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REVIEW ARTICLE

BEHAVIORAL TESTS USED IN THE EVALUATION OF LEARNING AND MEMORY IN EXPERIMENTAL ANIMALS

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ABSTRACT

Learning and memory regulate the necessary mental processes such as evaluating stimuli from the environment and developing appropriate behaviors. People consciously or unconsciously resort to memory functions in every process they perform. Experiences that emerge through interaction with the environment lead to changes and shaping of behaviors. Thus, learning of different behavioral phenomena takes place. Memory ensures that this learned behavioral information is stored and used when necessary. The central nervous system is capable of storing and processing information in mammals. Research on learning and memory in humans is limited due to ethical rules. Therefore, different experimental tests evaluating learning and memory states in rodents have been designed to find effective treatment strategies. The aim of this review is to provide information about the most commonly used learning and memory tests in experimental animals.

Keywords: learning and memory, behavioral tests, rats and mice

INTRODUCTION

Learning and memory is one of the main topics studied in neuroscience. Learning is acquired knowledge, while memory is the process of coding, storing and recalling when necessary (1). In the formation of learning and memory; There are processes such as receiving information with sense organs, processing, storing, coding of this information in the central nervous system, establishing a relationship between current information and previously acquired information, and remembering this information when needed (2). The central nervous system has the potential to store and use information in mammals.Studies show that the cerebral cortex, association areas and limbic system play a role in the learning and memory process (3). Among these areas, the parieto-occipito-temporal association center has functional sub-regions such as analyzing spatial coordinates, comprehending language, preprocessing visual language (reading), naming objects. The prefrontal association area is defined as an important structure for the processing and maturation of thoughts (2).

The limbic system consists of the hippocampus, hypothalamus, parahippocampal area, gyrus cinguli, subcallosal formations, anterior nuclei of the thalamus, septum and amygdala (4). There are two centers in the limbic system that affect behavior;reward center, penalty and escape areas.Animal experiments have shown that behaviors that do not lead to reward or punishment are very difficult to understand (5). If the stimulus causes reward or punishment, the cortical response is not extinguished by repeated stimulus and becomes stronger; this is called re-enhanced response (6). Experimental studies have shown that

Table 1. Tests of learning and memory and number of related articles in PubMed (searched in June 2021)

Behavioral Task	Primary Measure	Operant Schedule	Primary Measure	Number of articles in the last 1 year (2020-2021)
Morris Water Maze	Spatial memory	Diferential reinforcement: high responding	Attention and initiation of motor output	1,173
Passive Avoidance	Conditional avoidance learning	Stimulus detection	Sustained efort, motor output	255
Y-Maze Test	Working and reference memory	Diferential reinforcement: high responding	Sustained efort, motor output	228
Novel object recognition test	Recognition memory	Stimulus detection	Attention and initiation of motor output	149
Barnes maze	Spatial memory	Diferential reinforcement: high responding	Sustained efort, motor output	90
Radial arm maze	Working and reference memory	Diferential reinforcement: high responding	Sustained efort, motor output	53

damage to the limbic system has significant effects on behavior. With the damage of certain areas of the limbic system, memory is also damaged (4).

The hippocampus is very important in storing new information. Because the hippocampus is one of the most important pathways from the limbic reward and punishment system. The limbic reward center is stimulated by a sense of pleasure, happiness, and reward. The limbic punishment center is stimulated by stimuli and thoughts that cause pain and discomfort. Especially the hippocampus, thalamus dorsa-medial nucleus are effective in deciding which thoughts are important enough to be kept as memories on the basis of reward and punishment (1). While patients with left and right hippocampus removed do not have a serious effect on previously recorded information, the ability to transfer verbal and symbolic memories from short-term memory to long-term memory is greatly impaired in these patients. These patients are unable to reconstruct long-term memory to form the basis of intelligence. This is called anterograde amnesia. The hippocampus developed as part of the olfactory cortex. It plays an important role in determining which foods to eat in the most primitive animals, which objects can be dangerous because of their smell, sexually inviting odor, and taking many vital decisions (7).

The amygdala is a gray matter mass and is one of the medial temporal lobe structures located between the uncus and the parahippocampus. In human and animal studies, it has been shown that the amygdala is necessary for the learning of fear and emotional processing, the formation of conditioned experiences and fear response, the storage and acquisition of memory (5).

There are many animal behavior experimental models described to date for the evaluation of cognitive and locomotor abilities of rodents. With these models, many features such as anxiety, autonomic functions, learning, memory and locomotor activity are evaluated in rats. There is no behavioral test model that has all the criteria of scientific proficiency at the same time, and most of these models cannot provide results with 100% accuracy. However, with the developments in scientific technology, behavioral tests can be done in more and more ideal criteria. The purpose of this review is to give information about the most commonly used learning and memory tests in experimental animals (Table 1).

Learning Memory Tests

1. Morris Water Maze

The Morris Water Maze (MWM) is perhaps the best

known and most widely used spatial learning and memory test in rodents. It was developed by Richard Morris at St Andrews University in (8).

The MWM consists of a circular pool of water containing an escape platform and is surrounded by visual spatial cues that the rodent can use to help locate the escape platform (9). The standard protocol requires rodents to swim from an initial position to a previously unknown escape platform that is submerged below the opaque water surface and therefore hidden from view (10). This task is based on the rodent's dislike of the aquatic environment. Therefore, it is not difficult to motivate animals to swim around the pool in search of a submerged platform that provides a temporary escape from the water. The convenience of the task to the researcher is that the water prevents them from using proprioception to assess the distance traveled by the animals, forcing them to rely on the visual landmarks given in their surroundings. The trick is to release the animals from four different zones (9). The size, location, and visibility of the escape platform often vary depending on the lab or MWM protocol. In general, the test is completed with 3-5-day trial. The advantage of the 5-day trial is that the 1st and last trial of the day starts at the same place, allowing separate predictions for intraday or inter-day learning / memory (11,12). All trials are recorded as video. Data is analyzed via the Etho-VisionXT system from Noldus Information Technology (EthoVision XT, Noldus Information Tech, Wageningen, The Netherlands). In addition, data such as the animal's swimming path, distance traveled, search strategy/efficiency (eg, direct and circular swims) are also evaluated in this test (13). The dependent variables used for the analysis are the latency (seconds) to the platform for the learning trials, the number of platform transitions, the platform position first pass latency (seconds), and the speed (cm/sec) for the research trial (14).

Spatial learning can be assessed by measuring the latency to find the escape platform in many trials, and memory is often assessed by removing the platform and measuring a preference for the quarter in which the platform was previously located (10, 15). In addition to the standard protocol, reverse learning requires rodents to learn a new location of the platform and is used for lesion studies of the prefrontal cortex, striatum, and cerebellum (11,15,16).

2. Passive Avoidance Test

Passive Avoidance test is a classic test for long-term memory. It is a highly productive behavioral test as it is based on one-attempt learning and does not require prior training. The basis of the test is the passive avoidance task. It is a fear-motivated test applied to evaluate fear-based conditional avoidance learning and memory in rodents based on the relationship established between a specific deterrent event and a specific environmental context (17).

In this task, animals tested immediately after the experience show low memory and the rate of memory retention increases as the task is repeated (18). Therefore, administration involves taking the animal back to the home cage after exposure and returning it for testing the next day. The test is carried out in light (white and 24 V, illuminated with a 10 W bulb) and dark (black and dark) boxes, which are two equal sizes and have a passage between the two compartments. The test consists of the acquisition (training) phase and the testing phase. The animal is first placed on the well-lit box side and access to the dark compartment through the passage is allowed for 5 minutes. When the animal enters the dark side by its natural instinct, the opening closes and receives a 2 s mild foot shock (0.5 mA) with the door closed. The animal is allowed to stay in the dark box for 20 seconds after the foot shock for habituation. The animal is then taken into its own cage and on the second day, if the animal that is placed in the light compartment realizes that it will receive an electric shock and does not enter the dark compartment, it is considered to have learned the event.Input lag is taken as a measure of memory power(18). The data wereanalyzedusing ANY-maze video tracking software (Stoelting, WoodDale, IL) (17).

The results of this test may give accurate results regarding memory. However, differences in results can also be caused by a number of unrecognizable reasons. For example, genetic or drug induced hyperactivity or reduced anxiety will lead to shortening of delayed entry, while hypoactivity and increased anxiety appear to be superior memory in appearance. In addition, groups may differ in their sensitivity to shock. The task is very powerful when used to test drug effects on memory consolidation. In this case, drug administration follows the training session and therefore there is no significant drug concentration in either the training or testing phase.

It is also an excellent test to identify whether a drug affects memory encoding, consolidation, or retrieval depending on the time of drug administration. However, when used to compare the effects of chronic drug treatments or genetically modified mouse strains, the results need to be interpreted with caution (17).

3. Y-Maze Test

The Y-maze test is used in short-term spatial working memory studies and evaluating spontaneous change performance. The Y-maze apparatus is manufactured from three Plexiglass arms (50 × 10 × 30 cm3) called A, B, C, which are adjusted at an angle of 120 ° to each other. It does not require any special training for the animal and it is easy to perform this task (13,19).

If the animal chooses a different arm than the one it came from, that choice is called change. While this is considered the correct answer, returning to the previous arm is considered an error (13). The test has many variations. Alternatively, a single arm can be blocked initially, then unblocked on subsequent attempts. In this case, the result could simply be whether the rodent entered the previously blocked arm first (20).

Test results are usually reported as% change. The most common primary results are the total number of branch entries and the number of "spontaneous changes" or "triples" defined as entering each of the three arms without returning to a previously discovered arm (13,20).

In the test, the animals are placed in the middle of the apparatus and allowed to roam freely in the maze for an 8-minute period. The number of spontaneous changes is recorded using a monitoring system (EthoVision XT, Noldus Information Tech, Wageningen, The Netherlands) or manually. Spontaneous change (%) is taken into account when animals enter the three arms without repeating and the rate of spontaneous change; The% change is calculated by the formula = [(change count) / (total weapon entries-2)] 100 (15,21).

Brain regions associated with Y-Maze performance may vary depending on the experiment. Studies have suggested that regions including the hippocampus, prefrontal cortex,basal forebrain, striatum, and cerebellum are included (20).

One inherent problem with the task is that it is self-paced. If the animal is afraid, tired, or otherwise unwilling to explore, depending on the circumstances, it can stay on each arm for minutes, which weakens the memory of the previous place and also makes it less dependent on real working memory. On the other hand, if the animal is hyperactive, it may start to rotate. This gives 100% change points in the task without creating any demands on the working memory. Therefore, in addition to the percentage change in this task, the most important factor is the direction deviation (eg, the number of turns in the dominant direction as a% of all turns). However, this is rarely reported (20).

4. Novel Object Recognition Test

The new object recognition (NOR) test is a commonly used behavioral test to evaluate hippocampal-linked recognition memory, based on the tendency of animals to spend more time searching for a new object than a familiar object (17). The NOR test is widely used as it does not require extensive training, exposure to deterrent stimuli, water or any food deprivation. However, animals need to get used to the test environment (22).

To test recognition, rats are placed in an empty box (40x50x50 cm) for habituation 1 day prior to testing. On the first day of the test (the familiarization phase), mice are given 5 minutes to discover two identical objects in the recognition box. On the second day (after 24 hours, the testing phase), the researcher brings the mice back to the open field recognition box, which contains two objects, the first object discovered in the familiarization phase and a newly introduced object.At this stage, each animal is given 5 minutes to explore two objects freely. A time spent exploring object with any video software is recorded (23,24).

In the recorded test phase, the contact time with objects is measured and a preference index is calculated. The discovery of an object is defined as placing the animal's nose within 2 cm of the site of the object. The assumption is that the animal will spend more time exploring the new object than the familiar one. However, if both objects are spending equal time exploring, it indicates that the animal does not remember the object it has seen before. The discrimination index is calculated according to the following formula: Discrimination index (time spent on new objects / total time spent exploring both objects) 100% (25).Data is analyzed via the Etho-VisionXT system from Noldus Information Technology (EthoVision XT, Noldus Information Tech, Wageningen, The Netherlands).Performance in the Novel Object Recognition test and similar tests of

recognition memory are thought to involve function of the hippocampus as well as cortical areas (26).

5. Barnes Maze Task

The test was first developed by Carol Barnes in 1979. The Barnes maze (BM) is based on the assumption that the animal placed on the surface of a platform must learn and remember the location of the escape box. Thus, the evaluation of hippocampal-dependent spatial learning and memory in animals is realized. Experimental BM has also been found to affect both short-term and long-term memory (27).

The Barnes maze consists of a circular platform with a diameter of 122 cm and a height of 100 cm with 18 holes in series around it, and a black escape box (20*15*12 cm) hidden under any of these holes. The platform is illuminated by incandescent light (500 W, 1000 lux) located 1 meter above its surface. There are four different signs with different shapes around the table to facilitate learning. In the test, a mild deterrent stimulus, such as a light or fan above the apparatus, provides enough motivation for rodents to find the escape hole and seek the hole in the top of the escape box to escape (28).

The task is divided into 2 stages: 1st stage training trials, 2nd stage research test. Rodent searches the escape box for 3 minutes with the effect of stimuli. If he cannot find the escape box, the researcher slowly prompts him to locate the box. Each animal makes 1 trial per day during the training phase. On the test day, the escape box is removed, and the time spent in the quadrant where the escape box was originally placed is recorded with an overhead video camera, and the data obtained is analyzed. After each test, the platform is purged with 70% ethanol.

The first part of the mission, namely the acquisition phase followed by the acquisition research trial, allows the evaluation of spatial learning and spatial memory. This part is thought to be related to hippocampus function (27, 29,30, 31). The second part of the task (i.e. adverse learning trials) allows the assessment of cognitive flexibility associated with frontal cortex function (32, 33).

While the BM is not as popular as the Morris water maze or the radial arm labyrinth, it has some advantages that make it an alternative. Carol Barnes' original idea was not to use external rewards or punishments to motivate animals to flee from a brightly lit platform. Thus, performance in BM is not affected by individual variability in hunger or thirst, unlike the radial arm labyrinth. BM is also less stressful for animals (particularly mice) than the Morris water maze because there is no immersion in water and the subsequent high corticosterone increase that can affect the animals' performance.

The recorded data is used to compare the behavior of different animals, to measure the total distance traveled or the time spent searching for escape and unavoidable holes. One of the systems in which data is used is Any-maze. With this digital tracking software, the delay in reaching the target hole, the distance traveled and the time spent in the target quadrant can be measured. Another widely used program is Ethovision, which measures the escape delay, the total distance traveled, the number of errors and the running speed of the animal. Another program in the BM where data is recorded is the Opto-Varimex-Magnus animal monitoring system. This program records the position of the animal, the distance it moves, and the time the animal does not walk (twitch, make circular movements, etc.) despite walking, resting and moving (28).

The most frequently cited criticism of the BM mission is that it is less susceptible to genetic changes than the Morris water maze (34). There were also comparative studies showing differences in the efficacy of various mouse and rat strains in the BM task, so this should be taken into account when choosing this task.

6. Radial Arm Maze

The Radial Arm Maze (RAM) test consists of a raised platform with several evenly spaced arms (mostly 8) radiating from a small, open central area and visual cues placed around the maze (35). Both spatial memory (location learning) and non-spatial memory (relational learning) can be assessed in RAM, which uses rodents' tendency to discover, learn, and remember different spatial locations of a food supplement (36).

Although the number of arms used today is different from the original, the conceptual basis of the test is the same in all cases. More number of arm aim to make the task more difficult and improve the method (15). However, the experimenter should also be aware of the disadvantages of RAM. Animals should be modestly deprived of food (to sufficiently increase motivation for optimum performance) and the adaptation and learning process can be quite laborious (36).

Traditionally, during habituation, the animal is placed on the central platform of the RAM and is free to

explore the maze and receive rewards for a period of 10-20 minutes. During the test, a prize is placed at the end of each arm (working memory paradigm) or selected arms (combined working / reference memory paradigm). The animal is free to explore the maze and collect rewards until all the prizes are collected or 10-15 minutes have passed. Revisited branches are scored as errors (i.e. failures in remembering that a branch was already visited in that trial). An experiment usually takes 10-20 days in a row (working memory paradigm) or 7-14 days (combined working / reference memory paradigms). Data is record and analyzed via the Etho-VisionXT system from Noldus Information Technology (EthoVision XT, Noldus Information Tech, Wageningen, The Netherlands) (38).

Since the rewards on the arms are collected for each test and the maze is cleared after each training session to remove the odor clues, there is no memory in which arms will be visited in what order in the next test. Thus, only short-term, empirical memory provides information about which arms should be visited and which arms were visited first. Since the test measures empirical memory, it is an assessment of working memory, and more specifically spatial working memory, because the basic clues that guide arm choices are outside the maze (15). Once an animal has learned the protocol, its performance remains stable and the effects of treatments on memory can be tested (eg, drugs, seizures, genetic changes, and various other manipulations) (36,37).

The problem with this test is that animals can solve the maze without using their spatial working memory. One is the use of chain or a series of strategies (i.e. entering each arm in a systematic sequence). An example would be to always turn right or always turn left and enter the adjacent arm. This strategy is effective but spoils the purpose of the test. It is common to run the test this way with free access to all arms, but it may not measure working memory. Performance on RAM depends on the integrity of the hippocampus, frontal cortex, and forebrain cholinergic pathways (36).

CONCLUSION

Behavioral tests applied to various experimental animal models are frequently used to examine the effects of the chemicals under investigation or the different behavioral exposures established. It is anticipated that these tests will be used more and more in the near future. It is important to select the appropriate test according to the experimental protocol, to consider human or environmental factors that may affect animal behavior, and to set a standard. Today, these studies will likely remain a central methodology for studying the mechanisms underlying how the brain captures, integrates, and retrieves information, but the use of tests that combine different behavioral tests will become more common, as they offer a more comprehensive analysis option and large numbers of animals can be tested simultaneously.

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