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Experimental models of anxiety for drug discovery and brain research

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Abstract

Animal models have been critical in recent advances in experimental neuroscience, including the modeling of common human brain disorders, such as anxiety, depression, and schizophrenia. As mice express robust anxiety-like behaviors when exposed to stressors (e.g., novelty, bright light or social confrontation), these phenotypes have clear utility in testing the effects of psychotropic drugs. Of specific interest is the extent to which mouse models can be used for the screening of new anxiolytic drugs and verification of their possible applications in humans. To address this problem, the present chapter will review different experimental models of mouse anxiety, and discuss their utility for testing anxiolytic and anxiogenic drugs. Detailed protocols will be provided for these paradigms, and possible confounds will be addressed accordingly.

Key words: anxiety, experimental animal models, anxiolytic drugs, anxiogenic drugs, biological psychiatry, exploration

1. Introduction

Animal models are widely used for simulating human brain disorders, and for providing insight into their neurobiological mechanisms (1-4). The latter is of great interest today, given the increasing use of laboratory animals for screening various classes of psychotropic drugs (5, 6). The use of mice has been particularly beneficial, since fine-tuned manipulations of selected genes has led to new animal models relevant to drug discovery (3, 4, 7, 8).

It is important to understand, however, that any animal experiment in the laboratory is an artificial situation, and it may be biologically different from the natural behavior of the animal. Thus, it is crucial to correctly interpret the animal behavior observed in an experiment in order to identify parallels with specific human brain disorders. Although there are many other conceptual and methodological limitations of working with mice, this animal shows much promise for future psychopharmacological research.

In order for animal models to be useful, researchers must follow certain practices and methods which will optimize the translatability of data from animal models to human affective disorders. Here, we will review some reliable methods of analyzing mouse anxiety, and their utility for screening for anxiolytic therapeutic agents. We will also discuss how these protocols can be applied correctly, in order to avoid confounding experimental data.

2. Materials

2.1 Animals

1. Various inbred, selectively bred, and genetically modified (mutant or transgenic) mice may be used, and some searchable online databases, such as Mouse Phenome Project or Mouse Genome Informatics, may provide appropriate strains for studying mouse anxiety.
2. Researchers should also take other factors (age, weight, sex, stage of estrous cycle, and housing situation) into account when designing experiments. In addition, they should use

the most updated detailed nomenclature for mouse strains, and obtain the animals from certified vendors or other reliable sources.

2.2 Housing (see (9, 10) for details)

1. If mice are obtained from a commercial vendor or another laboratory, allow at least 1 week acclimation from shipping stress. In most cases, a much longer time will be required.
2. Housing animals in groups will help avoid social isolation stress/anxiety, but keeping groups small enough (e.g. not more than 5 animals per cage) will be necessary to avoid overcrowding stress.
3. The room in which mice are housed should be kept at approximately 21°C, on a 12/12 hr light cycle. As mice are nocturnal, the light cycle may be inverted if spontaneous activity measures are needed.
4. Food and water should be freely available, unless the intake is being controlled for experimental purposes.
5. Utilize plastic, solid-floored cages with sufficient space for mice to exercise and fully rear up. Note that enrichment items, such as cardboard tunnels, can improve general welfare but may also affect experimental outcomes or increase territorial aggression.
6. All experimental procedures (including handling, housing, husbandry, and drug treatment) must be conducted in accordance with national and institutional guidelines for the care and use of laboratory animals.

2.3 Drugs

1. All experimental protocols described here are compatible with drug testing. Researchers may choose from various antidepressants, anxiogenics, anxiolytics, or other psychotropic drugs, administered with a vehicle (e.g., saline).

2. Common routes of injection include *systemic* (intraperitoneal (i.p.), intramuscular (i.m.), intravenous (i.v.), per oral (p.o.), subcutaneous (s.c.)] and *local* [intracerebral (i.c.) or intracerebroventricular (i.c.v)). Route of administration, dose, and pre-treatment time vary depending on strain sensitivity and the drug being used.

2.4 Observations, Video Recording and General Procedures

1. A computer, digital camera mounted above the test apparatus, and video-tracking software will aid researchers in the collection of accurate behavioral data.
2. In addition to video-tracking, an observer with a timer and data sheet to tally behaviors will allow comparison of data if video-tracking is unreliable due to poor detectability (poor angle or bad contrast; e.g., if fur color matches the background).
3. Observers must refrain from making noise or movement, as their presence may alter animal behavior. Assess intra- and inter-rater reliability for consistency.
4. Allow at least 1-h acclimation of animals after their transfer from the animal holding room to the experimental room.
5. Mice should be introduced to the testing environment during their normal waking cycle, to prevent possible confounds. When performing ethological analysis as part of a battery of tests, consider how effects of these tests (such as habituation) may confound the mouse performance and drug sensitivity.
6. After each testing session, clean the equipment (e.g., with a 30% ethanol solution) to eliminate olfactory cues.

2.5 Data Analysis

1. Behavioral data may be analyzed with the Mann-Whitney U-test for comparing two groups (parametric Student’s t-test may be used only if data are normally distributed), or analysis of variance (ANOVA) for multiple groups, followed by an appropriate post hoc test.

2. Some experiments may require one-way ANOVA with repeated measures, while for more complex studies (e.g., those including treatment, genotype, sex, and/or stress) n-way ANOVA may be used.

2.6 Requirements for experimental models

2.6.1 Elevated-Plus Maze (EPM) (1, 9, 11):

- Elevated maze with 2 open and 2 closed arms in the shape of a plus. A commercially available mouse apparatus is made of steel, fiberboard, or Plexiglas and is either transparent of painted matte black. Arms are typically 30 cm long and 5 cm wide. The apparatus is usually elevated 40-60 cm on sturdy legs.

2.6.2 Open Field (10, 12)

- Enclosed 50 x 30 cm wood, plastic, or Plexiglas arena, marked into 10-cm squares. Gray or black arenas are typically used. If an arena is not available, a large animal cage marked into squares with indelible ink may be used.

2.6.3 Marble Burying Test (13-16)

- Woodchip bedding (e.g., aspen chips), up to 20 marbles (15 mm in diameter). Animal cages (e.g., large cage 30 x 20 cm for 20 marbles, smaller cages for 6-8 marbles).

2.6.4 Defensive Shock-Prod Burying test (6, 17)

- Familiar test cage or home cage with plentiful bedding and a hole in the wall 2 cm above bedding. Electrical probe connected to a shock source. Ruler for measuring depth to which prod is buried. Optional: Large (e.g., 10 cm) object associated with shock.

2.6.5 Grooming Analysis Algorithm (8)

- Small (e.g., 20 x 20 x 30 cm) transparent observation box. Stressors to induce grooming (e.g., novel environment, predator exposure, bright light), or other means of inducing

grooming (e.g., water mist). Optional: video-camera for subsequent frame-by-frame analysis.

2.6.6 Startle Response (1, 7, 18)

- Observation box (similar to the open field test). Conditioned stimulus (e.g., a light, paired with a footshock). Startle stimulus, such as an air puff or loud noise.

2.6.7 Social Interaction Test (1)

- Low-anxiety version: Test apparatus (similar to the open field test) familiar to the animals, with low illumination. Mid-low anxiety version: Familiar test apparatus with high illumination. Mid-high anxiety version: Unfamiliar test apparatus with low illumination. High anxiety version: Unfamiliar test apparatus with high illumination.

2.6.8 Suok Test (19, 20)

- Test apparatus (2.6-m aluminum tube, 2 cm in diameter, marked into 10-cm segments with indelible ink) with fixed 50 x 50 x 1 cm Plexiglas side walls to prevent escape, elevated 20 cm from a cushioned floor. Optional (the light-dark version of the test): several 60-W light bulbs suspended 40 cm above one half of the test apparatus.

2.6.9 Light-dark box test (5)

- Test apparatus (a 2-compartment box, 30 x 30 x 30 cm each; with one black, and one transparent brightly illuminated boxes, separated by a sliding door).

2.6.10 Stress-Induced Hyperthermia (SIH) (21)

- Oiled rectal thermometer with rounded tip, up to 3 mm thick. Cage or box (as in the open field test) to which mice can be transferred.

2.6.11 Hole Board Test (22)

- Test apparatus (similar to the open field test) with hole-board insert. The floor has 4 or more identical holes approximately 3 cm in diameter.

2.6.12 Rat Exposure Test (23)

- Medium (e.g., 40 x 30 x 30 cm) transparent observation box, with a wire mesh separating the two halves of the box. Small (e.g., 8 x 8 x 12 cm) black Plexiglas box, serving as the starting placement (home chamber for the mouse). Transparent Plexiglas tube (e.g., 4.5 cm in diameter, 13 cm in length) connecting the small black box to the medium transparent box.

2.6.13Novel Object Test (24)

- Test apparatus similar to the open field test (see above), novel objects (e.g., Mega Bloks structures).

3. Experimental Procedures

3.1 Elevated-Plus Maze (EPM) test

Possessing good face-, construct-, and predictive validity, the EPM is a reliable and pharmacologically sensitive paradigm based on the conflict between innate rodent desire to explore, and the fear of open, elevated areas (3, 5). Anxious mice generally have a lower ratio of open arm entries to total arm entries, and display fewer explorative measures such as rears, wall leans, or head dips. Anxiety also increases EPM freezing and stretch-attend postures. After administration of anxiolytic drugs, mice display more exploratory behaviors, a greater number of open arm entries, and an increased duration of time spent on open arms (9, 11). Anxiogenic drugs produce the opposite behavioral effects in this model.

1. Place rodent on the central platform of the EPM facing either an open arm or a closed arm consistently.
2. The open arm, closed arm, and total (open + closed arm) activity can be recorded for 5-10 min using a video-tracking system, while the researchers simultaneously document the number of arm entries (all four paws are on the arm) and time spent on each open arm.

Notes:

1. As some mice may fall off an open arm of the EPM, the data from these animals must be excluded from further analyses.
2. To avoid excessive freezing, testing environment should be kept quiet without disruptions. If the mouse freezes for more than 30% of the total test time, researchers should note of this abnormality, but continue testing. In case of unexpected or loud noises or other disruptions, the data should be discarded from analyses.
3. Age, gender, and strain differences affect EPM performance. Young, intact females generally spend less time on the open arms than males, although this varies with the estrous cycle. Pro-estrus rodents spend significantly more time on open arms than di-estrus females (or male mice) (9).
4. Since lighting can affect behavior in the maze, make sure it is consistent on all arms. Red light is preferable.

3.2 Open Field Test

This test is based on the balance between the animal's natural drive to explore novelty, and its aversion to open illuminated areas. Measuring exploratory behaviors and generalized motor activity, the open field test is simple and the most frequently used model of mouse anxiety (2). In general, anxious mice exhibit more freezing, less time spent and a lower percentage of ambulation in the center of the arena (thigmotaxis), and fewer exploratory behaviors. Anxiolytic drugs generally increase exploration, and reduce freezing and thigmotaxis (12).

1. After the apparatus is divided into central and periphery zones, mice are placed in a corner (or the center) of the open field arena, and allowed to explore for 5-10 min.
2. Behavioral measurements can be recorded automatically with appropriate software, and include: time spent in the central area, distance travelled in the center as a ratio of total

distance travelled, ambulation duration, time spent immobile (freezing), defecation score, and vertical activity such as rearing and wall leans (12).

Notes:

1. In some strains, reduced anxiety can be mistaken for hyperactivity. A minute-by-minute analysis of exploration and activity may aid in distinguishing these two different domains (25).
2. It is important not to misinterpret reduced locomotion due to high habituation as an anxiogenic response. The mouse learning/memory phenotypes should be assessed in separate tests (25).
3. If mice have poor habituation, this may result in increased "exploration" that should not be misinterpreted as decreased anxiety (25). Consider testing mouse cognitive functions in a separate study.

3.3 Marble Burying Test

While not a direct model of anxiety *per se*, this simple test represents a pharmacologically sensitive method assessing digging activity - a species-typical response to anxiogenic stimuli (13, 15, 26). Digging behavior is attenuated by low (non-sedative) doses of anxiolytic benzodiazepines and other ligands (14, 16). Control mice can be expected to bury roughly 75% of marbles, whereas drug-treated mice show a marked decrease in digging activity (27, 28).

1. Cages should be filled with wood chip bedding approximately 5 cm deep. The bedding must be flattened to create an even surface. Use the same volume (e.g., 300 ml) of bedding in each cage.
2. Marbles (up to 20 for big cages; 6-8 for smaller cages) should be placed on the surface of the bedding in a regular pattern, roughly 4 cm apart.

3. Place one mouse in each cage. After 30 min, count the number of buried marbles. Any marble covered 2/3 of its depth with bedding is considered “buried” (14). Alternatively, count fully covered (1/1) and partially covered (2/3) marbles separately, also calculating the sum of the latter two categories.
4. Use a new clean cage with fresh bedding for each animal.

Notes:

1. Some strain differences are apparent in digging behaviors. Slow or inactive mouse strains (e.g., 129) may be replaced with more active strains (e.g., C57) to achieve recordable amounts of burying data. Additionally, younger mice (2-4 months old) tend to show enhanced digging behaviors as opposed to mice over 1 year old (14).
2. If mice continue to display low burying activity, it may be useful to assess the environment for confounding factors. Unnecessary noise or stress should be eliminated and mice should be undisturbed throughout the experiment. Testing on cage-cleaning days may also cause mice to be less responsive to the new bedding (14).
3. Some strains with low burying/digging activity may require a longer (e.g., 45-60 min) testing time that may help reveal their phenotype.

3.4 Defensive (Shock Prod) Burying Test

Similar to the marble burying test, this paradigm is another pharmacologically sensitive method to assess rodent anxiety. Mice usually bury noxious stimuli posing an immediate threat (e.g., electrified shock-prod). The test has pharmacological validity, as benzodiazepines and the serotonergic anxiolytics potently suppress shock prod burying in a dose-dependent manner, whereas anxiogenic drugs have been proven to increase this behavior (6).

1. In a cage with bedding 5 cm deep, insert a wire-wrapped prod (6-7 cm long) through a hole 2 cm above the bedding surface.

2. After the initial contact with the bare wires and the subsequent shock, record the behavior of the animal for 10-15 min. Behavioral measures of activity may include: prod-directed burying, burying latency, height of pile at prod base, prod contacts (number, duration), prod contact latency, and stretch-attend postures directed at the prod (6).

Notes:

1. Troubleshooting is the same as in the marble burying test.

3.5 Grooming Analysis Algorithm

Anxious mice tend to display a disorganized behavioral sequencing of grooming (higher percentage of incorrect transitions, more interrupted bouts) and a longer duration of this behavior. In contrast, anxiolytic benzodiazepines normalize mouse grooming sequencing by significantly reducing interrupted bouts and incorrect transitions (8).

1. Induce grooming through exposure to novelty or a stressor. Alternatively, mist the animal with water using a spray bottle. Place the animal in a small transparent observation box for 5 or 10 min.
2. If using a video camera, begin recording. With a stopwatch, record cumulative measures of grooming activity, such as: latency to onset, time spent grooming, and total number of bouts. A new bout takes place after an interruption of greater than 6 s; bouts containing interruptions of less than 6 s are deemed “interrupted.” Additionally, record the patterning of each bout using the following scale: 0- no grooming, 1-paw licking, 2-nose/face/head wash, 3- body grooming, 4- leg grooming, 5- tail/genitals grooming.
3. There are several types of incorrect transitions, including skipped (e.g., 1-4, 3-5), reversed (e.g., 3-2, 5-3), prematurely terminated (e.g., 3-0, 4-0), and incorrectly initiated (e.g., 0-3, 0-5) transitions. Calculate the percentage of interrupted bouts, and the percentage of incorrect transitions; see (8) for details.

Notes:

1. Abnormally high grooming activity may be due to a strain-specific compulsive-like phenotype (consider using a more appropriate strain), or due to unintended stress in the animal facility (which may be assuaged by improved husbandry or enrichment).
2. High baseline or transfer anxiety may lead to unusually low grooming activity. This may be alleviated by using smaller observation boxes and dimmer lighting, as well as by improving handling techniques. Reduced grooming activity may also be due to a strain-specific low-grooming phenotype (for example, due to abnormal neurological/vestibular/motor phenotype) or overall inactivity of the stain being tested.
3. Detection of different stages of grooming behavior may sometimes be difficult. If using a video camera, replaying in slow motion will make the detection of transitions and interruptions much easier.

3.6 Startle Response:

The startle response test pairs a conditioned stimulus (sound, light) with a footshock to induce an anxiogenic "startle" response in mice. While the sensitivity of this test to many drugs is yet to be established, benzodiazepine and serotonergic anxiolytics have been effective in reducing the startle response (1). Since this model seems to be unaffected by motor phenotypes, activity levels, or neurological deficits, this test (unlike many other anxiety models discussed here) allows researchers to study mouse anxiety without these confounding factors.

1. In a conditioning trial, a conditioned stimulus (usually a light) is paired with a footshock. The timing of the conditioned stimulus and footshock can be controlled by the data acquisition software for consistency (7).
2. In a separate trial 24 h later, the animals are presented with a startle stimulus (e.g., loud noise or air puff) and their activity is recorded as a baseline. The startle stimulus can be

presented in 4 blocks of 5 startles each, with 30-35 s between each startle stimulus (7). Peak and amplitude of the startle response can be recorded (e.g., using a piezoelectric accelerometer) and digitized (18).

3. 24 h later in testing trials, the conditioned stimulus is displayed immediately prior to the startle stimulus, and the observed response is compared to the baseline startle response. Stimuli should be presented when the animal is quiet and inactive (7).

Notes:

1. If the startle stimulus is auditory, some mouse strains may be insensitive to this test because of hearing deficits. To rule out this possibility, mice should be tested for hearing problems. If the mouse strain shows abnormally poor hearing, consider using a physical startle stimulus (e.g., air puff or bright light) or a different strain.
2. If the mouse does not show a heightened response to the startle stimulus in the testing trials, it may have cognitive deficits. Memory should be examined in separate, specific tests to ensure accurate data interpretation.
3. Some mice may show an abnormally high startle response. High responses may be a result of brain pathological over-excitation, and this abnormality should be investigated further. Also, consider baseline brain activity as well when administering drugs. For example, due to the floor/ceiling effect, anxiogenic drugs can be tested on mice with a low baseline startle response, whereas anxiolytics would yield clearer results if tested on mice with high baseline responses.

3.7 Social Interaction Test

The social interaction test is a useful drug-sensitive approach to assessing anxiety in mice. There are four testing conditions which introduce varying levels of stress: 1) familiar test apparatus and low illumination; 2) familiar test apparatus and high illumination; 3) unfamiliar test apparatus

and low illumination; and 4) unfamiliar test apparatus and high illumination. The level of anxiety across these conditions ranges from low to high, respectively. Overall, the duration and frequency of social interactions negatively correlate with anxiety. Because this test successfully isolates levels of anxiety (high vs. low) in the subjects, it has been used for pharmacological screening of both anxiolytic and anxiogenic drugs in their effectiveness for increasing or decreasing social interaction, respectively (1).

1. The test environment should be the same in all conditions, except for the test apparatus (familiar or unfamiliar) and the lighting (low or high illumination).
2. Introduce the two mice into the test environment for 5 or 10 min, recording the duration and frequency of all social interactions (e.g., sniffing, following, chasing, touching, and biting).
3. After obtaining baseline data for each condition, administer an anxiolytic or anxiogenic drug to the mice. The same test (step 2) can be conducted and analyzed relative to baseline data. Alternatively, compare drug-treated with saline-treated groups (only one animal in the interacting pair receives the drug).

Notes:

1. Certain strains of mice may be more likely to engage in social interaction because of their high sociability phenotype (unrelated to their anxiety or emotionality profile *per se*. In this case, consider using other strains for this test. Additionally, the use of some strains should be avoided as their autism-like behavior may prevent the relevance of this test as a model of anxiety.
2. In performing the social interaction test for screening anxiolytic and anxiogenic drugs, it is suggested that the same two mice are not re-introduced into the same environment together,

as this may eliminate the social novelty of the condition, and will affect their test performance.

3. Mice with abnormally poor or abnormally good cognitive abilities may produce aberrant behavior in this test (e.g., increased or decreased social interaction, respectively). To rule out this possibility, consider testing mice in some additional memory paradigms.

3.8 Suok Test

The Suok test simultaneously examines anxiety**,** vestibular**,** and neuro-muscular deficits by combining an unstable rod with novelty. To analyze anxiety, the threats of height, loss of balance, and novelty are presented and animal exploration is recorded. Anxiolytic or anxiogenic drugs will increase or decrease animal exploration, respectively. Risk assessment and vegetative behaviors are generally higher in anxious mice. The model is also sensitive to anxiety-evoked balancing deficits, since administration of anxiogenic drugs increases the number of falls and missteps, while anxiolytics generally improve balancing (19, 20). A light-dark modification of the test may also be employed, as the illuminated environment will represent an additional stressor.

1. Place individual mice in the center of the apparatus facing either end (or, in the light-dark modification, orient the animal facing the dark end).
2. From approximately 2 m away, record the following behavioral measures (for 5-10 min per animal): horizontal exploration activity (latency to leave central zone, number of segments visited with four paws, distance traveled, number of stops, time spent immobile, average inter-stop distance, number of stops near border separating light-dark areas of the apparatus), vertical exploration (number of vertical rears or wall leans), directed exploration (head dips, side looks), risk assessment behavior (stretch-attend postures), vegetative responses (number of defecation boli and urination spots), and vestibular/motor

indices (number and latency of hind-leg slips and falls from rod). If the animal falls, replace it in the same position.

Notes:

1. Low motor or vertical activity may be a strain-specific phenotype. Inactive strains will produce less activity overall, and may not be suitable for this model. Likewise, hyperactive strains generally display less non-horizontal exploration and may have difficulties with balance. A narrower apparatus will encourage the animal to show less horizontal activity, enabling it to focus on other behavioral responses.
2. If the mouse displays abnormally high transfer anxiety, gently support it for approximately 5 s to facilitate a solid grip. If the animal continues to display high transfer anxiety, exclude it from the experiment. A dimly lit experimental room may help reduce anxiety.
3. Differences in mouse size should also be addressed. Use animals of similar size, age, and weight to accurately compare between groups.
4. Some strains have difficulties balancing on an aluminum rod, and a more textured surface (e.g., wood) may help stabilize the animal. Increasing the diameter of the rod is another possible solution. If mice continue to struggle with balance or motor abilities, assess motor and vestibular functions separately as these behaviors may be due to a neuromuscular or motor coordination problem unrelated to vestibular deficits or anxiety.

3.9 Light-Dark Box Test

This ethological model of anxiety measures the activity and time spent in brightly lit vs. dark compartments of the apparatus, and is based on the animal's innate desire to explore novel areas (5, 12). Anxious mice exhibit a profound preference for the dark area and display fewer exploratory behaviors (e.g., horizontal activity, vertical rears or wall leans) in the light. Increased

duration of time spent in the light area and more exploratory behaviors can be seen following anxiolytic drug administration.

1. Place one animal into the dark compartment of the box for 5 min for acclimation
2. Lift the shutter to allow the mouse to move freely between the dark and light compartments for 5 min.
3. Measure the latency to initial transition into the light box. Record the duration of time spent in each compartment, the number of transitions between them, and the distance travelled in each box. Additional indices may be vertical rearing, wall leans (in the light compartment), and the number of defecation boli.

Notes:

1. Certain strains of mice may be less inclined to explore the test environment, such as mice with anxiety- or depression-like phenotypes. Allow a longer acclimation and/or test time (e.g., 10 or 15 min) to reduce this factor.
2. Some mouse strains display visual deficits, and may not be a suitable model for this test. Consider other mouse strains for the light-dark box testing.

3.10 Stress-Induced Hyperthermia (SIH)

This test relies on the evolutionarily important role of hyperthermia, a rise in body temperature upon encountering stressful stimuli which occurs across many species, including humans. In mouse SIH test, the insertion of a rectal thermometer records a 0.5-1.5°C increase in body temperature. SIH is reduced or prevented by different anxiolytic drugs, however, it seems to be unable to detect anxiogenic and antidepressant effects (21).

1. Animals should be put in individual cages the day before testing to avoid effects of acute isolation stress.

2. Baseline body temperature should be recorded. To test mouse rectal temperature, carefully insert a probe with a rounded tip (up to 3 mm thick) after dipping it in any kind of oil for lubrication. The probe should be inserted consistently (approximately 2-2.5 cm) for 10 s.
3. Present the mouse with a stressor, such as a novel cage, and document the change in internal temperature.
4. After testing, mice may be re-socialized in grouped housing. They may be retested after in 1-week intervals.

Notes:

1. While most strains respond consistently to this paradigm due to its independence from motor activity, specific mouse strains (e.g., FVB/N) have considerably higher baseline body temperatures, and should be avoided in this model (21).
2. In the group-housed mice, the last mice to be tested show an increase in body temperature (compared with the first mice) due to anticipatory anxiety. Therefore, animals should be tested individually, with at least 10 mice in each experimental group (21).
3. Baseline body temperature is significantly higher during the night. If testing occurs during the dark phase, there may be an interference with the amplitude of hyperthermia when the stressor is presented (21).
4. Mice should be kept undisturbed before the experiment with proper handling and opening of cages to ensure accurate results.

3.11 Hole-Board Test:

Conceptually similar to the open field test, the hole-board test focuses on specific head dipping behaviors. Head dipping, an indication of directed exploration, can be vigorously affected by various drug classes, including anxiolytic and anxiogenic drugs. Due to its short duration and

quantifiable behavioral measures, this test is a readily available method for the testing of classic or novel drugs (22).

1. Place mice individually in hole-board apparatus and record behavior for 5-15 min, documenting traditional exploratory behaviors (as in the open field test, see above) and the number of head dips.

Notes:

1. Certain drugs (e.g., ethanol) are known to be strain-dependent in their effects and may not produce consistent results in the hole-board test (22).
2. Many commonly used drugs (e.g., fluoxetine) have pronounced dose-dependent effects on head dipping behavior, and therefore, dosing should be carefully considered (22).
3. For some other recommendations, see the open field test above.

Rat Exposure Test:

This test utilizes the natural defensive "avoidance" behavioral response of mice to signs of potential danger, such as a natural predator (e.g., rat). Defensive behaviors include stretch-attend posture, stretch approach, freezing, burying, and hiding, and are measured as a function of risk assessment. This test has proven useful to determine strain differences in defensive behaviors and relative levels of anxiety in response to predators. Additionally, the defensive behaviors measured are sensitive to anxiolytics, making this paradigm useful in pharmacological screening (23).

1. Introduce the mouse into the small black box, which will serve as a "home chamber" (safe environment). The Plexiglas tunnel should allow free movement between the home chamber and the observation box.
2. On the first 3 days of testing, allow the mouse to explore the observation box for 10 min to become familiar with the environment. In these sessions, there should be no rat present.

3. On the fourth day, insert the mouse into the home chamber and the rat into the observation box for 10 min. The rat should be placed in the opposite side of the cage, isolated from the mouse by the wire mesh.
4. In every testing session, record the number of stretch-attend postures, stretch approaches, freezes, and number of times the mouse retreats to the home chamber. Also, measure the amount of time spent in the home chamber and observation box, as well as time in contact with the wire mesh.

Notes:

1. If the mouse tested is very inactive and anxious, it may not even leave the home chamber, and this test will not work. In this case, use a milder stressor, such as an anesthetized rat, a toy rat, or rat odor. However, it may also be recommended to use a different mouse strain.
2. Although this test is very useful for comparing defensive behaviors between mouse strains, some strains are not suitable for this test. For example, mice with sensory deficits (e.g., poor vision or olfaction) or with particular cognitive problems (e.g., poor working memory) will not provide reliable data in this paradigm. As mentioned above, it may help to use a different mouse strain.

3.13 Novel Object Test:

This model investigates the approach-avoidance behaviors of mice in response to novel stimuli. Typically, mice tend to explore a novel object longer than a familiar one, and prior exposure to a stimulus increases consecutive approach behavior and decreases avoidance behavior. This robust behavior, as well as the simplicity of this model, makes this test particularly useful for measuring anxiety in a battery of tests (24). Anxiolytics have been shown to increase exploratory behavior of mice in novel environments (29), suggesting that the use of anxiolytics would similarly increase this behavior with novel objects.

1. On Day 1, introduce the mouse into the test apparatus, allowing it to explore the environment for 30-60 min.
2. On testing day, insert the novel object into the center of the testing apparatus prior to introducing the mouse. Record the frequency and duration of exploratory behavior, such as approaches, sniffing, physical contacts (e.g., touching, licking, biting, etc.), wall leans, vertical rears, head dipping, and time spent near the novel object, for 10-30 min. Also record amount of avoidance behavior as time spent in the perimeter.
3. A video tracking system may be useful for measuring amount of movement and position within the test apparatus. Conversely, the apparatus may be sectioned off, and duration in each section can be recorded, comparing the perimeter sections to the novel object section (24).

Notes:

1. Similar to some other previously described tests, mouse strains with sensory or cognitive deficits may not provide reliable data in this model. In addition, some mouse strains can exhibit strong neophobia, which would also confound behavioral data. Test mice prior to this experiment to screen for such defects, and consider using alternate strains and/or extending the observation time.

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