Object familiarization and novel-object preference in rats

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Abstract

We investigated whether object familiarization was related to novel-object preference in the novel-object preference (NOP) test in rats. In Experiment 1, we found that no significant correlation existed between the time spent investigating 2 identical copies of a sample object and the degree of preference for a novel object. In Experiment 2, rats investigated 2 identical sample objects for a total of 5, 30, 60, 90 or 120 s. Investigatory preference for the novel object was compared to chance expectancy as well as between the groups. Only the 90-s group and the 120-s group displayed above-chance investigatory preference for the novel object, but novel-object preference for these 2 groups did not differ from each other, suggesting that a minimal amount of sample object investigation is necessary for rats to develop a novel-object preference, beyond which no increase in novel-object preference was found. In Experiments 3 and 4, normal rats and rats with hippocampal lesions were given repeated test trials, with the same sample object presented with a different novel object, at 24-h and (Experiment 3) and 35-s intervals (Experiment 4). In both experiments, novel-object preference did not increase in magnitude with repeated sample object exposures, suggesting that increased familiarity with the sample object does not result in increased novel-object preference. Rats with lesions of the dorsal hippocampus showed an unreliable investigatory preference for the novel object. These results are discussed in terms of the potential limitations of the NOP test as a tool for the assessment of object-recognition memory in rats.

Keywords: Exploratory preference Novelty preference Object recognition Hippocampus Lesions

The NOP test (Ennaceur et al., 1989; Ennaceur and Delacour, 1988) is widely used to assess object-recognition memory in rodents (Ainge et al., 2006; Clark et al., 2000; Gaskin et al., 2003; Hughes, 2007; Mumby et al., 2002, 2005). It can be used to test the effects of various pharmacological treatments (see Dere et al., 2007 for a review) and brain damage (Ainge et al., 2006; Clark et al., 2000; Gaskin et al., 2003; Hughes, 2007; Mumby et al., 2002, 2005). The NOP test takes advantage of rats’ propensity to investigate a novel object significantly more than one they have previously encountered (sample object) during a period of familiarization, when both objects are presented simultaneously. This behaviour is inferred to involve object-recognition memory, because it is assumed that in order to preferentially investigate the novel object, rats must be able to detect the familiarity of the sample object.

The details of the NOP test procedure vary across studies, with no consensus as to which familiarization and test conditions are most conducive to novelty preference (Ainge et al., 2006). The effects of brain damage or pharmacological treatments may differ depending on several factors such as the retention delay between familiarization and testing (Clark et al., 2000) or the time spent investigating sample objects during familiarization (Ainge et al., 2006). In experiments investigating the effects of brain damage, lesion size may also be an important factor (Broadbent et al., 2004).

The statistical evaluation of the performance of rats with hippocampal lesions on the NOP test may also vary. For example, in some studies evaluation of novel-object preference is done by computing an investigation ratio (IR) (Gaskin et al., 2003; Clark et al., 2000) composed of the amount of time rats spend investigating the novel object relative to total object-investigation time (sample + novel) during the test. A difference-score that only takes into account the difference in time spent investigating the novel and sample object is sometimes used (Albasser et al., 2009).

In some studies, object recognition is inferred if the average IR obtained by a group of rats is significantly above what could be expected by chance, regardless of whether this IR is of lesser magnitude than that obtained by another group of rats (Gaskin et al., 2003; Mumby et al., 2002; Piterkin et al., 2008). In other studies, a statistically significant difference between a control and experimental group(s) is emphasized in order to determine whether a group of rats have an object-recognition impairment following some treatment (Clark et al., 2000).

However, when a statistical difference between the average IR of different groups is emphasized, one tacit assumption is that the magnitude of the IR is indicative of the strength of memory for the sample object. To our knowledge, there is little evidence to support this assumption and it rests mostly on intuition. Nevertheless, if the strength of memory for objects is reflected in IR magnitude,
1. Experiment 1

1.1. Methods

1.1.1. Subjects

Subjects were 44 Long–Evans male rats weighing 300–400 g, housed, individually in standard clear–plastic cages (“shoebox” 45.7 cm × 25.4 cm × 20.3 cm). Rats were given a daily ration consisting of 25–30 g of standard lab chow and had continuous access to water. The colony room was on a 12:12 light–dark cycle with light onset at 20:00. All procedures were approved by the Concordia University Animal Care and Use Committee, and were in compliance with the guidelines of the Canadian Council on Animal Care. The rats were used in a previous study that assessed the effects of global-cerebral ischemia on a variety of learning and memory tests, and were in corpulence with the guidelines of the Canadian Council on Animal Care.

1.1.2. Apparatus and materials

The apparatus and materials were identical for all experiments of the present study. The apparatus consisted of a square open-field arena constructed of gray PVC plastic and had dimensions of 70 cm × 70 cm with walls 60 cm in height. A stainless-steel tray served as the floor and was covered with wood shavings. The floor could be removed through a slot at the bottom of one wall to facilitate changing the shavings between each trial. A video camera was positioned over the arena and familiarization and test phases were videotaped for later analysis.

The test stimuli consisted of objects made of glass, porcelain, or glazed ceramic. The objects varied in height between approximately 6 and 12 cm, and in width between 6 and 10 cm. Attached with epoxy to the bottom of each object was a small glass jar (6 cm high), and attached to the floor of the arena were two inverted jar lids, each positioned 27 cm from opposing corners of the arena. Objects were fixed in place by screwing the jars into the lids. There were 3 copies of each object, which were used interchangeably. The objects were washed after each use with a 70% alcohol solution.

The shape of the objects was such that it was difficult for the rats to sit or climb onto them. All objects used for NOP testing in our laboratory have been tested for investigatory preference. Objects that elicited abnormally high levels of spontaneous investigation were discarded from our inventory of objects. Novel/sample object-pairs are pre-selected on the basis that each object in the pairs elicits a similar amount of spontaneous investigation.

1.1.3. Procedures

1.1.3.1. Habituation

Subjects were 44 Long–Evans male rats weighing 300–400 g, housed, individually in standard clear–plastic cages (“shoebox” 45.7 cm × 25.4 cm × 20.3 cm). Rats were given a daily ration consisting of 25–30 g of standard lab chow and had continuous access to water. The colony room was on a 12:12 light–dark cycle with light onset at 20:00. All procedures were approved by the Concordia University Animal Care and Use Committee, and were in compliance with the guidelines of the Canadian Council on Animal Care. The rats were used in a previous study that assessed the effects of global-cerebral ischemia on a variety of learning and memory tests, and were in corpulence with the guidelines of the Canadian Council on Animal Care.

The rats were permitted to explore the open-field arena, with no objects, by being individually placed in it for 10 min on 2 consecutive days. NOP testing began within 2 days of the second exploration session.

1.1.3.2. Familiarization

Rats were placed into the open-field arena, which contained two copies of the sample object, for 5 min.

1.1.3.3. Test

The test consisted of placing the rats back in the open field with a third copy of the sample object and a novel object. The retention intervals between familiarization and testing were 15 min and 24 h for the SHAM-operated group and 3 h for the un-operated intact rats. A rat was considered to be investigating an object when its head was oriented within 45° of the object and within 4 cm of it. Rearing with the head oriented upward was also included if at least one forepaw was in contact with the object. Climbing over or sitting on an object was not included.

1.2. Results and discussion

One-sample t-tests were used to evaluate whether the SHAM-operated and intact un-operated rats had average IRs that were significantly above chance. IRs consisted of the time spent investigating the novel object relative to total object investigation \( t_{\text{novel}}/(t_{\text{novel}} + t_{\text{familiar}}) \) during the first 2 min of the test, as investigation of the novel object subsides as the trial progresses (Dix and Aggleton, 1999). SHAM-operated rats displayed IRs that were significantly above chance with a 15-min \( t_{(23)} = 3.194, p = .004 \) and 24-h \( t_{(22)} = 3.407, p = .002 \) retention interval between familiarization and testing.

Pearson product-moment correlation coefficients were computed to assess the relationship between the total time spent investigating the sample objects during familiarization and average IRs, as well as between sample objects and difference-scores, based on the time rats spent investigating the novel object minus the time spent investigating the sample object \( t_{\text{novel}}/(t_{\text{novel}} - t_{\text{sample}}) \) during the test, conducted 15 min, 3 h, or 24 h later. One of the SHAM-operated rats unexpectedly died before the 24-h test.

Figure 1 summarizes the results. There was no significant correlation between the time spent exploring the sample objects during the familiarization phase and the subsequent IRs with either the 15-min \( r_{(21)} = -.12, p = .55 \) or 24-h \( r_{(22)} = .13, p = .53 \) retention interval (Fig. 1A and B respectively). In addition, no significant correlation was found between the time spent investigating the sample objects during the familiarization phase and difference-scores, with either the 15-min \( r_{(21)} = .03, p = .89 \) or 24-h \( r_{(22)} = .36, p = .08 \) retention interval (Fig. 1A and B respectively).

The intact un-operated rats displayed an average IR that was significantly above chance, with a 3-h retention interval between familiarization and testing \( t_{(19)} = 2.717, p = .013 \). There was no
significant correlation between the amount of time spent exploring the sample objects during familiarization and subsequent IRs during the test \( r(18) = -0.22, p = 0.345 \) or between sample object investigation during the familiarization phase and difference-scores \( r(18) = -0.14, p = 0.54 \) (Fig. 1C and C1 respectively).

No evidence of a correlation was found between the amount of time rats investigated the sample object during a 5-min sample familiarization phase and the magnitude of their subsequent IRs or difference-scores, obtained with either a 15 min, 3-h, or 24 h retention interval between familiarization and testing. The data were taken from two large groups of rats, both of which displayed significant novel-object preference under their respective testing condition. These results do not support the hypothesis that the magnitude of IRs during the test is related to how well the sample object was encoded during familiarization.

2. Experiment 2

Given the lack of correlational evidence that the magnitude of average IRs relates to sample object investigation during familiarization, we proceeded to further investigate this issue through experimentation. In this experiment, we manipulated the time that rats spent investigating the sample objects during familiarization and later measured the effects of such manipulation on the magnitude of IRs.

2.1. Methods

2.1.1. Subjects

Subjects were 36 male Long-Evans rats (Charles River, St. Constant, Quebec), weighing 275–400 g at the beginning of the
Fig. 2. Average IRs (bars) and average difference-scores (line) for rats that were permitted to investigate the sample object for 5, 30, 60, 90 and 120 s during familiarization. Average IRs are plotted along the y-axis situated to the left. Average difference-scores are plotted along the y-axis situated to the right. The asterisk above the connectors on the bar graph indicate the statistical differences between the 90-s group and the 5, 30 and 60-s groups (hatched) and between the 120-s group (regular) and the 5, 30 and 60-s groups (asterisk above the bars represents a significant difference from chance, $\alpha = .05$). Error bars indicate the standard error of the mean. The dashed line indicates chance levels of novel-object investigation.

The rats were housed and fed in a manner identical to that of Experiment 1.

2.1.2. Procedures

2.1.2.1. Habituation. Rats were permitted to explore the open-field arena, with no objects, by being individually placed in it for 10 min on 2 consecutive days. NOP testing began within 2 days of the second exploration session.

2.1.2.2. Familiarization. For the familiarization sessions, rats were placed into the open-field arena, which contained two copies of the sample object. Time spent investigating sample objects was measured, and rats were removed from the open field once they had accumulated 5 s ($n = 7$), 30 s ($n = 6$), 60 s ($n = 6$), 90 s ($n = 8$), or 120 s ($n = 7$) of object investigation, after which they were returned to the colony room.

2.1.2.3. Test. Following a retention interval of 3 h, rats were reintroduced to the open field, which now contained a copy of the sample object and a novel object, and permitted to explore for a period of five minutes. This procedure was repeated twice, for each rat, on 2 consecutive days, with different pairs of (sample/novel) objects. This resulted in each group of rats having two IRs, one for each day of testing. Because the results of these tests were virtually identical, the data were combined.

The main dependent measure was the IR based on the first 2 min of the test phase, averaged over the two test sessions. Only the first 2 min of the test phase were analyzed, as rats were shown to gradually decrease their amount of object investigation as the trial progresses beyond this time (Dix and Aggleton, 1999).

2.2. Results

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2.2. Results

One rat in the 120-s group failed to investigate one of the objects during the test; it was therefore eliminated from the analysis. Fig. 2 shows the results when test ratios (Left Y-axis) were averaged over both trials and the initial two minutes of testing were examined. A one-way analysis of variance (ANOVA) with sample object-investigation time (SIT) as the single independent variable and IRs as the dependent variable revealed a significant effect of SIT $[F(4,29) = 8.591, p < .001]$.

Post hoc multiple comparisons using Tukey (HSD) tests with inflation of $p$-values corrected for, using the Bonferroni procedure (Keppel, 1991; Tabachnick and Fidell, 2001), revealed a significant difference between the IRs obtained by the 120-s and 30-s group [$p = .049$], 120-s and 5-s group [$p = .001$], 120-s and 60-s group [$p = .018$], 90-s and 5-s group [$p = .001$] and the 90-s and 60-s group [$p = .022$]. There was no significant difference in IR between the 90-s and 120-s group [$p > .05$].

One-sample $t$-tests revealed that the average IRs from the 5-s, 30-s and 60-s groups did not significantly differ from chance (.50). Average IRs were significantly above chance only for the rats in the 90-s [$t(7) = 7.278, p < .001$] and 120-s group [$t(6) = 5.069, p = .002$].

Also shown in Fig. 2, is a line graph (right Y-axis) representing the difference-scores obtained by the same groups of rats. A one-way ANOVA with sample object-investigation time (SIT) as the single independent variable and Difference-Scores as the dependent variable revealed a significant effect of SIT $[F (4,29) = 5.860, p = .0014]$. Post hoc multiple comparisons using Tukey (HSD) tests with inflation of $p$-values corrected for using the Bonferroni procedure (Keppel, 1991; Tabachnick and Fidell, 2001) revealed a significant difference between the differences-scores obtained by the 120-s and 5-s group [$p < .05$], 90-s and 5-s group [$p < .01$] and the 90-s and 30-s group [$p < .05$] and the 90-s and 60-s group [$p < .05$]. There was no significant difference in investigation time between the 90-s and 120-s group $[p > .05]$.

One-sample $t$-tests revealed that the average difference-scores from the 5-s, and 30-s groups did not significantly differ from chance (0). Average difference-scores were significantly above chance only for the rats in the 60-s [$t(5) = 3.834, p = .012$], 90-s [$t(7) = 8.791, p < .001$] and 120-s groups [$t(6) = 3.315, p = .020$].

We also analyzed the average time each group of rats took to achieve the required object-investigation time in each condition during familiarization. A one-way ANOVA revealed a significant main effect of Group (5, 30, 60, 90 and 120 s) $[F (4,29) = 16.516, p < .001]$. Post hoc multiple comparisons using Tukey (HSD) tests with inflation of $p$-values corrected for using the Bonferroni procedure, revealed a significant difference between the time taken to achieve the required object-investigation time by the 120-s group and the 5, 30 and 60-s groups [$p < .01$] respectively. The 90-s group also differed significantly from the 5, 30 and 60-s groups [$p < .01$, $p < .01$, $p = .011$] respectively. In the time taken to achieve the required object-investigation time during familiarization. However, the 90-s group did not differ significantly from the 120-s group and the 5, 30, and 60-s groups did not differ from each other.

These results do not support the hypothesis that greater exposure to the sample objects will result in progressively higher IRs. Rather, these results show that a significant preference for the novel object may only occur if a minimal amount of object investigation during familiarization is achieved. In the present study, this minimal amount of object investigation was between 60 and 90-s. Beyond this minimal amount, additional sample object investigation did not result in a significant increase in the magnitude of the IR.

The pattern of average difference-scores nearly replicated the pattern of average IRs across the conditions. However, the pattern of statistical differences was different than the ones observed with the average IRs. This could partly be accounted for by the larger variability associated with difference-scores than with IRs.

The finding that the highest average IRs were found in the groups that spent more time in the arena (90- and 120-s group), suggests that exposure to the context in which the sample objects are situated may also contribute to the subsequent discrimination of a sample and novel object.

3. Experiment 3

In Experiments 3 and 4, we first tested rats using the conventional NOP testing procedure (Day 1 and Test 1 respectively). We
then increased the time rats were exposed to the same sample object by subjecting them to repeated test trials with the same sample object varying the novel object on each of the test trials.

3.1. Methods

3.1.1. Subjects
Subjects were 18 Long–Evans, male rats weighing 300–400 g at the beginning of the study (Charles River: St. Constant, Quebec). The rats were housed and fed in a manner identical to that of Experiments 1 and 2.

All rats were previously used in a study that assessed the effects of dorsal hippocampal lesions on spatial memory. Subjects were treated in accordance with the standards set by the Canadian Council on Animal Care, and Concordia University’s Animal Ethics Committee approved all procedures.

3.1.2. Procedures
3.1.2.1. Surgeries. Rats were subjected to excitotoxic lesions of the dorsal hippocampus (DHPC [n=9]) or sham surgeries (SHAM [n=9]). Rats were anesthetized with 3% isoflurane (Jaassen, Toronto, Ontario, Canada) in 81 l/min of oxygen at 14.7 psia at 21 °C (Benson Medical Industries, Markham, Ontario, Canada). Animals in the group that received lesions were infused with N-methyl-D-aspartate (NMDA [Sigma Chemical Co., St-Louis, MO], 7.5 μg/μl) at the rate of 0.15 μl/min for a total volume of 0.45 μl at 4 sites in the dorsal hippocampus according to stereotaxic coordinates ([AP −3.1, −3.1, −4.1, −4.1; ML ±1.0, ±2.0, ±3.5; DV −3.6, −3.6 −4.0, −4.0]; Paxinos and Watson, 2005).

The injections were done through an injection cannula attached to PE-20 tubing, using 10 μl Hamilton syringes mounted on a microinjection pump. The injection cannula was left in the injection site for 2.5 min after the injection to facilitate diffusion of the drug. Rats in the SHAM group received the same treatment except that no damage was done to the skull or brain. Scalp incisions were closed with wound clips and an antibiotic powder was applied to the wound. As the rats regained consciousness they received an injection of diazepam (0.3 cm3; 10 mg/ml, s.c.; Hoffman La-Roche, Mississauga, Ontario) as a prophylaxis against seizures. Food restriction was suspended for 7 days following surgery for all animals.

3.1.2.2. Histology. At the completion of behavioral testing all rats were sacrificed using a lethal dose of sodium pentobarbital (100 mg/kg, i.p.). They were perfused with a saline solution followed by a 10% formalin solution. Their brains were excised and...
stored in a 10% formalin/30% sucrose solution until sectioning. The brains were frozen-sectioned at 40 μm; every 10th section through the hippocampus was mounted on a glass microscope slide, and stained with cresyl violet.

3.1.3. Behavioral procedures
3.1.3.1. Habituation. Rats were habituated to the open field by placing them in it, individually, for 10 min, on 2 consecutive days.

3.1.3.2. Familiarization and testing (inter-session). Fig. 3A illustrates the inter-session familiarization and testing procedure. Testing was carried out over 5 days.

3.1.3.3. Day 1 (standard test, 2-h retention delay). The rats were familiarized, individually, with two identical copies of a sample object for 5 min in the open field. They were then removed from the open field for a 2-h retention interval. The rats were then placed back in the open field for a period of 5 min, with a third copy of the sample object and a novel object. The time spent investigating both the sample and novel object was recorded. The same pair of objects was used for each rat. However, the object that served as sample for half the rats served as novel for the other half.

3.1.3.4. Days 2–5 (24-h retention delay with repeated sample object exposure). Twenty-four hours after the first test, the rats were placed back in the open field for 5 min, with a copy of the sample object used on Day 1 along with a different novel object. The same steps were repeated three more times at 24-h intervals; thus, following the first (standard) NOP test after a 2-h retention interval on Day 1, there were four more tests, 24 h apart, and each test involved pairing the same sample object with a different novel object. The position of the sample and novel object was counterbalanced across tests in order to control for possible location preferences. The same novel objects were used for all the rats and were presented in the same sequence across test sessions.

3.2. Results and discussion
3.2.1. Histological results
Fig. 4 shows drawings of coronal sections representing the largest and smallest lesions for the rats in the DHPC group. Lesions were quantified by assessing the number of pixels taken up by the drawings of the normal dorsal hippocampal area and drawings of the lesioned area using Adobe Photoshop®. These data were then used to compute the percentage of lesioned area. Nearly 100% of the dorsal hippocampus was destroyed in each rat in the DHPC group, no discernable damage beyond the DHPC was observed.

3.2.2. Behavioral results
3.2.2.1. Familiarization. We tested whether the DHPC or SHAM group spent more time investigating one object over the other during familiarization.

Planned comparisons revealed that neither the DHPC nor SHAM group spent more time investigating one object over the other [\( t(7) = 2.223, p = .0615 \)] and [\( t(7) = 1.686, p = .135 \)] respectively.

Day 1. Independent samples t-tests were used to compare the average IR obtained by the SHAM and DHPC groups during Day 1 of testing, when a 2-h retention interval was used. One-sample t-tests were also used to compare each of the groups’ performance to chance (.50) on Day 1 and on each of the 4 remaining days of testing. Fig. 5A shows the average IRs for the SHAM and DHPC groups on the first day of testing. The SHAM group had a significantly higher IR than the DHPC group [\( t(14) = -2.202, p = .0449 \)]. However, both groups had an average IR that was significantly above chance (DHPC [\( t(7) = 9.466, p = .0001 \]) and SHAM [\( t(7) = 3.460, p = .0001 \])].
Planned comparisons, using t-tests, were used to compare each of the groups’ IRs to one another for each of the test days. The SHAM group had significantly higher average IRs than the DHPC group on days 3 \( t(14) = -2.232, p = .0215 \) and 5 \( t(14) = -1.830, p = .0443 \).

One-sample t-tests were used to determine whether the SHAM group and DHPC group had average IRs that were significantly above chance. The SHAM group had an average IR that was significantly above chance on all but Day 2 of testing (Day 3 \( t(7) = 6.982, p = .0002 \), Day 4 \( t(7) = 3.643, p = .0083 \), Day 5 \( t(7) = 2.951, p = .0214 \)). In contrast, the DHPC group only had a significantly above-chance IR on Day 4 \( t(7) = 4.463, p = .0029 \).

A one-way repeated measures ANOVA with Test Days as the repeated factor was used to find out whether a significant upward trend existed in the IRs across test days for both the SHAM and DHPC groups. There was no trend suggesting that IRs gradually improved across Days 2–5 for either the SHAM or DHPC group \( F(3,21) = 4.024, p = .020 \) and \( F(3,21) = 1.412, p = .2671 \) respectively. The significant F-value for the SHAM group was only due to a significant difference between the IRs obtained during Days 2 and 3 \( p < .05 \). Multiple comparisons, using the Bonferroni correction procedure, revealed no significant differences between the IRs of any other combination of test days.

Total object investigation. We investigated whether any differences in total object investigation between the SHAM group and DHPC group (sample + novel) existed across all of the tests. Planned comparisons showed that the SHAM and HPC groups only differed in their total amount of object investigation on Day 3 \( t(14) = -4.316, p = .0007 \).

Correlations. A Pearson product-moment correlation was computed to determine whether a correlation existed between the times spent investigating the sample objects during the familiarization phase and subsequent IRs during the initial test with a 2-h retention interval. There was no significant correlation between the time spent investigating the sample objects and the subsequent IRs for the SHAM \( r(6) = .17, p, n.s., \) or DHPC \( r(6) = .08, p, n.s., \) group. When a difference-score was used instead of IRs, no significant correlation was found with the SHAM group \( r(6) = .14, p = .72 \) or with the DHPC \( r(6) = .45, p = .26 \) group.

These results do not support the hypothesis that repeated exposures to the sample object will result in progressively greater investigation ratios. Therefore, it is unlikely that, in the NOP test, the magnitude of IRs reflects the strength of memory for the sample object. These findings also support the findings of Experiment 1, showing that a correlation between object investigation during familiarization and novel-object preference during the test, is not a necessary condition for rats that display a significant preference for the novel object in the NOP test.

Concerning the role of the hippocampus in novel-object preference, the results suggest that rats in the DHPC group could discriminate between the novel and sample object in the conventional tests on Day 1, as indicated by their significantly above chance average IR. However, the fact that the same rats did not show a significant average IR on 3 out of the 4 tests with the repeated sample object may signify that novel-object preference is unreliable in rats with damage to the HPC despite the initial evidence that the rats could discriminate between the objects.

4. Experiment 4

In Experiment 4 we tested whether the rats from Experiment 3 would display a similar pattern of results with a retention delay of 35-s between each test, within the same session, with an identical sample object and a different novel object.

As in Experiment 3, the results of the initial NOP test using the conventional testing procedure (Test 1), with a familiarization phase in which they investigated two copies of an identical sam-

![Fig. 6.](image)

**Fig. 6.** (A) Average IRs obtained by the DHPC and SHAM groups in the standard test with a 35-s retention interval (the asterisk denotes a significant group difference, \( \alpha = .05 \)). (B) Average IRs for the DHPC and SHAM groups on each of the tests with the repeated sample object with a 35-s retention interval (a single asterisk signifies a significant difference between the groups; a double asterisk denotes a significant group difference as well as a significant difference from chance, \( \alpha = .05 \)). Error bars indicate the standard error of the mean. The dashed line indicates chance levels of novel-object investigation.

4.1. Methods

4.1.1. Subjects

Subjects were the same as in Experiment 3.

4.1.2. Apparatus and materials

The apparatus and materials were the same as in Experiments 1 and 2. In addition, an upside-down open cardboard box (20 cm x 16 cm and 24 cm in height) was used to confine the rats during retention intervals.

4.1.3. Behavioral procedure

4.1.3.1. Habituation. Rats were re-habituated to the apparatus in a manner identical to Experiment 3. However, during this time rats were also exposed to the cardboard box, by being confined under it for a period of 35 s.

4.1.4. Intra-session procedure

4.1.4.1. Familiarization and testing. Fig. 3B illustrates the familiarization and test phases for Experiment 4. The procedure was similar to that of Experiment 3, with the exception that the 5 test trials were given within the same session on a single day.

4.1.4.2. Test 1 (standard test, 35-s retention delay). A rat was placed in the open field with two identical copies of an object for 5 min. While still in the open field, an upside-down open cardboard box was placed over the rat (see inserts, Fig. 6A and B) while one of the sample objects was replaced with a novel object and the other sample object was replaced with a cleaned copy. This procedure was completed in time to respect the 35-s retention interval. At the end of the retention interval the box was lifted, releasing the rat, free to investigate the objects. The same pair of objects was used for each rat. However, the object that served as the sample for half the rats, served as the novel for the other half and the object that served as the novel for half the rats served as the sample for the other half.
4.1.4.3. Tests 2–5 (35-s retention delay with repeated sample object).
Following Test 1, the rat was again covered with the box for a period of 35 s while the experimenter replaced the novel object with a different novel object and the sample object was replaced with another copy. This procedure was repeated 3 more times resulting in 4 novelty preference tests, each with a different novel object. The position of the sample and novel object was counterbalanced across tests in order to rule out the possibility that a side preference accounted for the preference of one object over the other. The same set of novel objects was used for all the rats, and was presented in the same sequence across test sessions.

4.2. Results and discussion

4.2.1. Familiarization

Planned comparisons revealed that neither the DHPC nor SHAM group spent more time investigating one object over the other \([t (7) = 1.276, p = .127]\) and \([t (7) = 1.024, p = .339]\) respectively.

4.2.1.1. Test 1. Independent samples \(t\)-tests were used to compare the average IRs obtained by the rats in the SHAM and DHPC groups during Test 1. Fig. 6A shows the average IRs for the SHAM and DHPC groups for the first Test-Session. Independent samples \(t\)-tests revealed that the SHAM group had a significantly higher IR than the DHPC group \([t (14) = -2.197, p = .0453]\). The SHAM group displayed a non-significant preference for the novel object \([t (7) = 2.171, p = .0665]\). The DHPC group had an average IR that did not significantly differ from chance \([t (7) = -0.772, p = .465]\).

4.2.1.2. Tests 2–5. A two-way mixed design ANOVA with Lesion-Type (SHAM or DHPC) as the between subjects factor and Test-Session as the within subjects factor was used to analyze the rats’ performances across Tests 2–5. Fig. 6B shows the IRs for the SHAM and DHPC groups on Tests 2–5 with a retention interval of 35 s, with repeated presentations of the sample object. There was an effect of Lesion-Type that approached statistical significance \([F (1,14) = 3.959, p = .066]\) and a significant effect of Test-Session \([F (3,42) = 3.333, p = .0284]\) but no significant Lesion-Type \(\times\) Test-Session interaction \([F (3,42) = .737, p = .5361]\).

Post hoc comparisons revealed that the SHAM group had a significantly higher average IR than the DHPC group on Test 3 \([t (14) = -1.886, p = .0401]\) and a higher average IR than the DHPC group on Test 5 at a level that approached statistical significance \([t (14) = -1.637, p = .0619]\).

One-sample \(t\)-tests were also used to compare each of the groups’ performance to chance (.50) on test 1 and each of the remaining four tests. The SHAM group had average IRs that were significantly above chance on all Tests except for Test 2 (Test 3 \([t (7) = 2.467, p = .0430]\), Test 4 \([t (7) = 5.623, p = .0008]\), Test 5 \([t (7) = 2.395, p = .0476]\)). In contrast DHPC rats never had an average IR that was above chance on any of the tests.

One-way ANOVAs performed separately on the SHAM and DHPC group revealed that there was no trend suggesting that IRs improved from Tests 2–5 for either the SHAM or DHPC group \([F (3,21) = 2.239, p = .113]\) and \([F (3,21) = 1.864, p = .166]\) respectively.

4.2.1.3. Total object investigation. A two-way mixed design ANOVA with Lesion-Type as the between subjects factor and Test-Session as the within subjects factor was used to determine whether total object-investigation time (sample + novel) differed between the SHAM group and DHPC group across all tests. There was no significant effect of Lesion-Type \([F (1,14) = 2.282, p = .153]\), a significant effect of Test-Sessions \([F (4,56) = 6.755, p = .0001]\) and no significant Lesion-Type \(\times\) Test-Day interaction \([F (4,56) = .4718, p = .756]\).

4.2.1.4. Correlations. A Pearson product-moment correlation was computed to determine whether a correlation existed between the times spent investigating the sample objects during the familiarization phase and subsequent IRs during the initial test with a 35-s retention interval. There was no significant correlation between the time spent investigating the sample objects and the subsequent IRs for the SHAM \([r (6) = -.29, p = .084]\) or DHPC \([r (6) = -.51, p = .195]\) group. In addition, no significant correlation was found when a difference-score was used instead of IRs for both the SHAM \([r (6) = -.05, p = .89]\) and DHPC \([r (6) = -.66, p = .07]\) group.

Similarly to Experiment 3, these results do not support the hypothesis that repeated exposures to the sample object will result in progressively greater investigation ratios. This lack of support for the hypothesis was made evident by the lack of a progressive increase in the magnitude of average IRs across tests with repeated sample object exposure. No correlations were found between sample object-investigation time and either IRs or difference-scores during the test.

However, contrary to Experiment 3, rats in the DHPC group could not discriminate between the sample and novel object during the initial test as indicated by an average IR that was not different from chance, further demonstrating the unreliability of novel-object preference in rats with hippocampal damage.

5. General discussion

Our hypothesis stated that the amount of time rats spent investigating the sample object during the familiarization phase of the NOP test procedure would be related to the subsequent magnitude of IRs, obtained during the test phase. This hypothesis was based on the assumption that if IR magnitude reflects better memory for the sample object, then better encoding of this object during familiarization should produce greater IRs during the test. The results of the present study did not support this hypothesis. We found that no significant correlation existed between the amount of time rats spent investigating the sample object during the familiarization phase and the magnitude of IRs, or difference-scores, obtained during the test phase, on all occasions in this study. These results suggest that a correlation between sample object-investigation time during familiarization and the amount of novel-object preference during the test is not a necessary condition for novelty preference to be observed in the NOP test.

The results of the present study did not support the findings of Albasser et al. (2009) who showed a positive correlation between the time spent investigating the objects during familiarization and subsequent difference-scores during the test. However, we do not deny the possibility that such a correlation can occasionally be found.

Rather, the results of the present study suggest that the amount of investigation of the sample objects during familiarization is an unreliable predictor of the rats’ novel-object preference during the test and that it is not likely that there is a direct relationship between the amount of encoding of the sample object during familiarization and preference for the novel object during the test. This, in our opinion, mitigates NOP as a test of “pure” object-recognition memory.

In Experiment 2 of the present study, when the amount of time spent investigating the sample object during familiarization was manipulated, above-chance preferential investigation of the novel object depended on a minimal amount of sample object-investigation time during familiarization (90 s), beyond which no increase in the magnitude of the average IRs was found. This result is consistent with the findings of Albasser et al. (2009). In their study
the amount of preference for the novel object increased sharply with sample object-investigation times above 90 s Albasser et al. (2009) (Fig. 3A).

Manipulating the amount of time rats investigated the sample objects during familiarization in Experiment 2 did not result in a gradual significant increase in the magnitude of IRs or difference-scores from the 5-s to the 120-s group. This finding provides experimental support for the results of the correlational analyses in Experiment 1.

However, Experiment 2 was limited by the fact that only one sample-investigation condition (120 s) followed the step function marked by the 90-s condition. It is unknown whether even more sample object investigation during the familiarization phase would have resulted in subsequent IRs of greater magnitude. The addition of such a condition would be impractical for two reasons: first, rats require increasing amounts of time to achieve the target investigation time during the familiarization phase; second, we found that rats seemingly lose interest in the objects when investigation time goes beyond 120 s. Instead, we used an alternate way to manipulate sample object exposure in Experiments 3 and 4.

The results of Experiments 3 and 4 showed that repeated exposures to the same sample object, either across days or within a single session, did not result in progressively larger IRs. This was the case for both the SHAM and DHPC groups. Moreover, there was no correlation in either group between the amount of time rats spent investigating the sample object during the initial familiarization phase and their subsequent IRs or difference-scores on Day 1 of Experiment 3 and Test 1 of Experiment 4 when rats were tested following a regular familiarization session, providing further support for the findings of Experiment 1.

Altogether, these findings suggest at least two factors that must be taken into consideration when interpreting the results of NOP testing: (1) that directly comparing the IRs or difference-scores across groups of rats comes with at least one important limitation. When comparing groups of rats based on average IRs, one must assume that IR-magnitude is reflective of how well rats encoded a representation of the sample object, an idea not supported by the present findings. Instead, we propose that it may be more appropriate to evaluate rats’ memory for the sample object based on whether a group’s average IR significantly differs from chance or not. (2) The NOP test is not likely to be a pure measure of object-recognition memory. That a group of rats obtains a greater IR than another may reflect more than object-recognition memory in that group. The IR may also reflect the amount of habituation, dis-habituation (Vinogradova, 2001) as well as neophilic or neophobic responses to the objects (Ennaceur et al., 2006a, 2009, 2006b). In return, this may drive the IR more or less further above chance. Investigatory preference for the sample object during the test may also involve behaviors that are not directly observable by the experimenter that may only be detectable by physiological measures (Jeewaje et al., 2008).

5.2. The influence of context

5.2.1. The role of contextual cues

The IR may also partly reflect the extent to which contextual cues play a role in the retrieval of information about an object. For example, reactivation of the familiarization context during the test may trigger the representation of the sample object. This may occur through pattern completion (Kirwan et al., 2005; Rudy and Sutherland, 1995), in which even partial retrieval of the context could result in the retrieval of the entire familiarization episode, including a representation of the object.

Related to this idea, it is proposed that novelty preference may be induced by a violation of expectancy (Fortin et al., 2002; Kumaran and Maguire, 2007a, 2007b). In the NOP test, expectancy may develop through the exposure to contextual cues during familiarization. Through pattern completion (Kirwan et al., 2005; Rudy and Sutherland, 1995) these cues are retrieved during the test but a mismatch, induced by the novel stimulus, is detected by the hippocampus. This mismatch may then be translated into automatic orientation towards the mismatching stimulus.

There is some evidence that, during familiarization, the amount of exposure to contextual cues may play a role in how well rats can discriminate between objects during the test, independently of the time spent investigating the objects themselves. In one study (Wilkinson et al., 2006), rats were familiarized with the test environment with no objects for either 1, 3 or 10 min prior to being exposed to sample objects. Another group of rats was familiarized to an environment that differed from the one they were subsequently tested in. Rats exposed to the test environment for 3 and 10 min spent significantly more time with the novel object during the test than rats pre-exposed for only 1 min or rats pre-exposed to a different environment. The results of these studies suggest that discrimination between the novel and sample object depends on both environmental familiarization and familiarization to the objects.

In another study (Besheer and Bevis, 2000), rats were given different amounts of exposure to the test environment before being exposed to the sample objects. In one condition, the rats were exposed to the sample objects during all 3 min of a 3-min familiarization phase (3/3). A second group of rats was exposed to the objects for the last 3 min of a 10-min familiarization phase (3/10) and a third group was exposed to the objects for the full 10 min (10/10). Only the rats in the (3/10) and (10/10) condition showed a significant preference for the novel object during the test. The results of both of these studies are consistent with the findings of Experiment 2 of the present study, in which only the 90-s and 120-s group, the two groups that spent significantly more time in the environment that contained the objects than other groups, were the only ones to have average IRs that were significantly above chance.

5.2.2. Role of the hippocampus

Although the DHPC group in the present study generally had IRs that were below that of the SHAM group, the DHPC group showed evidence that they retained some ability to discriminate between the sample and novel object. The DHPC group in Experiment 3 had a lower average IR than the SHAM group during the initial test, using a 2-h retention interval, but at the same time this average IR was significantly above chance, indicating that they could discriminate between the objects. However, in general, rats with hippocampal lesions produced unreliable preference for the novel object across the present experiments.

This general impairment may not have only been due to an object-recognition memory deficit as is often asserted (Clark et al., 2000) but to a deficit in processing other types of information dependent on the hippocampus, such as the processing of con-
textual information. Therefore, deficits in processing this type of information would impair rats with hippocampal lesions in the detection of a mismatch between the context of familiarization and that of the test, as described above (Kirwan et al., 2005; Rudy and Sutherland, 1995).

These results are interesting in view of the controversy that surrounds the role of the hippocampus in object-recognition memory (Mumby, 2001). The results of the present study do nothing to settle this controversy. Instead, they suggest that the interpretation of rats’ performance on the NOP test, after being subjected to various treatments, may vary depending on which measure one chooses to use or emphasize, sometimes rendering the results ambiguous or difficult to interpret. For example, what is perceived to be an object-recognition deficit in rats subjected to any form of treatment may be inferred if rats do not show an investigatory preference for the novel object. This can be done by comparing a group’s average IR to what could be expected by chance. On the other hand, a significantly above chance IR signifies that the rats spent significantly more time investigating the novel object than the sample object, possibly suggesting that they recognized the sample object. This could be inferred irrespective of whether a significant difference exists between the IR of that group versus another. This indicates that, in the NOP test, whether a group of rats have above-chance IRs can be an important factor in determining the effects of treatments involving pharmacological, genetic, neurological and other manipulations on what is believed to involve object-recognition memory.

6. Conclusions

The results of the present study provide converging evidence that IR-magnitude does not constitute an accurate measure of memory strength in the NOP test. The results suggest that factors other than the encoding of an object representation during familiarization must play a significant role in retrieving that representation during the test. Also, a lack of investigation of the novel object may not necessarily indicate an impairment in the retrieval of the representation of the sample object, as manipulations aimed at increasing the familiarity of the sample object had no effect on the preference for the novel object during the test. Furthermore, no correlation was found between the amounts of time spent investigating the sample object during familiarization and the subsequent novel-object preference during the test.

Given the widespread use of the NOP test in many fields of study, we feel that the issues raised in the present study are of utmost importance if one is to make a proper assessment of rats’ ability to recognize objects. There is little, if any, evidence that the NOP test reliably indicates how well an animal has learned about an object.

Although the NOP test may be an excellent laboratory paradigm for studying novelty preference, its usefulness as a test of recognition memory is limited to situations where a group of subjects display novel-object preferences that are significantly different than what would be expected from chance. In such instances, it can be safely inferred that some or all of the rats in the group were able to recognize the sample object. Caution should be used, however, when drawing conclusions about why a group of rats fail to display a significant preference for the novel object, as this may not always be a reliable sign that rats are unable to recognize the sample object. Finally, the purpose of the present study was not to discourage the use of the NOP test in the assessment of memory but to raise awareness of the many factors that may contribute to misinterpretations of the findings derived from the use of this test.

Coming to a pure measure of object-recognition memory devoid of all confounds may be impossible to achieve. However, the mechanisms that underlie novel-object preference in the NOP test may go beyond those involved in object recognition, such as those associated with non-declarative memory processes. We therefore suggest that the results obtained with NOP testing be corroborated with better measures of object recognition per se. Such measures could be obtained from the use of tasks in which animals engage in reinforced behaviour, such as in the delayed matching and non-matching-to-sample task (Mumby, 2001). Although other issues are associated with the use of these tasks, such as the necessity for food deprivation and rule learning, considerably less alternative mechanisms other than object recognition can be postulated to explain the observed behaviour.

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