

Insignificant influence of test order on cognitive behavior in Wistar rats

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Abstract

Objectives: The aim was to evaluate the effects of test order in behavioral performance of Wistar rats in cognitive performance tasks. **Materials and Methods:** The rats were tested with both object recognition and object location tasks in different test orders with an inter-test interval of 1-day. **Results:** An open field box sized 60 × 60 × 40 cm was associated with better experimental outcome compared with box sized 100 × 100 × 50 cm. Test order has insignificant effects on the exploration activity of rats with the 1-day inter-test interval. **Conclusion:** Test order of the cognitive performance tasks with inter-test interval of 1-day did not influence the performance of Wistar rats.

Key words: Exploration, novelty-preference, object location, object recognition

INTRODUCTION

Novel object recognition (NOR) task has been extensively used to assess and evaluate the recognition ability in animal models of memory, particularly in rodents.^[1] It exploits the innateness of the rodents, which is the spontaneous exploration behavior on novel objects in preference to familiar objects.^[1,2] NOR task has become the test of choice in recognition studies as this task requires no rule learning, food reinforcement or reward, but requires a little pretraining or habituation.^[3,4] Object location task employed the novelty-preference paradigm same as NOR task for assessment of spatial working memory, which is part of memory responsible for spatial environment and location or placement.^[5]

Testing history or test order might influence the performance of animals from one test to another.^[6,7] Few studies have addressed the effects of test order on

performance. However, most of these studies are limited to test of anxiety and exploration activity, and lesser extent to cognitive performances.^[6-9] The effects of test order on cognitive tasks are unclear. Hence, in this study, the influence of test order on cognitive performance of rats had been evaluated in NOR and object location tasks.

MATERIALS AND METHODS

Animals

A total of 18 male Wistar rats were purchased at aged 2 months with weight of 200-220 g on arrival. They were kept under controlled environment conditions (23°C ± 1°C, 50-60% humidity, 12 h light/12 h dark cycle) with free access to food and water. The rats were housed in groups of three and allowed to acclimatize for 1-month before the start of the experiment. All experimental procedures were carried out during the light phase. All animal care and handling were conducted in accordance with approved use by Animal Ethics Committee of university.

Optimization method prior to experiment

Before the experiment, optimization method had been done to select the open field arena size that suitable for Wistar rats. Six rats were used and assigned to two session of NOR task in two different boxes with inter-test interval of 1-day. The NOR task described in this study

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is a modification of the protocol used by Ennaceur and Delacour.^[2] The test was performed in two open boxes with different dimensions and wall paints: (i) box P: 100 × 100 × 50 cm (width × length × height), made of Plexiglas with the inside walls painted with matt grey and a clear Plexiglas floor;^[5,10] and (ii) box Q: 60 × 60 × 40 cm, made of Plexiglas with the inside walls painted with black and a clear Plexiglas floor.^[11] Any rat that exploring the objects for 12 s or more within 3 min period of sample phase was included and rats with a minimal level of object exploration of 5 s (novel + familiar <5s) during choice phase were excluded in the experiment.^[11]

Test order effects

Testing comprised of two tasks, namely NOR task and object location task. Twelve rats were randomly assigned into two groups with two different orders of test; test order 1 (NOR → object location task) or test order 2 (object location task → NOR). All these tasks were run in the selected open field arena from the optimization method with an inter-test interval of 1-day. The objects used for discrimination were beverage cans (7 × 7 × 12 cm) covered with colored papers and filled with sand in order to ensure that they could not be displaced by rats. The objects were placed in two adjacent corners of the arena that approximately 10 cm from the sidewall.

The day before the behavioral test, the animals were habituated in the boxes for 5 min. On the test day, the animals were placed in the dark experimental room at least 30 min before testing. Subsequently, animals were allowed to explore two identical objects (A1, A2) in a sample phase (T1) and then followed by a choice phase (T2) in which they are reintroduced to a familiar object (A3) that identical to object in the first trial (A1), and a novel object (B) that replacing the familiar object (A2) [Figure 1]. Each trial lasted for 3 min with an interception of 5 min inter-trial interval (spent in the home cage). The boxes and objects were cleaned between each trial using 10% ethanol to avoid olfactory trails during the habituation session and experiments. The position of objects was arranged in a balanced manner and objects were randomly exchanged to reduce potential place and object preferences effects.

Exploration was defined as the animal directing the nose to the object at a distance ≤2 cm and/or touching it with the nose. Turning around or sitting on the object was not considered as exploratory behavior.^[2]

For object location task, the procedures were similar as NOR task except for the choice phase. In the object location task, two identical sample objects (A3 and A4) were used in the choice phase, in which object A3 replaced

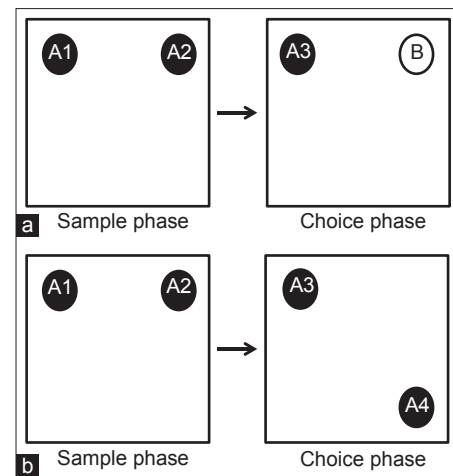


Figure 1: Schematic representation of sample and choice phases in (a) novel object recognition task and (b) object location task. Different object is represented by different symbols that is, A and B

in the same position as A1 and A4 was placed at corner adjacent to original position of A2 (two objects were in diagonal corners) [Figure 1].

Measurements and statistical analysis

The basic measure was the total time spent by rats exploring each object (namely A1, A2, A3 and B or A4) in both trials (T1 and T2). The following variables were also calculated according to the method of Ennaceur and Delacour:^[2] $e1 = A1 + A2$; $e2 = A3 + (B \text{ or } A4)$. These measures represent the total time spent by rats in exploring both objects during T1 and T2, respectively.

Statistic data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 21 (IBM Corporation, Armonk, New York, US) and normality of data were tested. Student's paired *t*-test (or Wilcoxon signed-rank test if data not normally distributed), two-tailed, was performed within the same experimental group to compare time spent for exploring the two identical objects (A1, A2) during sample phase, the time spent for exploring the familiar object (A3) and novel object (B) or novel location (A4) during choice phase or the total exploration time in the sample ($e1$) versus choice phase ($e2$). Student's *t*-test (or Mann-Whitney U-test if data not normally distributed), two tailed, was performed for intergroup comparison to test any significant differences in $e1$ and $e2$ values. In all statistical tests, statistical difference was set at a probability level of $P < 0.05$.

RESULTS

Optimization method prior to experiment

In this experiment, the number of rats included after exploring in both open field box *P* sized 100 × 100 × 50 cm and box *Q* sized 60 × 60 × 40 cm

were 3 (50%) and 6 (100%) respectively. The data of three rats after exploring box P were excluded due to lack of exploratory activity. All rats spent no significantly different in time exploring both objects during sample phase, as well as in time exploring familiar and novel object or location during choice phase [Table 1]. By comparing e1 and e2 values of both tasks, the behavioral responses of rats in both groups were no significant difference in both test orders [Table 1]. However, the rats had higher frequency of approach to the object during sample phase in box Q (10.8 ± 1.4) compared with box P (6.0 ± 0.0) ($\chi = -2.02, P < 0.05$) as revealed by Mann-Whitney U-test. The rats showed absolute longer latencies of first approach to the object during choice phase in box P (55.3 ± 39.6 s) than the rats in box Q (10.7 ± 4.2 s), but yet this difference was not significant.

Test order effects

Under the effect of different test order, all rats spent no significantly different in time exploring both objects during sample phase, as well as in time exploring familiar and novel object or location during choice phase [Table 2]. By comparing e1 and e2 values of both tasks in different

groups, the explorative activity of rats were no significant different in both test orders although the rats had low exploration activity in NOR task in test order 2 compared to those in test order 1.

DISCUSSION

Different open field arenas have been exploited in NOR tasks to assess recognition ability of rats. The open field arenas used in previous studies varied from $100 \times 100 \times 50$ cm to $60 \times 60 \times 40$ cm.^[2,5,10-12] Therefore, the exploratory activities of rats were evaluated in the bigger box (box P: $100 \times 100 \times 50$ cm) and the smaller box (Box Q: $60 \times 60 \times 40$ cm) prior experiment in order to optimize the behavioral performance of Wistar rats for these behavioral tasks.

High exclusion rate for rats that exploring in box P indicates the rats have a lower tendency to explore in a bigger arena. In addition, the rats in box Q demonstrated higher frequency of approach during sample phase. This showed that Wistar rats have higher exploratory activities in smaller open field arena. Hence, box Q was selected for the subsequent experiment.

Table 1: Exploratory activity of the rats in different open field arenas in NOR task

Open field size	n	Sample phase			Choice phase		
		A1	A2	Total exploratory time (e1)	A3	B	Total exploratory time (e2)
Box P	3 ^a	9.0±4.5	5.3±3.5	14.3±1.5	11.3±8.3	13.7±1.9	25.0±9.2
Box Q	6	5.3±1.7	11.7±1.6	17.0±1.0	6.7±1.6	10.0±2.5	16.7±2.4

The size of open field arenas does not demonstrate significant changes in these exploratory activities. Data express as mean in seconds (\pm SEM); n=Number of animals per group. The size of box P and Q are $100 \times 100 \times 50$ cm and $60 \times 60 \times 40$ cm respectively. ^an=3 had been excluded due to lack of exploratory activity. A1, A2: Identical objects; A3: Familiar object; B: Novel object; SEM: Standard error of mean; NOR: Novel object recognition

Table 2: Exploratory activity of the rats in different test orders

Exploration activity in seconds (mean±SEM)	Sample phase		
	A1	A2	Total exploratory activity (e1)
Test order 1: NOR→object location test (n=6)			
NOR	5.3±1.7	11.7±1.6	17.0±1.0
Object location task	8.3±1.9	7.7±1.0	16.0±1.8
Test order 2: Object location test→NOR (n=6)			
NOR	2.3±1.0	5.2±1.0	7.5±1.3
Object location task	4.3±2.5	6.8±2.4	11.2±3.9
Exploration activity in seconds (mean±SEM)	Choice phase		
	A3	B or A4	Total exploratory activity (e2)
Test order 1: NOR→object location test (n=6)			
NOR	6.7±1.6	10.0±2.5	16.7±2.4
Object location task	5.2±1.4	8.8±1.8	14.0±2.5
Test order 2: Object location test→NOR (n=6)			
NOR	2.8±0.6	9.7±3.5	12.5±3.8
Object location task	5.5±2.5	5.3±2.0	10.8±3.1

The test order does not demonstrate significant changes in exploratory activities during (i) sample phase and (ii) choice phase ($P \geq 0.05$). NOR: Novel object recognition task; A1, A2: Identical objects; A3: Familiar object (in NOR task) or identical object at familiar location (in object location task); A4: Identical object at novel location (in object location task); B: Novel object (in NOR task); SEM: Standard error of mean

There are advantages to use these both cognitive behavioral tasks to assess the recognition and spatial memory for same rodents. Multiple behavioral responses can be evaluated in the same animal and this allows studying the correlation of central nervous system function with different behavioral response simultaneously.^[6] In ethical viewpoint, using the same animal for multiple behavioral assays can help to reduce the number of animal needed in a particular study.^[6] Besides, this is also beneficial in term of economic criteria.

However, behavioral performance of animal may be influenced by the previous testing experience due to prior training effects when evaluating multiple behavioral responses in the same animal.^[6,8,9] It had been reported there were possibility of training history effects that influence the responses of animals from one test to another in certain tasks, such as open field tests and elevated plus-maze tests.^[6-8] In order to reduce this prior training effect, the order of tests within a study is recommended to arrange in such a manner from least to most invasive.^[6] Namely, anxiety and exploratory activity tests should be carried out first and followed by cognitive behavioral tests in latter experiment.^[7] In cognitive tasks, most of the studies had been carried out with NOR task firstly and followed by object location task.^[5,10,12] The effects of testing history in these cognitive tasks are unclear.

The current study investigated the effects of test orders on cognitive tasks in Wistar rats. The findings showed that the behavioral responses of the rats in cognitive tasks, namely NOR and object location tasks were not altered by prior test history. This indicates the test order appeared to have no significant impact on cognitive performance of rats. Controversially, it have been demonstrated that the test order has influences on behavior in mice, particularly by reducing exploration activity.^[6,7] However, in current study, the exploration time (namely e1 and e2) were not significant different in both tasks between different test orders. Hence, it is suggested that the test variables in the NOR and object location tasks are not affected by test order.

Although the results suggested that test order may not have substantial impact on behavioral response in Wistar rat, it is important to aware that behavior can be easily affected by other confounding influences such as genetic factor.^[13,14] Other strains of rodent might sensitive to test order on cognitive tasks. Hence, studies on different strains are needed to reconfirm the test order effects on cognitive tasks. In addition, inter-test interval can also be one of the confounding factors for cognitive performance. Although little is known regarding the appropriate inter-test interval for each strain of rodents, in this study, an inter-test interval of 1-day was used. Generally, it has been suggested that an inter-test interval of 1-2 days has little effect on overall

performance.^[15] It is unclear whether the rapid inter-test interval that <24 h can influence the result of test orders. On the other hand, the results showed that the rats failed to discriminate either the novel objects from familiar object in NOR tasks or the novel location from familiar location in object location task significantly although the rats tend to spend more time to explore the novel objects in choice phase of both tasks. This may be due to the limited samples in this study, which potentially attributed to false negative result.^[16]

CONCLUSION

In summary, the present study showed that the test order of the cognitive tasks with inter-test interval of 1-day has no significant effect on the performance of Wistar rats. Such optimization studies are needed to confirm the effects of test order on cognitive performances for commonly used strain of rodents in order to produce a reliable experimental data.

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