

Elevated Plus Maze Test to Assess Anxiety-like Behavior in the MouseLuciana M. Leo¹ and Fabricio A. Pamplona^{2*}

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[Abstract] The elevated plus maze task is a simple method to assess anxiety-like behaviors in rodents. This version describes the procedure used in mice. However, the protocol may also be applied to rats, considering a proportionally larger apparatus (arms: 10 x 50 cm; height: 55 cm). Briefly, the test is performed on a plus-shaped apparatus with two open and two closed arms. The animal is allowed to freely explore the maze for 5 min while the duration and frequency of entries into open and closed arms is recorded. The task is based on an approach-avoidance conflict, meaning that the animal is faced with a struggle between a propensity to explore a novel environment and an unconditioned fear of high and open spaces. Consequently, an anxiety-like state is characterized by increased open arm avoidance, compared to control animals. On account of being a very popular test, there can be considerable variations in the procedures applied across different laboratories. Here we provide a working protocol that has been able to detect both anxiogenic and anxiolytic drug effects under the specified conditions. Protocol originally published in (Leo *et al.*, 2014).

Materials and Reagents

1. Laboratory-bred plus maze-naïve mice

Note: Mice housed in groups of 4-5 per cage, kept in an environment with controlled temperature (around 23 °C) and humidity under a 12-12 h light-dark cycle with food and water ad libitum.

2. Paper towels and 70% ethanol for cleaning

Equipment

1. Elevated Plus maze (either customized apparatus or commercially available one)
2. Video camera (placed directly above the maze)
3. Indirect white light
4. Digital lux meter (Instrutherm, Mastech, Dr. Meter or similar)

5. Digital chronometers for manual analysis, or computer software (Any-maze, Ethovision or others) for automated analysis

Note: In our laboratory, we use a custom made plus-shaped maze sized for mice (Leo et al., 2014), which is elevated 50 cm above the ground, and consists of two opposite closed arms, two opposite open arms and a central square of 5 cm sides (see image below). The closed arms measure 30 x 5 cm and are constituted of white-painted wood, as are the 15 cm high walls that enclose the arms. Glossy painting should be avoided as to prevent excessive glare, thus a matte finish is recommended. The open arms measure 30 x 5 cm and are made of transparent acrylic; a 0.3 cm high transparent acrylic railing prevents animals from falling while exploring the open arms. The apparatus can be disassembled and moved to and from the experiment room where it is set up for use. Wood-made apparatuses covered with wood lacquer, painted in black or transparent Plexiglas apparatuses are also frequently found. Commercially available apparatuses, such as those from Stoelting Co. or Panlab S.L.U., are recommended as alternatives. The room illumination and apparatus light reflection strongly affect the elevated plus maze test. In our laboratory, a Samsung Flashcam digital camcorder is used, as it is more appropriate for posterior off-line analysis. One should always run pilot experiments to establish reliable baseline parameters and standardize the experimental environment before starting the experimental manipulations (drugs, procedure, transgenic animals, etc.).

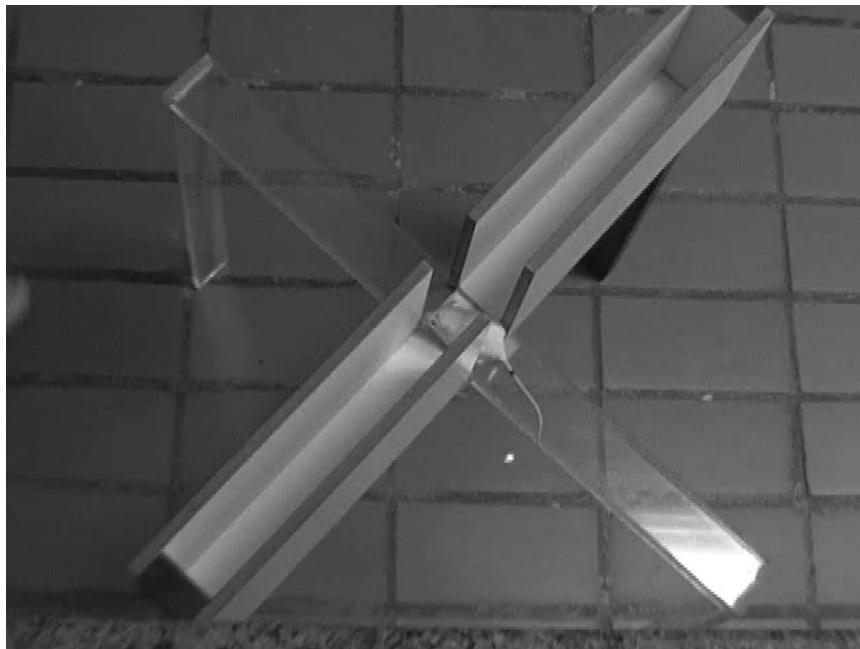


Figure 1. Plus-shaped maze

Software

1. Automated computer software (Any-maze, Ethovision and others)

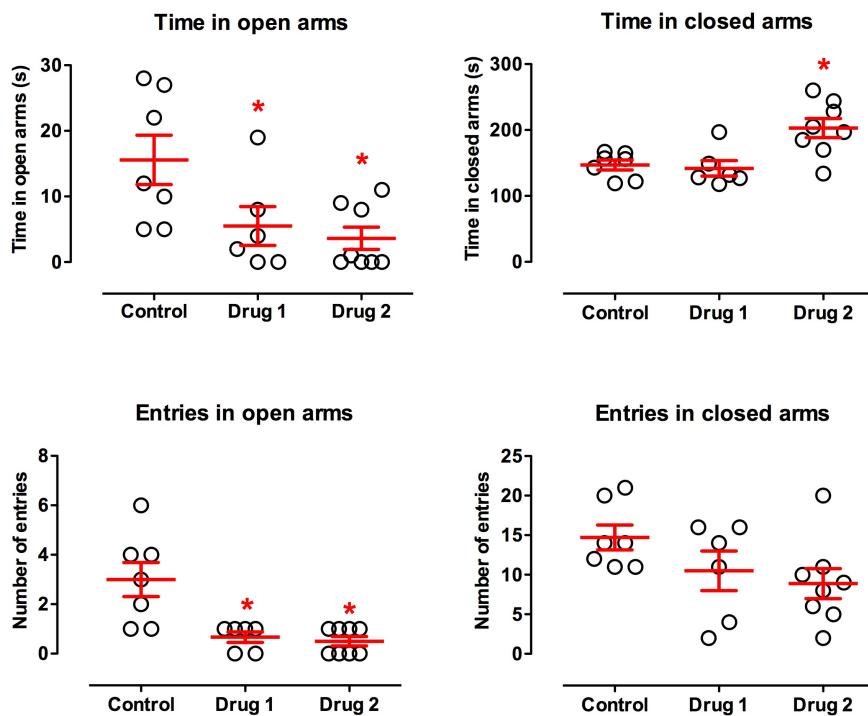
Procedure

1. The maze should be assembled in an isolated room away from any extraneous interference of noises, scents or movement. You may choose to place a source of low-intensity white noise in the behavioral experiment room. It should be noted that guidance cues, such as drawings on the walls around the room might skew the animal's activity to a certain area of the maze. Therefore, any sort of paintings or artifacts that may serve as cues must be removed.
2. The experimenter must restrain from making any excessive noise or movement during the entire trial and from wearing perfumes, colognes or any product with a strong smell, since it could act as anxiogenic stimulus for mice.
3. Illumination in the room must be measured with the aid of a lux meter, kept constant and controlled according to the analysis that is to be performed. Given that low-intensity luminosity reduces open arm avoidance (Morato and Castrechini, 1989; Bertoglio and Carobrez, 2002; Cosquer *et al.*, 2005; Pereira *et al.*, 2005), to analyze an anxiogenic effect low-intensity lighting (5-30 Lux) should be preferred, whereas an anxiolytic effect should be analyzed under higher intensity lighting (200-400 Lux or more).
4. After these experimental conditions are adjusted to a standard, the animals will be brought into the experiment room, where they will be left in their home cages for 45 to 60 min in order to recover from the stress of being moved.
5. Clean the maze with 70% ethanol before starting the test in order to remove any dirt or smells accumulated on the apparatus.
6. Turn on the video camera and place the first mouse in the center square of the maze facing one of the open arms, preferably the one opposite to the experimenter.
7. The experimenter will stand as far away as possible from the maze and out of sight of the test animal, outside of the room if necessary. He must also avoid making unnecessary movement or sounds.
8. After 5 min of free exploration, the mouse may be moved out of the maze and back into its home cage.
9. All the urine and fecal boli must be removed and the maze cleaned entirely with 70% ethanol to remove any residual smell from the first mouse. Afterwards, the next mouse may be submitted to the test.
10. Repeat steps 6-9 until all the animals have been tested.

11. The recorded videos can be analyzed by automated computer software or manually with the aid of a chronometer. Several parameters can be considered:
 - a. Entries in closed arms and time spent in closed arms: Measures of total locomotion on the maze throughout the experiment (Carola *et al.*, 2002; Walf and Frye, 2007).
 - b. Entries in open arms and time spent in open arms: May be used as inverse measures of anxiety, that is to say, reduced open arm avoidance reflects lower levels of anxiety (Carola *et al.*, 2002; Walf and Frye, 2007; Griebel *et al.*, 1993).
 - c. Risk-assessment behavior: the frequency and the duration of head-dipping (downward movement of the head towards the floor while on the open arms) and stretch-attend posture (stretched posture with the head and two or three paws on the open arm and retraction to previous position) can be a direct measure of anxiety, *i.e.* increased risk-assessment behavior indicates higher levels of anxiety (Carola *et al.*, 2002; Weiss *et al.*, 1998; Carobrez and Bertoglio, 2005).
12. Statistical analysis: Parameters should be examined separately by analysis of variance across all treatment schedules. The data can be further analyzed by a *post-hoc* test, if applicable.

Representative data

SAMPLE DATA



The figure above represents typical data from the elevated plus maze (EPM) in our settings, adapted to investigate anxiogenic responses (10 Lux). The behavior of the control group is relatively distributed, but decent statistically valid data has been gathered with a sample of 6-8 mice per group. Drug 1 is a clear anxiogenic drug, displaying decreased open arm time and open arm entries, without alterations in the time spent and number of entries in the closed arms. Drug 2 suggests a relatively flawed interpretation of an anxiogenic-like pharmacological profile, as treated-mice displayed decreased locomotion, as revealed by the number of entries in each arm. In such case, we recommend a slight reduction in drug dose in order to identify a dose allowing distinction between emotional and locomotor effects induced by the drug. A representative video from one of our experiments published in (Leo *et al.*, 2014) is also available.

Notes

1. Typically, male mice are used when performing behavioral experiments, as variations of estrous cycle often influence performance of females in such tests (Simpson *et al.*, 2012; da Silva *et al.*, 2014).
2. Baseline activity may vary according to mouse strain, gender and age (Leo *et al.*, 2014; Carobrez and Bertoglio, 2005; Hogg, 1996).
3. Animals should be allowed at least one week for habituation to the home cage, as they tend to present high anxiety levels upon arrival from the animal breeder facility.
4. In our experiments, animals are only handled for cage cleaning, weighing, drug treatment and during the testing. Also, animals are only tested during the light cycle; testing in the dark cycle may produce different results (Bertoglio and Carobrez, 2002).
5. In order to ensure uniform luminosity, an indirect light should always be used to avoid the production of hard shadows, which can be a place of preference for the mouse, skewing its locomotion activity to that area of the maze. This may be done with an appropriate light reflector.
6. The maze should always be cleaned after the testing of each animal.
7. Make sure the maze is fully dried after cleaning, or a strong smell of the cleaning agent may alter mouse behavior.
8. In the event that the mouse falls off the open arm during the test, the experimenter must immediately pick it up and place it back on the open arm. This should be noted and taken into consideration when performing the experiment analysis. This animal can be excluded from the final analysis.
9. An entry in the open or closed arm is only considered when the animal places all four paws out of the center square and onto one of the arms. However, two paws out of a

- given arm, is already sufficient to stop counting the time spent into the arm. If the animal return with these two paws into the arm, the chronometer should be continued, but no entry is registered. A new entry is only registered after a full exit occurs.
10. There is no evidence of age-related restrictions for behavioral testing in the elevated plus maze. Despite data supporting increased anxiety-like state in adolescent and aged animals (Lynn and Brown, 2010; Bessa *et al.*, 2005; Chen *et al.*, 2007), the task has been validated for successful assessment of anxiety-like behavior in adolescent, adult, middle-aged and aged rodents (Leo *et al.*, 2014; Warneke *et al.*, 2014; Carrasco *et al.*, 2013; Joshi and Pratico, 2011; Pietrelli *et al.*, 2012; Doremus *et al.*, 2006).

Acknowledgments

The protocol described here has been adapted from a previous study (Leo *et al.*, 2014), which succeeded in identifying anxiogenic-like as well as anxiolytic-like behavior for mice. The project received financial support from FAPERJ-Brasil (E-26/112.448/2012) and CNPq-Brasil (474443/2012-4).

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