > 0.05$), whereas d1 in the 2nd session was lower ($p < 0.001$) and d1 in the 3rd session was higher ($p < 0.001$) compared to all other sessions.

One-sample *t*-tests showed that all conditions in the 1 h familiarization protocol had d2 and d3 values that were significantly higher than 0 and 0.5, respectively (Figure 4B). Like d1, ANOVA showed that the d2 and d3 measures (Figure 4B) were significantly different between sessions; $F_{(4,2215)} = 62.13$ for d2 and $p < 0.001$ $F_{(4,2215)} = 61.94$, $p < 0.001$ for d3. Bonferroni *t*-tests showed that the d2 and d3 values in the 2nd session were significantly lower compared to all of the other sessions ($p < 0.001$). This is in contrast with the 3rd session, in which both were significantly higher than all the other sessions ($p < 0.01$), except the 4th ($p > 0.05$). No further differences were found.

3.1.2. *24 h familiarization protocol*

The first two sessions in the '24 h familiarization protocol' were the same sessions as those in the 1 h familiarization protocol. The protocols diverge after the 2nd familiarization session. Hence, in both protocols the same data was used for the analyses of the 1st and 2nd session. In

the 24 h familiarization protocol, the '3rd session' was a 24 h interval ($n = 96$) with saline injection(s), after this the experiments started. The '4th session', which is the firsts 24 h vehicle test session, was also included ($n = 48$) in the analyses and the remaining 24 h vehicle test sessions were combined in the '≥5th session' ($n = 267$). The e1, e2, d1, d2 and d3 measures of the different sessions are graphically presented in Figures 5 and 6.

In Figure 5, e1 and e2 are shown for each session. ANOVA demonstrated that exploration varied significantly between the different sessions, e1 $F_{(4,1293)} = 69.76$ and e2 $p < 0.001$ $F_{(4,1293)} = 24.29$, $p < 0.001$. Post hoc analysis with Bonferroni correction showed that, compared to all other sessions, e1 was higher in the 1st session ($p < 0.001$) and lower in the 4th session ($p < 0.001$). No e1 differences were found between the 2nd, 3rdand ≥5th session ($p > 0.05$). The e2 value of the 3rd session was higher than that of the other sessions ($p < 0.05$), whereas e2 in the 4th session was lower ($p < 0.001$). Furthermore, e2 in the ≥5th session was lower than in the 1st and 2nd session ($p < 0.05$). No difference was found between the e2 measure of the 1st and 2nd session ($p > 0.05$).

Within each individual session, e1 and e2 were compared using paired-samples *t*-tests. It was found that e2 was higher than e1 in the 2nd, 3rd and 4th sessions $t_{(443)} = 9.22$, $p < 0.001$, $t_{(95)} = 5.37$, $p < 0.001$ an $t_{(47)} = 3.56.37$ $p < 0.001$, respectively. On the other hand, in the 1st session e2 was lower than e1 $t_{(442)} = 8.37$, $p < 0.001$ and in the ≥5th session no statistically significant difference was found $t_{(266)} = 1.01$, n.s.

The mean d1 values of the 24 h familiarization protocol and subsequent test sessions are graphically presented in Figure 6A. One-way ANOVA showed that d1 values are significantly different between sessions $F_{(3,728)} = 3.50$, $p < 0.05$. Post hoc analysis was performed using Bonferroni correction. The d1 value was shown to be higher in the 1st session, compared to the rest of the sessions ($p < 0.001$). Additionally, the 2nd session was found to have a higher d1 than the 4th and ≥5th sessions ($p < 0.05$). Finally, d1 was higher in the 3rd session compared to the ≥5th session ($p < 0.01$).

The d2 and d3 measures of the 24 h familiarization sessions are shown in Figure 6B. ANOVA showed significant differences between familiarization sessions on d2 $F_{(4,1293)} = 68.25$, $p < 0.001$ and d3 $F_{(4,1293)} = 67.56$ $p < 0.001$. Bonferroni *t*-tests revealed that d2 and d3 were higher in the

1st session, compared to all subsequent sessions ($p < 0.001$). Furthermore, both measures were found to be higher in the 2nd session, compared to the 4th and ≥5th session ($p < 0.05$). Finally, the 3rd session was found to have higher d2 and d3 values compared to the ≥5th session ($p < 0.05$).

3.2. Novelty

In our experimental ORT setup, 4 sets of objects are used. These 4 sets are rotated over the animals in a way that the familiar object from a certain test session serves as novel object 3 test sessions later. For example, if a particular object was used as familiar object in test session 1, the animal will encounter it again in test session 4. During analysis of the 24 h familiarization protocol we found that animals only do not discriminate between objects anymore (in a 24 h retention interval) in the 4th test session (section 3.1.2.). To investigate whether this phenomenon was related to the re-use of the object-sets we performed a supplementary experiment in which animals ($n = 24$) performed two consecutive 24 h retention sessions. These animals had already encountered all 4 sets of standard objects and showed no discrimination in a 24 h retention interval. In the first 24 h test session of this experiment two completely new sets of objects were used, meaning that the animals had never before encountered these objects. The new objects are shown in Figure 2. In the 1st session the novel object encountered by the animals in T2 was absolutely novel (i.e. never before encountered) to the animals. The 2nd 24 h test session was exactly identical to the 1st session for each individual animal, meaning that the exact same objects were presented as being familiar or novel. Thus, in the 2nd test session the novel object is not novel anymore in an absolute sense, but it is novel relative to the familiar object from that particular test session. The exploration measures of the 1st and 2nd test session are presented in Figure 7 and discrimination measures are graphically presented in Figure 8.

3.3. Treatment effects of MK-801 and scopolamine

To assess the effect of different treatment conditions on behaviour in the ORT, only familiarized animals were incorporated. The data used in the analysis of the treatment effects are graphically presented in figures 9 and 10.

Comparison of the different treatment conditions with ANOVA revealed that there were differences in e1 $F_{(3,1678)} = 7.94$, $p < 0.001$ and e2 measures $F_{(3,1678)} = 9.61$, $p < 0.001$. Post hoc

analysis showed that in T1, untreated animals explored less in T1 compared to saline (1 h) treated animals ($p < 0.001$). In T2 no difference between these groups were found. Also, in comparison with saline (1 h) treated animals, e2 was lower in scopolamine treated animals ($p < 0.001$). Furthermore, paired-samples *t*-tests pointed out that e2 was increased compared to e1 in all 1 h intervals. The effects of the different treatments on exploration are visualized in Figure 9.

The effects of the different treatments on discrimination are depicted in Figure 10. Significant differences were found on the d1 $F_{(3,1678)} = 222.64$, $p < 0.001$, d2 $F_{(3,1678)} = 266.29$, $p < 0.001$ and d3 measures $F_{(3,1678)} = 266.29$, $p < 0.001$. Post hoc analysis revealed no significant differences ($p > 0.05$) between the untreated animals and animals in the saline (1 h) condition on any of the discrimination measures. Also, saline (1 h retention interval) treated animals showed significantly more discrimination than animals treated with scopolamine or MK-801 ($p < 0.001$). This was the case for d1 (Figure 10A), d2 and d3 measures (Figure 10B).

3.4. Relationships between exploration and discrimination measures

To investigate the relationship between the level of exploration in the learning and test trial and the three different discrimination measures, only the treatment conditions in which animals discriminated were included. Thus, only sessions from the 1 h untreated and 1 h saline conditions were included and overall Pearson's correlations were calculated between e1 and e2, as well as their correlations with d1, d2 and d3. Additionally, the same correlations were analyzed separately for familiarized animals and animals that were not yet fully familiarized to check whether there was a difference in this respect between fully familiarized animals and animals that still underwent familiarization (Table 3).

Cohen [42] postulated the following guidelines for the interpretation of the correlation coefficient; $r = 0.1 - 0.23$, small effect; $r = 0.24 - 0.36$, medium effect; $r = 0.37 - 1$, large effect. According to these criteria a positive, large overall correlation was found between e1 and e2. There was no significant correlation between e1 and d1, whereas the negative correlations between e1 and d2/d3 were only very low. A large positive overall correlation was found between e2 and d1. Correlations between e2 and d2/d3 were also positive, but very low. The same was found when familiarized and non-familiarized animals were separately analyzed (data not shown). The relations between d1/d2 and e2 are graphically presented in Figure 11,

illustrating the clear relationship of d1 with e2, but not for d2, which corrects for exploratory differences.

Within the familiarized and non-familiarized groups, subdivisions were made based on the total amount of exploration of the individual animals, to investigate the relationship between exploration and discrimination in further detail. For each exploration time bin group, one-sample *t*-tests were performed assessing whether d1 and d2 were significantly higher than zero and whether d3 was different from 0.5.

Figure 12A and 12B show the discrimination measures plotted against subsequent time bins of e1 and e2 in familiarized animals, thus showing the relationship between the level and the discrimination measures. Table 4 shows the frequencies per time bin for e1 and e2 of both the familiarized and non-familiarized animals, as well as the minimal amount of exploration that was required for reliable discrimination performance. Meaning that, when exploration further increased the d1 and d2 values remained significantly higher than zero, or 0.5 in case of the d3 measure. It was found that non-familiarized animals required 9–10 s of exploration in T1, whereas familiarized animals only needed 6-7 s of exploration. Both non-familiarized and familiarized animals generally needed more exploration in T2, as compared to T1. In T2 non-familiarized animals also needed more exploration compared to familiarized animals, 11-12 s and 9-10 s respectively. This means that, in T1, 3.7% of the non-familiarized animals and 0.4% of the familiarized animals did not show sufficient exploration for reliable discrimination performance. In T2, 4.2% of the non-familiarized animals and 1.5% of the familiarized animals did not reach the exploration threshold. Thus, the proportion of animals that showed a sufficient amount of exploration was bigger in the familiarized group.

3.5. Trial duration

The within-trial development of exploration and discrimination was assessed in familiarized animals using a 1 h interval ($n = 36$), the total exploration in T1 and T2 was divided over 6 time bins of 30 s each. Exploration was distributed over the consecutive time bins as follows; 27.8%, 17.5%, 18.5%, 12.7%, 10.8% and 12.7% in T1, and 29.7%, 21.8%, 16.3%, 12.3%, 9.9% and

10% in T2 (Figure 13A). The cumulative exploration was also calculated after 30 s, 60 s, 90 s, 120 s, 150 s and 180 s of trial duration (Figure 13B). Exploration kept increasing with time, ANOVA showed that the differences between trial durations were significant in both T1 $F_{(5,210)} = 48.65$, $p < 0.001$ and T2 $F_{(5,210)} = 44.15$, $p < 0.001$. The results from Bonferroni post hoc analysis are displayed in Figure 13A.

Development of d1, d2 and d3 measures was analyzed in the same cumulative fashion as e1 and e2 (Figure 14). The d measures were calculated for the hypothetical situations that the trial ended after 30 s, 60 s, 90 s, 120 s, 150 s and after the actual duration of 180 s. One-sample *t*-tests showed that d1 and d2 measures were significantly higher than zero for all theoretical trial durations. ANOVA revealed significant differences in the d1 measure between trial durations $F_{(5,210)} = 4.49$, $p < 0.001$, but not in the d2 $F_{(5,210)} = 0.53$, n.s. and d3 measures $F_{(5,210)} = 0.53$, n.s. Post hoc analyses were performed using Bonferroni *t*-tests, results are displayed in figure 14A and 14B.

4. Discussion

4.1. Exploration and discrimination

We found a large positive correlation between e1 and e2, indicating that there is consistency in the amount of exploration of individual animals. Correlation coefficients found between e1 and the d measures were slightly negative, indicating that higher exploration levels in the sample trial T1 are not associated with better discrimination performance, a phenomenon previously reported by Gaskin et al. [2]. This may suggest that the amount of exploration in T1 does not reflect the quality of memory encoding. On the other hand, it is also possible that discrimination ratios are not a valid estimate of memory strength, as was argued by Gaskin et al. [2]. The slightly negative correlation that was observed in our study might be explained by the assumption that rats with higher e1 values spent less time familiarizing with the environment (contextual information), which has been shown to increase novelty preference [37, 38]. During each trial, animals distribute their attention over the objects and the arena. Therefore, a higher e1 measure might imply that animals spent less time exploring the arena and therefore acquired relatively less contextual information. If the context is less familiar in the test trial, the contrast between the novel object and the context might be a fraction lower. However, it should be noted that the low

correlations only turned out significant because of the high number of subjects in our analysis, that is, the explained proportion of the total variance is very low.

In the test trial T2, exploration (e2) and d1 had a high positive correlation, whereas e2 only had low positive correlations with d2 and d3. Therefore, d2 and d3 are less biased by exploratory activity. The latter was also observed in an object location test (OLT) using a protocol similar to ours, but, in T2, the location of one of the familiar objects is changed instead of replacing it by a novel object [43]. These findings make the d2 and d3 measures more suited for conditions that may affect the exploratory activity in animals, e.g., when testing pharmacological compounds or the effects of stress. Of note, again the low correlation had turned significant due to the high number of subjects.

Non-familiarized animals required at least 10 s of object interaction in T1 and 12 s in T2 for reliable object discrimination. This amount was lower for familiarized animals which needed 7 s and 10 s of object exploration in T1 and T2, respectively. The majority of the animals reached this amount of exploration. In T1, but 3.7% of the non-familiarized animals and 0.4% of the familiarized animals did not show sufficient exploration. The same pattern was found in T2, with insufficient exploration in 4.2% and 1.5% of non-familiarized and familiarized animals, respectively. This indicates that when using non-familiarized animals a substantial drop-out due to insufficient exploration levels has to be considered.

Within our 3 min trials, exploration kept increasing significantly over the first 90 s. Both in T1 and T2, the first 90 s contributed roughly 66% of the total exploration, this corresponds to the findings of Dix and Aggleton [44] who found that most discriminative exploration was displayed in the first 2 minutes of a trial. In our experiments d1 and d2 measures were already significantly higher than zero after a trial duration of 30 s in T2. The same holds for d3 with respect to its reference value of 0.5. Within this 30 s trial duration, the level of exploration in T2 was already 8 s, which was approximately 30% of the total exploration and close to the required minimum amount of exploration (9-10 s) that we found to be necessary for reliable discrimination performance in familiarized animals. After a trial duration of 60 s, the minimum exploration criterion is always met in T2. Indicating that, using our protocol, a trial duration of 60 s is already sufficient in T2. In a recent study using a similar test protocol, but for object location memory, Ozawa et al. [43] observed the same minimum trial durations.

In contrast to Dix and Aggleton [44], we found that the d1 measure kept increasing with trial duration of T2, meaning that longer trials will automatically produce better discrimination performance. On the other hand, after a 1 h delay, the d2 and d3 measures were insensitive to the trial duration of T2 and remained stable during the whole trial duration of 3 min. In a recent OLT study using a similar test protocol, it has been shown that at longer delays, when the memory trace is 'weaker', the d2 measure will decrease after 1-2 min with increasing trial duration of T2 [43]. Nevertheless, the above observations make d2 and d3 the preferred measures for inter-experimental comparisons. However, when comparing experimental designs with a fixed exploration level, d1 may also be a useful measure.

4.2. Familiarization

In the '1 h familiarization protocol', animals discriminated significantly between the objects in T2 in all familiarization sessions. This finding indicates that naïve animals are intrinsically capable of object discrimination, so the ORT indeed appears to utilize the rat's natural tendency towards novelty [1]. The first two familiarization sessions were identical in both the 1 h familiarization protocol and 24 h familiarization protocol. It was found that e1 decreased from the 1st to the 2nd familiarization session, whereas the level of e2 remained the same. In addition, we observed that e1 was higher than e2 in the first familiarization session. It has been shown that exposure (3 min) to a novel environment is sufficient to elicit a stress response in rats [45. 46]. The decrease in e1 from the 1st to the 2nd familiarization session, as well as the decrease in e1 to e2 in the 1st familiarization session could be explained by behavioural habituation [47], since the stress response has been shown to decrease with repeated exposures to a stressor [45]. On the other hand, the 1st familiarization session is the first time the animals encounter objects in the arena. Therefore, e1 in this session may also be elevated out of curiosity or arousal.

In the 1 h familiarization protocol and the 24 h familiarization protocol, there was an increase in e2 in the 3rd session whereas e1 did not change from the 2nd to the 3rd session. In both protocols, the e2 value of the 3rd session was higher than the e2 value in any of the other sessions. The '3rd' familiarization session was the first session in which the animals were tested in combination with (saline) injections. This is likely to induce a stress reaction due to the discomfort of the injection procedures [48, 49]. In the our studies, animals received (minimally) one saline injection 30 min before T1 in the 1 h retention studies and in the 24 h retention studies animals received a vehicle

injection between 0 min and 3 h after T1. It is, however, unlikely that e2 was directly affected by stress, since in the 3rd session, e1 remained unchanged compared to the prior session in which animals did not receive injections. Furthermore, in the 24 h familiarization protocol T2 was performed at least 21 h after the time of injection. After this interval the stress response will be normalized [45].

The increased exploration in T2 might the result of a memory effect. That is, the detection of a novel object may increase arousal and thereby exploratory activity. Indeed, all discrimination measures of the 3rd familiarization session were higher compared to any of other individual sessions except the 4th. Many studies have shown that stress can have varying effects on memory functioning [50-55]. Memory can be facilitated when stress is experienced in the same context and around the time of learning [56]. Therefore, experiencing the injections close to T1 for the first time might have enhanced memory formation during T1.

From the 3rd session onward, e1 measures did not significantly decrease anymore in the 1 h protocol and e2 values of the 1st, 2nd and 4th sessions were all equal to that of the ≥5th session. In the 24 h training protocol, we observed an exploratory drop in the 4th familiarization session. In this session, both e1 and e2 were significantly lower than in any of the other sessions. In the 5th test session of the 24 h training protocol, e1 returned to the level of the 3rd session. Also the level of e2 increased to a level identical to that of e1, indicating that when the familiar object is not remembered the objects in T2 were equally interesting to the animals as those in T1. The drop in exploration in the 4th session of the 24 h training protocol is difficult to explain, since animals underwent exactly the same procedure as in the ≥5th session. However, this finding might be incidental, since this condition only incorporated animals from 2 small experiments.

4.3. Novelty

In the 24 h familiarization protocol, all discrimination measures of the first 3 sessions were significantly above chance level, indicating that the animals discriminated between the objects. In the 1st session this is to be expected, since a 1 h retention interval was used. In the 2nd and 3rd session discrimination measures were significantly above chance level, indicating that the animals remembered the familiar object over a 24 h retention delay. However, from the 4th session on the rats stopped discriminating between the novel and familiar object after a 24 h

delay. This observation might be explained by the manner in which the 4 sets of objects were presented to the animals, as was therefore investigated into detail.

In our experiments, the object sets were rotated over the individual animals in such a way that, after the 3rd session, the novel object in T2 was the same as the familiar object from 3 sessions before. This might imply that Wistar rats are able to remember object information much longer than 24 h, since in every experiment at least 9 days were interposed between the 1st and 4th test sessions. It is even possible that rats permanently retain object information after a single test trial and after the first acquaintance only temporal information is updated. We found support for this view in the supplementary experiment in which we used two completely new sets of objects in a 24 h retention interval. In the first of 2 consecutive sessions, the fully familiarized animals again discriminated between the objects. In the following session, which was identical to the first one, discrimination had disappeared again. This supports the notion stated by Ennaceur [57] that after the first single encounter with an object, rats only reconsolidate or perform a 'familiarity update' in successive encounters. We also found a marked decrease in overall exploration from the 1st to the 2nd test session, which indicates a habituation effect that cannot be explained by environmental factors, since these remained the same. Animals only stopped discriminating between objects after a 24 h retention interval if they had had an encounter with the novel object in a previous session. Hence, natural 'forgetting' in a 24 h interval might not reflect the forgetting of the total object representation, but only that of the temporal aspect of the object representation. After the initial encounter with an object, animals probably reconsolidate the object information each time that an object is encountered, adding new contextual information (object location, last time encountered) to the already existing object representation. Therefore the object that was more recently encountered (i.e. the familiar object) is likely to be more familiar compared to a relatively less familiar object (i.e. the novel object), that is an object which's representation was less recently updated. This relative difference in familiarity is forgotten within 24 h.

4.4. Pharmacological deficit models

In the experimental phase of our experiments, untreated and saline treated animals were well capable of discriminating between the novel and the old object and no differences were found between the d measures of both conditions. As mentioned above, data from the familiarization

protocols showed that animals that discriminate between objects have an increased exploration level in T2, as compared to T1. This was also the case in fully familiarized untreated and saline treated animals.

In the $\geq 5^{th}$ session of the 24 h familiarization protocol, animals showed no discrimination above chance level and e2 remained equal to e1. This exploration pattern was also observed in scopolamine treated animals, which also did not discriminate between objects in T2. Scopolamine is a muscarinic receptor antagonist which, amongst others, is used to mimic cognitive deficits in Alzheimer's disease [9]. The increase in e2 was markedly reduced compared to the e2 of saline treated rats in the 1 h retention interval. However, the difference still turned out significant, due to the large dataset (n = 715).

MK-801 treated animals were also unable to discriminate between objects in T2, yet e2 was significantly increased compared to e1, which was similar to the e1 of saline treated animals. MK-801 is a non-competitive NMDA receptor antagonist that is used as a model for schizophrenia because it mimics both positive and negative symptoms of the disorder [58]. MK-801 is a potent cognition impairer, but sensory, locomotor and toxicological side effects have been shown to become problematic with doses higher than 1 mg/kg and may already influence ORT performance at lower doses [40, 59]. Young Wistar rats have even been shown susceptible for non-cognitive side effects like increased locomotion and stereotypic movements at a dose of 0.5 mg/kg [60]. With age, animals become more resistant to these side effects (Pesic, et al.). MK-801 reaches maximum brain concentrations, 30 minutes after (i.p.) administration [61], thereby exactly coinciding with our T1. Increased locomotion has been reported to last up to 90 min after injection, whereas other side effects like ataxia and head weaving have shorter durations, of around 30 min and 60 min, respectively [60-62]. It might therefore be that, in T1, increased locomotion was masked by other side effects that are related to distorted perception and lack of coordination. In T2, on the other hand, these other side effects will have faded. Therefore, the increase in e2 might be explained by locomotor effects of MK-801 treatment.

4.5. Considerations when using the object recognition task

The ORT is a sensitive, reliable memory assessment tool that is able to detect subtle behavioural and cognitive effects. However, although the basic drive behind the task is very straightforward, i.e. rats prefer novel objects, there are more underlying factors influencing behaviour and

memory performance. For example, injection stress increased exploration when animals experienced it for the first time and thus, may directly affect discrimination measures. It is therefore highly recommended that animals are familiarized to the complete testing and administration procedure during familiarization, to prevent bias during the experiment.

We found that, in our protocol, familiarized Wistar rats only require 7 s of exploration in T1 and 10 s of exploration in T2, to achieve reliable discrimination performance. Both in T1 and T2, animals already achieved these exploration levels after a trial duration of 60 s. Additionally, only a weak negative relationship was found between e1 and the d measures. Therefore, it would be advisable to use fixed trial durations, since testing with an exploration threshold will lead to different trial durations for each animal which in turn will cause differences in context familiarization between animals. This familiarization bias [37, 38] can be minimized by using a fixed trial duration that is long enough for the animals to reach reliable discrimination performance. Using our testing conditions, both T1 and T2 should last at least 60 s.

It is commonly believed that Wistar rats are unable to remember objects over a 24 h retention period, our findings, however, suggest that at least some object information is retained for a longer period as Ennaceur suspected [57]. Animals only stopped discriminating between objects after a 24 h retention interval if they had previously encountered the novel object. Taking these findings into account, one should familiarize the animals to all the different objects during familiarization or provide completely novel sets of objects for each test session to prevent confusing consolidation with reconsolidation processes.

Several different discrimination measures are used to report findings in the ORT and different names are used to indicate them. Basically, two types of discrimination measures can be used, absolute and relative measures. We found that the absolute discrimination measure d1 is positively correlated to exploratory activity. Hence, more active/exploratory animals are assumed to show better discrimination compared to animals that are less exploratory active. This makes absolute discrimination measures vulnerable to any factors affecting exploration, like (injection) stress and drug-induced side effects. Relative discrimination measures such as d2 and d3 correct for a potential bias in exploratory activity and thus might be more suited for comparing ORT studies between laboratories.

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Tables

TABLE 1 FAMILIARIZATION PROTOCOLS

The 1 h and 24 h familiarization protocols are initially the same, this is indicated by the dotted line. Starting with 3 min in the empty arena for 2 consecutive days, followed by a 1 h retention test session and a 24 h retention test session without any injections. After this animals were familiarized the injection procedures by one or more saline injections, using the route and volume required by the intended experiment. After this the protocols diverge, with only 1 h test sessions being performed in the 1 h familiarization protocol and only 24 h test sessions in the 24h familiarization protocol. From the 3rd session on, the full experimental procedure is applied and from the 4th session on saline injections are replaced by vehicle injections. Familiarization is completed when animals show no more object discrimination (24 h protocol) or good object discrimination (1 h protocol), which is normally in the 3rd session.

TABLE 2 MEASURES IN THE OBJECT RECOGNITION TASK

The output variables e1, e2, d1, d2 and d3 can be calculated from the time spent exploring both identical objects (a_1 and a_2) in the first trial (T1), and the time spent in exploring the familiar (a_3) and novel (b) object in the second trial (T2).

TABLE 3 PEARSON'S CORRELATIONS

Pearson correlations (*r*) between d1, d2, d3, e1 and e2. Animals from the untreated and 1 h saline conditions were pooled together and overall (all, $n = 1896$) correlations were calculated. Additionally, familiarized (fam., $n = 948$) and non-familiarized (non-fam., $n = 948$) animals were analyzed separately. The corresponding significance level is shown below each individual r value.

TABLE 4 EXPLORATION AND DISCRIMINATION FREQUENCIES

Frequencies and percentages of familiarized (fam.) and non-familiarized (non-fam.) animals reaching the criteria for subsequent exploration bins in a 1 h retention experiments. The total amount of e1 or e2 is depicted in the header row for subsequent time bins, ranging from its own value to that of the prior exploration bin. Threshold exploration values, i.e. minimal exploration required for discrimination to remain significant in subsequent time bins, are indicated by a vertical line.

Figures

FIGURE 1 STANDARD OBJECTS USED IN THE ORT
The different objects used in the ORT were; 1) a bottle; 2) a cube; 3) a cone and 4) a bullet shaped piece of aluminum. Rats were unable to displace the objects.

FIGURE 2 NEW OBJECTS USED IN SUPPLEMENTARY EXPERIMENT
Two completely novel sets of objects were introduced to test the relativity of novelty. Object set 1 consisted of red tin cans and set 2 contained white porcelain cups.

FIGURE 3 1H FAMILIARIZATION PROTOCOL - EXPLORATION
The development of e1 and e2 in the 1 h familiarization protocol. The x-axis shows the familiarization sessions in chronological order, additional information about particular sessions is specified within brackets. All sessions used 1 h retention intervals, except for the 2nd session, in which a 24 h retention interval was used. With the exception of the 3rd session, animals did not receive injections during familiarization (session 1-3), during testing (4th and ≥5th session) animals did receive injections. The 4th session represents the first 1 h saline test sessions after familiarization, session ≥5 is a collection of the remaining 1 h saline test sessions. A difference between e1 and e2 is indicted with asterisks (paired-samples *t*-test, *: $p < 0.05$; ***: $p < 0.001$).

FIGURE 4 1H FAMILIARIZATION PROTOCOL - DISCRIMINATION

The d1 measure (A), d2 and d3 measures (B) of the different familiarization sessions in the 1 h familiarization protocol. The x-axis shows the familiarization sessions in chronological order, additional information about particular sessions is specified within brackets. All sessions used 1 h retention intervals, except for the 2nd session, in which a 24 h retention interval was used. With the exception of the 3rd session, animals did not receive injections during familiarization (session 1-3), during testing (4th and ≥5th session) animals did receive injections. The 4th session represents the first 1 h saline test sessions after familiarization, session ≥5 is a collection of the remaining 1 h saline test sessions. A difference from the 1st familiarization session is indicated with asterisks (Bonferroni *t*-tests, ***: $p < 0.001$). Significant differences from zero are indicated with hashes (one-sample *t*-tests, ### $p < 0.001$).

FIGURE 5 24H FAMILIARIZATION PROTOCOL - EXPLORATION

The development of e1 and e2 in the 24 h familiarization protocol. The x-axis shows the familiarization sessions in chronological order, additional information about particular sessions is specified within brackets. All sessions used 24 h retention intervals, except the 1st session, in which a 1 h retention interval was used. With the exception of the 3rd session, animals did not receive injections during familiarization (session 1-3), during testing (4th and ≥5th session) animals did receive injections. The 4th session represents the first 24 h vehicle test sessions after familiarization, session ≥5 is a collection of the remaining 24 h vehicle test sessions. A difference between e1 and e2 is indicted with asterisks (paired-samples *t*-test, ***: $p < 0.001$). Of note, because the familiarization protocols are identical up to the 3rd session, the 1st and the 2nd familiarization sessions in the 24 h familiarization protocol are the same as those in the 1 h familiarization protocol.

FIGURE 6 24H FAMILIARIZATION PROTOCOL - DISCRIMINATION

The d1 measure (A), d2 and d3 measures (B) of the different familiarization sessions in the 24 h familiarization protocol. All sessions used 24 h retention intervals, except the 1st session, in which a 1 h retention interval was used. With the exception of the 3rd session, animals did not receive injections during familiarization (session 1-3), during testing (4th and ≥5th session) animals did receive injections. The 4th session represents the first 24 h vehicle test sessions after familiarization, session ≥5 is a collection of the remaining 24 h vehicle test sessions. A difference from the 4th session (first test session) is indicated with asterisks (Bonferroni *t*-tests, *: $p < 0.05$; ***: $p < 0.001$). Significant differences from zero are indicated with hashes (one-sample *t*-tests, ### $p < 0.001$). Of note, because the familiarization protocols are identical up to the 3rd session, the 1st and the 2nd familiarization sessions in the 24 h familiarization protocol are the same as those in the 1 h familiarization protocol.

FIGURE 7 ABSOLUTE AND RELATIVE NOVELTY - EXPLORATION

Exploration of objects that are absolutely novel or relatively novel. Animals performed two 24 h retention sessions containing the exact same objects, divided by a 4 day period. The x-axis shows the consecutive sessions in which they were confronted with objects they had never seen before (absolute novelty) and the same objects again (relative novelty). A difference from the 1st session (absolute novelty) is indicated with asterisks (Bonferroni *t*-tests, ***: $p < 0.001$).

FIGURE 8 ABSOLUTE AND RELATIVE NOVELTY - DISCRIMINATION

The d1 (A), d2 and d3 measures (B) of the same objects in two consecutive 24 h retention sessions. Animals were confronted with objects they had never seen before (absolute novelty) in the 1st session and the same objects again in the 2nd session (relative novelty). Sessions were separated by a 4 day period. Differences from the 1st session (absolute novelty) are indicated with asterisks (Bonferroni *t*-tests, *: $p < 0.05$) and significant differences from zero are indicated with hashes (one-sample *t*-tests, ##: $p < 0.01$; ###: $p < 0.001$).

FIGURE 9 AMNESIC TREATMENTS - EXPLORATION
Effects of different treatments on exploratory behaviour in T1 (e1) and T2 (e2) in a 1 h retention interval. The x-axis shows the different treatment conditions and the inter-trial interval. Differences from the saline (1 h) condition are depicted with asterisks above the error bars (Bonferroni *t*-tests, *: $p < 0.05$; ***: $p < 0.001$), underlined asterisks indicate a difference between e1 and e2 within the treatment conditions (paired-samples *t*-tests, ***: $p < 0.001$).

FIGURE 10 AMNESIC TREATMENTS - DISCRIMINATION
The effects of the different treatments on the d1 (A) and d2/d3 (B) in a 1 h retention interval. Different treatment conditions and corresponding inter-trial interval are shown in the x-axis. Hashes (one-sample *t*-test, ###: $p < 0.001$) denote differences of d1 and d2 from 0, or 0.5 in case of d3. Significant differences from the saline (1 h) treated group are indicated by asterisks (Bonferroni *t*-tests, ***: $p < 0.001$).

FIGURE 11 CORRELATION OF EXPLORATION AND DISCRIMINATION MEASURES
Relationship between exploration in the test trial (e2) and the d1/d2 measures for familiarized and non-familiarized animals pooled together, the best fitting straight line is defined by Y=bX+a, where Y=d1 or d2, X=e2, b= the slope of the line and a= the intercept, r = the Pearson's correlation coefficient.

FIGURE 12 DISCRIMINATION MEASURES PLOTTED AGIANST EXPLORATION LEVEL
Exploration in the learning trial (e1, Figure 8A) and test trial (e2, Figure 8B) plotted against d1, d2 and d3, for familiarized animals. Subsequent time bins range from the previous value to the maximally shown value. For d1 and d2 groups that significantly differed from 0 (one-sample *t*-test, $p < 0.05$) are encircled with a black line, when the difference was insignificant a grey color was used as outline. The d3 measure was compared to 0.5 and indicated with triangles. For significance indication, the same color coding is used as with the d1 and d2 measures. Vertical black lines show the point from which discrimination remained stable.

FIGURE 13 DISTRIBUTION OF EXPLORATION WITHIN TRIALS
Distribution of exploration in T1 and T2 presented in time bins of 30s. Figure 11A shows the cumulative exploration, given the hypothetical situation if the trial would have been stopped after the time bin depicted on the x-axis. Figure 11B shows the exploration per time bin as a percentage of the total exploration over 180s. A significant increase from the preceding time bin is indicated by asterisks (Bonferroni *t*-tests, (*): $p < 0.06$; *: $p < 0.05$; **: $p < 0.01$).

FIGURE 14 DISTRIBUTION OF DISCRIMINATION WITHIN THE TEST TRIAL
Cumulative d1 value (A), or d2/d3 value (B) given the hypothetical situation that the test trial T2 would have been stopped after the time bin depicted on the x-axis. Differences from zero (d1 and d2) or 0.5 (d3) are indicated with hashes (one-sample *t*-tests, ### $p < 0.001$). A significant difference from the first time bin is indicated with asterisks (Bonferroni *t*-tests, (*): $p < 0.06$; *: $p < 0.05$; ***: $p < 0.001$).