Video Article

Testing Animal Anxiety in Rats: Effects of Open Arm Ledges and Closed Arm Wall Transparency in Elevated Plus Maze Test

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Abstract

The elevated plus maze test is a behavioral test for assessing animal anxiety in rodents. Although this test is widely applied in the field of behavioral science, conflicting outcomes are often provided from different laboratories. To identify reasons for the different outcomes, we previously focused on arm features, which differ between laboratories, most notably the presence/absence of ledges at the sides of open arms and the transparency/opaqueness of closed arm walls. In a previous report, we used a custom designed container to compare rat behavior on different combinations of open and closed arm designs under otherwise identical experimental conditions, and showed that differences in arm features interfere with experimental outcomes. In brief, open arm ledges significantly increased anxiety-like behavior in rats, while transparent arms may also have decreased this behavior. Furthermore, we verified a higher detection sensitivity of the effect of an anxiolytic drug in a combination of no-ledged open arm + opaque walled closed arm compared with a combination of ledged open arm + transparent walled closed arm. In this report, we introduce our protocol for the elevated plus maze test, together with discussion of the key results from the previous report and our experimental experience. We believe this report will provide useful information for researchers who have employed or who plan to use the elevated plus maze in their studies.

Video Link

The video component of this article can be found at https://www.jove.com/video/56428/

Introduction

The number of patients suffering from mood disorders, including anxiety-disorder and depression, is increasing. Although the development of these disorders involves both genetic and environmental factors, the detailed pathogenic mechanisms are not yet fully understood[1]. Therefore, research needs to focus on uncovering the underlying mechanisms of mood disorders, as well as the development of effective drugs that have fewer side effects.

Behavioral tests for laboratory animals are useful for investigating brain higher function, including emotion. As animal behavior is highly variable, experimental outcomes often differ between laboratories, even if an identical inbred strain was used[2]. Thus, researchers also need to understand the basic features of the experimental devices they adopt in order to provide reliable, precise outcomes in the laboratory.

The elevated plus maze test was originally established to examine emotional reactivity in rodents[3,4]. The entire apparatus is usually located 500 mm above the floor, and consists of two open and two closed arms that make a plus shape. Measurements of arm exploration (including duration and frequency of entries on each arm), distance traveled, and specified behaviors such as stretched-attending posture of subjected animals are recorded during observational periods. By these measures, animal anxiety is estimated as an experimental outcome.

The elevated plus maze test is used throughout the world, but not all laboratories use the same apparatus design[5,6], and these differences may affect the experimental outcomes. For example, differences include the presence or absence of ledges along the open arms and/or the use of transparent or opaque walls for closed arms[5,6]. The ledges along open arms and the use of transparent walls for closed arms were developed to prevent animals falling off the apparatus[7], and to facilitate behavioral observations inside the closed arms, respectively[8,9]. Although there have been some studies that examined how individual arm structure differences may affect the ability to detect animal anxiety[1,2,5,10,11], the combined effects of open (with/without short ledge) and closed (transparent/opaque walls) arm features on animal behavior has not yet been investigated.

In a recent report, we discussed the effect of these combinations of arm designs on anxiety-like behavior in rats[12]. In brief, open arm ledges significantly increased anxiety-like behavior in rats, while transparent arms may also have decreased this behavior. Furthermore, we verified higher detection sensitivity of the effect of an anxiolytic drug in a combination of no-ledged open arm + opaque walled closed arm compared with a combination of ledged open arm + transparent walled closed arm. In this report, we will introduce our protocol for the elevated plus maze

test, together with discussion of the key results from the previous report. We believe this report will provide useful information for those who have employed or who plan to use the elevated plus maze in their studies.

Protocol

All experimental procedures described here have been approved by the Institutional Animal Care and Use Committee of Meiji University (IACUC 14-0002(1)). Animals used in the representative results were prepared as follows: Male Sprague-Dawley rats were purchased from a laboratory animal company and housed in groups of 4 animals per standard rat cage (W200 × D410 × H250 mm) for at least one week before the start of the behavioral experiment. The animal housing room was maintained at standard ambient conditions for light (12:12 light/dark schedule with lights on at 10:00 h), room temperature (25.0 ± 0.5 °C), humidity (55 ± 10%), and ventilation (10 times/h), and animals had ad libitum access to food (Oriental Yeast Co. Ltd., Tokyo, Japan) and water. The animals were kept in a standard rat cage (W410 x D250 x H200 mm, Toyoriko Co., Ltd., Aichi, Japan) with paper bedding (alpha-dri, LSG Corporatin, Tokyo, Japan).

1. Elevated Plus Maze Test

1. Set up the elevated plus maze apparatus in a customized container (W1,500 × D1,500 × H2,000 mm) (**Figure 1A**) with a plus-shaped metal framework 500 mm above the floor to support the run way arms (**Figure 1B**), lighting in the container ceiling to illuminate the surface of the maze to the required light intensity (**Figure 1C**), and a digital video camera in the center ceiling to enable live video monitoring of the behavioral tests (**Figure 1C**).
2. Fix the arms (L500 x W100 mm) on the plus-shaped framework, and then fix the square central platform (W100 x D100 mm) at the center of the four arms (**Figure 1D - F**).
   NOTE: In our laboratory, we have two types of open arms (L500 x W100 mm) and two types of closed arms (L500 x W100 x H450 mm) (**Figure 1D**). The closed arms have either transparent or opaque walls (**Figure 1D**), and the open arms are designed with or without a short ledge (H5 mm, **Figure 1E**). The square central platform (W100 × D100 mm) is placed at center of the four arms (**Figure 1D - F**). Thus, the following four combinations of arms can be prepared according to experimental purpose: open arms with ledges + closed arms with transparent walls (Ledges/Transparent), open arms with ledges + closed arms with opaque walls (Ledges/Opaque), open arms without ledges + closed arms with transparent walls (No-Ledges/Transparent), and open arms without ledges + closed arms with opaque walls (No-Ledges/Opaque). The surface of the arm runway is black, to enable better animal tracing by the automated behavior analysis software, as described in Section 2, Behavioral Analysis.
3. Adjust lighting intensity on the surface of the distal end part of each arm and central platform to approximately 100 Lux.
4. Before each test, clean the entire apparatus with 70% ethanol solution and wipe with distilled water.
5. Click the "record button" on the hard disk recorder to start the digital video camera that will record the rat's behavior for the video imaging analysis described in Section 2, Behavioral Analysis.
6. Place an animal on the central platform facing an open arm and allow free exploration on the apparatus for 5 min.
7. Click the "stop button" on the hard disk recorder to finish the recording.
8. Count the number of feces and urinations after returning the tested animal to the animal housing room.
9. Repeat from step 1.4 until the end of the experiment.
   NOTE: A dry test run is always carried out before starting each experiment to check that all the experimental settings are correctly set up before any experimental animals are exposed to the experimental conditions. Otherwise, some animals, especially the first animal may experience different environmental conditions (*e.g.*, noises possibly induced by the experimenter during manipulation and/or animal cues possibly left by the previously tested animal).
   NOTE: The experimenters are near the container when an animal is being tested. Thus, even though the experimenters are not visible the rat may still be able to sense them via smell and/or sound.

2. Behavioral Analysis

Note: Behavioral analysis is performed by manual observation and automated analysis software.

1. **Manual observation**
   1. After the animal is placed on the ledge, observe the following: number of entries (an entry onto an arm is defined as any instance when all 4 paws are on the arm), time spent on each arm, and number of stretched-attending postures facing an open arm, while backing off the central platform towards a closed arm.
   2. Measure duration and frequency of the behaviors defined above by stopwatch and count for 5 min, starting from when the doors of the container are closed.

2. **Automated analysis**
   1. Using the available software program, record the following: distance traveled, time spent in different positions on open arms (proximal, middle, and distal segments from the central platform), and a minute-by-minute analysis of the time spent on open and closed arms.
   2. Click the "start button" of the automated analysis software at the same time as the manual observation begins (the recording will stop automatically if recording duration is set).
      NOTE: The behavioral analysis software used in this study tracks the animal via its contrast with the background field; the animal, which is assigned a fixed size, is identified as the brighter object and the locomotion of its center point can thus be used for animal tracking.

Representative Results

In the previous study, we compared the combined effects of open arm ledges and transparency of closed arm walls on the exploratory behaviors on the maze[12]. Rats significantly spent much less time on open arms without ledges than on open arms with ledges ($F[1, 60] = 13.49$, $P = 0.0005$) (**Figure 2A**). Similar results were also found for the number of entries into open arms ($F[1, 60] = 11.17$, $P < 0.0014$)[12], suggesting that the presence of ledges increases rat exploratory behavior on open arms. Rats significantly showed less stretched-attending posture on the apparatus that had closed arms with transparent walls than on apparatus that had closed arms with opaque walls ($F[1,60]= 4.03$, $P = 0.0492$) (**Figure 2B**), suggesting that transparent closed arm walls decreased anxiety-like behavior in rats. Rats spent more time on the distal segment of open arms on the apparatus that had transparent closed arm walls than on apparatus with opaque closed arm walls ($F[1,60] = 8.00$, $P = 0.0983$) (**Figure 2C**), suggesting that transparent walls may also decrease rat open arm exploratory behavior. Furthermore, open arm exploration on the distal segment was significantly lower in the No-ledges/Opaque apparatus compared to the Ledges/Transparent apparatus (**Figure 2C**). Taken together, these results indicate that detection sensitivity for anxiety-like behavior could differ depending on the types of arm design features used, and the No-ledges/Opaque apparatus and Ledges/Transparent apparatus may have a relatively large difference in the detection sensitivity.

To verify differences in detection sensitivity between different types of arm design, a further experiment was performed using the anxiolytic drug diazepam (**Figures 3A-D**). In brief, the No-Ledges/Opaque apparatus detected the effect of high-dose diazepam treatment on the time spent in the open arms, whereas the Ledges/Transparent apparatus did not (**Figure 3A-B**). Similar results were also found for the number of entries into open arms[12] (data not shown). The lack of any significant effect on the Ledges/Transparent apparatus may have been due to the fact that the basal activity (vehicle treatment) on this apparatus was higher than on the No-Ledges/Opaque apparatus, but that open arm exploration never exceeded 33% of the time at any stage on either apparatus. Therefore, this narrower open arm exploration time range made it more difficult to distinguish the anxiolytic effect of diazepam on the Ledged/Transparent apparatus (**Figure 3C-D**).

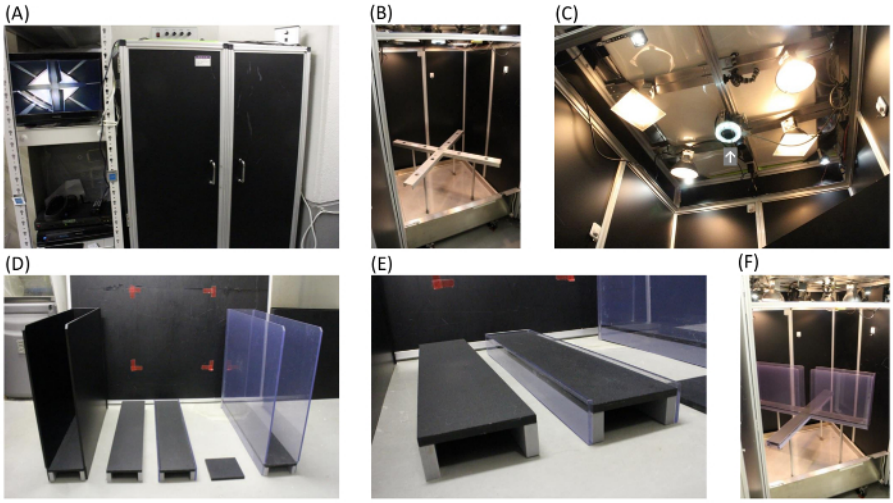

Figure 1: Custom-designed equipment for elevated plus maze test. Panel (**A**): Elevated plus maze test container. The apparatus is placed in the black container, and a monitor next to the container projects live movie images recorded during the experiment. Panel (**B**): Plus maze framework in the container that supports fitted arms. Entire apparatus is located 500 mm above the floor. Panel (**C**): Ceiling lighting that can be adjusted for intensity. A digital video camera (see arrow) surrounded by lighting records animal movements. Panel (**D**): Elevated plus maze arms and central platform that are fitted to framework; from left-to-right, opaque wall closed arm, no-ledge open arm, ledged open arm, central platform, transparent wall closed arm. Panel (**E**): Close-up view of open arms; from left-to-right, open arm without ledges, open arm with ledges (H5 mm). Panel (**F**): An example of a constructed maze (Ledges/Transparent apparatus). Please click here to view a larger version of this figure.

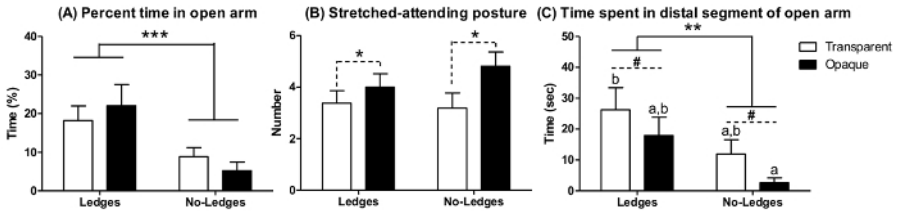

Figure 2: Open arm ledges reduce anxiety-like behavior, and transparent walls partially decrease anxiety-like behavior. Male rats underwent the elevated plus maze test with different combinations of arm structures (Ledges/Transparent, Ledges/Opaque, No-ledges/Transparent, and No-ledges/Opaque). Testing order was normalized across the experimental groups (each combination of apparatus type). This procedure normalizes unexpected effects that could have occurred due to the time of day of each test. Panel (**A**)**:** Time spent on open arms; Panel (**B**)**:** Number of stretched-attending postures; Panel (**C**)**:** Time spent in the distal segment of open arms. Solid and dashed lines indicate significant main effects of open and closed arm structures, respectively (Two-way ANOVA, $^{\#}P < 0.1$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Different letters beside the bars in Panel (**C**) indicate a significant difference (Bonferroni multiple comparison test, a < b, $P < 0.0125$). The data were expressed as mean with SEM (n = 16 per experimental group). These figures have been modified from our previous report[12]. Please click here to view a larger version of this figure.

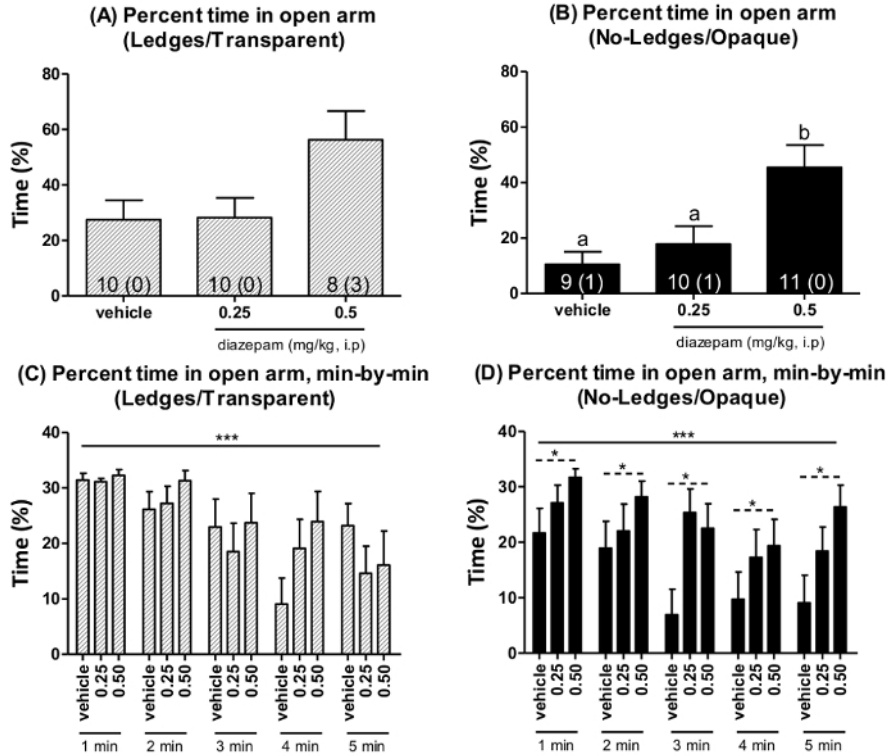

Figure 3: No-Ledges/Opaque apparatus shows higher detection sensitivity of anxiolytic effect of diazepam. Male rats underwent the elevated plus maze test, with either No-Ledges/Opaque or Ledges/Transparent apparatus, 30 min after being treated with either vehicle (0 mg/kg), low (0.25 mg/kg), or high (0.5 mg/kg) doses of the anxiolytic drug diazepam (intraperitoneal injection). Diazepam solution was dissolved in 0.9% normal saline solution with a surface-activating agent. In this experiment, the testing order of the three drug treatment groups and two types of apparatus design were randomized to account for any effects that may occur due to the time of day of each test. Panels (**A** & **B**): Total percent time (5 min) spent on open arms of Ledges/Transparent and No-ledges/Opaque apparatus, respectively. Panels (**C** & **D**): Minute-by-minute percent time spent on open arms of Ledges/Transparent and No-ledges/Opaque apparatus, respectively. Different letters beside bars in Panel (**B**) indicate a significant difference between treatments (Bonferroni multiple comparison test, a < b, $P < 0.0167$). Solid and dashed lines in Panels (**C** & **D**) indicate significant main effects of the time-lapse and diazepam treatments, respectively (repeated measures ANOVA, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). The data were expressed as mean with SEM. Numbers in the columns indicate animals that completed the experiment (numbers in parenthesis indicate animals falling off the apparatus). These figures have been modified from our previous report[12]. Please click here to view a larger version of this figure.

Discussion

As the number of patients suffering from psychiatric disorders, including anxiety-disorder, increases, it is important to develop cutting-edge studies to improve understanding of the basic mechanism of anxiety. To achieve this, it is also important to optimize the experimental procedures. Therefore, to improve the application of the elevated plus maze test, we have demonstrated the effect of arm features on experimental outcomes using rats.

The elevated plus maze was originally established to test animal anxiety in rodents[4], but although the basic structures are usually consistent (*e.g.*, elevated 500 mm above the floor with a plus shape), various arm designs are often used. Originally, the apparatus consisted of open arms without ledges, and closed arms with wooden opaque walls. However, more recently open arms have been developed with ledges to prevent animals falling off the apparatus, and closed arms have also been developed with transparent walls to facilitate observation of behavior on the closed arms. However, there have been no reports that addressed the combined effects of these arm features on the experimental outcomes in the elevated plus maze test. As shown by our results, the presence of open arm ledges and the transparency of the closed arm walls decreased avoidance of open arm exploration in rats (**Figure 2**), which may decrease the detection sensitivity of the anxiolytic effect of diazepam (**Figure 3**). The Ledges/Transparent apparatus appeared to encourage greater exploration on the open arms by vehicle treated rats (**Figure 3**). This reduced behavioral discrimination in open arm exploration activity between vehicle and diazepam treated rats, thus making it more difficult to detect differences in anxiety levels. Therefore, the lower sensitivity of Ledges/Transparent apparatus may mean that more animals would need to be tested to demonstrate significant treatment effects compared with using No-Ledges/opaque apparatus.

In our protocol, the duration and number of entries onto each arm was manually measured by well-trained experimenters. This procedure enables accurate measurement of arm entries, defined as all 4 paws placed on an arm. Although it is true that automated animal tracking systems are available for some behavioral parameters, such as traveling distance, in our experience they cannot always accurately distinguish between entries and non-entries onto arms, especially when animals perform stretched-attending postures near the border between the central platform and an arm. Thus, we recommend using a combination of both manual and automated observation systems, based on their respective strengths for each behavioral parameter.

There are some other protocol differences between laboratories. For example, some researchers place animals on the central platform facing a closed arm[13,14], whereas others place the rats facing an open arm[15,16]. In addition, some researchers test behavior immediately after taking rats from their home-cage, whereas others allow the rats some time to acclimatize to the behavior testing room before starting the test. With respect to former case, in preliminary tests we experienced how the outcomes differed depending on which arm the rat initially faced; there was an increased incidence of entering onto the arm that the rat was placed facing. Also, when the rats were placed facing a closed arm, some animals did not move out of that closed arm after the first entry. In such a case, it is difficult to distinguish whether the rats experience conflict between the open and closed arms or that they merely prefer to be in a closed place without ever recognizing the presence of the open arms. Although in our study we chose to place the rats facing an open arm, to ensure a high incidence of being under conflict, we recommend that further studies be conducted to ensure that the effect of this protocol difference on experimental outcomes is properly investigated.

The measurements of exploration of the distal segment of open arms and minute-by-minute analysis provided extra detailed information about rat anxiety-like behavior. These measurements helped to show the differences in detection sensitivity between the different arm designs. Despite this, reported results of anxiety research using the elevated plus maze test often do not report these types of detailed measurements[13,17,18]. Therefore, we highly recommend that researchers also report further information about rat exploratory activities, including distal exploration, minute-by-minute analysis, and also stretched-attending posture.

In conclusion, our findings suggest that different types of apparatuses may have their own characteristics. From these tests, we have demonstrated that No-ledges/Opaque apparatus can better detect the effect of drugs potentially having an anxiolytic effect. In contrast, it is conceivable that Ledges/Transparent apparatus may have a similar advantage when testing potential anxiogenic drugs. To date, behavioral tests have been used not only for drug screening, but also for phenotyping assays in mutant animals. Indeed, advanced genome editing techniques allow rapid production of mutant animals. Therefore, it is very important to develop testing procedures with better precision and sensitivity to better detect the variations in behavior. This report demonstrates an improved application of the elevated plus test for animal anxiety research based on the expected functions of the drug or target genes. Thus, we believe that our efforts will help to advance research employing the elevated plus maze.

Disclosures

The authors have nothing to disclose.

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