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Jaanus Harro *Editor*

Psychiatric Vulnerability, and Mood and Anxiety Disorders

Tests and Models in Mice and Rats, Volume 2

Humana Press

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Psychiatric Vulnerability, and Mood and Anxiety Disorders

Tests and Models in Mice and Rats, Volume 2

Edited by

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Preface to the Series

Experimental life sciences have two basic foundations: concepts and tools. The *Neuromethods* series focuses on the tools and techniques unique to the investigation of the nervous system and excitable cells. It will not, however, shortchange the concept side of things as care has been taken to integrate these tools within the context of the concepts and questions under investigation. In this way, the series is unique in that it not only collects protocols but also includes theoretical background information and critiques which led to the methods and their development. Thus it gives the reader a better understanding of the origin of the techniques and their potential future development. The *Neuromethods* publishing program strikes a balance between recent and exciting developments like those concerning new animal models of disease, imaging, in vivo methods, and more established techniques, including, for example, immunocytochemistry and electrophysiological technologies. New trainees in neurosciences still need a sound footing in these older methods in order to apply a critical approach to their results.

Under the guidance of its founders, Alan Boulton and Glen Baker, the *Neuromethods* series has been a success since its first volume published through Humana Press in 1985. The series continues to flourish through many changes over the years. It is now published under the umbrella of Springer Protocols. While methods involving brain research have changed a lot since the series started, the publishing environment and technology have changed even more radically. Neuromethods has the distinct layout and style of the Springer Protocols program, designed specifically for readability and ease of reference in a laboratory setting.

The careful application of methods is potentially the most important step in the process of scientific inquiry. In the past, new methodologies led the way in developing new disciplines in the biological and medical sciences. For example, Physiology emerged out of Anatomy in the 19th century by harnessing new methods based on the newly discovered phenomenon of electricity. Nowadays, the relationships between disciplines and methods are more complex. Methods are now widely shared between disciplines and research areas. New developments in electronic publishing make it possible for scientists that encounter new methods to quickly find sources of information electronically. The design of individual volumes and chapters in this series takes this new access technology into account. Springer Protocols makes it possible to download single protocols separately. In addition, Springer makes its print-on-demand technology available globally. A print copy can therefore be acquired quickly and for a competitive price anywhere in the world.

Saskatoon, Canada ***Wolfgang Walz***

Preface

The burden of mood and anxiety disorders on society is enormous and continues to grow despite advances in medicine and psychology. The negative impact of emotion-centered disorders is aggravated by comorbidity with other psychiatric disorders as well as with many somatic diseases. Animal models remain indispensable in combat with pathological mental processes because they enable precise experimentation on the brain in its physiological complexity of regulation. With their aid, the understanding of the molecular neurobiology of brain disorders will become much more detailed. Behavioral models also help predict whether new drugs in development will be effective and whether they might be liable to central nervous system (CNS)-based side effects. Animal modeling has undergone major technological developments, and this has even affected the range of species in use; nevertheless, given the organization of affect in the mammalian CNS, mice and, increasingly again, rats retain their position at the center of all efforts. Obviously, human mood and anxiety disorders cannot be reproduced in rodents. However, the neural "building blocks" for the mood and anxiety disorders have evolved in evolution and can be studied in animals for the benefit of humankind by carefully established behavioral techniques and related, neurobiology-informed methods.

This book is a complement to Volume I of *Psychiatric Vulnerability, and Mood and Anxiety Disorders: Tests and Models in Mice and Rats*, published as Neuromethods Vol. 190 in 2023. The previous collection of chapters aimed at refreshing the view on classic tests and models and providing detailed protocols and useful hints for more novel methods that are likely to join the mainstream of studies on mood and anxiety disorders and to support the development of precision medicine. Volume I also promoted the concept that in animal modeling one should consider the hierarchical inner structure of psychiatric disorders, as much of what we observe in behavioral pathology reflects general psychiatric vulnerability. Volume II includes detailed protocols for classic tests such as the elevated mazes, the novelty suppressed feeding test, and fear-potentiated startle. To assess reward sensitivity, tests relying on both natural reward seeking and operant behavior are presented, as well as more recent assays of affective bias and reward learning. Evolution-shaped avoidance of looming stimuli has been addressed with a novel ethoexperimental fear and anxiety test. The translational value of anxiogenic chemical compounds is highlighted, and the marble burying test has received a further technological refinement to aid interpretation. Prenatal stress, disrupted caregiver–infant interactions, predator-based stress, and witness social stress broaden the range of evolutionarily relevant challenge-based paradigms, as does the neuroinflammation by immunological provocation. Going beyond behavioral readouts, the volume includes infrared thermography, electrophysiological recording, and calcium imaging for the measurement of emotion via neurophysiology and interpretation of what is the meaning of observed behavior, and where in the brain anxiety and mood disorders reside. Naturally occurring variation in emotional and cognitive reactivity can be employed by using well-established animal strains. Together with Volume I, this book offers a comprehensive resource for researchers and students who

wish to establish animal model (batteries) for drug discovery or the study of mental processes, as well as for professionals who wish to broaden their understanding of how this frontier of psychiatric research operates.

Tartu, Estonia *Jaanus Harro*

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Chapter 1

Testing Anxiety-Like Behaviors in Rodents with the Elevated Plus and Elevated Zero Mazes

Laura B. Tucker and Joseph T. McCabe

Abstract

The elevated plus and elevated zero mazes (EPM and EZM) are behavioral paradigms frequently employed in neurobiological research to investigate the mechanisms of anxiety and to develop effective pharmacological treatments for psychiatric disorders. Each apparatus has regions that are anxiogenic (illuminated and open/exposed) and regions perceived as safe (darkened and enclosed), with rodents showing a preference for the latter. The reliability of these assays depends on rigorous, standardized procedures. Factors such as maze construction, illumination, circadian timing, prior handling, and test order can meaningfully alter behavioral outcomes. Even subtle changes (e.g., transport method, environmental noise, cleaning protocol) can shift exploration patterns or drug sensitivity. Experimental design must also account for species, strain, and sex differences, as well as the influence of prior behavioral experience on subsequent performance. In this protocol, we outline critical procedural considerations and discuss how differences between the EPM and EZM may inform assay selection. By applying these methodological controls, researchers can generate high-quality, reproducible data while making informed choices between the two tests based on experimental goals.

Keywords Elevated plus maze, Elevated zero maze, Anxiety-like behavior, Rodent behavioral testing, Behavioral phenotyping

1 Introduction

For many decades, preclinical behavioral testing has played a critical role in neurobiological research and the search for efficacious treatments for anxiety disorders. Many studies measuring anxiety-like behaviors take advantage of rodents' approach-avoidance conflict between their curiosity about new environments and their fear of bright, open spaces [1]. Along with the light–dark box (LDB) test, the elevated plus and elevated zero mazes (EPM and EZM, respectively) are the assays that are most frequently used in preclinical research that aim to evaluate state anxiety-like behaviors (*see* **Note 1**). As of 2025, a search on PubMed for "elevated zero maze" or "elevated

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plus maze" produced over 11,000 publications. (The LDB appears in about 1000 results.)

The EPM, first introduced in 1984 [2] and pharmacologically validated in rats the following year [3] and later adapted for use in mice [4], consists of a raised, plus-shaped platform with two enclosed arms and two open arms joined at a central square (Fig. 1A). The animal is placed on the maze and allowed to freely explore for 5–10 min while behavior is recorded by an observer or tracking software. Rodents typically favor the enclosed arms, emerging only briefly into the open, illuminated regions that are considered anxiogenic. Experimental manipulations can shift this balance: stressors such as early-life adversity, chronic stress, corticosterone level elevation, or immune activation (e.g., lipopolysaccharide administration) reduce time in the open arms, whereas anxiolytic drugs (e.g, benzodiazepines, serotonergic agents, cannabinoids) increase it (e.g., [5, 6]; but see [7]). The central platform, however, introduces interpretive challenges, as time spent there may reflect hesitation or decision-making rather than anxiety per se.

The EZM, developed in the 1990s [8] as a refinement of the EPM, was specifically designed to eliminate the central platform. Its continuous annular track, divided into two enclosed quadrants and two open quadrants, allows uninterrupted exploration (Fig. 1B). By removing the central square, the EZM reduces decision-related ambiguity and provides a more direct measure of approach-avoidance conflict. Behavior is recorded in the same manner as in the EPM, but the circular design may yield more consistent exploration across trials and improve sensitivity to both anxiolytic and anxiogenic manipulations.

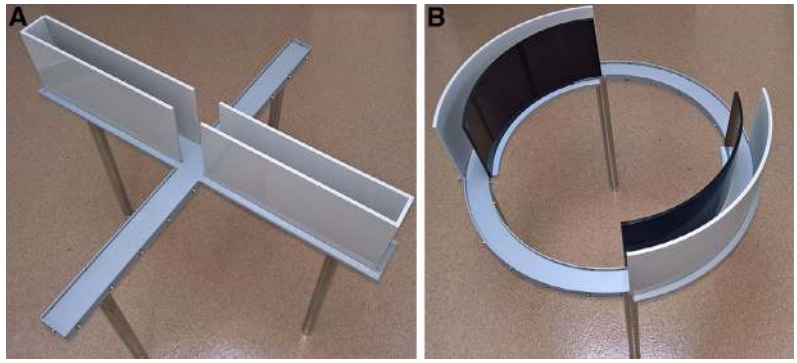


Fig. 1 The elevated plus maze (EPM; **A**) and elevated zero maze (EZM; **B**), both from Stoelting Co. (Wood Dale, IL). Both mazes are elevated 50 cm above the floor. The EPM (**A**) has two open, exposed arms; perpendicular to those arms are two darkened and enclosed arms. The four arms meet in a 5-cm square region. The EZM (**B**) consists of four equal quadrants; two opposite quadrants are darkened and enclosed, and the remaining two are open and exposed. See Tables 1 and 2 for all dimensions. (Adapted from Tucker and McCabe, 2017; CC-BY, Frontiers in Behavioral Neuroscience)

In addition to assessing conflict-related behaviors, ethological variables such as stretch-attend postures (SAPs) and head dips (HD) can capture a broader range of natural, anxiety-like behaviors in rodents in both the EPM and EZM. They provide a more nuanced understanding of behavioral responses and anxiety-like states, reflecting not only spatial preference but also actions that indicate stress (SAPs) or exploration (HD). SAPs are defined by the rodent elongating its body with the fore- and hindlimbs remaining fixed in place, and are considered a risk-assessment behavior reflective of hesitation, increased caution, and heightened anxiety. Head dips are an exploratory behavior used as an index of reduced anxiety and are characterized by an animal investigating the edge of the open region of the apparatus, extending its nose or head below the plane of the maze platform. Both SAPs and HDs can be protected, occurring while the body of the animal is within the enclosed arms, or unprotected, which occur when the animal's body is fully on the open regions of the apparatus.

Although spatial preference and ethological behaviors in the EPM and EZM are relatively simple to assess, procedural differences that exist between research groups make replication and comparison of results very difficult. Factors such as apparatus size, lighting condition, animal handling, and definitions of ethological behaviors (e.g., stretch-attend postures) can all affect the behavior of the animals and interpretation of anxiety-like behaviors. This chapter aims to offer standardized methods for using both the EPM and EZM in rodents. Although protocols for the EPM have been previously published (e.g., [9–11]), we also include details about the EZM (see Sects. 2.1 and 2.3) and provide comparisons between the tests that may aid investigators choosing between the similar assays (see Sect. 7). The goal is to help behavioral researchers be more consistent, improve reproducibility, and generate data that is comparable between laboratories when studying anxiety in clinical models.

2 Material and Setup

To ensure consistency and reproducibility across studies, successful completion of an elevated maze (EM) experiment requires careful attention to apparatus design, environmental factors, and animal treatment to ensure consistency and reproducibility across studies. This section outlines physical specifications of the EMs, as well as essential environmental and technical considerations needed to standardize testing conditions between laboratories and species.

2.1 Apparatus

The EPM and EZM (Fig. 1A, B, respectively) are designed according to a similar concept—two opposing dark and enclosed regions, alternating with open and exposed regions. The devices are elevated about 50 cm from the surface (e.g., floor) and can be constructed

Table 1
Example dimensions (Stoelting Co.) of the elevated plus maze apparatus for mice and rats

Elevated plus maze	Rat	Mouse
Open and closed arm lengths	50 cm	35 cm
Open and closed arm widths	10 cm	5 cm
Center square region	10 cm^2	5 cm^2
Height of walls	40 cm	15 cm
Leg height	50 cm	50 cm
Rim height for open arms	5–10 mm	2–5 mm

Apparatus dimensions may vary slightly by vendor; investigators should confirm specifications before comparing across studies

with plastic, wood (fiberboard), or metal. Dark or neutral colors are typically used, and the surface should be non-reflective (matte) and non-slip to avoid distress or injury to the animal.

2.2 Elevated Plus Maze (EPM) Apparatus

Elevated plus maze apparatuses are available from numerous companies supplying behavioral neuroscience equipment, including but not limited to Stoelting Co., Maze Engineers, San Diego Instruments, Harvard Apparatus, and Med Associates. Although exact material composition, features, and dimensions vary by vendor, the common design of EPMs includes a plus-shaped design with two opposing open arms without walls, with the remaining two arms enclosed with walls (but open at the top). The four arms intersect at a central square. The EPM available from Stoelting Co. is composed of ABS hard plastic (aluminum prior to 2022) in either dark gray or light gray, depending on the color of the animals to be tested (for automated tracking by software). Representative dimensions of the EPM, based on the apparatus sold by Stoelting Co., are in Table 1. It should be noted that characteristics of the apparatus can vary by vendor. For example, CleverSys (Reston, VA) offers a plastic blue EPM for mice that is higher above the surface (59 cm) with shorter (30 cm) and wider (7 cm) arms.

2.3 Elevated Zero Maze (EZM) Apparatus

The EZM is designed with the same concept as the EPM, but instead of a plus-shape, the EZM is an elevated circular "zero" track with two opposing open and exposed quadrants and two closed quadrants. Like the EPM, the material of the EZM from Stoelting Co. is gray ABS plastic or aluminum. Dimensions of the EZM available from Stoelting Co. are found in Table 2.

2.4 Video Recording and Tracking System

Automated tracking for most EM measures is preferred for speed, reduction of experimenter bias, and for more precise replication of analysis between laboratories. Many options are available for video

Table 2
Example dimensions (Stoelting Co.) of the elevated zero maze apparatus for mice and rats

ELevated zero maze	Rat	Mouse
Outer diameter	120 cm	60 cm
Inner diameter	100 cm	50 cm
Width of lanes	10 cm	5 cm
Height of walls	30 cm	15 cm
Leg height	50 cm	50 cm
Rim height for open quadrants	5–10 mm	2–5 mm

Apparatus dimensions can differ slightly among vendors, and specifications should be verified to ensure consistency when comparing across studies

recording and automated tracking for EPM and EZM testing. Typically, a digital video camera is mounted to the ceiling directly above the apparatus and connected to a standard desktop or laptop computer. Videos can be stored for post hoc analysis and curation. Many commercially available software packages are available for real-time animal tracking (e.g., ANY-maze [Stoelting Co.] and EthoVision XT [Noldus]). Alternatively, no-cost open source programs such as ToxTrac and DeepLabCut are increasing in popularity.

2.5 Environmental Setup

Mazes should be placed in a dedicated behavioral testing room, separate from housing facilities or other procedure rooms. Ideal temperature is 21°C–23°C with 40%–60% humidity. Room ventilation should have quiet and consistent airflow, and direct drafts on the maze should be avoided. Testing should occur in a quiet and low-traffic environment. A white noise machine can be employed if ambient sound is unavoidable.

Testing Room

Lighting Conditions

Lighting levels (lux) vary widely between individual investigators' protocols, but a typical range is 5–20 lux at maze level. This relatively low level of illumination reduces stress and encourages exploration (*see* **Note 2**). Overhead fluorescent lights should be avoided; indirect and diffuse lights are ideal for avoiding casting shadows on the maze. In our laboratory, we use non-heat-emitting dimmable floor lights with flexible necks, allowing the light to be aimed upward and away from the apparatus for even illumination without glare. Reporting illumination conditions is an important data element for publication.

Maze Placement

The apparatus should be placed away from lab benches or other large objects, such as equipment or furniture, to minimize distractions and reduce the risk of the animal attempting to jump toward nearby surfaces. Ideally, the maze will be approximately in the center of the

room, equidistant from walls, doors, and other large objects. Avoid placing the maze near windows or any reflective surfaces. Opaque curtains or partitions can be used for isolation from visual cues. The floor or platform should be even and without vibrations.

2.6 Subjects

Although occasionally employed in other species (e.g., gerbils, guinea pigs, tree shrews), mice and rats are the most common animals tested in the EPM and EZM. Rodents are typically group-housed with enrichment (e.g., tunnels, nestlets, huts) and maintained on a 12:12 light–dark schedule, with food and water available *ad libitum*. Temperature and humidity should remain relatively constant, and animals should acclimate to housing for at least 7 days following any environmental change prior to beginning experimental manipulations or behavioral testing. Bedding changes should be coordinated with husbandry personnel or performed by the investigator. Bedding should never be changed prior to testing; if needed, it can be changed the day before testing or immediately after.

Individual animals can be identified using long-term methods (e.g., radio frequency identification devices [i.e., microchips], ear tags or punches, tattoos) or short-term methods (e.g., fur clipping or tail marks with non-toxic permanent marker). If long-term methods are employed, short-term marking is also recommended for faster identification, but marking should not be performed just prior to testing. The procedures described here apply to both mice and rats. Typically, young adult rodents (mice 8–12 weeks old, rats 8–16 weeks old) are used as they are large enough to explore the apparatus confidently. Ideally, the subjects will be naïve to the EPM or EZM, although repeated measures can be employed with some considerations (see Sect. 6.4).

2.7 Additional Equipment and Supplies

In addition to the apparatus and camera with a computer and tracking software, several items are essential for a successful EM experiment. A digital luxometer helps ensure the lighting is consistent across testing sessions and evenly distributed within the maze, especially in the open arms or quadrants where differences in brightness can affect animal behavior. If manual scoring of behaviors not integrated with tracking software will be performed, a silent timing device, stopwatch, and/or cell counter will be needed, as well as a notebook, data sheet, or digital tablet. Personal protective equipment (e.g., lab coat and gloves) must be worn, and cleaning supplies (70% ethanol, paper towels, and germicidal wipes [e.g., Sani-Cloth® Plus]) are necessary for cleaning the maze between subjects and at the end of the experiment. Soft padding may be placed beneath the maze to minimize risk of injury or stress in the event of a fall, particularly when the apparatus is elevated to a greater height. Depending on the species, transport containers or devices are employed to minimize stress of the subject prior to placement on the apparatus [12]

(see Sect. 6.3). If pharmacological agents are administered prior to testing, an animal scale should be available, ensuring accurate dosing based on animal weight.

3 Procedures

3.1 Apparatus Setup and Calibration

Place the EPM or EZM in the desired location of the testing room, and ensure it is level and stable on its base of support (to prevent wobbling). Using a lux meter, adjust lighting to predetermined brightness, and ensure no glares or shadows fall on the maze. Within the exposed regions (open arms or quadrants), brightness should be at precise and equal levels. Set up the video camera directly above the apparatus (*see* **Note 3** for comment on camera placement for the EZM) and test the field of view, focus, and brightness (appropriate contrast is necessary for most automated tracking software). Within the tracking software (e.g., ANY-maze), calibrate maze dimensions and define zones of interest (open/closed regions for both mazes, center square for the EPM). If the experimenter is remaining in the room for manual scoring, ensure they are positioned out of sight of the animal being tested but maintaining full view of the computer monitor. Ideally, the investigator will quietly leave the testing room and observe from a nearby location.

3.2 Animal Preparation and Handling

Animals should be brought to the holding or testing room approximately 1 h prior to testing. They are typically transported in their home cages on a quiet (e.g., plastic) cart with vibration-minimizing pads. During the 1-h acclimation, animals may be kept in the testing room or held separately in an anteroom or adjacent holding area (keeping lighting consistent with the testing room). In some laboratories, the animals remain in the testing room for the duration of the day's testing session; in others, they are transported individually or in small groups just prior to testing to minimize the effects of social cues or environmental exposure. If injections are required prior to testing, they should not be performed in the testing room.

One animal is tested at a time, and carried gently from the cage to the testing apparatus using a transport cage or other handling device (e.g., enrichment tunnel; see Sect. 6.3). To avoid stress or excitement, direct handling by the base of the tail should be minimized. For the EZM, place the mouse or rat at the border of a closed and open quadrant, ensuring the animal walks into the closed region (*see* **Note 4**). In the EPM, the animal is typically placed in the center, facing the opening to a closed arm. Some laboratories, to encourage exploration, choose to place the animal in the center facing an open arm, or at the distal region of an open arm. Start the timer or recording software as soon as the animal is released, and leave the testing room

to a location where the mouse or rat can continue to be monitored, or, if performing live manual scoring, move to a region of the room out of sight of the animal but within view of the computer monitor.

3.3 Testing in the Elevated Plus Maze

Allow the animal to explore the maze for 5 min (e.g., [10, 11, 13]). Behaviors can be scored by the investigator in real time, but the session should not be disrupted by movement or by noises from timers or counters. Key behaviors to be recorded are entrances to and amount of time spent in open and closed arms (with care given to the definition of an arm entry; see Sect. 4.1), and ethological indications of anxiety, such as SAPs and HDs. These behaviors can be classified as protected (occurring while the body of the animal remains in the center or closed arms), or unprotected, when the animal is fully positioned in the open arms. Gross behaviors such as entries and time spent in arms are typically scored in real time by video tracking software (if available), but ethological behaviors are often quantified post hoc from recorded videos.

Interventions of animal behavior are to be avoided and should only be performed for safety; if the animal climbs a wall or falls from the maze, gently guide it back or lift it back onto the maze, noting in the lab notebook the occurrence of the event (*see* **Note 5**). At the end of the trial, stop the video recording and gently remove the subject from the apparatus and carry it using a transport object or container back to the home cage away from the testing area. Between animals, lightly spray the apparatus with 70% ethanol to remove olfactory cues and allow it to dry before the next animal's trial. After all subjects have been tested, the apparatus should be treated with a germicide (e.g., Sani-Wipes Plus®)

3.4 Testing in the Elevated Zero Maze

Testing procedures in the EZM are very similar to those for the EPM. In the EZM, the mouse or rat is placed at the boundary between an open and closed quadrant, ensuring the animal fully enters the closed quadrant. The trial duration is typically 5 min (e.g., [6, 8, 13]); the subject can freely move in either direction around the circular track. Entries to and time spent in the open and closed quadrants are scored manually or with automated software (see Sect. 4.1 regarding quadrant entry definition), and protected/unprotected SAPs and HDs can also be quantified. Unlike the EPM, there is no center zone to act as a transition, and the animal is always positioned in either a closed or open quadrant, simplifying scoring and interpretation. Similar to the EPM, interference by the investigator should be minimal and only be performed if the subject falls or climbs on the walls of the maze. After 5 min, stop the session and gently carry the animal in a transport container or object to the home cage away from the maze. As described for the EPM, the maze should be cleaned with 70% ethanol and allowed to fully dry prior to testing the next animal, and a germicide should be used after completion of the cohort.

3.5 Pharmacological Manipulations

The EMs are often employed to assess the impact of drugs on anxiety-like behavior. If administering a drug via injection at a predetermined time shortly prior to EM testing, ensure the time between injection and testing is identical for all subjects. Injections should always be performed in an anteroom and never in the testing room. Acute anxiolytic or anxiogenic agents are often administered 30 min prior to testing (e.g., [8, 14, 15]), but exact timing will depend on the drug's known onset of action. The control group should receive an equivalent volume of the vehicle solution, at the same predetermined time prior to testing. To avoid biases in animal handling, the experimenter should be blinded to the treatment condition of the subject. (Drug vials can be coded.) If cohorts are large and testing will take several hours on a given day, treatments should be randomized or counterbalanced to avoid time-of-day effects. During the post-injection period prior to testing, subjects can be kept in their cages in a quiet anteroom or in the testing room. After the post-treatment interval has elapsed, testing can proceed as described.

4 Data Acquisition

4.1 Scoring Behavioral Measures

Although early scoring for the EZM and EPM was performed manually by stopwatches and counters, modern software has automated the analysis of most measures of interest in the EMs and provides a greater level of accuracy and reproducibility. The most traditional behaviors scored in the EMs include the amount of time spent in the open and closed arms, as well as the total number of arm entries. These measures can be automatically calculated based on pre-defined zones in the software, and care should be given to operational definitions of entry to a zone. During manual scoring, the traditional definition of zone entry required placement of all four paws in the arm or quadrant. Automated scoring allows greater precision of this definition. While the location of the center point of an animal may provide an efficient and objective means of determining zone entries, lingering behavior and/or rapid back-and-forth movements at zone borders can lead to inflated counts of zone entries. A practical substitution for the traditional "all four paws" criterion employed in hand scoring is considering 80% of the animal's body (based on whole-body tracking) within a new zone as an entry. For example, within the settings for zones in ANY-maze software, "zone entry settings" allows a user to control how the software scores a zone entry. One option is "use the animal's entire area," and to enter 80 as the "minimum percentage of the animal that must be in the zone for an entry to occur." It must also be specified as the "minimum percentage of the animal that must remain in the zone for an exit

NOT to occur, and an entry of 80 ensures that an exit from the zone and entry into another zone follows the "all four paws" criterion.

For the EPM, the amount of time in the center zone is also recorded. This value can be reported separately or employed to report time spent in the open/closed arms as a percentage of total arm exploration time, depending on analysis preferences. The EZM does not have a center zone, simplifying analysis.

4.2 Additional Ethological Measures

Beyond the basic open/closed zone analyses, many ethological measures of behavior provide further insight into anxiety-like states and results of anxiolytic or anxiogenic manipulations [8]. The most common measures to quantify are HDs, referring to the number of head dips over the edge of the apparatus, and SAPs, in which the body of the animal stretches forward without movement of the hindlimbs. HDs are thought to reflect exploratory risk assessment, and SAPs represent hesitation based on the approach/avoidance conflict. Both of these behaviors can be further classified as protected, meaning they occur within the safety of the closed zones (typically at borders between open and closed regions), or as unprotected, observed while the animal is located in the open and exposed zones.

To date, automated analysis of these subtle exploratory behaviors by standard tracking software is challenging and requires a manual review or more specialized software. HDs require knowledge of the head's position in the Z plane, as the vertical motion of the head to a position below the surface of the maze is required to be defined as an HD. A single overhead camera and tracking systems such as ANY-maze and EthoVision XT only capture the animal's position in the X-Y plane. Additional cameras with side views of the apparatus can be helpful, but the behavior is nuanced and cannot be accurately assessed by logging coordinates crossing a line. Open-source software for 3D pose estimation based on machine learning, such as DeepLabCut (MATLAB-based) and Social LEAP Estimates Animal Poses (SLEAP; Python-based) is increasing in popularity, but they also require multi-view synchronized cameras in order to provide estimates [16, 17]. Detection of SAPs presents similar challenges. Although less dependent on 3D positioning, SAPs are subtler and more complex than HDs, requiring recognition of the length and contour of the animal's body, as well as the spatial relationship between body parts. SAPs typically include a lowering of the body toward the floor of the maze, which cannot be detected by an overhead camera. To date, most commercially available tracking software packages lack interpretive algorithms and pose estimation needed to distinguish SAPs from similar behaviors such asslow walking.

Quantifying HDs and SAPs is typically performed manually, using cell counters or event-recording software. Both Stoelting's ANY-maze and Noldus' The Observer XT allow for detailed event logging; BORIS (Behavioral Observation Research Interactive

Software) is free and open-source software designed for event logging from live observations or post hoc from recorded videos. For manual scoring, operational definitions of the behavior of interest must be established in advance. HDs are relatively straightforward, though some extended observation and practice may be required to accurately assess from an overhead view the vertical motion of the head below the surface of the maze. Defining SAPs is more challenging due to their subtle, transitional nature, individual variability in expression, and reliance on nuanced postural cues that are difficult to detect with overhead video or standard scoring criteria. While SAPs are classically defined as forward elongation of the body with stationary hindlimbs [18], laboratories vary in their criteria for duration, degree of extension, and scoring method, leading to differences in how SAPs are identified and quantified. For example, some groups describe a 1–2 s minimum duration of the posture to be recorded, and/or do not count a posture that ends with the animal taking steps forward.

Due to the subjective nature of hand-scoring SAPs and HDs, it is highly recommended that the investigator be blinded to the experimental condition of the animal. Additionally, particularly when taking on HD and SAP scoring for the first time, it is advisable to use two blinded, independent observers and calculate inter-rater reliability to ensure the behavior is defined clearly and can be identified consistently across scorers. If a single investigator is performing the scoring after definitions have been agreed upon, the same investigator should carry out the analysis for all subjects within a given experiment to ensure consistency.

4.3 Locomotor Activity

The number of entries to open and closed zones of the apparatus provides a measure of locomotor activity of the subject, and assessment of general activity is especially critical if a pharmacological agent may result in sedation or hyperactivity. Closed-zone entries represent general activity, where more entries indicate a greater level of exploration. Open-zone entry counts are more representative of anxiety-like states, where fewer open-zone entries suggest a greater anxiety-like state. Together, the frequency and pattern of entries into closed and open zones are commonly used to infer the animal's balance between exploratory drive and anxiety-related avoidance. For example, a rodent with a normal level of exploratory drive but high anxiety may make many closed zone entries with very few forays into the open regions. Ratios, such as "open/(open + closed entries)," can be employed to more accurately interpret anxiety-like behavior independent of general locomotion [3].

Automated software also allows for extraction of numerous measures that may be of interest and value depending on the experimental questions. Like closed-zone entries, total distance traveled in particular can be very helpful as it gives an idea of the overall

exploratory drive of the animal. While the number of zone entries provides a general measure of locomotor activity, this is only a binary view of movement, counting transitions rather than reflecting the full extent of exploratory behavior. Distance traveled is a continuous variable that more accurately reflects exploratory drive, capturing within-zone movement and/or pacing behavior that is not reflected in zone entry data.

However, distance traveled should not be interpreted as a direct measure of anxiety-like behavior. Rather, it serves as a complementary index of overall locomotor activity, providing essential context for interpreting time spent in open or closed regions. For instance, low open-zone time in an animal with low total distance traveled may indicate hypoactivity or sedation, rather than increased anxiety. Alternatively, an animal with increased open-zone entries and high total distance may be exhibiting hyperactivity, which can also confound anxiety-related interpretations. Including distance traveled alongside open/closed entries and ethological measures strengthens the behavioral analysis by helping distinguish true anxiety-like behavior from general changes in activity level.

4.4 Data Validation

Prior to formal analysis, the raw data should be reviewed for irregularities that may indicate tracking errors or technical artifacts, especially if the automated tracking was not monitored on the computer screen for the duration of the test. Automated systems have occasionally been observed to misidentify the animal's location, particularly during rapid movements, immobility near zone borders, or when lighting or contrast is suboptimal (*see* **Note 6**). Visual inspection of track plots is particularly helpful in confirming that the position of the animal was accurately recorded at all times. Track plots should appear smooth, lacking sharp angles that may be indicative of tracking errors. Furthermore, consistency across behavioral measures (e.g., high open-zone time accompanied by high open-zone entries) can serve as an internal check. Outlier analysis may be used to identify animals with values falling well outside the typical range, though exclusion should be applied cautiously and transparently, following pre-established criteria. When possible, video recordings can be referenced to resolve discrepancies or re-score ambiguous behavior. Finally, all data validation procedures should be performed blind to the experimental group to minimize bias.

4.5 Statistical Analysis

Statistical methods should be established prior to data collection in accordance with the experimental design. Wherever possible, sample size determination should be guided by power analysis, as this improves the interpretability of results and reduces the likelihood of both false negatives and excessive animal use. After data collection, normality and homogeneity of variance should be assessed via visual inspection and the Shapiro–Wilk test and Levene's test, respectively.

If the assumptions for parametric analysis are not met, data transformations (e.g., log, square root, or Box–Cox) can be performed to stabilize variance or approximate normality. If parametric assumptions are met, analysis of variance (ANOVA) is recommended to assess between-group differences. One-way ANOVA (or *t*-test, if appropriate) is employed for single-factor designs (e.g., drug dose), and higher-level ANOVAs are recommended when the effects of multiple factors are assessed (e.g., a two-way ANOVA for Sex × Treatment). If multiple EPM or EZM tests are performed on the same animals, a repeated-measures ANOVA or mixed model may be used, depending on data structure and sphericity. Covariates such as body weight, age, estrus cycle, or level of locomotor activity may be included in mixed models if biologically relevant.

In the event of significant differences when more than two levels are represented within factor(s) or when interaction effects are present, post hoc testing will identify specifically which groups are significantly different. Like statistical tests, post hoc comparisons should be determined a priori and corrections for multiple comparisons (e.g., Bonferroni, Tukey's HSD) should be applied to reduce the risk of Type 1 errors (false positives).

Although parametric testing is preferred due to greater statistical power, precision, and rigor, nonparametric alternatives such as the Mann–Whitney *U* test or Kruskal–Wallis can be used if data continue to violate assumptions of normality or equal variance despite transformation. In this case, investigators should carefully assess and document any outliers using standardized residuals or graphical methods (e.g., boxplots). As described previously (Sect. 4.4), outliers are to be removed only by predefined criteria or in the event of procedural anomalies, and there should be transparency regarding outlier removal in any report of the data.

All statistical procedures should be performed by validated software packages such as SPSS, GraphPad, SAS, or R, with statistical significance set at $p < .05$ unless otherwise specified. Data are typically reported as mean ± standard error of the mean (SEM), and exact p values and effect sizes (e.g., Cohen's d or eta squared [η^2]) should be provided where possible.

5 Anticipated Results

5.1 Baseline Performance and Test–Retest Effects

Properly executed and analyzed, the EMs reliably produce approach-avoidance behavior consistent with the rodents' conflict between exploration and the aversion of bright, open spaces. The majority of our experience with the EMs is in C57BL/6J mice. Figures 2 and 3 show behavior of male and female mice naïve to the apparatus in the EPM and EZM, respectively [13]. In the first trial,

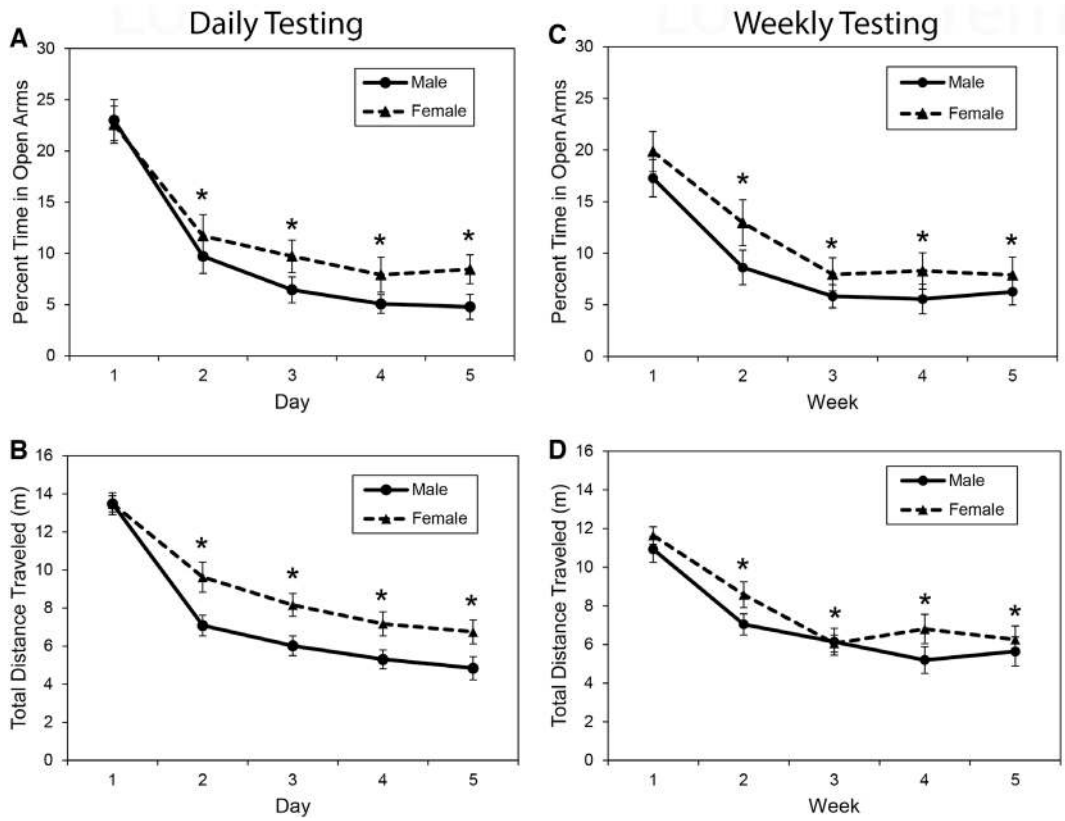


Fig. 2 Behavior of male and female mice in the EPM during repeated daily (**A, B**) or weekly (**C, D**) testing. Illumination was 1600 lux and 200 lux in the open and closed quadrants, respectively. All mice habituated rapidly to the apparatus as evidenced by a significant decrease in exploration (distance traveled) after the first trial (**B, D**). When the mice were tested daily, female mice ambulated greater distances on the second day of testing (**B**). In addition, the amount of time spent exploring the open, anxiogenic regions of the EPM (**A, C**) was significantly decreased on all days following the first exposure. These changes did not depend on the test interval. The asterisk (*) denotes a significant difference on the denoted day compared to Day 1. The pound sign (#) denotes a significant effect of sex on the indicated day. EPM, elevated plus maze; EZM, elevated zero maze. (Adapted from Tucker and McCabe, 2017; CC-BY, Frontiers in Behavioral Neuroscience)

total distance traveled was similar in the EPM and EZM (~12 m), but the time spent in the anxiogenic (bright and open) zones differed by apparatus, with mice tested in the EZM spending ~40% of the session in the open quadrants and mice in the EPM spending about half that time (~20%) in the open arms. Following the first EPM trial, both locomotion and time spent in the open arms declined significantly, regardless of the interval between trials—a well-documented pattern known as the one-trial tolerance phenomenon (OTTP) [15, 19, 20]. In the EZM, however, open-quadrant exploration remained relatively constant across multiple trials when testing was conducted either daily or weekly. Sex differences were observed only in locomotor activity, with females exhibiting higher activity levels than males.

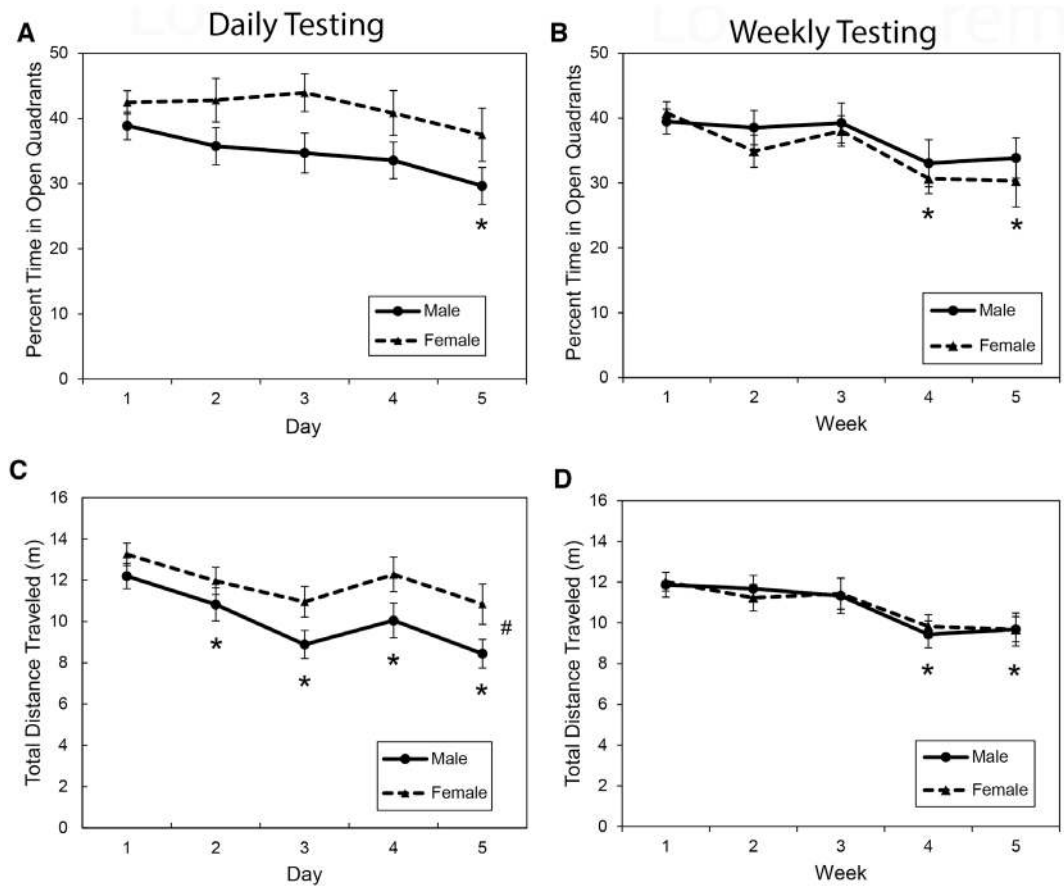


Fig. 3 Behavior of male and female mice in the EZM during repeated daily (**A, C**) or weekly (**B, D**) testing. Illumination was 1600 lux and 200 lux in the open and closed quadrants, respectively. There was a main effect of sex on ambulation in the maze during daily testing, with female mice exploring the apparatus more than male mice (**C**). With daily testing, the amount of exploration decreased after the first trial (**C**), but this measure remained consistent until the fourth week, when the interval was increased to 1 week (**D**). The time spent in the open quadrants also remained consistent with further exposure to the EZM, with the percent of time not decreasing until Day 5 or Week 4 (**A, B**). The asterisk (*) denotes a significant difference on the denoted day compared to Day 1. The pound sign (#) denotes a main effect of sex. EPM, elevated plus maze; EZM, elevated zero maze.(Adapted from Tucker and McCabe, 2017; CC-BY, Frontiers in Behavioral Neuroscience)

5.2 Pharmacological Validation

Although the EPM and EZM may differ somewhat in sensitivity to specific experimental manipulations (see Sect. 6), both reliably detect the direction of anxiety-related changes: anxiolytic conditions (e.g., benzodiazepines and environmental enrichment) increase open-zone exploration, whereas anxiogenic conditions (e.g., high doses of caffeine and chronic stress) reduce it. Benzodiazepines such as diazepam and chlordiazepoxide, which act as $GABA_A$ receptor agonists, consistently increase open-area exploration in single-trial sessions across both mazes (e.g., [4, 6, 8, 21–23]). While Braun and colleagues reported greater open-zone occupancy in the EZM compared to the

EPM, both were equally sensitive to diazepam's anxiolytic effects [6]. In the EPM, however, the development of one-trial tolerance substantially limits benzodiazepine detection in repeated testing. The EZM tends to show more stable baseline behavior across sessions, yet whether pharmacological responsiveness persists under repeated exposure remains uncertain, warranting further investigation.

The influence of serotonergic agents on maze performance is more complex, varying with dose, brain region targeted (if applicable), treatment duration, and receptor subtype specificity. Acute administration of selective serotonin reuptake inhibitors (e.g., fluoxetine) typically produces anxiogenic-like effects in rodents [24, 25], whereas anxiolytic effects only emerge with subchronic or chronic treatment [5, 26], paralleling the delayed therapeutic onset seen clinically. Receptor-specific manipulations yield distinct outcomes: antagonists at 5-HT_2C, 5-HT_3, and 5-HT_4 receptors have been shown to reduce anxiety-like behavior in the EZM, while paradoxically, a 5-HT_4 agonist also showed an anxiolytic-like effect, suggesting an inverted-U-shaped dose-response profile [23].

In contrast, interventions that heighten anxiety decrease open-area exploration. For example, yohimbine (alpha-2 adrenergic receptor antagonist) or a high dose of caffeine (adenosine A_1 and A_2 receptor antagonist) significantly reduce the percentage of time in open zones of both the EPM and EZM [6]. Some environmental stressors, including acute restraint, likewise lead to reductions in open-zone exploration [27, 28]. Increased anxiety-like behavior is also evident in genetic models predisposed to high anxiety, such as the 5-HT_1A receptor knockout mouse or selectively bred high-anxiety rat lines [29]. Across species and manipulations, anxiogenic conditions reliably produce less time in open areas, indicating heightened anxiety-like behavior in both mazes.

6 Experimental Variables

Behavioral outcomes are shaped by a combination of internal (e.g., species, strain, sex) and external (e.g., illumination, handling methods, time of day) factors. Careful recognition and control of these variables are essential for reliable data interpretation and cross-study comparison. This section highlights the key biological and environmental influences on EM performance, summarized in Table 3.

6.1 Biological Factors: Species, Strain, and Sex

Rats and mice share core behavioral and neurobiological responses in EPM/EZM tasks, including increased corticosterone levels in response to maze exposure and sensitivity to anxiolytics [30, 31]. However, differences in size, learning capacity, and social behavior may influence test suitability. For example, rats are better suited for surgical manipulations and some cognitive paradigms, whereas mice

Table 3
Recommended reporting variables for elevated maze studies

Category	Variable to report	Example/notes
Maze characteristics	Dimensions (length, width, height)	Open-arm length, wall height, platform width
	Shape and design	Plus (+) vs. zero (O), transparent vs. opaque walls
	Material	Wood, plastic, metal, etc.
	Vendor	Stoelting Co., CleverSys, etc.
Environmental conditions	Illumination (lux at maze surface)	Report measurement location (open arms, closed arms, center)
	Room lighting conditions	Overhead, indirect, red light, etc.
	Noise level	dB reading if controlled
	Time of day	With respect to light/dark cycle
Animal variables	Species, strain, vendor	Jax C57BL/6, Charles River Sprague–Dawley, etc.
	Sex and age	M/F, age in weeks/months
	Weight at testing	Especially if varied across groups
Testing parameters	Trial duration	Typically 5–10 min
	Inter-trial interval (if repeated testing)	Hours, days, weeks
	Handling/habituation protocol	Pre-exposure, transport method, handling procedures
Pharmacological manipulations	Drug, dose, route, timing	i.p. diazepam, 1 mg/kg, 30 min prior

are preferred in genetic models [32, 33]. There is limited direct comparison of EM behavior between species, though both exhibit robust avoidance of open arms and pharmacological sensitivity to anxiolytics [6, 22, 23, 34]. Notably, species-specific responses to certain agents—such as 5-HT_6 agonists—highlight the need for pharmacological validation in both species [35, 36].

Strain influences behavior in both mice and rats. In mice, BALB/c lines often show reduced exploration and heightened anxiety-like behavior compared to C57BL/6, though findings vary across labs and sub-strains [37–39]. In rats, Long–Evans typically show lower anxiety than Sprague–Dawley rats [40, 41]. Strain also modulates drug sensitivity and stress responsiveness [34, 42, 43].

Sex differences in EM performance are well documented, with females often showing reduced anxiety-like behavior but increased locomotion [44–46]. Hormonal fluctuations across the estrous cycle may modulate these effects and influence drug responsiveness [47–49], though findings are sometimes inconsistent [50]. Including both sexes and documenting estrous stage can enhance the rigor and interpretability of results.

6.2 Environmental Factors

Illumination

Illumination level is a key environmental parameter in EM paradigms, grounded in the principle that rodents find brightly lit, open spaces anxiogenic—a central feature of the unconditioned conflict these tests aim to exploit. Rodents naturally avoid brightly lit, open spaces, and changes in ambient illumination can influence both baseline anxiety-like behavior and pharmacological responsiveness. Despite its theoretical importance, light level is frequently underreported or inconsistently controlled across studies [51].

In rats, Garcia and colleagues described an "all-or-none" pattern of behavior, with anxiety-like responses increasing sharply between 1 and 3 lux, with no further change at higher intensities (up to 300 lux) [52]. Similar findings have been reported in other studies examining both baseline behavior and pharmacological sensitivity [53–55].

In mice, fewer studies have directly manipulated illumination. Male NIH Swiss mice displayed reduced open-arm exploration under ~20 lux compared to ~1 lux, suggesting light-dependent modulation of anxiety-like behavior [56]. In contrast, Shoji and Miyakawa reported no significant behavioral or physiological effects (including corticosterone levels) across three light levels (5, 100, and 800 lux) in C57BL/6J mice [31]. These findings raise the possibility of a similar "all-or-none" threshold in mice, where illumination may affect behavior only below a certain intensity, though additional studies—particularly at <5 lux and across strains—are needed to clarify this effect.

To reduce variability and improve reproducibility, light intensity should be measured at the surface of the apparatus and standardized across subjects. Illumination levels should also be reported explicitly in all protocols.

Circadian Rhythms

Rodents are nocturnal, with peak activity occurring during the dark phase. Despite this, behavioral testing is typically performed during the light phase, primarily for practical reasons. While testing during

the active (dark) phase often increases general locomotion, the effects on anxiety-like behavior in elevated mazes are mixed and may depend on species/strain and sex.

Several studies have demonstrated that circadian phase does not significantly alter traditional anxiety-related measures. Jones and King reported that Sprague–Dawley rats showed more locomotor activity during the dark phase, but there were no differences in open-arm activity of the EPM [57]. Similar results were reported in C57BL/6 mice tested in the EZM, with increased locomotion in the dark phase but no significant change in anxiety-like behavior [49]. Likewise, a similar study employing both C57BL/6 and 129S1/SylmJ mice reported no phase-related differences in EPM behavior [58].

However, interactions between circadian phase and sex have been found in Sprague–Dawley rats: sex differences, with females showing lower anxiety evidenced by greater time in the open arms, were only apparent in the light phase. Furthermore, among females, those tested in the dark phase spent less time in the open arms of the EPM [59].

Together, these findings suggest that while anxiety-like behavior may be stable across the circadian cycle in many contexts, phase-dependent effects can emerge under specific conditions. Also, importantly, corticosterone—an established biomarker of stress and arousal—follows a strong circadian rhythm, peaking around the onset of the dark phase [60–62]. Even in the absence of behavioral differences, fluctuations in corticosterone may reflect underlying shifts in physiological stress reactivity across the cycle. For reproducibility, testing should occur within a consistent time window, and the phase of the light–dark cycle should be reported explicitly.

Maze Construction

The physical properties of the EMs—such as arm width, wall height, and presence/absence of a ledge—can significantly influence behavioral outcomes, and these features vary across vendors. A recent comparison of performance of C57BL/6J mice in EPMs purchased from Stoelting Co. or CleverSys reported that anxiety-like measures were not affected, but overall exploration was greater in the CleverSys apparatus [63]. The devices from the two vendors vary in dimensions, color, material, and whether a ledge is present on the open arms. Notably, the CleverSys maze has wider arms compared to the Stoelting maze (7 vs. 5 cm), which may increase perceived safety and encourage exploration, although a previous study found no effect of arm width (5 vs. 7 vs. 9 cm) on the behavior of C57BL/6J mice [64]. Another key difference is that the CleverSys device includes a 4-mm ledge along the edges of the open arm, whereas the Stoelting maze did not in this study (*see* **Note 7**). The absence of a ledge in the Stoelting device may have reduced animals' confidence during ambulation in the anxiogenic zones, which was in turn associated with reported falls [63].

Several studies have examined the impact of closed arm opacity and/or the presence of a small ledge around the open arms. Transparent closed arms encourage greater exploration of open arms in C57BL/6, 129/Sv, and C3H/He mice [31, 65] and Sprague–Dawley rats [66]. Horii and colleagues specifically manipulated transparency of the walls of the closed arms (opaque vs. transparent), as well as the presence or absence of a ledge on the open arms of the EPM. They reported that closed arms with transparent walls combined with open arms featuring a ledge produced the lowest levels of anxiety-like behavior, as indicated by greater percent time in open arms, fewer SAPs, and more time spent in the distal regions of the open arms [66]. Greater levels of anxiety resulting from a lack of an edge on the open arms and opaque walls resulted in the highest sensitivity to a low dose of the anxiolytic diazepam. These findings confirm an earlier report that transparent arms encourage greater exploration of open arms, although the investigators reported no effect of arm design on the effectiveness of diazepam [65]. Thus, the completely unprotected open arm along with a very safe-feeling "closed-in" opaque created a large anxiety contrast that an anxiolytic can reduce. Making the closed arms less private (transparent) and adding safety features (ledges) to the open arms blunted the maze's sensitivity to the drug. This interplay highlights that researchers should consider open vs. enclosed design elements together, taking research goals into account.

In addition to wall transparency, maze color and contrast can influence behavioral outcomes. Shoji and Miyakawa compared behavior of C57BL/6J mice in EPMs with either clear, transparent blue, white, or black walls in the closed arms, and showed that the clear walls encouraged greater exploration of the open arms, confirming previous results [31, 65, 66]. Black walls resulted in the least activity in the anxiogenic regions, likely because they enhanced the perception of the closed arms as secure, enclosed spaces, amplifying the natural preference of rodents for dark, protected environments compared to clear walls, which may diminish this safety cue.

Visual contrast between the open and closed regions also alters maze activity. C57BL/10J mice had reduced ambulation in an EPM (and complete avoidance of the open arms) with a white floor compared to mice tested in a maze with a gray floor, suggesting that a gray maze is less aversive [64]. If the floor of the open arms is gray and the center/closed regions are white, the mice overcame their aversion to the anxiogenic regions, suggesting that visual discrimination of the open versus closed zones can alter the animals' risk assessment [64].

Taken together, apparatus features such as arm width, floor and arm colors, and visual contrast interact in complex ways to influence rodent behavior. These findings underscore the importance of considering open versus enclosed design elements in combination, with careful attention to the goals of the experiment. The effectiveness of pharmacological agents may depend heavily on whether the apparatus amplifies or dampens anxiety-like responses.

6.3 Animal Handling and Habituation

Routine handling procedures of rodents have a dramatic impact on anxiety-like behaviors (e.g., [67]. The traditional method of picking mice up by the tail is now strongly discouraged as it is aversive to the animal. Seminal work by Hurst and West in BALB/c, C57BL/6, and CD-1 mice demonstrated that compared to tail handling, non-aversive handling techniques such as cupping (in the palm of the hand) or guiding and carrying the mice in a tunnel resulted in greater interaction with experimenters and markedly lower anxiety responses in the EPM [12]. Despite multiple handling sessions, tail-handled mice avoided interactions and remained anxious. Moreover, repeated exposure to aversive procedures, including immobilization or subcutaneous injections, did not negate the beneficial effects of tunnel handling [68]. This work was more recently confirmed by a separate laboratory; BALB/c and C57BL/6 mice handled with tunnels showed significantly reduced anxiety-like behaviors in the EPM compared to mice handled by the tail [69]. In our laboratory, we use tunnel handling for mice; a standard enrichment tunnel is placed in the cages immediately after arrival to our facilities. The mice acclimate to the facilities (unhandled) for 3–5 days, after which they are handled very briefly with the tunnels for another 3–5 days prior to any behavioral testing. As reported in the literature, mice require very little experience with tunnel handling before they become comfortable and docile. The size of the tunnel (5 cm diameter × 10 cm length) does not allow direct transfer of the mouse to the apparatus; cupping is employed very briefly for final placement on the maze.

Like mice, rats should not be handled by the tail. There are several acceptable methods for handling rats; most of which involve initially lifting the animal with one hand, placing the hand around the rat's thorax just behind the forelimbs ("thoracic support hold"), and lifting while supporting the body with the other hand. Once lifted, the weight of the animal should be supported on the investigator's arm or against their side. Rats that are regularly and gently handled become acclimated and can usually be picked up without protest [70]. An additional positive handling technique for rats includes tickling, which mimics dorsal play wrestling that juvenile rats naturally engage in [71]. Tickling by the handler significantly reduces fearful reactions to handling and improves rat welfare in the long term [72].

Regardless of specific handling techniques employed by investigators for rats or mice, consistent and compassionate handling allows behavioral testing to be conducted in a way that respects their well-being while still meeting experimental needs. Specific handling procedures should be recorded and reported.

6.4 Testing Order and Repeated Testing

If a battery of behavioral tests is employed in a study, test order requires careful consideration. A widely accepted guideline is to start with the least stressful test and end the study with the most stressful assay, an approach that minimizes the chances that an early high-stress experience will alter performance on later tests [73].

Tests that rely solely on exploration in a relatively benign environment, such as the open field and the EMs, are typically performed first, with assays including stressful stimuli (e.g., fear conditioning, Morris water maze) saved for the end of the study [74]. Behavior in the EPM is particularly sensitive to prior testing experience, whether that prior experience is in the EPM or in other tests such as the open field (e.g., [13, 31, 54, 75]).

The sensitivity of the EPM test to prior experience has been well documented and described in both mice and rats as the "one-trial tolerance phenomenon" (OTTP), in which after an initial exposure to the maze, rodents show a marked reduction in open-arm exploration during subsequent trials, and anxiolytic compounds typically fail to increase time spent in anxiogenic areas (e.g., [13, 15, 19, 20, 76, 77]). Waiting 4 weeks to retest animals may partially reduce the OTTP [78], but a context change (different room) is required to restore benzodiazepine sensitivity on the second trial [79].

The expression of the OTTP in the EZM is less well established, in part due to the limited number of studies compared to the EPM. However, data at present suggest that the OTTP is markedly reduced in the EZM (but see [80]). In a direct comparison of the two mazes, male and female C57BL/6J mice were tested in either the EPM or EZM across five sessions at daily or weekly intervals. In the EPM, mice tested at both intervals exhibited a pronounced OTTP, with significantly reduced exploration and open-arm activity in trials after the first exposure. By contrast, mice tested in the EZM maintained more consistent exploration and open-arm activity across sessions, regardless of interval [13]. The markedly reduced OTTP has also been demonstrated in rats; EZM behavior remains reliable for at least three to four daily trials [18, 81] or when the testing interval is 1–2 months [82, 83].

Given these differences, test order should be carefully planned with consideration of each assay's susceptibility to prior experience. For studies requiring repeated testing, the EZM may offer greater reliability than the EPM, particularly when multiple exposures or longer intervals are unavoidable.

7 Comparison of the EPM and the EZM

As discussed above, the OTTP is a critical factor in repeated measures designs, and its effects are generally more pronounced in the EPM than in the EZM. The reduced OTTP in the EZM can make it advantageous for studies that require repeated testing, but other factors continue to influence apparatus choice. Key structural and procedural differences between the two mazes, including OTTP considerations, are summarized in Table 4 for quick reference.

The EPM remains the gold standard for assessing rodent anxiety-like behaviors. Its longer history of use, compared to the EZM,

Table 4
Comparison of the elevated plus maze (EPM) and elevated zero maze (EZM)

Feature	EPM	EZM
Historical precedent	Gold standard; in use since the 1980s; large body of comparative data across species, strains, drugs, and models → strong interpretive context	Increasingly adopted, but smaller historical footprint; fewer legacy datasets for direct comparison
Design	Four arms with a central square (neutral zone) → rodents may spend substantial time here, creating ambiguous behavioral interpretation	Continuous circular track with alternating closed/open quadrants; no central zone, so time is always in the open or closed areas, and transitions are clearly defined
Ambiguity of scoring	Neutral zone behavior difficult to categorize (risk assessment, hesitation, indecision) → complicates interpretation	Removes ambiguity by eliminating neutral zone; scoring and data interpretation more straightforward
Baseline exploration	Lower baseline open-arm exploration, especially in high-anxiety strains → may be prone to floor effects	Typically, higher baseline open-quadrant exploration in both mice and rats → can make subtle anxiolytic effects easier to detect
Open-arm risk	Central square offers a pause point; open arms may feel more aversive relative to EZM	Continuous design encourages movement; lack of pause points increases time in open quadrants
One-trial tolerance to prior experience (OTTP)	Strong OTTP; repeated exposure reduces responsiveness → less suitable for repeated-measures designs	Reduced OTTP; more consistent behavior across repeated testing → advantageous for longitudinal designs
Pharmacological sensitivity	Extensive history of pharmacological validation; reliably detects benzodiazepine and anxiolytic effects; may produce larger effect sizes or detect lower doses	Also detects benzodiazepine and anxiolytic effects; higher baseline exploration may reveal effects of broader drug classes, including non-benzodiazepines
Overall strengths	Strongest precedent, interpretive context, and pharmacological sensitivity; best for single-trial designs with naïve animals	Structural clarity, reduced OTTP, higher baseline exploration, and streamlined scoring; better for repeated-measures design
Overall limitations	Ambiguous central zone behavior; floor effects in high-anxiety strains; reduced utility in repeated testing	Smaller literature base; limited historical comparability; may yield smaller effect sizes for some compounds.

has produced a vast amount of comparative data across species, strains, pharmacological classes, and experimental paradigms, giving it unmatched interpretive context. While the EZM has been increasingly adopted, it has a smaller historical footprint, which can limit direct comparisons with older studies when continuity is important.

Structurally, the most obvious distinction is the EPM's central square, where all arms meet. Rodents can spend substantial time in this neutral zone, particularly if they hesitate to commit to open or closed arms. This behavior is difficult to interpret; it may reflect risk assessment, conflict, or indecision, but it does not fit into traditional anxiogenic or anxiolytic categories. The EZM removes this ambiguity by eliminating the center square entirely; rodents are always in either an open or closed segment, and transitions are more clearly defined. By reducing intervals that cannot be reliably scored, this approach improves the clarity of behavioral data and facilitates more direct comparisons across subjects and treatment groups.

Baseline exploration also differs. The EZM often produces higher baseline time in anxiogenic (open) regions in rats and mice [6, 13], likely due to its continuous track and lack of pause locations. In rats, open-area times may be more similar between mazes, although direct comparisons are limited. These baseline differences can shape sensitivity; in high-anxiety strains or conditions, the EPM may be susceptible to floor effects, whereas the EZM's higher baseline exploration can make subtle anxiolytic effects easier to detect.

The relative sensitivity of the mazes to different classes of pharmacological agents is complex, discussion of which is beyond the scope of this protocol, but see Rosso and colleagues for a systematic review and meta-analysis [7]. Both EMs reliably detect increases in open-area exploration following administration of benzodiazepines and other anxiolytics in a single-trial design [6]. The EPM's long history of pharmacological validation and extensive literature base provide a strong reference point for interpreting drug effects, and some evidence suggests it may yield larger effect sizes or detect drug effects at lower doses than the EZM for certain compounds. For studies prioritizing maximal pharmacological sensitivity, especially in between-subjects designs with naïve animals, the EPM may be preferable. The EZM, however, often produces higher baseline open-area exploration and may reveal effects of a broader range of compounds, including non-benzodiazepine agents, while also offering reduced OTTP and greater consistency across repeated sessions. Additionally, trial scoring and data interpretation may be more streamlined in the EZM due to the absence of the central platform. Although additional comparative studies are warranted, for research requiring repeated testing, the EZM's reduced OTTP may help maintain drug responsiveness across sessions.

In summary, the EPM offers great historical precedent, strong pharmacological sensitivity, and broad comparability with past

literature. The EZM has structural advantages that reduce ambiguous behavior, may improve the detection of mild anxiolysis in high-anxiety conditions, and is less affected by repeated testing. Apparatus choice should be based on study goals, strain, characteristics of independent variables, and the balance between historical continuity and practical design considerations. Key distinctions between the two mazes are summarized in Table 4.

8 Conclusion

Both the EPM and EZM are robust tools for assessing anxiety-like behavior in rodents, but careful procedural control is essential to ensure valid, reproducible results. While each maze has unique strengths and limitations—including differences in sensitivity to pharmacological manipulations, prior testing effects, and exploratory patterns—selecting between them should be guided by specific experimental goals. Standardized apparatus specifications, consistent handling, and thoughtful test sequencing remain critical to data integrity. By applying these considerations, investigators can maximize the interpretive value of each assay and make informed decisions across studies.

9 Notes

1. In both human and rodent anxiety research, a distinction is drawn between *state anxiety*, which reflects a temporary response to temporal stressors, and *trait anxiety*, which represents a more enduring disposition in the animal [84, 85]. The EMs are most commonly used to assess state anxiety, though trait anxiety arising from genetic manipulations can also be detected with the EPM and EZM.
2. As a general principle, investigators are advised to minimize stress in rodents prior to behavioral testing with environmental habituation and non-aversive handling techniques. These procedures help stabilize baseline behavior and improve reproducibility across cohorts. However, it has also been noted that deliberately withholding habituation—such as by testing animals immediately upon exposure to a novel room, or following brief handling stress—can heighten baseline anxiety levels and thereby increase the apparent sensitivity of the EPM to anxiolytic compounds [21]. While such manipulations may facilitate detection of pharmacological effects, they reduce ethological validity and should be used cautiously, with clear reporting of pre-test conditions.

3. A single camera placed over the center of the EPM works well, but for the EZM this setup creates blind spots in portions of the closed quadrants of the EZM, obscuring the animal from view and limiting reliable tracking. To address this, two cameras can be positioned—each mounted above one of the closed quadrants—allowing the full maze to be captured. The two video streams can be merged within tracking software such as ANY-maze, providing continuous coverage across the entire apparatus. Alternatively, infrared (IR)-transparent inner walls may be used for the closed quadrants. When paired with appropriate IR lighting and an IR-sensitive camera, these walls allow the animal to remain visible to the tracking system even while enclosed.
4. During placement on the EZM, be sure the animal fully enters the closed quadrant before testing begins. This standardizes the starting condition across subjects, prevents ambiguity in zone assignment by tracking software, and avoids bias from partial exposure to the open quadrant. Most importantly, if the animal is set too far into the open quadrant, it may freeze at the start of the session—a stress response that artificially inflates the recorded time in the open zone without reflecting true exploratory behavior. Being fully in the closed quadrant not only prevents this artifact but also preserves the ethological validity of this assay, as the animal must actively choose to leave a protected environment and enter an anxiogenic space, thereby maintaining consistency and reliability in approach-avoidance measures.
5. While some protocols recommend returning an animal to the maze after a fall, the investigator's response should ultimately follow institutional guidelines. Animal care and use policies may require a veterinary evaluation and, in some cases, a quarantine period to monitor for injury or pathogen exposure. To ensure compliance, investigators should be familiar with their institution's requirements and document all falls in the study record.
6. Our experience has shown that the greatest sources of software tracking errors are poor lighting and/or insufficient contrast between the animal and the background. A medium-gray background color for the apparatus is ideal for detecting either dark (e.g., C57BL/6 mice) or light (e.g., Sprague–Dawley rats) animals. Strains with mixed coat colors (e.g., Long–Evans hooded rats) can present tracking challenges; however, many software packages include settings designed to optimize detection of animals with multicolored coats. General lighting should be uniform, as glare resulting from direct illumination can interfere with tracking, particularly in white animals. If testing is conducted under very low-light conditions, the addition of

red lighting (> 650 nm wavelength) and/or IR lighting paired with IR-sensitive cameras can improve overall illumination.

7. The Stoelting EPM apparatus for mice in our laboratory includes an adjustable ledge, which we maintain at 4 mm. Our mouse EZM initially lacked a ledge; in this configuration, animals were very reluctant to leave the closed quadrants and occasionally fell from the open regions. Adding a 4-mm ledge to the open quadrants substantially increased the amount of time the mice spent in the open quadrants, and completely eliminated falls.

Author Disclosure Statement

No competing financial interests exist. JTM is a federal employee, but the opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army, Department of Defense, the U.S. Government, the Uniformed Services University of the Health Sciences, or the Henry M. Jackson Foundation for the Advancement of Military Medicine, Inc. The use of trade names does not constitute an official endorsement or approval of the use of reagents or commercial hardware or software.

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Chapter 2

Anxiogenic Drug Effects in Behavioral Test Batteries: A Pharmacological Translational Approach

Jaanus Harro and Margus Kanarik

Abstract

Animal models and tests of anxiety rely on evolutionary continuity between humans and other animals in their neurobiological substrates. These substrates can be affected chemically by a variety of drugs known to elicit in humans subjectively experienced and reported anxiety or panic. Behavioral changes in response to anxiety-provoking substances are more reliably interpreted as anxiety-related than those provoked merely by environmental stimuli, yet this pharmacological approach is applied quite infrequently. This chapter is devoted to the principles, details, and some caveats for experiments in rodents that apply anxiogenic drugs such as the ß-carbolines that act as $GABA_A$ receptor (partial) inverse agonists (e.g., DMCM, FG 7142), pentylenetetrazole, m-chlorophenylpiperazine, yohimbine, caffeine, and cholecystokinin CCK_B receptor agonists.

Keywords Anxiogenic drugs, Behavioral test batteries, Route of administration, Dose, Environmental conditions

1 Introduction

Anxiety, as we all know it, is a subjectively felt human phenomenon. We can, however, assume with some confidence that the neural substrates underlying the emergence of fear reactions have been obtained in evolution and hence largely shared with other mammals [1]. In animals, neural activity in such circuits must, for the time being, be assumed in observation of the behavioral responses or deficits that result from presumably anxiety-producing stimuli. The gap of uncertainty between the subjectively felt anxiety states and behavioral manifestations upon exteroceptive stimuli is attempted to be filled with a variety of clever test designs and, in case of a more rigorous approach, additional validation by administration of anxiolytic drugs [2]. Another road to anxiety would be to use interoceptive stimuli, and for the time being, its feasibility and translational validity together would be the highest for chemical compounds that can elicit anxiety and fear in humans. Extrapolating from humans, who can experience

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existential fear upon brain stimulation, one can hypothesize that the intrinsic nature of the drug stimulus would produce in animals, for obvious reasons not having been informed about what to expect, a species-specific mode of experience of imminent ambiguous threat.

Anxiety-provoking or anxiogenic drugs come with divergent chemistry and pharmacology. Because anxiety can emerge in neural circuits that are served by a vast diversity of neurochemical communication [3], an occasional anxiogenic-like effect has been described for a large number of drugs that act through multiple mechanisms. Some of these can even have an anxiolytic-like action under other conditions. Nevertheless, a number of chemical compounds do elicit anxiety much more consistently than others. Anxiety can be very reliably produced by compounds that reduce GABAergic inhibition, either by binding to the $GABA_A$ receptor-chloride channel complex and reducing the efficacy of GABA or inhibiting chloride flux more directly. Early studies in animals characterized the anxiogenic effects of the convulsants pentylenetetrazole [2] and picrotoxin [4] that prevent chloride flow by blocking the channels in a GABA-independent manner. Low doses of pentylenetetrazole had been found capable of inducing very severe anxiety in humans when it was applied as a convulsant to treat psychiatric disorders, and the selected dose had been too low for causing seizures [5]. After their introduction, the anxiogenic β-carboline derivatives, such as β-CCE (β-carboline carboxylic acid ethyl ester), DMCM (methyl-4-ethyl-6,7-dimethoxy-9H-pyrido[3,4-b]indole-3-carboxylate) and FG 7142 (*N*-methyl-5*H*-pyrido[3,4-*b*]indole-2-carboxamide) that bind to the benzodiazepine site of the $GABA_A$ receptor and behave as inverse agonists became more commonly used [6]. DMCM is a full inverse antagonist of the $GABA_A$ receptor, and the anxiogenic doses are close to those that have proconvulsant action; therefore, the partial inverse agonists such as FG 7142 and β-CCE have become preferred. These drugs (and several others such as ZK 90,886 and ZK 132,556) require for their action that the majority of their binding sites be occupied [7]. Administration of FG 7142 caused severe anxiety in humans [8] and thereafter this compound has been the most frequently applied anxiogenic. In these human studies, it was noted that the dose had to be individually adjusted, and that intravenous administration of a benzodiazepine anxiolytic immediately abolished the drug-induced anxiety [9]. Another substance, commonly applied to rodents as an anxiogenic, is yohimbine. Yohimbine has long been available for clinical use as a treatment for male erectile dysfunction [10], but it does elicit anxiety quite reliably in overly sensitive humans, notably patients with panic disorder [11]. The mechanism of anxiogenic action of yohimbine is not entirely clear, but distinct from the drugs acting on the $GABA_A$ receptor-chloride channel complex. It is probable that the blockade of α_2-adrenoceptors plays a major role, because yohimbine has its highest affinity for all

three subtypes of α_2-adrenoceptors and this action through presynaptic autoreceptors enhances noradrenaline-mediated arousal, but the additional contribution of other targets, such as some subtypes of 5-HT receptors, is not excluded. Yet another class of anxiogenic drugs is represented by m-CPP (m-chlorophenylpiperazine), which is a 5-HT agonist [12]. m-CPP can elicit anxiety in both humans and animals [13]. Similar effects have been shown in animal experiments with other 5-HT agonists such as TFMPP (1-[3-(trifluoromethyl) phenyl]piperazine) [14, 15] that is abused by humans. TFMPP is usually taken together with 1-BP, and if consumed alone, its stimulant effect is accompanied by increased tension that easily transforms to anxiety [16]. While mCPP has multiple targets, its anxiogenic effect is mostly associated with agonism at the 5-HT_{2C} receptors [17]. Of other reliable anxiety-provoking drugs, those acting on several of the neuropeptide receptors should be listed. Neuropeptides are recruited to modify signaling at higher neural activity [18] and may therefore be less robust across conditions but target more specifically the anxiety-producing substrate. Anxiogenic action is elicited by corticotropin-releasing factor after intracerebral administration [19]. Cholecystokinin (CCK) peptides that activate the CCK_B receptor are anxiogenic in humans [20, 21] as well as in animals [22, 23] after systemic administration. Anxiogenic action is also characteristic to neuropeptide Y Y_1 receptor antagonists [24], and to substance P [25] acting through neurokinin NK_1 receptors [26]. Figure 1 presents the chemical structures or representative drugs of the most commonly used anxiogenic drug classes.

Multiple behavioral readouts have been employed to detect the anxiogenic action of chemical compounds. Anxiogenic drugs reduce

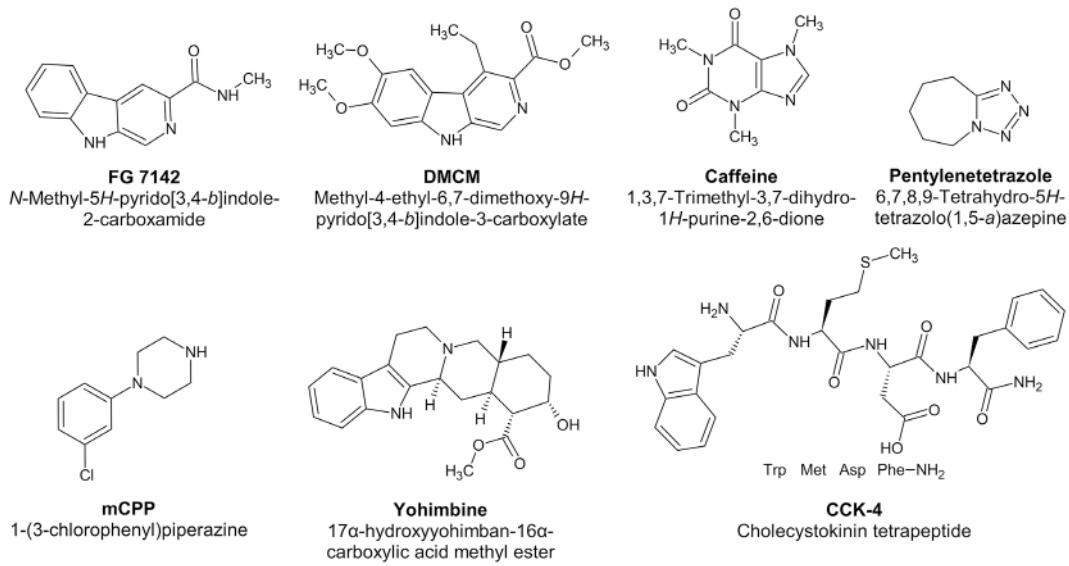


Fig. 1 Chemical structures of the more frequently used anxiogenic drugs

exploratory activity at lower doses than those producing sedation-related loss of locomotion, increase thigmotaxis [27], reduce time spent in mirrored chamber [28], reduce social interaction with unfamiliar conspecific, increase ultrasonic vocalization induced by isolation of pups [29], increase hyponeophagia [30], inhibit lever pressing for food in the conflict task [31], reduce social competition in dominant rats [32], increase risk aversion [33], alter the microstructure of grooming [34], and, if administered to a trapped rat, increase the probability of trap opening by its cagemate, hypothetically owing to emotional contagion [35]. Other anxiogenic drugs generalize to DMCM- or pentylenetetrazol-induced discriminative stimuli [36]. This also holds for anxiogenics with mechanisms of action distinct from the directly GABA-negative, such as m-CPP [13], and so does withdrawal from benzodiazepine anxiolytics [2]. Anxiogenic drugs also produce conditioned place aversion [37] and can, after administration of a single dose, have carry-over effects to drug-free sessions in a similar context [38]. Behavioral tests that can be used to detect anxiety-like responses are, however, not equal in the study of anxiogenic drugs. Fear potentiation appears to be particularly unpredictable with regard to the direction of effect of anxiogenics [39]. This may be related to multiple interference with the whole complex of physiological systems involved. Neither is stress-induced hyperthermia potentiated by the anxiogenic drugs [40].

Anxiogenic drugs have been given either by systemic or intracerebral administration. Where to administer the drug depends on the purpose of the study. Systemic treatment can be made with very limited stress involved, while intracerebral administration includes anesthesia and surgery for the guide cannula placement. Surgery-related procedures can alter behavior in subsequent tests. On the other hand, systemically administered drugs act indiscriminately (or usually without known bias) on their molecular target(s) throughout the brain and elsewhere. The effect of intracerebrally administered anxiogenic (and anxiolytic) compounds has occasionally been reported to be qualitatively dependent on the locus of injection, but for the time being, limited information is available on this matter for each drug class and anxiety test [41].

While making the selection of which anxiogenic drug to use, one should be aware that such drugs come on a continuum with regard to the universal reliability of their anxiety-provoking action. Literature does not always make any distinction, and the label “anxiogenic” may be applied to compounds that elicit anxiety only in certain, not well-defined conditions and not in all individuals. For example, caffeine is quite often mentioned as an anxiogenic, while obviously this would go against it being the worldwide most broadly used psychotropic substance [42]. Caffeine antagonizes the inhibitory neurotransmitter action of adenosine, and its effect can be perceived as anxiogenic, much more so in some individuals, including those

with a diagnosed anxiety disorder [43, 44]. In humans, both genetic factors and sex interact with habitual caffeine intake in determining the sensitivity to its anxiogenic properties [45] and, together with the dose administered, these factors relate to the fact that both mood-enhancing and anxiogenic effects can be observed [46]. In animals, caffeine can elicit a reliable anxiogenic effect [47], but not in all conditions, and similarly to humans, caffeine may be anxiogenic mostly in highly anxious rodents [48]. However, it is conceivable that mice and rats, being under the control of much bigger creatures, can, despite all gentle handling, have a good reason for more anxiety than humans sipping their coffee, hence the high potential for an anxiogenic effect in experiments. There is more, however, in the differential sensitivity to caffeine than the momentary anxiety, as caffeine can antagonize the anxiety-provoking effect of yohimbine and benzodiazepine withdrawal, while not that of pentylenetetrazol or FG7142 [49]. Nonetheless, systemically administered caffeine can by itself elicit taste and place aversion in the rat [50], suggestive of a significant anxiogenic potential.

Other drugs with even stronger psychostimulant and more prominent euphorigenic action, such as amphetamine and its derivatives [51, 52], cocaine [53, 54] or phenylethylamine [55] can also elicit anxiogenic effects in mice and rats. Studies in humans have suggested that, similarly to caffeine, subjects with higher trait anxiety are more prone to react with anxiety to the stimulation of the dopamine system [56], and this may well hold true also for rodents [57]. An interesting dissociation between such anxiogenic drugs such as FG 7142, pentylenetetrazole, and mCPP from psychostimulants such as amphetamine and caffeine was demonstrated by measurement of 50-kHz ultrasonic vocalizations in amphetamine-primed rats, whereas the former group of drugs reduced vocalization rate while the latter increased the vocalization [58]. Fifty-kHz ultrasonic vocalizations are thought to reflect a positive affective state [59] and are reliably produced by psychostimulants with addictive potential [60]. Yohimbine had no robust effect on 50-kHz ultrasonic vocalization, but if anything, the trend of vocalizations was upwards [58]. Yohimbine is a proconvulsant, while the mechanism of the latter does not appear mediated through its actions as an α_2-adrenoceptor blocker [61], and this is also the case for other stimulants. Administration of yohimbine has been found to reduce sensitivity to reward outcomes [33]. Yet, yohimbine can increase risky choice-making [62], doses of yohimbine that elevated corticosterone levels could also condition place preference, and rats can acquire yohimbine self-administration, similarly to addictive psychostimulants [63]. It thus appears that the action of yohimbine as well as caffeine varies qualitatively, but for both appropriate experimental conditions can be found to facilitate their use for induction of anxiety.

For sure, many additional chemical compounds can elicit an anxious state, but with even more contingency with confounding factors. For example, endocannabinoid release moderates both excitatory glutamatergic and inhibitory GABAergic neurotransmission [64]. Cannabis is abused for intoxicated relaxation, so it is expected that endocannabinoid CB_1-receptor antagonists can be anxiogenic [65], but acute intoxication with cannabinoids can also be anxiogenic via CB_1-receptor stimulation [66]. Such opposite types of effects are much less unpredictable with proper anxiogenic drugs, but still, involvement of confounding mechanisms can lead to unexpected outcomes. For example, drug interactions within the neural substrate of exploratory motivation can cause false anxiogenic/anxiolytic-like effects [67]. Drugs producing anxiety through separate primary mechanisms have distinct types of confounding effects. The effects of compounds that act relatively nonspecifically on the serotonin system are particularly sensitive to contingent stimuli; for example, metergoline, a 5-HT antagonist that can abolish anxiety in animal tests [15], prevents the anti-anxiety action of other drugs [68], and in human volunteers speaking in front of a video camera, increased anxiety [12].

2 Materials

2.1 Animals

Rats and mice of a variety of strains have been used successfully in studies with anxiogenic drugs. In principle, all strains should be suitable for observing anxiogenic drug action, but their relative sensitivity may differ drug- and, more importantly, test-wise [69]. Differences within the strain can be related to vendor-specific epigenetic and transcriptomic differences that have been found in stress studies [70] and theoretically may interfere with anxiogenic drug efficacy in a mechanism of action-dependent manner. Standard group-housing conditions generally apply, and especially drugs with psychostimulant properties, e.g., nicotine, may have variable effects in anxiety tests that depend upon housing conditions [71]. Isolation rearing may reduce the behaviorally apparent anxiogenic effect [72]. If the drug is expected to reduce the behavioral readout, young adult animals that display more vigor should be used. Nevertheless, adolescent rats have been shown to be less sensitive than adults to a selection of anxiogenic drugs [73]. Whether animals can be used for more than one experiment depends on the anxiety test [74].

Interest in sex differences in the neurobiology of anxiety continues to be on the rise [75, 76]. Limited comparative information on anxiogenic drug effects is available, as studies have mostly been conducted in male animals; the increase in the use of female mice and rats in behavioral neuroscience is relatively recent. But several studies

are suggestive that sex is a factor to consider if planning experiments with anxiety induced by pharmacological means, especially with drugs that have less predictable behavioral effects such as psychostimulants and endocannabinoid receptor ligands [77–79].

2.2 Anxiogenic and Other Compounds

Drugs can be obtained from a number of providers. Pay attention to the molecular weight of salts and consider this for calculation and selection of the dose. Follow the suggested conditions for storage. Stabilities of the chemical compounds are variable. The safe option is to prepare solutions *ex tempore*. This may not be feasible owing to, e.g., a very small quantity needed for a single experiment; on this occasion, aliquots of the preparation can be stored frozen. In case the drug is not water soluble, take care to administer a proper vehicle to control animals, and keep in mind that some vehicles may produce internal cues. Emulsifiers and solubilizers such as polysorbates (Tween) can be efficient. We prefer Tween 80 or 85 with their significant resistance to electrolytes and strong hydrophilicity.

2.3 Equipment for Behavioral Testing

After selecting the test or forming a test battery (*see* **Note 1**), advice can be sought from specialized literature, e.g., from subject-devoted chapters of this or its preceding volume [80]. True anxiogenic drugs in appropriate doses should allow anxiety readout without nonspecific effect owing to, e.g., motor retardation (Fig. 2).

3 Methods

3.1 Selection of the Anxiogenic Drug and Dose

This depends on other experimental conditions, and the drug effect and its dose-dependency should be evaluated in pilot experiments. Initial selection of the dose should be based on what can be learned from the literature, on the results of previous experiments with the species and strain of interest, and then a dose range should be tested in own animal house conditions. Examples of effective doses are presented in Table 1. In studies with psychotropic drugs, an inverted U-shape dose effect on the behavioral readout can occur, because higher doses recruit additional neural circuits and the behaviors under investigation are usually shaped by more than one motivation.

3.2 Group Size

We have eight animals per group as a standard. Power analysis could be helpful, but these often are to be made on unfirm presumptions. A biologically significant effect should be observable with a limited number of animals, but the control group may display considerable inter-individual variability, and if this is not random but grounded in neurobiology of different adaptive strategies, an interaction between behavioral trait and drug action may take place [115], so be prepared to observe possibly meaningful peculiarities.

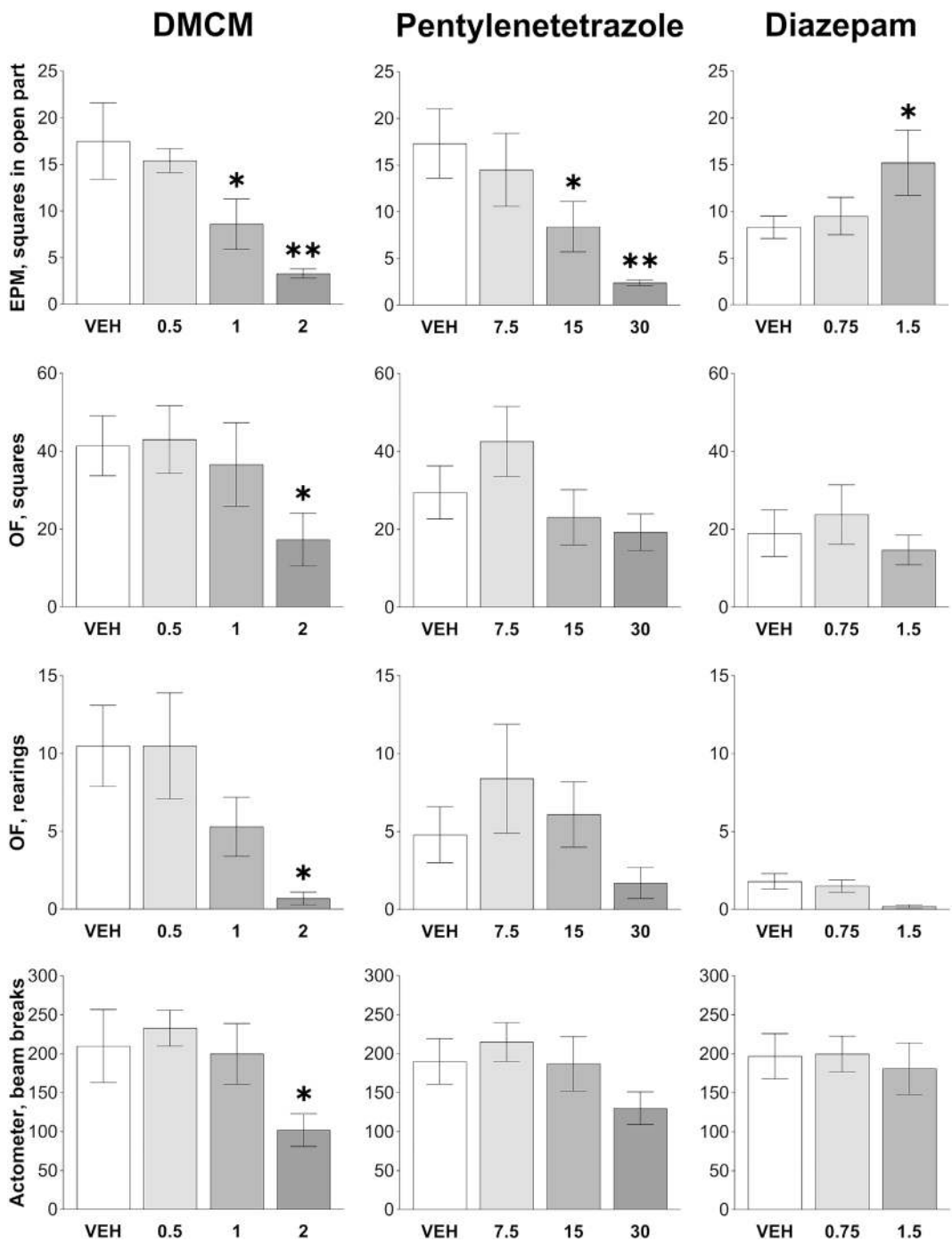


Fig. 2 Effect of DMCM, pentylenetetrazole, and diazepam on mouse behavior in successively performed elevated plus-maze (EPM; observation for 4 min), open field (OF; 30×30 cm; 2 min), and actometer (cylinder with a diameter of 30 cm; 30 min) tests. VEH, vehicle; drug doses in mg/kg. *$P < 0.05$; **$P < 0.01$ as compared to vehicle; Duncan post hoc tests after statistically significant ANOVA. Note the different sensitivity to the drug and the relevance of the measure to the anxiety state. Previously unpublished data from the senior author's doctoral dissertation

Table 1

Examples of doses of chemical compounds for the induction of an anxiogenic effect in mice and rats after systemic administration, in different laboratories. These substances have been used with success in more than one lab and, except for the convulsant DMCM, have shown an anxiety-provoking effect in humans

Drug	Species	Dose	Reference
FG 7142	Rat	5 mg/kg	[81]
		7.5 mg/kg	[82]
		10 and 20 mg/kg	[83]
		10 mg/kg	[7]
	Mouse	5 and 10 mg/kg	[84]
		5 mg/kg	[85]
		10 and 20 mg/kg	[86]
DMCM	Rat	0.25–1 mg/kg	[87]
		1 mg/kg	[88]
		1 mg/kg	[89]
		0.5 mg/kg	[90]
	Mouse	0.5–1.5 mg/kg	[91]
		0.4 and 1.0 mg/kg	[92]
		1.5 mg/kg	[28]
Pentylenetetrazole	Rat	15 mg/kg	[82]
		10 mg/kg	[93]
		10 and 20 mg/kg	[94]
	Mouse	15 mg/kg	[95]
		20 and 30 mg/kg	[28]
		20 mg/kg	[96]
mCPP	Rat	0.5 and 1 mg/kg	[97]
		0.5 and 1 mg/kg	[98]
		1 and 2 mg/kg	[99]
	Mouse	1–4 mg/kg	[14]
		2 and 4 mg/kg	[100]
		1 mg/kg	[101]
Yohimbine	Rat	2.5 mg/kg	[82]
		1–2.5 mg/kg	[4]
		2.5–5 mg/kg	[102]
	Mouse	0.2–2 mg/kg	[103]
		5 mg/kg	[104]
		1 mg/kg	[105]

Table 1 (continued)

Drug	Species	Dose	Reference
Caffeine	Rat	20 and 40 mg/kg	[106]
		30 mg/kg	[99]
		10–40 mg/kg	[107]
	Mouse	60 mg/kg	[84]
		30 mg/kg	[108]
		20 and 30 mg/kg	[109]
CCK-4	Rat	25 and 50 μg/kg	[110]
		10 μg/kg	[111]
		200 μg/kg	[112]
	Mouse	50 μg/kg	[113]
		300 μg/kg	[114]

3.3 Time to Observation

It is recommended to exploit the neophobia that would be synergistic to the anxiogenic drug effect, enabling the use of a relatively lower dose and, by this means, reducing the side effects of the drug. For this purpose, animals should be placed in the test condition as soon as the drug can be expected to have taken effect.

3.4 Validation of the Anxiogenic Effect by Administration of an Anxiolytic Drug Is Advisable (Fig. 3)

The dose of the anxiolytic should be kept low. Parenthetically, validation of the anxiety-provoking effect by administration of an anxiolytic drug should be conducted in all anxiety models, including genetically modified animals that often are tested drug-free but have developmental differences in the CNS, meaning that the behavioral readout in the given test condition may not reflect what had been expected (*see* **Note 2**). Some anxiety-provoking manipulations have the potential to alter drug effects: Sensitization to stress can reverse anxiolytic-like effects of drugs acting on the serotonin system [116].

3.5 If Comparison of Results Between Experiments Is Foreseen, Keep Experimental Conditions Invariant

This holds for e.g., housing of animals between drug administration and all procedures that testing involves. The presence of an experimenter is usually recommended to be as modest as possible [117]. If not avoidable, movements must be smooth, avoiding sudden onset of motion. A frightened animal will not recover easily within the testing condition, and should best be excluded from the analysis. If a sudden sound occurs, drug effect can influence the reaction (*see* **Note 3**).

3.6 Control for the Effect on Locomotion

Either learn in pilot experiments which dosage normally does not elicit this, or use consequent tests that are differentially sensitive for anxiogenic effect. For example, if an elevated plus-maze test is followed by open field, a proper anxiogenic dose can reduce open arm

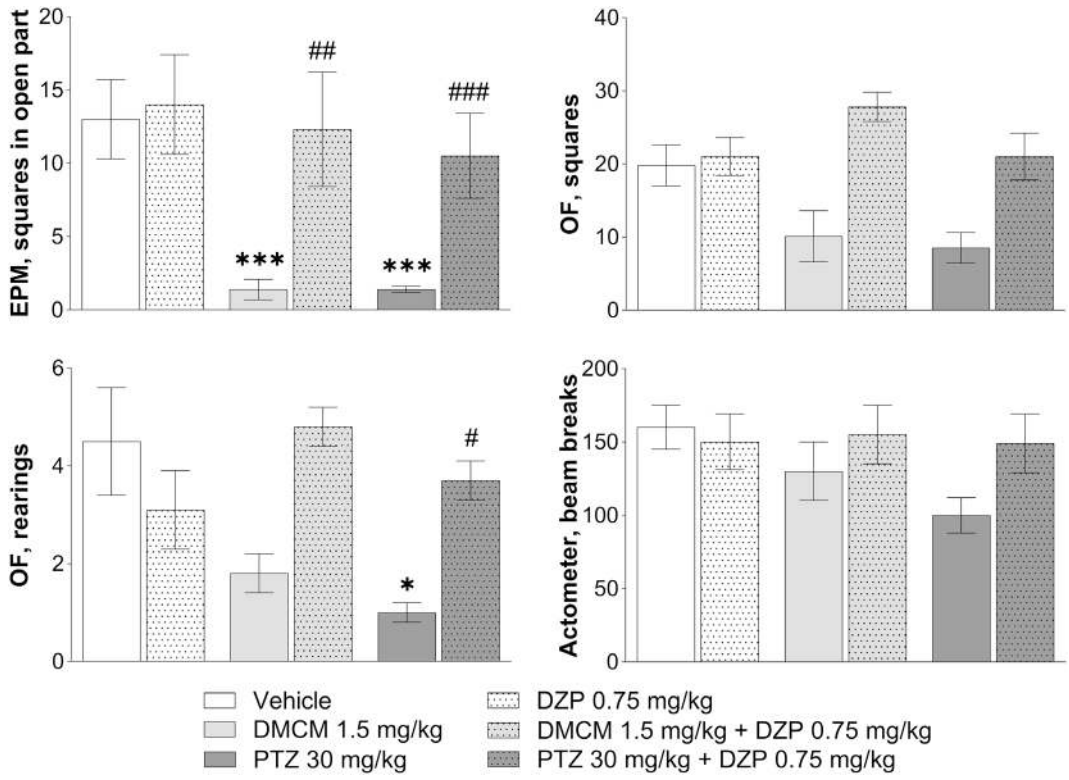


Fig. 3 Antagonism of the anxiogenic effect of DMCM and pentylenetetrazole (PTZ) by the anxiolytic benzodiazepine diazepam in the mouse. If the reduction of open-arm activity induced in the elevated plus-maze by DMCM and PTZ was owing to motor depression, then diazepam, which is also a sedative compound, would have, if anything, rather potentiated their action. EPM, elevated plus-maze test for 4 min; OF, open field test for 2 min. $^{*}P < 0.05$; $^{***}P < 0.001$ as compared to vehicle; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ as compared to the respective anxiogenic-only treatment group; Duncan post hoc tests after statistically significant ANOVA. Previously unpublished data from the senior author's doctoral dissertation

exploration in the plus-maze without a decline of locomotion in the other test (Fig. 2). In open field conditions, rearing is more sensitive to anxiogenic effect than horizontal locomotion (*see* **Note 4**).

3.7 Avoid Floor Effect

In most tests, anxiogenic drugs reduce active behavior. If the experimental setup (e.g., level of illumination, background ultrasonic noise, nervousness in the experimenter) itself elicits anxiety, the anxiogenic drug can not add enough for measurement.

3.8 Dark–Light Cycle

The dark–light cycle does not need to be reversed, but since animal activity is already changing before the anticipated change of lighting, experiments should be avoided 1–2 h before that. Timing of the experiments should be held constant throughout a series (*see* **Note 5**).

4 Notes

1. Some behavioral paradigms may be resistant to the appearance of an anxiogenic drug effect, such as fear-potentiated startle [118].
2. If different regulatory domains are affected by a drug, then the behavioral readout may surprise the observer. An example of this was provided by early studies with the partial serotonergic lesion by 3,4-methylenedioxymethamphetamine (MDMA or *Ecstasy*) treatment that could cause either an anxiolytic-like or an anxiogenic-like effect observed after the elevated plus-maze test, dependent on rat strain and its baseline activity [119]. Converging evidence suggests that the elevated plus-maze is not an optimal anxiety test if the treatment interferes with impulsivity [120].
3. While rarely, an anxiogenic drug can elicit atypical behavioral expression, e.g., a flight reaction. One of the authors has observed a few times how a rat, administered an anxiogenic compound and placed into an elevated plus-maze, would rush to the end of the open arm and freeze there until the end of observation time. It seems fair to exclude such cases from data analysis.
4. A low-dose anxiogenic drug may stimulate behavior. In a rare observation made in a dose optimization experiment in mice, a combination of a low dose of DMCM and a low dose of diazepam caused a synergistic increase in horizontal activity on an open field. This would likely be explained by somewhat different molecular targets in the $GABA_A$ receptor complex, whereas the anxiogenic ß-carboline excited the CNS and the benzodiazepine prevented anxiety.
5. Randomization is important for several reasons, including the effect of circadian rhythm on other spontaneous behaviors that can interfere with the behavioral readout of the drug effect. Curiously, it has been observed in experiments of long duration that the readout may change even within an experiment: During earlier hours, the anxiogenic drug may reduce exploratory behavior, this effect being dose-dependently reduced by an antagonist, and at later hours, control group activity declines; the anxiogenic treatment now instead increases locomotion, and the pharmacological dose-dependent antagonism is present even for this effect.

Competing Interests

The authors have no conflicts of interest to declare that are relevant to the content of this chapter.

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Chapter 3

The Novelty-Suppressed Feeding Test in Mice

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Abstract

Hyponeophagia is a behavioral phenomenon seen in rodents, including mice, where exposure to a novel environment leads to inhibition of feeding behaviors. This naturalistic behavior has been exploited in behavioral neuroscience research for several decades, in a range of behavioral paradigms designed to study anxiety and depression-like behaviors in particular. More recently, the novelty-suppressed feeding test has emerged as a more standardized version of the test, where food-restricted mice are presented with a known food item in a novel and stressful environment. Here, the latency to initiate feeding behavior is recorded and compared between experimental groups as a measure of anxiety and/or depression-like behavior. As a behavioral paradigm with high face, predictive, and construct validity, while at the same time being low-cost and relatively easy to implement, the novelty-suppressed feeding test has become a widely established test in neuropsychiatric research. This chapter will provide an overview of the test and describe the procedure in detail, as well as highlighting important considerations and offering experimenters suggestions for troubleshooting.

Keywords Hyponeophagia, Anxiety, Depression, Mouse behaviour, Chronic antidepressant

1 Background and Overview

The novelty-suppressed feeding test (NSFT) is a widely used behavioral paradigm in modern neuroscientific research based on hyponeophagia, a behavioral phenomenon whereby rodents display a reduction in feeding behavior when exposed to a novel environment. Calvin S. Hall first described this concept in 1934, when he observed an inverse relationship between the defecation and feeding behavior of rats when placed into a novel arena [1]. Over the past several decades, hyponeophagia has been instrumentalized in a variety of paradigms in preclinical neuropsychiatric research. Collectively, this body of work has demonstrated that experimental manipulations associated with anxiety- and depression-like phenotypes, including

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genetic, pharmacological, and environmental interventions, modulate the expression of this behavior in rodents [2–8]. These findings have consequently established the widespread use of hyponeophagia-related behavioral tasks in neuroscientific research. Indeed, in the published literature, hyponeophagia and associated behaviors have been attributed to both anxiety- and/or depression-like behaviors, illustrating the view that both of these neuropsychiatric symptom complexes are thought to be partially modeled in this particular test. This overlap represents one of the strengths of hyponeophagia in terms of its face validity, as major depressive disorder and generalized anxiety disorder are highly comorbid in patients, with around two-thirds of patients with either current diagnosis having experienced the other disorder in their lifetime [9–11].

Early pharmacological studies established that hyponeophagia in rodents is sensitive to acute and chronic treatment with anxiolytics such as benzodiazepines [12], while common antidepressants are effective in reducing this behavior when administered chronically, but not after subchronic or acute treatment [13, 14]. The latter finding accurately reflects the time course of the antidepressant response in patients, conferring important predictive validity to hyponeophagia-based paradigms. In addition, transgenic rodent models associated with anxiety- and/or depression-like behaviors, such as the serotonin transporter knockout (KO) mice [15], the 5-HT_{1A} receptor KO mice [8], or the substance P receptor KO mice [7], all show increased latencies to feed in a novel environment compared to control animals. Rodent stress models such as chronic corticosterone treatment [14, 16] or chronic unpredictable mild stress [6], which are used to induce depression-like behavior, also lead to increased feeding inhibition in a novel environment. Neurobiologically, the control of hyponeophagic behavior in rodents is highly complex. The involved neural circuits include those relevant to threat evaluation, behavioral flexibility, emotional control, reward valence, and feeding control. Perhaps unsurprisingly, various neurotransmitter systems have been implicated to date. These include those classically associated with antidepressant and anti-anxiety treatments, namely monoaminergic [6, 8, 13] and GABAergic [12] systems, respectively. However, hyponeophagia and associated behaviors have also been shown to depend on stress- and fear-related circuits, such as corticotropin-releasing hormone afferents to the amygdala [17], as well as glutamatergic signaling [18] and endogenous opioids [19]. Crucially, these systems substantially overlap with those that have been shown to be disturbed in anxiety and depressive disorders, strongly supporting the use of hyponeophagia-based paradigms in this area of research due to their construct validity.

While various rodent behavioral tests based on hyponeophagia exist [2, 20], this chapter will focus exclusively on the NSFT, a paradigm increasingly used across behavioral neuroscience research [16, 21–25]. During the NSFT, rodents are food-deprived for a period of time before being offered a known and palatable food in a

novel and anxiogenic environment. The time taken until initiation of feeding (latency) is recorded and used as the primary variable to evaluate levels of hyponeophagia displayed by individual mice, and ultimately to compare depression- and/or anxiety-related responses between experimental groups. However, measuring and controlling for body weight loss during the fasting period and food consumption in the home cage following the test are crucial secondary measures which serve to control for the potentially confounding influence of metabolic or hunger-related physiological alterations in driving the feeding behavior [2, 6]. Due to its reliance on hyponeophagia, a naturalistic and ethologically relevant rodent behavior, the NSFT does not require animals to undergo any type of training and is relatively simple to carry out. However, there are several experimental parameters which can strongly influence the behavioral outcomes, which therefore require special consideration. The current chapter will provide a detailed overview of the required materials, equipment, the procedure, data analysis, as well as a discussion of troubleshooting suggestions. The aim is to offer experimenters a comprehensive manual and useful tools to successfully implement this test in their neuroscientific research practice.

2 Materials

- Experimental arena (e.g., box 60 cm × 60 cm × 60 cm)
- Standard bedding used in home cage (e.g., wood chip bedding) [*see* **Note 1**].
- Precision scales
  - to weigh mice (minimum accuracy 0.1 g)
  - to weigh individual chow pellets (minimum accuracy 0.1 mg)
- Food platform
  - Whatman filter paper (circular, or cut into a circular shape ~12 cm diameter) to hold food pellet
  - tape to affix food pellet onto the filter paper
  - standard chow pellets in similar sizes for the latency test
- Standard chow pellets in similar sizes (pre-weighed, one per experimental mouse)—for Consumption test
- Number of (holding) cages and lids is equal to the number of mice to be tested
- Recording sheet
- Timer
- Video recording system (if used)

- Floodlights x 2 (LED strongly preferable) [*see* **Note 2**]
- Luxmeter

3 Methods

3.1 Setup

Environment

Experimental Arena

- Any large box can be used. We use a square box measuring 60 cm × 60 cm × 60 cm, which is appropriate to guarantee well-defined central vs periphery areas (Fig. 1). It is important that the walls are high enough to constrain the mouse to the apparatus without interference by the experimenter, as some strains of mice can attempt to escape the arena. Opaque walls are preferable to decrease any distracting environmental influences, and we also suggest that the arena be elevated off the ground (e.g., on a table or trolley) to reduce the possibility of the animals being able to see the experimenter during the task.



Fig. 1 Arena setup for the novelty-suppressed feeding test. The feeding platform, consisting of a circular filter paper with a single food pellet attached, is placed in the center of the brightly lit (~750 lx) arena, which is filled with standard bedding. Shadows along walls and in corners should be avoided

- Fill the box with the bedding of use (e.g., wood chip) up to 2–4 cm. We suggest mixing clean bedding with a handful of soiled bedding, removing fecal pellets, from each cage that will be tested on that day/batch (from the same sex). This decreases the confounding factor of novel mouse odors, which the first mouse to be tested may otherwise have less exposure to than the last mouse in the batch. [*see* **Note 3**]

Food Platform

- Stick a food pellet on the center of a circular Whatman filter paper using double-sided tape and place the platform in the center of the arena. The tape should not have any strong adhesive odor as this could influence the decision of the mice to approach the food pellet.
  [*see* **Note 4**]
- The platform can be reused for the subsequent trial if it remains clean (free of urine and feces). The arena should be carefully inspected for food crumbs, which should be removed before the next trial.
- Replace the tape if any tested mouse has managed to grab the food pellet from the filter paper. The main goal of this platform is to keep the food pellet in the center, forcing the mouse to eat in the most exposed area of the arena, as their instinct will be to grab the pellet and take it to a corner before initiating feeding.

Room

- Turn on floodlights and use a luxmeter to measure illuminance at the center of the box, aiming for 750 lux. Adjust by altering light intensity and height. Aim to keep lighting as consistent as possible in the different corners and arena edges, i.e., there should not be one side that is darker than the other, and shadows along walls should be avoided (Fig. 2). [*see* **Note 5**]
- Make sure the room remains quiet during the testing time.
- A curtain is useful to hide the experimenter and guarantee that they will not interfere with the test. As mentioned above, elevating the arena above the ground will also guarantee fewer distractions for the tested mouse. If visual observation and manual scoring are required, we recommend the experimenter stay at least 2 m away from the testing arena, remain silent and hidden to the greatest extent possible.
- Strong and/or novel odors should be avoided (e.g., perfumes).
- If any interruption occurs during the test, take detailed notes in the recording sheet and consider these later during the analysis. Sudden loud noises during trials may require the collected data to be discarded, as acute stress can influence the outcomes of the NSFT.



Fig. 2 Room setup for the novelty-suppressed feeding test. The arena is placed below a video camera to assist with live scoring of trials and for video recording purposes (optional). It is lit from above to achieve ~750 lx in the center, with additional standing LED floodlights used if necessary. The arena is elevated off the ground and separated from the experimenter by a curtain to minimize visual interference

Video Recording System (Optional)

- While this test can be done without a video recording system, by simply using timers, physical recording sheets, and visual observation, this additional approach can bring several benefits. It might be challenging to determine if the mouse has bitten the food pellet at first, particularly for inexperienced researchers. Saving video files allows a second independent experimenter to score the trials, or they may simply revisit those trials that they are unsure about themselves.
- In addition, if a curtain is in place and the video is displayed live while the test is running (behind the curtain), this can be used to assess when the mouse bites on the chow pellet, avoiding the physical interference of the researcher during the trial.
- We recommend using high-definition image acquisition cameras to ensure a detailed visual of the food consumption is possible.

Holding Cages

- Prepare cages and lids for each cage of mice.
- Label with cage IDs.

- The holding cages should contain standard bedding used in home cage and in the test arena (e.g., wood chip bedding) [*see* **Note 6**].

Food Pellets

- For Latency testO
  - Prepare food pellets by affixing them to a filter paper platform using low-odor double-sided tape. Handle food pellets with gloves at all times to avoid transfer of odors.
- For Consumption test
  - OPrepare one per tested mouse, pre-weigh each food pellet using precision scales (0.1 mg precision), and record its weight on the data sheet. All pellets should be of a similar size. We suggest including extra pellets in case of experimental errors, e.g., if a pellet drops to the ground inadvertently.
  - OWe suggest keeping all the pre-weighed food pellets organized in a box appropriately identified with trial ID numbers before the test. We have found pill organizer boxes to be very useful for this purpose (Fig. 3). After the test, pellets can be placed back into the appropriate box compartment, and all pellets can be weighed at the same time.

Recording Sheet

- Print a comprehensive recording sheet with the appropriate columns (IDs, pre- and post-bodyweights, latencies, pre- and post-pellet weight…) and spaces for notes (Fig. 4).

3.2 Mice

All experiments conducted using animals require permissions and approval from the local Animal Ethics Committee and should follow international animal research guidelines.

To reduce the influence of handling stress on the results of the NSFT (and other tests, if performed as part of a behavioral battery), we recommend that experimenters handle each mouse prior to starting behavior tests to ensure mice do not experience being handled as stressful. If mice are raised in a facility and regularly handled (e.g., weekly weighing) by the same person who will be performing the test, this is not considered necessary. However, if mice are purchased externally or if husbandry procedures are performed by a different person, we recommend that the experimenter handle each mouse twice (1 min each on non-consecutive days) in the week preceding behavior tests.

It should be noted that different strains of mice show varying levels of baseline anxiety and may therefore behave differently in the NSFT [5, 26]. The current protocol is optimized for C57BL/6 mice. Notably, another well-studied high-anxiety strain, DBA, does not seem to respond in the same manner to this testing paradigm [27–29]. On the other hand, BALB/c mice appear to perform similarly to C57BL/6 [29]. When using a less common mouse strain,



Fig. 3 Organization of pre-weighed food pellets for use during the novelty-suppressed feeding test. For the food consumption part, one standard chow pellet of similar size is weighed per mouse (with extra contingency for errors). Storage in a marked container, such as a pill organizer box, makes it easy to keep track of individual pellets for later weighing

			Body weight (g)			Food consumption test		
Mouse ID	Time fasted	Time tested	Pre fasting	Post fasting	Latency to feed (s)	Pellet weight pre test (g or mg)	Pellet weight post test (g or mg)	Additional notes

Fig. 4 Example data recording sheet for use during the novelty-suppressed feeding test. Important parameters (bodyweight, latency to feed, food consumption) should be recorded for each mouse in a spreadsheet during the test. Detailed notes should also be taken in case any deviation from the protocol occurs (e.g., sudden loud noise during trial) to enable appropriate data evaluation during data analysis stages

or if a strain is known to exhibit low levels of baseline anxiety, we recommend a pilot study to confirm that the mice show the expected performance. Depending on results, adaptations to the fasting

protocol and the testing environment can be made (see Experimental variables and troubleshooting).

Randomization/Blinding of Experimenter

- All experiments following good research practices should involve a blinding step to ensure researchers are blind to experimental conditions of individual mice. This is crucial to avoid any bias, particularly because this test involves manual scoring.
- Carefully randomize the order of individual mice from different experimental groups throughout the batch. Potential circadian rhythm influences on behavior should be kept in mind, as testing mice at different times can influence behavioral outcomes [30].
- Do not run males and females in the same batch to avoid distracting odors/pheromones interfering with the test.
- If males and females need to be run on the same day, we suggest running males first, but cleaning the arena with ethanol and changing the bedding completely between batches.
- We suggest running no more than 20 mice per day (especially if the test is carried out with one mouse at a time).
- Since this test is based on the principle of hyponeophagia, mice should be tested only once as the testing environment will lose its novelty aspect when exposed a second time. If the test needs to be repeated (e.g., after a treatment), we suggest at least 10 days of interval between trials and a change in the test condition to guarantee the novelty needed for the test—[see *Experimental variables & troubleshooting section*.

3.3 Procedure

Fasting (Day Prior to Testing)

- Fasting the mice 24 h before the experiment is the standard protocol for this test [31]. Other fasting protocols might be possible—*see Experimental variables & troubleshooting section*.
- Record the body weight of each mouse before fasting.
- Remove all food from home cages. Ensure no broken food pellets have fallen into bedding, which may be consumed by the mice overnight. Leave the water supply in place.
- Fasting initiation should be staggered to ensure that each mouse has as close to 24 h fasting period as possible before testing. This is particularly important if the testing period will last more than 4–5 h when the study includes large cohorts of mice. We suggest fasting mice in 2–3 groups at several timepoints throughout the day.

Preparation

- Make sure all the above detailed materials and setup conditions are in place.

- Take mice to the behavior room for habituation (30–60 min prior to the start of the test).
- Place mice into a group-holding cage during the habituation period.
- Place the first pre-weighed pellet on the bedding inside the empty home cage for the later Consumption test.
- Weigh the mice and record their post-fast body weight [*see* **Note 7**].

Latency Test

- Have a timer ready in hand. Avoid timers that beep when started/stopped.
- Start recording video if the automated system is in use.
- Put the mouse into the corner of the arena (facing the corner; Fig. 5) [*see* **Note 8**].
  - We suggest running one mouse at a time to avoid the experimenter activity when one mouse finishes their test, which interferes with the other one still under experimentation.
- Start timer immediately and close the curtain/hide from the testing mouse line of sight [*see* **Note 9**].
- Observe the mouse on the screen (if an automated system is in use), stop the timer when the mouse grabs food pellet with forepaws (while sitting on their hind legs) and starts to eat. Record the latency to eat in seconds in the recording sheet.
  - It is expected that the mice will approach the center and sniff the food pellet (without biting) a few times before they properly bite the pellet to initiate feeding.
  - Tip: pay attention to the sound they make to be sure they are properly eating the pellet, not only sniffing. This is a distinct sound and can help in identifying the start of feeding.
- If a mouse does not eat the pellet within the 10-min test, then record 600 s in the recording sheet, but write down that the mouse did not feed.
- Remove the mouse immediately after the test is over (after starting to eat the pellet or 10 min has passed) and run the consumption test (see below) immediately.
- Stop and save the video into an appropriately identified folder (if automated system is in use).
- Collect fecal pellets from bedding in the arena (if any), re-mix and flatten the bedding surface (a spare plastic home cage can be used for that).
- Check the food pellet and food platform. Check the arena and bedding carefully and remove any crumbs if found. If the food pellet is still attached to the filter paper and clean, it can be reused. Otherwise, redo the setup.



Fig. 5 Initiation of trials during the novelty-suppressed feeding test. Placement of each experimental mouse facing alternating corners avoids potentially confounding effects of side differences within the arena, and ensures all mice start the trial without immediately seeing the presented food pellet

- Repeat the procedure above for the next trial.

Consumption Test

- After the Latency test is finished, measure food consumption over 5 min in the home cage [*see* **Note 10**].
- Place a pre-weighed food pellet into the center of the empty home cage prior to starting the Latency test to ensure the Consumption test can start immediately after the mouse has started feeding in the Latency test.
- Set the timer to 5 min.
- Once the mouse has initiated feeding in the main arena, transfer the mouse into the home cage.
- Start the timer.
- After 5 min, place the mouse into a new holding cage to ensure the home cage is free to use for the next Consumption test. This holding cage should contain food so that mice can immediately start re-feeding. Do not mix tested and untested mice in holding cages.

- Take the food pellet out of the home cage for Consumption test and weigh them once again using precision scales (0.1 mg precision), record this weight on the scoring sheet.
- Return all mice to the home cage from holding cages after all mice from the same home cage have been tested. Add food back into the home cage.

4 Analysis

This test generates three sets of data. The "Latency to feed" from the Latency test (Fig. 6A), the "% Change in bodyweight post-fast" (Fig. 6B), and the "Food consumed after fasting" from the Consumption test (Fig. 6C) (adapted from (22)).

- For the "% Change in Bodyweight post-fast":
  - Subtract the post-fast bodyweight from the pre-fast bodyweight and plot the "% change from baseline" (baseline: pre-fast bodyweight).
  - The statistical analysis will follow the particularities of your experimental design.
  - In the example above (Fig. 6), the data were parametric, two factors (sex and paternal treatment) and one random factor (litter) were in place. In this case, a generalized linear mixed model was used.
  - If significant differences are found in this parameter (as in the example above, Fig. 6B), this may suggest an influence on the food-seeking behavior in the novel aversive environment related to metabolic differences. It would be possible that increased hunger in one specific group (the group that lost more bodyweight) could be contributing to reduced latency to feed in this test, rather than reduced anxiety or depression levels, and hyponeophagia *per se*.
  - This outcome is crucial for the interpretation of this test, therefore consider it with due attention. It may indicate that a particular treatment, transgenic mouse model, is not suited for the NSFT due to differences in metabolism and hunger drive.
- For the "Food consumed after fasting":
  - Subtract the post-test pellet weight from the pre-test pellet weight and plot the "Food consumed" in grams (or milligrams if preferred).
  - The statistical analysis will follow the specifics of your experimental design.

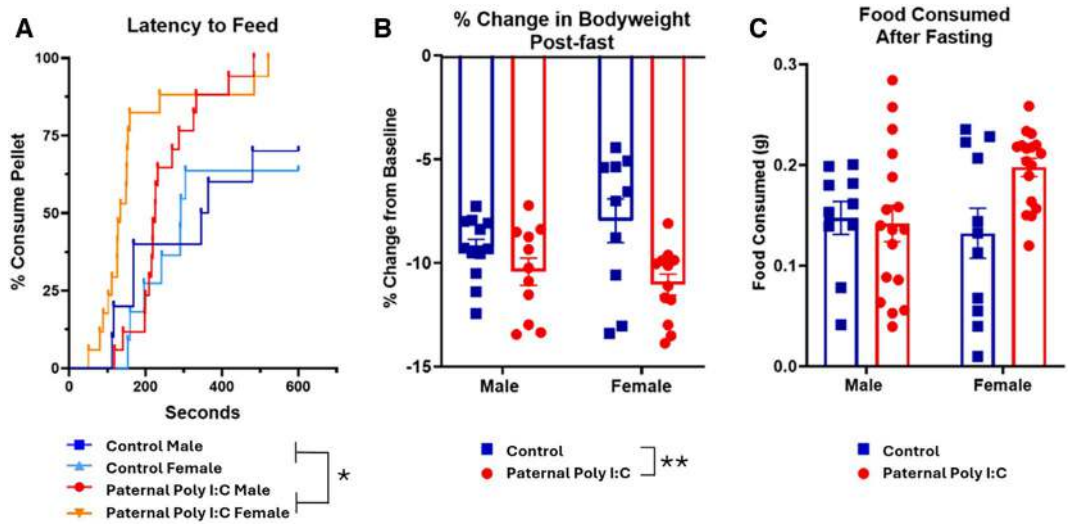


Fig. 6 Analysis of behavioral outcomes during the novelty-suppressed feeding test. Example data obtained from the NSFT is shown. Male and female animals bred from fathers that underwent Poly I:C (Paternal Poly I:C; polyinosinic:polycytidylic acid, a viral mimetic commonly used to model the acute phase of viral infections), or vehicle exposure (Control) were tested. (**A**) Latency to feed is represented using a survival plot, where food pellet consumption (or lack thereof) is defined as the binary event outcome, and the latency to initiate feeding is defined as the continuous outcome. (**B**) Fast-induced body weight changes and (**C**) food consumed after the test were analyzed for differences between treatment groups. Note that in this case, a significant difference in bodyweight loss was observed after a 24-h fast (**B**), therefore, the significant difference in latency to feed (shown in **A**) should not be interpreted as being related to anxiety and/or depression. Instead, the observed differences may be due to metabolic differences. Figures adapted and reproduced from Kleeman et al (2023)

  - In the example above, the data were parametric, two factors (sex and paternal treatment) and one random factor (litter) were in place. In this case, a generalized linear mixed model was used.
  - Similarly to the "% Change in Bodyweight post-fast" parameter, the food consumed post-test is a crucial control to exclude potential influences of differential hunger drive (and related metabolic factors) on the Latency parameter. This is necessary to ensure the timepoint of initiation of feeding is mainly under emotional control and can be confidently interpreted as depression- and/or anxiety-like behavior.

- For the "Latency to feed":
  - Use the time that the mice took to eat the food pellet in seconds.
  - Most often, a small number of mice do not eat within the 600 s, and the latencies do not fit into a parametric distribution for this test. In this case, non-parametric statistical

analysis is required, which can take into account the non-occurrence of events, such as survival analysis.

Kaplan-Meier estimators are commonly used to account for mice that did not consume the food pellet. These should be coded as non-events in survival analyses, using the maximum time (600 s) as the latency. Excluding these data points is not appropriate as the absence of feeding behavior within the set time period may be part of the observed phenotype.

In the example above, Cox regression (proportional hazards) was applied with clustered standard errors (clustering based on litters) with the factors (sex and paternal treatment) as fixed covariates.

5 Notes

1. Different types of bedding from the home cage, or no bedding at all, can be used to make the environment more anxiogenic if needed; and to guarantee novelty in case the test has to be repeated—see Experimental variables and troubleshooting section.
2. Ensure the set of lights does not heat up over time—this could make the environment aversive to the mice to approach the center, becoming an unwanted and uncontrolled additional anxiogenic factor, and changes in temperature over individual trials may cause unwanted variability in the data.
3. New bedding can be added after each trial if needed to increase the anxiogenic environment (though this is not very convenient for the experimenter)—see Experimental variables & troubleshooting section.
4. If low-odor tape is not available, the food pellet can be attached to the filter paper using cotton thread by carefully piercing a hole through the pellet and the paper and threading the thread through both– see Experimental variables & troubleshooting section.
5. If a more (or less) anxiogenic environment is needed, the illuminance of the arena can be adjusted accordingly—see Experimental variables & troubleshooting section.
6. We suggest using the same mixed bedding material used in the test arena—including the handful of each box that will be tested in that day/batch (all from the same sex). This approach aims to decrease the anxiety to be put in a new environment (new box with new bedding) that might influence their performance during the Consumption test.

7. It is important to weigh the mice before the start of the test to avoid the food pellet ingested during the test interfering with this measurement. Also, it is ideal that this measurement be done as close to 24 h post-initial body weight recording as possible.
8. Mice should be placed randomly in distinct corners to avoid potential differences in feeding behavior due to side preferences.
9. If the suggested approaches are not feasible due to room limitations, the experimenter should stay in the same position for all tested mice (and as far away from the arena as possible) to ensure the influence on the performance of individual mice is similar.
10. As mentioned above, this should be done immediately after the Latency test and before the start of the next mouse for the Latency test. Alternatively, the Consumption test can be done by a second experimenter—we only suggest the latter if a curtain is in place and this is done with minimal noise to avoid interfering with the parallel Latency test.

6 Experimental Variables and Troubleshooting

6.1 Important Variables to Consider

Antidepressant Treatment

One of the main strengths of the NSFT is that mice respond to chronic antidepressant treatment, reflecting the course of action of chronic antidepressant treatments typically observed in patients. This is not the case for many other popular tests of depression-like behavior and therefore represents a distinct advantage for the NSFT in its predictive validity.

- Chronic selective serotonin reuptake inhibitor (SSRI) treatment: oral fluoxetine [14, 32]
  - Treatment lasts 3 weeks
  - Fluoxetine hydrochloride (FLX) is added to the drinking water (concentration 160 μg/mL) in home cages for ad libitum consumption
  - Bottles should be opaque or covered in foil as FLX is light-sensitive.
  - Fluid intake should be monitored to determine the average dosage received by each mouse. At a concentration of 160 μg/mL, this should equate to around 20 mg/kg/day

Repeated Testing

- Due to its intrinsic reliance on novelty, the repeated exposure of mice to the testing paradigm is not recommended, as it will compromise the performance in the test. Rather than a longitudinal design where mice are repeatedly tested, we suggest control groups to compare performance in the NSFT across experimental groups at a single time point.
- **If the experimental design requires the test to be repeated in the same cohort of mice, we strongly advise performing a pilot experiment**, where adaptations to the test can be validated. We suggest the following conditions:
  - An interval of at least 10 days between trials.
  - A significant change in the testing conditions to guarantee the novelty required for the test. This may include one or a combination of the following adaptations:
  - Increasing the illuminance from the previous trial (e.g., 1000 lux).
  - Use of a different type of bedding. Alternatively, other materials can be used instead of bedding (e.g., newspaper sheet, changed between trials)
  - Changing the odor of the testing arena by adding 1–2 drops of essential oil to the center of the arena (e.g., lemon)

6.2 Troubleshooting

- **If all/most the mice approach and eat the pellet too quickly, leading to a potential floor effect**. This scenario indicates that the environment might not be anxiogenic enough, or the mice are too hungry. In that instance, a few adjustments to the test can be done and are listed below. We suggest validation tests be carried out before running the trial.
  - The illuminance can be increased to 1000 lux
  - The test can be performed without placing bedding into the experimental arena
  - The fasting period can be shortened. We have experienced good results with body weight loss in the range of 8%–12% after 24 h fasting, with a majority of mice initiating feeding within the 10-min experiment. If mice lose far more, consider fasting overnight only.
- **If all/most of the mice do not eat the pellet within the 600 s time-limit, leading to a potential ceiling effect**. This scenario indicates that the environment might be too anxiogenic or the mice are not hungry enough. In that instance, a few adjustments to the test can be made and are listed below. We suggest pilot tests be carried out before running the trial.
  - The illuminance can be decreased to 500 lux or less

- the fasting period can be extended to 48-h aiming to achieve at least 15% of body weight loss [33]

  In the event of requiring a 48-h food deprivation, mice should be provided a 2-h ad libitum feeding period after the initial 24 h.

  If even after the 48-h food deprivation, the mice still fail to lose at least 15% body weight, researchers have the option of excluding the individual animal from the experiment.

- If feeding initiation after 600 s is a feature of the particular strain or experimental paradigm used, the maximum duration for this test can be increased to 15 min if approved by the relevant animal ethics authority.

Conflict of Interest

The authors have no conflicts of interest to declare.

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Chapter 4

Differential-Reinforcement-of-Low-Rate (DRL) Operant Behavior: Methodological Issues and Effectiveness as an Antidepressant Assay

Joseph H. Porter , Fan Zhang , Herbert E. Covington III and Todd M. Hillhouse

Abstract

The Differential-Reinforcement-of-Low-Rate 72 s (DRL 72 s) operant assay has been used as a preclinical screen for antidepressant drugs since the early 1980s. It has been shown to have strong predictive validity for identifying drugs that have antidepressant-like effects. It is essential to evaluate treatment strategies not only for mood improvement but also for alleviating cognitive symptoms, which are strong predictors of long-term treatment outcomes and crucial to restoring day-to-day mental functions. A major advantage of the DRL 72 s test over other preclinical behavioral assays like the forced swim test and the tail suspension test is the lack of false positive results produced by stimulants. Also, the DRL 72 s assay appears to be measuring the cognitive process of impulsivity (which is disrupted in patients with major depressive disorder) and the ability of drugs to reduce impulsive behavior in this assay. One major disadvantage of all currently used preclinical behavioral assays for screening antidepressant drugs is that they use acute dosing regimens. Clinically, antidepressant drugs typically take 4–8 weeks to produce therapeutic effects. Thus, there is a need for the validation of current preclinical assays using repeated (chronic) dosing regimens to extend their validity as screens for the development of antidepressant drugs.

Keywords Antidepressant screen, Operant behavior, Predictive validity, Preclinical assay, Rats, Mice, Methodology

1 A Brief History of the Development and Use of DRL Schedules and Chapter Overview

What is Differential-Reinforcement-of-Low-Rate (DRL) Operant Behavior? DRL is an operant reinforcement schedule that requires an organism (typically rats or mice) to delay responding for some specified period of time before making an operant response to obtain a reinforcer. Thus, subjects are required to wait a specified period of time between responses to obtain a reward. More specifically, this operant schedule reinforces response inhibition over an interresponse

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time (IRT, the time between each operant response) by imposing a timed delay between reinforced responses. Thus, the DRL schedule represents a departure from the more traditional schedules of reinforcement that everyone is familiar with: fixed ratio (FR); variable ratio (VR); fixed interval (FI); and variable interval (VI). Specifically, FR and VR schedules of reinforcement require subjects to respond a specific number (FR) or a variable number (VR) of times to receive a reinforcer. In contrast, the FI and VI schedules have a time requirement and either a fixed (FI) or a variable (VI) amount of time must pass before a single response results in the delivery of a reinforcer. Unlike the DRL 72 s schedule of reinforcement, a premature response does not reset the FI or VI schedule's timer. The diagram in Fig. 1 provides a simple schematic for a DRL 72 s reinforcement schedule.

The history of reinforcing the interresponse interval instead of the operant response begins (not surprisingly) with B.F. Skinner in his 1938 book *The Behavior of Organisms* (pp. 306–307) [1]. Fast forward to 1953 and a study by Wilson and Keller [2] with rats found that as the IRT interval is increased from 10 s to 30 s, response rates decrease in a linear fashion, demonstrating an increase in the length of the time between successive responses (i.e., the interresponse interval) and a subsequent decrease in the number of earned reinforcers. As discussed by these authors, such changes in behavior were probably the result of an interaction between extinction and reinforcement effects, indicating an active learning process. One of the first demonstrations on the effects of drugs on "timing behavior" found rats trained on a DRL 21 s schedule displayed an inverted "U" function with the highest frequencies for the IRTs between 18 and 21 s [3]. When the stimulant DL-amphetamine (1.5 and 3.0 mg/kg)

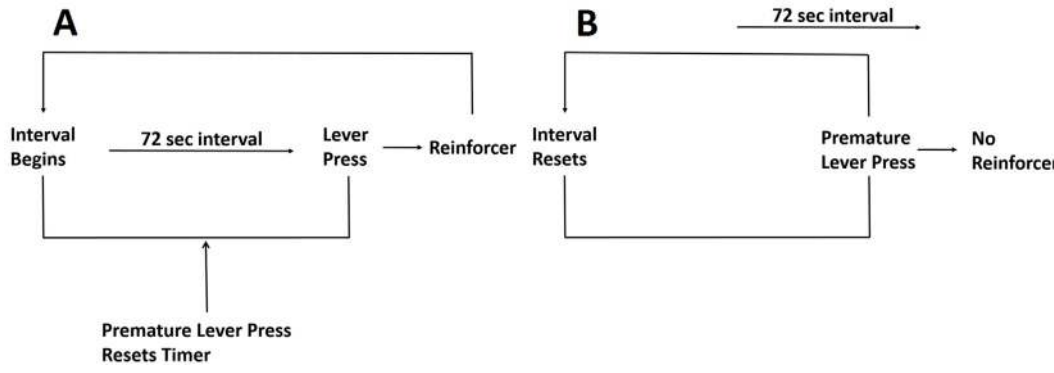


Fig. 1 Schematic of a DRL 72 s operant schedule. In panel (**A**), three events can start the 72 s timer: (1) the test session begins (house light is turned on); (2) a reinforcer delivery; and (3) a premature response during the 72 s interval. If the subject waits the entire 72 s interresponse interval (or longer) before making the next response, that response results in delivery of the reinforcer (food or water). In panel (**B**), a premature response (i.e., a response prior to the end of the 72 s interresponse interval) resets the interresponse timer to a new 72 s interresponse interval

was administered, there was a marked increase in the total number of lever presses that resulted in significantly reduced IRTs, and a leftward shift in IRTs (i.e., the majority of the IRTs became shorter—less than 18–121 s). Conversely, when alcohol (3 ml of a 10% ethyl alcohol solution) was administered, total responses were depressed, but the timing behavior of the rats was not significantly altered.

Over the next two decades, a number of behavioral pharmacology studies employed this DRL operant methodology to study a variety of different drug classes on elements of cognitive performance. For example, Ford and Balster [4] examined the acute and chronic administration of the analgesic morphine in rats using a DRL 15 s schedule. They reported an inverse correlation between morphine dose and both mean responses and mean reinforcers earned under an acute dosing regimen, effects portrayed with a general flattening of the IRT distribution. Effects considered to be a result of morphine's sedative effects. Interestingly, after 21 days of repeated dosing with 18 mg/kg morphine, the IRT distribution closely resembled that of saline, indicating tolerance to morphine's suppressive effects in this cognitive, behavioral realm, although overall response rates were still somewhat reduced. These results resemble the findings with another CNS depressant, alcohol [3]. Not surprisingly, CNS stimulants (D-amphetamine and phencyclidine) dose-dependently increase responding in mice trained on a DRL 10 s schedule and produced a leftward shift in the IRT distributions with the highest frequencies [5]. However, it should be noted that phencyclidine produced an "antidepressant-like" effect, as measured by a significant increase in reinforcers earned, decrease in responses emitted, and a rightward shift in the interresponse interval (IRT) when tested in rats trained on a DRL 72 s schedule [6]. Another, less potent stimulant, caffeine, has also been shown to produce increased responding in both mice (DRL 18 s [7]) and rats (DRL 20 s [8] DLR 72 s [9]).

This chapter will not attempt to review this literature in depth, as our focus is on the use of the DRL procedure for screening compounds for their potential and selective antidepressant-like effects. In the early 1980s, Lewis Seiden and colleagues published several articles demonstrating the utility of the DRL 72 s operant task as a preclinical assay for screening antidepressant drugs [10–12]. In these studies, they demonstrated the advantages of using a longer interresponse interval (i.e., 72 s) over shorter intervals and their ability to selectively identify antidepressant drugs from several different drug classes whose pharmacological mechanisms differed (also see [13]). These findings will be discussed in greater detail in Sect. 2.

A number of prominent reviews have compared behavioral assays (tests) that screen for antidepressant drugs with degrees of reliability and validity (e.g., [14–20]); however, the DRL assay has comparatively received less attention than most models. Unlike many other behavioral tests used to screen potential antidepressant drugs, the

DRL is not a "high-through-put" assay, in that it takes considerable time to train animals in the DRL 72 s operant procedure. However, it can be argued that this assay offers several advantages when examined in parallel with others, as we discuss in Sect. 3. To keep the length of this chapter manageable, the validity of the DRL 72 s assay will be compared systematically to both the forced swimming test (FST) and the tail suspension test (TST), which are the most commonly used preclinical behavioral tests for screening antidepressant efficacy (see Sect. 2). Sect. 3 describes the methodology for conducting DRL studies and the advantages and disadvantages of the DRL 72 s approach. Sect. 4 details an example of a reliable protocol for conducting the DRL 72 s procedure. Finally, Sect. 5 provides some conclusions and promising suggestions for future directions.

2 Validity of the DRL 72 s Behavioral Assay for Assessing Antidepressant-Like Effects of Drugs

In this section, we will examine the validity of the DRL 72 s assay. First, however, the distinction between *tests* and *models* should be considered. One of the earliest articles to discuss criteria for models of depression was by McKinney & Bunney [21]. In that article, they summarized a list of criteria that they felt were the minimum requirements for a valid animal model of depression:

1. *The symptoms of the depression so induced should be reasonably analogous to those seen in human depression.*
2. *There should be observable behavioral changes that can be objectively evaluated.*
3. *Independent observers should agree on objective criteria for drawing conclusions about the subjective state.*
4. *The treatment modalities effective in reversing depression in humans should reverse the changes seen in animals.*
5. *The system should be reproducible by other investigators (page 246)*

A review by Cryan et al. [22] points out that these criteria are widely used in this field and provides an insightful discussion about these five criteria. As they originally emphasized, important distinctions are made between models and behavioral tests. Harro [19] and Slattery and Cryan [23] argue that models involve the manipulation of an independent variable that "induces" the behavioral and/or biochemical change (i.e., the dependent variable) for the disorder of interest (depression in this case). In contrast, a test (or assay) only measures this change, which they called a "readout measure." Unfortunately, these behavioral tests (or assays) are often used as (thought to be) actual models of depression or at least measuring symptoms

seen in humans suffering from depression. This anthropomorphism has resulted in part from the naming of the tests based on human attributions (i.e., depression). Martin Seligman published several articles in the late 1960s and early 1970s in which he attributed the failure of dogs to escape shock after having been subjected to an inescapable shock procedure, "*learned helplessness*" [24–27]. This use of a human attribute to describe a behavior observed in animal studies has resulted in the assumption that learned helplessness in nonhuman animals is equivalent to depression in humans—i.e., that this procedure is a valid model of depression or depressive behavior. Similarly, Porsolt published a series of papers in the late 1970s identifying what he called "Behavioral Despair" [28–31] in a new behavioral test. This test was the FST, in which either rats or mice are placed into an escapable tank of water. After swimming vigorously at the beginning of the test session, the majority of animals will float for most of the remaining session. This behavioral "immobility" was described by Porsolt [29] as "a state of despair in which the rat has learned that escape is impossible and resigns itself to the experimental conditions." In the years since, this immobility behavior is often called "*behavioral despair*." In short, we frequently see human attributes being used to describe the behavior of nonhuman animals and the acceptance of the FST as a model of depression rather than as a behavioral test.

The validity of tests and models is an important consideration for their use. While there are a number of different types of validity, our focus is on *construct*, *face*, and *predictive* validity of the DRL 72 s test (with comparisons to the FST and TST). We will not be examining the large number of animal models, which have been developed to study depression (e.g., unpredictable chronic mild stress [UCMS], early life stress model, olfactory bulbectomy [OBX] model, social defeat model, chronic restraint model, glucocorticoid/corticosterone model, genetic models (for further discussion, see [17,18]. In the context of our discussion herein, *construct validity* refers to how well a behavioral test represents or models clinical depression (i.e., sharing common behavioral attributes). First, it is very difficult to argue that the human disorder of depression actually occurs in nonhuman animals, given that depression is a relatively recent complex clinical phenomenon with a limited understanding of its etiology, symptoms, and underlying neurobiological factors. As Gencturk and Unal [20] discuss, the immobility seen in the FST (and by inference the TST) is typically referred to as depressive-like behavior. In their article, they argue that behavioral tests "*fail to measure the higher-order constructs*" of depression. Similarly, no overt behaviors were shown by rats or mice in the DRL 72 s assay that can be argued to represent depression-like behavior in terms of any human symptoms. It is, however, arguable that the DRL-72 s procedure measures cognitive processes reflecting behavioral impulsivity (see [13]), which,

if true, provides an avenue towards measuring at least one specific symptom of depression. Control over impulsiveness is disrupted in many patients with major depressive disorder (MDD), and in many cases, cognitive dysfunction is a stronger predictor of poor long-term pharmacotherapeutic outcomes than affective symptoms alone ([32], see Sect. 3 for additional discussion on this topic).

If the behavior in an animal assay "extrapolates" to the human behavior or disorder, then that test might be considered to possess *face validity*. Thus, the immobility seen in the FST and TST "looks" like depressive behavior. However, it can easily be argued that floating (i.e., immobility) in an inescapable context (i.e., a tube) filled with water might also be interpreted as a coping strategy to conserve energy or learned behavior (i.e., the animal learns they can float with minimal effort for an extended period of time and still survive). Molendijk and de Kloet [33] argue that the "response to the forced swimming stressor should be considered for what it shows: a switch from active to passive behavior in the face of an acute stressor, aligned to cognitive functions underlying behavioral adaptation and survival." Obviously, the DRL 72 s assay is an operant procedure and not necessarily interpreted as having any face validity with regard to depression. So, what is left? *Predictive validity*, which in our context is defined as the ability of a behavioral test to accurately predict key therapeutic effects of antidepressant drugs in humans. Rupniak [15] also notes the failure to distinguish between "animal models of depression and screen[s] for antidepressant drugs" and argues that the most important "*question is not whether behavioral assays accurately model human disease, but whether they accurately predict antidepressant efficacy in human patients.*" Can behavioral tests be used to successfully screen for the prominent antidepressant effects of drugs?

To determine a potential for examining therapeutic efficacy, we first review here what an "antidepressant profile" looks like in the DRL 72 s, FST, and TST assays. First, the FST and TST approaches represent variations of tests designed to measure immobility, which is thought to represent depressive-like behavior in rodents. The ability of antidepressant drugs to decrease this immobility (i.e., decrease depressive-like behavior) is used as a prediction of antidepressant therapeutic effects in humans. Thus, a decrease in immobility represents an antidepressant-like profile in these two assays. How well do FST and TST accomplish this? As review articles have suggested, both the FST [34] and the TST [35] have good predictive validity for known antidepressants compared to other therapeutic drug classes. That said, these two tests are subject to false positive results with stimulants and other drugs often revealing positive effects in these assays but having failed in clinical trials [34,35]. In contrast, one of the major advantages of the DRL 72 s test is the small number of false positives that emerge across data sets (see below and Sect. 3 for further discussion of this). But what is an antidepressant-like

profile in the DRL 72 s assay and how does that contrast with a stimulant-like profile?

Figure 2 shows typical results for the tricyclic antidepressant imipramine, the selective serotonin reuptake inhibitor (SSRI) antidepressant fluoxetine, and the stimulant D-amphetamine in adult, male Sprague–Dawley rats in the DRL 72 s test [36]. Both imipramine and fluoxetine display what is considered to be an antidepressant-like profile, in which both drugs produced a significant increase in the number of reinforcers earned, a significant decrease in the number of responses, and a significant rightward shift in the IRT distribution (indicating a decrease in the number of premature responses). In contrast, the stimulant D-amphetamine produced a stimulant-like

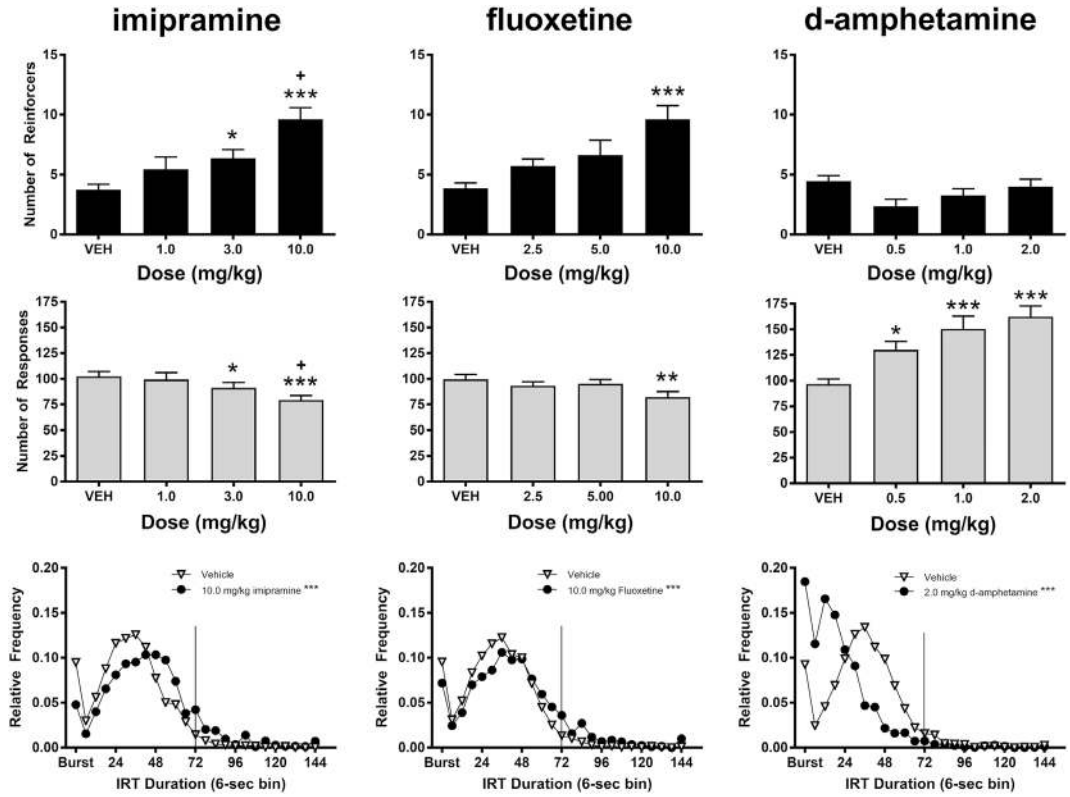


Fig. 2 Example of antidepressant-like profile for the tricyclic imipramine and the SSRI fluoxetine and a stimulant-like profile for the stimulant D-amphetamine. Effects of imipramine (left; $n = 11$), fluoxetine (center; $n = 11$), and D-amphetamine (right; $n = 11$) on the number of reinforcers earned (top), number of responses emitted (middle), and inter-response time (IRT) distribution (bottom). Only main effects are shown as these drugs failed to produce any significant interaction effects. Each IRT graph displays the vehicle and the highest effective dose for each drug. Significant shifts in the IRT distributions were assessed using the peak location analysis. Reinforcers and responses are expressed as means ± SEM. IRT data are expressed as means. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus vehicle; $+P < 0.05$ versus all doses. VEH vehicle. This comparison highlights how these drugs differently affect response rates and timing, important for understanding their behavioral impact. ([35,57]; reproduced with permission)

profile with a significant dose-dependent increase in responses and a significant leftward shift in the IRT distribution (indicating an increase in the number of premature responses). There was a small, but nonsignificant decrease in the number of reinforcers earned. The stimulant D-amphetamine was not, in this case, a false positive in the DRL 72 s assay (unlike results found with FST and TST).

In summary, it is clear that the DRL 72 s test has strong predictive validity for the acute effects of antidepressants. In fact, across many studies, a wide range of antidepressant classes, including tricyclic antidepressants, monoamine oxidase inhibitors, selective serotonin reuptake inhibitors, norepinephrine reuptake inhibitors, serotonin and norepinephrine reuptake inhibitors, NMDA antagonists, and electroconvulsive therapy, have been evaluated using the DRL 72 s test. With the exception of bupropion, all of these treatments consistently produce a behavioral profile characterized by an increased number of reinforcers, a decreased number of responses, and a rightward shift in the IRT distribution (e.g., [12, 13, 35–38]).

3 Methodological Considerations

Like all preclinical behavioral assays, the DRL task has both advantages and disadvantages. One advantage is that the DRL-72s procedure selectively engages the cognitive process of impulsivity, which is disrupted in patients with major depressive disorder (MDD) [23, 32, 39]. For example, patients suffering from depression have increased "attentional impulsivity," which is defined as difficulty in focusing attention or concentrating and acting without considering the consequences, and "nonplanning/reactive impulsivity," which is defined as the lack of future orientations, tendency to focus on the present moment, and disregard for potential consequences. It is known that antidepressant drugs decrease impulsivity (or impulsive behaviors) in patients suffering from major depressive disorders [39]. The DRL test was originally designed to measure impulsivity at lower interresponse times (IRTs; e.g., 10–30 s) [40], which is defined as difficulty in delaying responses for an immediate reward, as opposed to obtaining greater rewards through delayed gratification, or as the inability to inhibit the urge for immediate rewards [41, 42]. An antidepressant-like effect in this procedure, which requires a decrease in premature responses (i.e., a decrease in impulsivity), may possibly be a result of the antidepressant drugs' ability to reduce reactive impulsivity. O'Donnell et al. [13] also suggested that the changes in DRL performance by antidepressant drugs might be related to their effects (i.e., an improvement) on the animal's ability to make a temporal discrimination (see Sect. 4 for further discussion about impulsivity). The external validity of the DRL procedure as a test for impulsivity has been supported by a series of studies conducted

by van den Broek et al. [43]. In these studies, when impulsive and non-impulsive human participants were asked to delay responding using an IRT of 10 s to earn a reward, impulsive participants earned significantly fewer reinforcers than non-impulsive participants in the absence of cues.

A second advantage of the DRL procedure is that it allows for within-subjects experimental designs, leading to two significant benefits. First, the within-subjects design controls for the individual differences between the subjects by allowing comparisons of an individual subject's baseline data to their drug treatment data. This increases the "power" of statistical analyses to predict reproducible outcomes as a result of reducing errors (variability), and increases the likelihood of detecting significant drug effects. Thus, the within-subjects design (as compared to between-subjects designs) provides greater statistical power and reduces the likelihood of producing detrimental type I errors [6, 37, 44, 45]. The second advantage of the within-subjects design is that this operant behavior (i.e., DRL 72 s responding) is stable for months, allowing the researcher to test multiple drugs in each rodent, provided a drug washout period is used. Also, baseline behavior can be reestablished prior to each new drug testing regimen. This permits a number of different drugs to be tested in the same animals, maximizing the amount of information from fewer animals. Importantly, this meets one guideline of the three Rs—*Reduction* [46–49]. As discussed in the *Guide for the Care and Use of Laboratory Animals* [50], the three Rs are important guidelines for researchers' ethical responsibility for the "humane care and use of these animals" (p. 4).

A third advantage is that the DRL assay has a very low potential for "false positive" findings typically seen with stimulants (e.g., amphetamine) in behavioral assays that use immobility as the primary dependent measure. FST and TST are both more vulnerable to false positive results with stimulants, as a general increase in locomotor typically leads to more mobility but not necessarily escape-related behaviors. For example, Hillhouse and Porter [37] examined the effects of ketamine and MK-801 in male Sprague-Dawley rats in a DRL 72 s study. They found that 10 mg/kg ketamine produced an antidepressant-like profile with a significant increase in the mean number of reinforcers earned, a significant decrease in mean responses, and a significant rightward shift in the IRT distribution. However, the more potent NMDA antagonist MK-801 (0.01 mg/kg) produced a stimulant-like profile with a significant decrease in reinforcers and responses and a significant leftward shift in the IRT distribution. In contrast, MK-801 produces very different effects in the FST. Li et al. [51] found that both ketamine (20 mg/kg) and MK-801(0.01 mg/kg) produced a significant decrease in immobility (i.e., an antidepressant-like effect) in male Kunming mice in the FST.

MK-801 does not appear to have antidepressant effects in patients with depression (unpublished data, Merck; see [52]).

A fourth advantage is that the DRL assay is an operant task, which is programmed and controlled with a computer program. That program records operant data in an objective manner; thus, it does not have the subjective aspect that is inherent with FST and TST. The test sessions for FST and TST are typically videotaped and then the data are "decoded" or scored by at least one (hopefully more) trained experimenter(s) who is "blind" to the experimental treatments. Some error in that scoring is expected, unfortunately, as there is some subjectivity involved and some bias associated with each individually trained behavioral observer. While the FST has been used extensively in both rats and mice, the TST is used primarily with mice (especially since the increase in the number of genetically manipulated mouse models in the past 20–25 years).

Finally, the DRL procedure has been successfully used in several different species of animals and also in human subjects. This allows a comparison of similarities and differences across species and increases the translational value of this procedure. While rats and mice have been used in the majority of DRL studies, pigeons (e.g., [53]), non-human primates (e.g., [54]), and humans (e.g., [55]) also have been tested with the DRL operant procedure. (Fig. 3)

One major limitation with the DRL-72s procedure, and other preclinical assays (e.g., FST, TST), is that traditional antidepressant drugs (e.g., SSRIs, tricyclics, etc.) can produce an "antidepressant-like" response after a single, acute injection [6, 37, 45, 56]. This does not align with clinical data as patients typically require a minimum of 4–8 weeks of antidepressant drug treatment before seeing a clinical response [57–60]; although ketamine and esketamine appear to be exceptions and can produce rapid and prolonged antidepressant effects in humans (see [61]). This discrepancy between the chronic treatment required for therapeutic efficacy in humans



Fig. 3 Med-Associates operant chamber in a sound-attenuating chamber (left picture), a Sprague-Dawley rat (middle picture), and C57Bl/6 mouse (right picture) in operant chambers.

and the acute effects measured in the DRL paradigm highlights a potential limitation in the translational validity of this test (a problem that exists for most preclinical behavioral assays used to screen for the antidepressant-like properties of drugs). Despite its high reliability in detecting the acute effects of existing antidepressants, there is no evidence that the behavioral effects measured by the DRL assay (as well as FST and TST) have construct validity as related to the therapeutic efficacy of antidepressant drugs or to depression. Unfortunately, there have been limited studies that examined the use of repeated dosing regimens with the DRL assay. Such experiments would greatly extend the validity of the DRL procedure.

Another disadvantage of using the DRL assay is the initial (startup) cost of the operant equipment and the computer-controlled software that controls the experimental sessions and records the data, as compared to the FST and TST assays. Usually, the largest cost associated with those procedures is a video camera(s) for recording the test sessions, and that cost increases significantly if an automated computer scoring software program is used to analyze behavioral data. That said, the use of an operant procedure removes all subjectivity seen with the decoding of FST and TST data (clearly an advantage). The automated data collection objectively records discrete lever presses and delivery of reinforcers during the test sessions. Precise information can be obtained about both the number and temporal patterning of these data during test sessions. Thus, there is greater objectivity for the DRL task and greater test-test reliability in data collection and analysis.

4 DRL 72 s Protocol

The exact methods used for training rats or mice will vary somewhat from lab to lab, but there are a number of common methodological procedures that should be used and considered in training and testing. The DRL 72 s Protocol presented in BOX 1 is primarily based on our experience with both rats and mice, and we also have incorporated some material/ideas that were presented and discussed in two previous chapters by other authors [62,63] – both are valuable resources.

BOX 1—DRL 72s Protocol

(1) **Equipment, Supplies, and Software**

(a) **Operant Chambers**

Operant conditioning chambers appropriate for rats or mice are used, and they should be housed in sound-attenuating cubicles. Our labs have used equipment from Med-Associates (see Fig. 2), but there are several other sources, including Camden Instruments, Conduct Science, Harvard Apparatus, Lafayette Instruments, and Maze Engineers. Our comments will be limited to Med-Associates equipment and software, but generally apply to equipment from any vendor.

A minimum of two operant chambers, but preferably more, should be purchased (recommend buying them in multiples of 2) as this allows more animals to be tested simultaneously. The cubicles have a ventilation fan that provides background white noise during test sessions. Each operant chamber is equipped with a house light, one or two levers (either fixed or retractable), and a food receptacle (trough) or a liquid dispenser for either water or liquid food (typically sweet). The house light is usually mounted on the back wall. On the front wall, one or two levers are mounted on one or both sides of the receptacle for reinforcer delivery. For rat studies, our lab has used 45 mg purified diet pellets (grain or sucrose) from Bio-Serv as food reinforcers. For the mouse studies, we use a sweetened-milk solution (by volume: 150 ml powdered milk, 150 ml sugar, and 500 ml water). Both of these reinforcers require the animals to be maintained on a restricted food diet in their home cages (we individually house the animals) in order to maintain their body weights at 85%–90% of their free-feeding weights (this is established prior to food restriction). If you decide to use water as the reinforcer, there are such procedures in the literature. One caution about water restriction regimens is that this requires diligent adherence to the restriction procedures, as animals can become seriously dehydrated in as little as 24 h with no water. We weigh animals daily while they are on food (or water) restriction, as body weight is one of the best indicators of an animal's overall health. A sudden decrease in body weight can indicate a potentially serious health issue.

(b) **Supplies and other equipment needed (not exhaustive)**

For rats—45 mg food pellets

For mice—sugar and powdered milk to make the sweetened milk solution

Small bench scale for measuring the body weights of the animals

Analytical Balance for measuring drugs

Spatulas, weigh boats, and weighing paper

Assorted glassware (beakers, graduated cylinders, etc.) for mixing drugs and measuring fluid volumes

Magnetic Stirring Hotplate and stir bars

Latex or non-latex gloves

1 ml tuberculin syringes with 23–25 g 3/8–5/8 (rats) and 25–27 g 3/8 (mice) needles

(c) **Software and computer**

Computer with Windows XP or 10

Med-State software (Med-PC, version 4 or higher; Med-Associates) controls test sessions and records data

Software for graphing data and statistical analysis (we use GraphPad Prism for Windows for both the current version is 10)

(2) **Training and Test Procedures**

(a) **Acclimation**

Acclimate the animals to the vivarium and laboratory for a minimum of 7 days. During this time, the animals are brought to the laboratory daily to be habituated to handling and being weighed.

(b) **Food Regulation**

Establish a free-feeding (100%) body weight (i.e., the highest weight) for each animal while the rats are on *ad libitum* food (body weights measured each day). For food restriction, we first remove all food from the home cage overnight. Then, the next day, the animals are weighed, and a small amount of food is placed in the home cage. The amount of food obviously depends on both the species and strain—more for rats (8–14 gm) than mice (3–6 gm). It normally takes about a week to stabilize a new, reduced body weight for each animal. Initially, we establish an 85% target of their 100% body weight. The amount of food fed in the home cages will vary slightly, and fairly precise body weights can be established for each animal. On training/testing days, daily food rations should only be given to the animals after the training/testing sessions. Approximately every 6 months, we reestablish animals' free-feeding body weights by allowing free access to food in the home cages.

(c) **Lever Press Training**

One day before starting training, give the animals a small amount of the reinforcer (i.e., food pellets or sweetened milk) in the home cage to avoid neophobia.

Magazine Training. Magazine training is done for two days in 30 min sessions in which reinforcers are delivered according to a fixed time 60 s schedule (no response required). The house light is turned on at the start of the 30 min training session and off at the end. During magazine training sessions, no lever is present in the operant chamber. It is easy to determine if the animals eat all of the food pellets at the end of each session. However, observation of the animals during the training sessions is necessary with a liquid reinforcer.

FR 1 training. A response lever is placed in the chamber. With retractable levers, it is easy to counterbalance the lever position (left or right) across animals and between each test session. The animals are first trained under a fixed ratio 1 (FR1) food reinforcement schedule in which each lever press results in the delivery of one reinforcer. We use a criterion of obtaining (and consuming) a minimum of 30 reinforcers in 20 min. Some animals will meet the FR1 training criterion in the first session, but some will require several training sessions.

DRL Training. Under the DRL schedule, a response produces a reinforcer only after a specified interresponse interval has elapsed. Responses emitted before the end of the inter-response interval reset the timer and will not produce a reinforcer.

The interresponse interval criterion is gradually increased from an initial value of 4.5 s to a terminal value of 72 s. All DRL training and testing sessions are set to end at 60 min or when the animal

receives 50 reinforcers. In our lab, the following training regimen is used:

5 sessions of DRL 4.5 s training.

5 sessions of DRL 9 s training. In rat studies, DRL 9 s is often used first.

10 sessions of DRL 18 s training.

10 sessions of DRL 36 s.

DRL 72 s training until performance is stable.

DRL 72 s performance is considered stable when the number of responses for each animal does not vary by more than 10% of the mean for five of six consecutive sessions. More than 70 responses and fewer than 8 reinforcers are considered ideal. If an animal struggles with the shift from 36 s to 72 s, the DRL schedule can be returned to 36 s for further training at that interval. Occasionally, it might be necessary to use one or two intervals between 36 s and 72 s to obtain stable responding on the DRL 72 s schedule.

(d) **DRL Testing**

The animals should be habituated to vehicle injections several times before the first testing session.

An example of a typical weekly testing schedule is below, after the DRL 72 s behavior is stable. There should be a minimum of one training session prior to each test session in order to determine that DRL 72 s performance is stable [6,45], as there is the possibility of overtraining rats and mice. Therefore, we recommend the schedule below that has incorporated training breaks to limit overtraining. If an animal is becoming too efficient on the DRL 72 s schedule, the number of test days can be reduced to one with only two training days. During the initial DRL acquisition period, training the animals 5 or 6 days per week is fine.

MON	TUE	WED	THU	FRI	SAT	SUN
Training	Testing	Break no training or testing	Training	Testing	Break no training or testing	Break no training or testing

For each dose-response curve, a vehicle test should be connected at the beginning and the end of each dose-response curve. Depending on the experimental design, it is ideal to counterbalance drug doses and the order in which the drugs are tested across animals. The table below shows a simple Latin square design for randomized testing of three doses of a single drug or for the randomization of testing three different drugs.

Rat 1	Veh	Dose 1 or Drug 1	Dose 2 or Drug 2	Dose 3 or Drug 3	Veh
Rat 2	Veh	Dose 2 or Drug 2	Dose 3 or Drug 3	Dose 1 or Drug 1	Veh
Rat 3	Veh	Dose 3 or Drug 3	Dose 1 or Drug 1	Dose 2 or Drug 2	Veh

On training and test days, the animals should be transferred to the testing environment one hour prior to the test. Drug solutions should be prepared ahead of time (or removed from refrigerated

storage) to allow the solution to reach room temperature before the injections.

Clean the operant chambers with 10% ethanol solution between each session.

(3) **Data analysis**

Dependent variables:

Total number of earned reinforcers during each test session

Total number of responses during each test session

IRTs for all responses during each test session.

All data should be expressed as means ± SEM.

It is standard to present the data (e.g., overall responses, earned reinforcers, and IRTs) for the complete 60 min session. This would be analyzed with a one-way repeated measures analysis of variance (ANOVA) for a study using a within-subjects design or a one-way between-subjects ANOVA for a study with between-subjects design. However, if the researcher is interested in the within-session time-course of effects for the tested drugs, the data can be recorded during the test session in several time bins (e.g., 10 or 20 min) (see [35]). In this case, both reinforcer and response data can be analyzed using a two-factor repeated-measures analysis of variance (ANOVA) with drug dose and time bin treated as within-subject factors.

IRT distributions for DRL 72 s require shorter time bins. This is typically done with twelve 6 s bins. IRT graphs are constructed by first determining the relative frequency for each 6 s bin (i.e., 1–6 s, 6–12 s, etc.) individually for each animal. The relative frequency for a bin is calculated by taking the number of responses for a given bin and dividing that number by the total number of responses for the entire session for that animal. These results are then averaged for each bin. The last bin is 72 s and beyond. To determine it if there were any significant changes in the IRT distribution, a peak location analysis can be performed (see [64]). The median (peak location) of the IRT distribution for each individual animal is determined after first eliminating burst responding (responses in the first 6 s bin) from the total number of responses for that animal. Finally, the medians (peak locations) were analyzed using one-way repeated-measures ANOVA across drug doses. This peak location analysis determines if there are any significant shifts (leftward or rightward) in the IRT distribution produced by the drug. If significant effects ($P < 0.05$) are obtained for any ANOVA, then an appropriate post hoc test (e.g., Tukey test) can be used to determine further information about dose or drug differences; although the appropriate post hoc comparisons will depend on whether or not the interaction in a two-way ANOVA is significant or not. If not, then only the main effects of the independent variables can be examined.

5 Conclusions and Future Directions

The use of non-human animals in the study of antidepressants is justified primarily for two reasons: to advance our understanding of the physiological mechanisms underlying depression and to facilitate the screening and development of more effective treatments.

To achieve these objectives, the behavioral tests employed in preclinical research must be rigorously validated for their predictive validity—not merely for their ability to detect any effects of existing antidepressant drugs, but specifically for their capacity to reflect the therapeutic effects of these treatments. Despite the time-intensive nature of training rodents on complex operant procedures like the DRL, such approaches are justified by their ability to model the stable, unrelenting cognitive disturbances characteristic of clinical depression—precisely the features that must be probed to advance translational relevance [65]. As Willner [14] emphasized, antidepressants exert not only therapeutic effects but also a range of side effects, which can complicate the interpretation of preclinical findings. Therefore, it is crucial to distinguish between behavioral changes that are indicative of therapeutic efficacy and those that may arise from non-specific or secondary pharmacological actions.

As discussed in Sect. 3, the DRL 72 s assay has strong predictive validity, but does not have strong construct or face validity. While it does not appear to be measuring depression per se, it may be measuring behaviors associated with symptoms found in human depression or possible side effects associated with acute antidepressant drug treatment. For example, a common side effect shared by antidepressant drugs in humans (with the exception of bupropion) is their acute dose-dependent sedative properties [66,67]. However, given the low rate of operant behavior in the DRL assay, this may not be a relevant issue. It is easy to test the same doses in a standard activity assay to determine if this is an issue to worry about. Also, several other classes of drugs with known acute sedative effects (alcohol, chlordiazepoxide, morphine, and pentobarbital) typically do not produce an antidepressant-like profile in the DRL 72 s assay [13]; although there have been conflicting reports [68,69].

In Sect. 3, we discussed the evidence that the DLR 72 s assay measures impulsivity behavior, which is a cognitive process that is known to be disrupted in MDD (i.e., a symptom of depression). Also, the DRL assay is less subject to false positive results from stimulants in contrast to other assays like the FST and TST—clearly a major advantage. The ability to use within-subjects designs is also important as it allows the testing of fewer animals to test more drugs. Thus, the DRL 72 s test has demonstrated high reliability in detecting acute drug effects and offers high predictive validity as an antidepressant screening test. The major limitation of the DRL assay and other preclinical assays is that there is very limited research determining their ability to capture clinically relevant treatment regimens with repeated/chronic dosing. The absence of this information needs to be resolved with preclinical studies designed to evaluate repeated dosing regimens.

Conflict of Interest

The authors have no conflicts of interest to declare that are relevant to the content of this chapter.

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Chapter 5

Video Analysis of the Marble Burying Task

Lauren Burgett and Marcy Kingsbury

Abstract

The marble burying task (MBT) is a behavioral assay used in rodents to assess obsessive-compulsive, restricted-repetitive, and anxiety-like behaviors and screen pharmacological compounds for anxiolytic and anti-compulsive effects. The task became widely adopted in the literature due to its simplicity, lack of training requirements, and ability for rapid throughput. However, contradictory results have raised questions about the validity of the MBT. There is widespread variation in the application of the MBT, which may have contributed to inconsistent results between laboratories. Non-marble-oriented digging behaviors have been shown to substantially influence marble coverage, leading to poor construct validity of the traditional MBT. Reliance on the number of marbles buried at the sole data point oversimplifies the complex behavioral repertoire exhibited during the task. To enhance the validity and translational utility of the MBT, this chapter outlines a video-based approach to the MBT, with a focus on distinguishing between marble-directed and non-marble-directed behaviors. Methodological considerations, including marble configuration, cage setup, duration, behavioral descriptions, and statistical approaches, are detailed. Recommendations for standardizing the MBT are provided. Video-based MBT analysis offers valuable information in behavioral analysis in murine models of anxiety, Obsessive-Compulsive Disorder (OCD), and Autism Spectrum Disorder (ASD) when used in conjunction with other behavioral assays. The traditional MBT still retains its utility as a pharmacological screening tool. This chapter provides a framework for refining the MBT and enhancing its reliability and validity in neurobehavioral research.

Keywords Marble burying, Anxiety, Pharmacological screening, OCD, ASD, Video-based behavioral analysis

1 Background and Significance

1.1 Historical Background

The marble burying task (MBT) is a behavioral test used to assess obsessive-compulsive, restricted-repetitive, and anxiety behaviors, as well as anti-compulsive and anxiolytic effects of pharmacological compounds in rodents, most frequently mice. Thus, this assay has been widely adopted in murine models of anxiety, obsessive-compulsive disorder (OCD), and autism spectrum disorder (ASD). In this task, an experimenter places a rodent into a cage with a set number of marbles placed on top of ample bedding substrate. The animal

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interacts with the environment for a set amount of time, usually between 20 and 30 min.

The first study to establish the use of the modern MBT in mice was conducted in 1986 [1]. In the context of defense burying (a behavior where an animal uses its forepaws to displace material toward a perceived threat, often an electrified probe or another noxious stimulus), it was discovered that mice would bury glass marbles [2]. Broekkamp (1986) used glass marbles to assess burying behaviors because the original defensive burying task is time-consuming and requires control for general inhibition of behavior, whereas glass marble burying does not. This study found differential effects of psychotropic drugs on marble burying and grooming behaviors. Chlordiazepoxide, clonazepam, diazepam, flunitrazepam, and ethanol were the most effective in selectively inhibiting marble burying [1]. Broekkamp concluded that compounds with a strong selective inhibitory effect on marble burying should be evaluated further as potential anxiolytic agents, thus establishing the MBT as a screening test for anxiolytic drugs. In subsequent years, the MBT became used as a rapid method for screening psychotropic compounds due to its simplicity, lack of need for training, and ability for rapid throughput.

Since 1986, the popularity of the marble burying task has rapidly increased. As of September 2025, a PubMed search for articles mentioning marble burying yielded 883 papers. Notably, 840 of these papers were published between 2006 and 2025, with 506 being published between 2018 and 2025. This increased usage of the MBT in the literature is partially due to the expansion of the MBT beyond its original application of pharmacological screening to broader neurobehavioral research in the mid-2000s. This shift was due to accumulating evidence that the marble burying task is influenced by innate rodent behaviors, such as object exploration and digging, that are independent of pharmacological interventions.

1.2 The Naturalistic Behavior of Digging

Digging, burrowing, and burying behaviors are innate, ecologically relevant, and genetically influenced complex behaviors observed in both wild and laboratory rodents. These behaviors can serve many goals, including burrowing for shelter, burying noxious and non-aversive objects, finding food, storing food, exploration, entrance sealing, and escape digging [2–8]. Mice will switch between digging behaviors depending on context. For example, after food restriction, male mice will switch from burrowing to exploratory digging [9].

Thus, burrowing, digging, and burying are useful behaviors to assess in mice for early detection of disease or pharmacological effects, as these complex behaviors are goal-directed and require executive function. The presence of aberrant behavior or absence of innate adaptive behaviors, like digging, during behavioral tasks can be used to assess the health of laboratory animals. Adaptive behaviors may decrease before aberrant behavior arises [7]. Therefore,

digging behaviors can be a sensitive variable in behavioral testing of mice. However, despite the diverse goal-directed behaviors exhibited during different digging behaviors, digging has been frequently reduced to a behavior that is interpreted as repetitive or anxiety-like, and motor function in behavioral assays, without distinction of the type of digging. This reductionist approach has led to criticism of the marble burying task [10–14].

Mice engage with marbles in the MBT with task-specific behaviors. Mice have been observed burying marbles by spraying bedding over the marble with their forepaws and nose, digging a hole and pushing the marble into the hole, and rolling a marble around, seemingly "playing" with the marble [13, 15]. These marble-directed behaviors provide an avenue for assessing persistent, restrictive, and repetitive behaviors in animals. However, care must be taken to differentiate direct interaction with the marbles, which may be an appropriate model for OCD and ASD, from naturalistic behaviors that accidentally bury marbles. During the marble burying task, mice have been observed "accidentally" burying marbles through non-marble oriented, normal exploratory behaviors [13, 15, 16]. The number of marbles buried at the end of the task correlates with general digging activity and does not correlate with marble-directed digging activity [13]. This finding highlights the importance of distinguishing different digging behaviors in the MBT, as the number of marbles buried is not an accurate proxy for marble-oriented behaviors, with naturalistic digging behaviors significantly contributing to the number of marbles buried.

1.3 Controversy Regarding Construct Validity, Predictive Validity, and Translational Value

Criticism of the MBT has focused on several key issues, including inconsistent methodologies, discordance with other anxiety measures, contradictory results, inconsistencies between human responses to pharmacological interventions and MBT results, and the oversimplification of diverse behaviors into a single metric.

Variations of the MBT are common across pharmacological and neurobehavioral studies, with inconsistencies in task duration, marble density within the testing apparatus, burying substrate, number of marbles, marble orientation, definition of a "buried marble", strain of mouse, assessment of locomotor activity, and repeat testing of mice. Reported durations range from 10 to 30 min, with a mode of 30 min [17]. Marble density has been reported as low as 23.8 cm^2 per marble [18] to as high as 112.58 cm^2 per marble [11, 19]. Burying substrates have included sawdust [20], corncob [21], and woodchip [22]. Researchers have used between 4 and 25 marbles [20, 23] arranged in various configurations, such as evenly spaced marbles [13, 20, 24], along the walls of the testing apparatus [23, 25], in close contact with other marbles in the center of the cage [15], or in a two-zone configuration to allow for avoidance of the marbles [26]. Some researchers utilize each mouse only once [27] or expose mice to repeated testing within the MBT [28].

Despite these inconsistencies between laboratories, the unifying characteristic of the MBT is the quantification of marbles buried at a fixed endpoint. Most researchers define a marble as "buried" solely on the percentage of the marble that is covered with bedding without analyzing the behaviors that led to coverage of the marble. Marbles have been classified as "buried" when they have been covered by bedding to at least one-half [21, 29], two-thirds [1, 13, 20], or completely [30]. Genetic effects on marble burying behavior are addressed later in this chapter. To control for the general motor effects of pharmacological compounds, some researchers measure locomotor activity to confirm that reduced marble burying is not secondary to a decrease in general movement [27, 29, 31]. However, locomotion is not always assessed in pharmacological studies [32]. While no clear consensus exists on the optimal parameters of the MBT, the variability in methodology emphasizes the need for standardization of parameters across future studies, as the impact of these differences on marble burying outcomes has not been systematically characterized.

The MBT has been hypothesized to measure anxiety-like responses to marbles when animals are introduced to these stimuli for the first time. This theory is supported by studies finding that known anxiolytic compounds, such as benzodiazepines (alprazolam, chlordiazepoxide, diazepam), selective serotonin reuptake inhibitors (fluoxetine, citalopram, paroxetine), and mood stabilizers (valproate, carbamazepine, lamotrigine), selectively reduce marble burying without altering locomotor activity [1, 27, 31, 33]. However, the MBT's specificity for screening for anxiolytic effects of drugs is limited, as non-anxiolytic compounds, including antipsychotics (haloperidol and chlorpromazine), also decrease marble burying behavior, but often alongside locomotor changes. Moreover, compounds known to induce compulsive behaviors in humans, such as pramipexole and methyl β-carboline-3-carboxylate, fail to increase marble burying [10]. Results from the MBT have been found not to correlate with other measures of anxiety-like behavior [21]. The presence of marble burying behavior persists even after repetitive exposure and when avoidance of the marbles is possible [21, 26]. Additionally, digging within the MBT is not influenced by the presence or absence of marbles, suggesting that the marbles may not be anxiogenic, and the task might be tapping into genetically influenced species behaviors [15, 21, 26, 34]. The persistence of marble burying behaviors, poor alignment with other anxiety measures, and limited drug specificity raise concerns about the reliability of the MBT as a tool for assessing animal models of anxiety when marbles are used as an anxiogenic stimulus.

While there is evidence against using the MBT in translational models of anxiety, the test has promise in studying the effects of experimentally induced anxiety-like behavior in rodents [11]. The

preceding discussion focused on marbles and/or the testing environment as the primary anxiogenic stimulus. However, when aversive stimuli such as acute immobilization [32] or foot shock [35] are applied, mice exhibit increased marble burying. Administration of anxiolytic agents reduces this stress-induced increase [35]. While the MBT itself should not be considered inherently anxiogenic, marble burying behavior following induced stress may provide insight into the anxiolytic effects of pharmacological interventions.

Conflicting and clinically inconsistent results from the MBT further cloud its usefulness in pharmacological screening and animal models of neurodevelopmental and psychiatric diseases. Anxiolytic compounds (alprazolam, diazepam, and chlordiazepoxide) and anxiogenic compounds (methyl β-carboline-3-carboxylate and D-amphetamine) have both been found to significantly reduce marble burying behavior [10, 27]. The administration schedules of these compounds are also frequently performed in a clinically inconsistent manner, reducing the translation value of the test. Pharmacological compounds that are only clinically effective for generalized anxiety disorder and OCD after chronic administration, such as SSRIs and second-generation antipsychotics, are tested in the MBT after 30–60 min [27, 36–39]. Beyond pharmacology literature, incongruencies have been reported in genetic models of neurodevelopmental disorders. In murine models of ASD, Shank-1 knockout mice have been reported to bury either more or fewer marbles than wild-type mice [40, 41]. These findings highlight the limited translational value of MBT as it is currently implemented.

Unlike other behavioral tasks, the MBT is not classically video recorded, and the number of marbles buried is often the only measure used to quantify behavior. This one-dimensional approach of behavior, when applied to translational models of neurodevelopmental and psychiatric disorders with diverse disease phenotypes, has drawn criticism. Although the applications of the MBT were expanded in the late 2000s and early 2010s, its behavioral analysis remains essentially unchanged from its original, simplistic pharmacological screening format introduced in 1986. The criticism and evidence against the validity of the traditional marble burying task raise the need for reform and standardization of the MBT. It is questionable whether a single static image after a test that lasts 20–30 min can accurately capture and quantify the rich array of behaviors exhibited. The MBT, as it has been classically applied, does not differentiate between inherent and aberrant behavior within mice. This lack of differentiation and standardization may account for the clinical inconsistencies and contradictory findings reported in the literature. Despite the criticism of the MBT outlined above, the use of digging behavior as a model for psychiatric disorders and for screening pharmacological interventions remains conceptually valuable. Description of the traditional marble burying assay in pharmacological screening can be found in

Neuromethods Volume 190 [42]. The inconsistent application of the MBT has led to legitimate concerns about the test's validity. This chapter outlines how to perform a video-based marble burying task with the aim of building upon the traditional MBT to improve its translational value.

2 Equipment, Materials, and Setup

2.1 Mice

Any genotype of mouse can be used in the marble burying task. Our lab has primarily utilized C57BL/6J mice (strain #000664 from Jackson Laboratory—Bar Harbor, ME, USA). Mice were 70–75 days old at the time of testing. The average weight of male mice was 24 g, and that of female mice was 19 g. Our mice were maintained under a 12:12 light-dark schedule. Mice prior to testing can be housed in group or individual cages. All mice should receive continuous access to food and water before testing.

2.2 Test Environment

Behavioral testing should occur in a room separate from the colony. The room should have minimal temperature variability across testing days and minimal noise. Male mice and female mice are tested on separate days. To minimize testing variability, experiments should be conducted at similar times daily. When possible, the same handlers should perform the task across experimental cohorts.

We consistently have performed social tasks (such as the three-chamber social interaction and social novelty tests) before the MBT and anxiety-like measures (such as the light-dark box and elevated zero maze) after the MBT. This is to reduce any potential for anxiety measures influencing other measures of behavior.

2.3 Equipment

Our laboratory utilizes the following equipment for the marble burying task:

- Standard-size mouse cage (28 × 20 × 12 cm), which is the same model as their home cage (*see* **Note 1**)
- Sani Chip Hardwood bedding. Our lab has sourced this bedding from ScottPharma (Marlborough, MA, USA)
- Glass marbles with a 1.42 cm diameter in two colors (*see* **Note 2**).
- Plexiglass covers for cages to prevent escapes
- Paper dividers to obscure the view between cages if testing more than one mouse concurrently
- Light diffuser over any overhead lighting source to decrease glare
- Video camera mounted above the cage with good visualization of all four corners of the cage
- Camera to obtain post-task images

2.4 Setup

Mice are habituated to the testing room for 1 h before testing. Fill the standard mouse cage with Sani Chip Hardwood bedding to a depth of 5 cm. Tamp the bedding down and create an even surface with a gloved hand.

For a one-zone configuration (Fig. 1A):

Evenly space 20 marbles into the testing cage. The marbles should be arranged in a 5 × 4 arrangement with alternating colors.

For a two-zone configuration (Fig. 1B):

Evenly space 10 marbles into one half of the testing cage. One half of the cage should be marble-free.

Clean bedding, marbles, and cages should be used for each mouse and test (*see* **Note 3**). If performing tests on multiple mice at once, place paper dividers between cages to block the mouse's view of other cages. In previous experiments, our lab has had all mice from the same home cage complete the marble burying task at the same time.

2.5 Dependent Variables

For rapid screening of the pharmacological effects of drugs, the number of marbles buried at the end of the task is analyzed. A marble is counted as buried if more than two-thirds of the marble is obscured by bedding substrate.

For video behavioral analysis, a list of behaviors frequently seen in the MBT is described in Table 1. The behaviors we have previously observed, scored, and analyzed include freezing, hugging side walls, huddling in corner, marble investigation, playing or pushing of marbles, moving bedding with nose, rapid digging, marble burying, other digging, face grooming, body grooming, uncovering of buried marbles, supported rearing, unsupported rearing, and moving. This list of behaviors was comprehensive of behaviors seen in our experiments. Video examples of these behaviors are provided in the

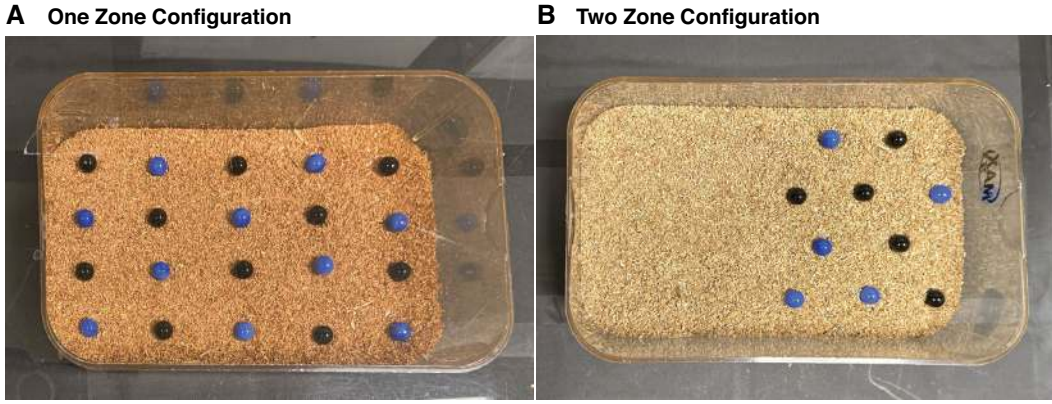


Fig. 1 Setup for marble burying task. (**A**) one zone configuration with 20 evenly spaced marbles in a 5×4 arrangement with alternating colors. (**B**) two-zone configuration with 10 evenly spaced marbles placed into one half of the cage

Table 1
Description of the behaviors frequently observed and hand-scored during the marble-burying task. Material from: Burgett et al., A revision to marble burying: video analysis during the marble burying task is imperative to understanding rodent behavior, Lab Animal, published 2024, Springer Nature

Scored behavior	Description
Freezing	Immobile for more than 0.2 s
Hugging walls	Walking next to the walls of the cage with no gap
Huddling in corner	Shortened posture, rear of the mouse backed into the corner of the cage
Marble investigation	Sniffing or poking the marbles
Playing and pushing marbles	Sustained interaction with a single marble involving slight or large movements of the marble
Moving bedding with nose	Bedding is disturbed or pushed away with nose
Rapid digging	Quick, aggressive, and sustained digging. Typically directed away from the corners of the cage
Marble burying	Controlled digging directed at a single marble, frequently proceeded directly by interaction with the marble or walking over the marble. Does not have to result in the marble being fully buried.
Other digging	Digging not otherwise categorized as rapid digging or marble burying
Uncovering of marbles	Digging to uncover a previously buried marble
Face grooming	Quick facial grooming only with forelimbs, typically lasting 0.2–0.4 s
Body grooming	Grooming bouts including both grooming with forelimbs and hindlimbs
Supported rearing	Rearing supported by the walls of the cage
Unsupported rearing	Rearing in the middle of the cage or otherwise not supported by walls
Moving	Walking around the cage and uncategorized behavior

supplementary materials of our 2024 Lab Animal publication [13]. However, this list can be expanded if other behaviors are observed in other laboratories.

3 Behavioral Procedure

3.1 Pretest Manipulations

No pre-task behavioral training is recommended or required for the MBT. After setup, weigh each mouse and mark tails for identification if necessary. If testing the effects of pharmacological compounds, dose each mouse based on weight. After dosing the agent, place the mice in individual holding cages. Set a time for the pretreatment interval.

3.2 Testing

Before handing the mice, start the recording. Place the mouse into the center of the testing apparatus, being careful not to disrupt any marbles. It is important to have the handler remove their hand from the testing cage quickly to minimize visual obstruction of initial mouse behaviors (*see* **Note 4**). An assistant should start a 20-min timer as soon as the mouse has been placed in the testing chamber. Have all personnel leave the room once the task has begun. Leave the mice in the testing chamber for 20 min. Return to the testing room after 20 min and remove the mouse from the testing chamber with special attention to not disrupt any marbles. End the recording and take a photo of the chamber. The entire test procedure lasts 20 min.

4 Data Analysis

4.1 Number of Marbles Buried

Two observers, blind to treatment groups, score the number of marbles buried. A marble is defined as buried if more than two-thirds of the marble is obscured by bedding substrate. The scorers should remain the same for a set of experiments to minimize variability between cohorts. The number of marbles should be analyzed at 5, 10, 15, and 20 min. The rationale for these timepoints is explained in Sect. 6.2. Generalized linear models should be used to analyze the number of marbles buried.

4.2 Video-Based Behavioral Analysis

A training period is necessary for scorers analyzing MBT videos. During this training period, two scorers blind to experimental conditions create a behavioral ethogram. Our lab's behavioral ethogram is described in Table 1 and serves as a baseline for common behaviors observed during the MBT. An atlas of video recordings exemplary of each behavior should be created for reference during scoring. The training period is completed once videos can be consistently scored with minimal differences between scorers. All videos are analyzed by classifying behavior at every 0.2 s interval. Our lab has scored behavioral videos from the MBT using Solomon Coder (RRID: SCR_016041). Other hand-scoring software, such as BORIS, can be used for video analysis based on laboratory preferences. The two scorers' durations and frequencies of each behavior are averaged to create the final data. Statistical analyses can be conducted using the R environment. Duration data from the MBT can be analyzed using linear mixed effects (LME) models or ordinary least squares (OLS) linear regression models with robust variance estimation. Count data from the number of marbles buried should be analyzed using generalized linear models, particularly negative binomial models (refer to Lazic for a more detailed explanation on statistical analysis of the number of marbles buried) [43]. Sexes can be combined during analysis if there are no differences in the distribution, variance, and no interaction effects. However, as with other behavioral

tests, biological context must be considered before removing sex as a variable. In C57BL/6J mice, it has been reported that there is no significant difference in marbles buried by sex [13, 44].

5 Results

5.1 Anticipated Results from Video Analysis

The MBT should yield data for the number of marbles buried and the duration spent engaging in each behavior defined by the behavioral ethogram. Our lab has found an average of 14.8 ± SD 3.92 marbles buried after 20 min in C57BL/6J mice. Correlations between behaviors and the number of marbles buried can be analyzed. The amount of time spent engaging in marble-directed behavior and self-grooming can be a helpful metric in describing repetitive, compulsive behaviors. Anxiety-like behaviors such as avoidance of marbles, reduced exploratory behaviors, freezing, reduced unsupported rearing, and reduced center exploration can be quantified. Behavioral tracking software (e.g., Ethovision) can be used to create heat maps and tracing of mice, similar to open field analysis.

5.2 Results Supporting the Use of Video-Based Analysis in the MBT

Our laboratory studied a triple-hit model of ASD that included a genetic predisposition for intestinal permeability (HP2-2 genotype), early-life maternal separation stress (MS), and fecal microbial transfer of ASD stool from human patients (ASD FMT) in multiple behavioral tasks, including the MBT. We hypothesized that mice exposed to all three factors (HP2-2, MS, ASD FMT, referred to as "triple hit mice") would show more restrictive, repetitive behaviors than control mice. After our first experimental cohort, we were surprised to discover that triple-hit mice buried fewer marbles than control wild-type mice. Female zonulin transgenic mice (HP2-2) have been found to bury more marbles than wild-type females [45]. Due to these unexpected results and criticism regarding the validity of the MBT, we recorded the MBT for a second cohort of animals to analyze behaviors exhibited during the task.

During our analysis of behaviors detailed by our behavioral ethogram (Table 1), we found a significant positive correlation between rapid digging (defined as fast, aggressive digging in the corners of the cage) and the total number of marbles buried by the end of the MBT (Fig. 2A, Pearson r: $^{**}P = 0.0028$). In contrast, the number of buried marbles showed no significant correlation with marble-directed digging behaviors. (Fig. 2B, Pearson r: $P = 0.1570$). These findings are consistent with previous reports demonstrating that overall digging behavior correlates with the number of marbles buried [15,21]. However, our results highlight the importance of video-based analysis to distinguish between digging subtypes, as the final count of marbles buried may not reliably reflect marble-directed behaviors. Notably, "rapid digging" behavior has been observed in

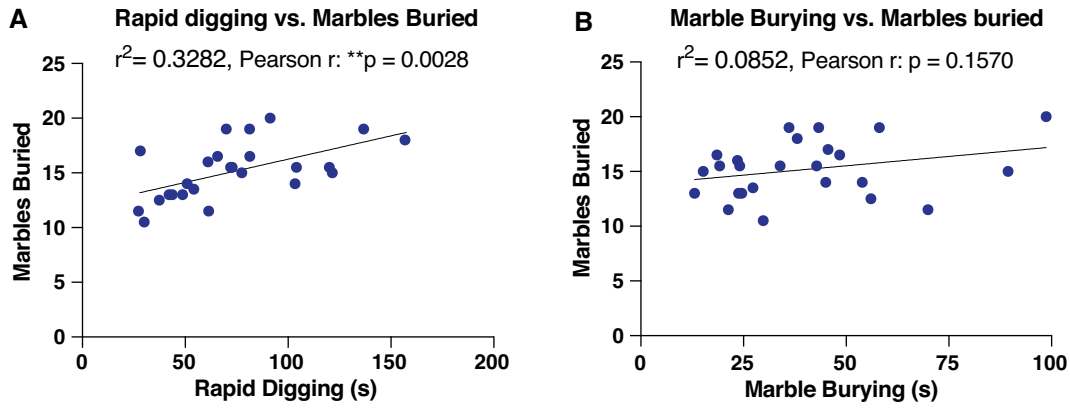


Fig. 2 Behavioral analyses of marble-burying in real-time. (**A**) The time spent rapid digging was positively correlated with the number of marbles buried at the conclusion of the marble burying test (Pearson *r*: $r = 0.5729$, 95% CI [0.2297, 0.7893], $^{**}P = 0.0028$. Simple linear regression: $r^2 = 0.3282$, $N = 25$). (**B**) The time spent on marble burying was not significantly correlated with the number of marbles buried at the conclusion of the marble burying test (Pearson *r*: $r = 0.2918$, 95% CI [−0.1168, 0.6159], $P = 0.1570$. Simple linear regression: $r^2 = 0.0852$, $N = 25$). ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). C57BL/6 mice (13 female, 12 male) were tested. (Material from: Burgett et al., A revision to marble burying: video analysis during the marble burying task is imperative to understanding rodent behavior, Lab Animal, published 2024, Springer Nature)

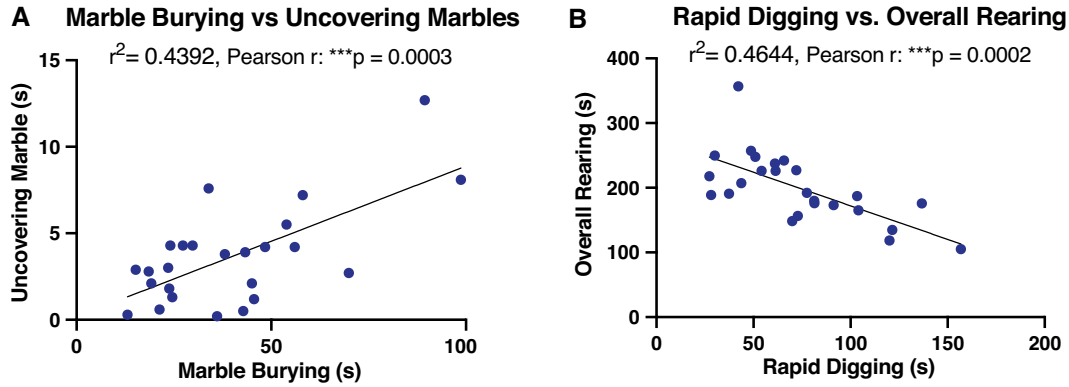


Fig. 3 Correlations discovered during the marble burying test. (**A**) Marble burying was positively correlated with the time spent uncovering marbles (Pearson *r*: $r = 0.6627$, 95% CI [0.3625, 0.8383], $^{***}P = 0.0003$. Simple linear regression: $r^2 = 0.4392$, $N = 25$). (**B**) Rapid digging was negatively correlated with overall rearing (Pearson *r*: $r = -0.6815$, 95% CI [−0.8482, −0.3919], $^{***}P = 0.0002$. Simple linear regression: $r^2 = 0.4644$, $N = 25$). ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). C57BL/6 (13 female, 12 male) were tested. Material from: Burgett et al., A revision to marble burying: video analysis during the marble burying task is imperative to understanding rodent behavior, Lab Animal, published 2024, Springer Nature

the absence of marbles [15]. Additionally, mice can unbury marbles, which could confound endpoint measurements. We found a significant positive correlation between marble burying and uncovering the marbles (Fig. 3A, Pearson *r*: $^{***}P = 0.0003$), suggesting a

behavioral phenotype characterized by increased engagement with the marbles. Other phenotypes, such as anxiety-like behaviors, may be observed during video analysis, as we found a significant negative correlation between rapid digging and overall rearing behavior (Fig. 3B, Pearson r: $^{***}P = 0.0002$). Collectively, these findings provide evidence of the necessity of video-based behavioral analysis in place of the traditional endpoint measure of marbles buried in the MBT.

6 Experimental Variables and Troubleshooting

6.1 Statistical Strategies

Statistical analysis of MBT data is complicated by predictions that produce impossible values, such as confidence intervals yielding negative marble counts or counts exceeding the total number of available marbles. The number of marbles buried is count data, which is bounded by zero and the total number of marbles placed in the arena. Additionally, the MBT may include censored data in which mice have buried all marbles present, and it cannot be measured whether they would have buried more marbles if they were available. Censored data can lead to loss of sensitivity, bias in estimates of treatment effects, and the breakdown of the underlying construct and the measured outcome [43]. Many statistical approaches commonly used to analyze marbles buried, such as t-tests and ANOVA, assume unbound data with normal distribution and homogeneity of variance. Because the marble burying counts are bounded and often affected by ceiling effects, the data frequently do not meet assumptions of normality. Use of normal linear models for data that does not meet the assumption of normality can lead to impossible confidence intervals, invalid p-values, and biased predictions [43]. To avoid these errors, researchers should optimize the duration of the task and the number of marbles to avoid censored data and use generalized linear models to analyze the number of marbles buried.

There is a significant main effect of observer in different burying substrates on the number of marbles buried [16], indicating that the scoring criteria are subject to observer bias and differential approaches to scoring application. Consistent observer training protocols should be implemented, and a minimum of two observers should score MBT outcomes to decrease observer bias.

6.2 Testing Time and Environment

It is critical to limit the duration of the MBT, as differences between results from different experimental manipulations may converge. With a limited number of marbles and a confined arena, the number of marbles buried may approach 20, leading to censored data. Decreasing the time mice interact with marbles can help to reduce

the number of censored observations (the number of times mice bury all marbles provided).

Sugimoto et al. observed the effects of milnacipran and fluvoxamine on marble burying at 10, 20, and 30-min intervals [46]. Notably, mice injected with the lowest dose of fluvoxamine (1 mg/kg) buried roughly one third of the number of marbles after 10 min in comparison to the control. After 30 min, this effect of low-dose fluvoxamine was no longer observed, with control and experimental mice burying the same number of marbles. Santini et al. observed a similar convergence in marble burying between experimental groups in a murine model of ASD using eIF4E-transgenic mice [47]. Although EIF4E-transgenic mice buried significantly more marbles at all time points, the difference between groups diminished over the course of the task, particularly when examined at 30 min. Notably, the control mice buried an average of approximately 20 out of 20 marbles at both the 20- and 30-min time points, indicative of range restriction and censored observations.

Given these findings and the above discussion of statistical considerations, we propose an abbreviation of the task to 20 min to minimize censored observations and improve the sensitivity of the MBT. If the number of censored observations is notable in an experiment, the duration of the MBT should be reduced further, or the methods of analyzing censored data should be implemented.

6.3 Repeated Testing

Optimization of research protocols to reduce the number of animals needed for an experiment is one of the fundamental ethical principles of research. A within-subject design of the MBT provides an avenue to achieve this aim. Partridge et al. found that the number of marbles buried and locomotor activity were consistent across four test sessions over two weeks [28]. This finding of consistency across multiple exposures has been repeated, noting that scores were most consistent when mice were tested at a similar body size and with a one to fourteen-day interval between tests [21, 48]. Thus, the MBT is suitable for a within-subject design, which can promote animal stewardship and increase statistical power.

6.4 Genotype Comparisons

Genetic background is an important factor in digging behavior, with studies observing significant strain differences in the MBT. Thomas et al. (2009) examined ten inbred mouse strains for marble burying behavior [21]. There was a significant effect of strain on the number of buried marbles, with CBA/J, FVB/NJ, and AKR/J mice burying the most, and 129S6/SvEv Tac, 129S1/SvImJ, and C57BL/6J burying the least. However, Nicholas et al. found that C57BL/6J mice buried significantly more marbles than CBA/J mice [27]. These conflicting results indicate that strain-specific variability within the MBT is also context dependent. Researchers should not assume behavioral consistencies across laboratories due to the irregularities

in MBT methodology across the literature. Future reporting should aim to characterize the strain differences in video-analyzed behaviors.

6.5 Experimental Chambers

The usefulness of a two-zone configuration of the MBT has been recently explored as an avenue to increase the test's validity in anxiety research. De Brouwer and Wolmarans conducted a study that investigated the effects of zone configuration on behavior within the MBT [16]. They suggest that the one-zone configuration is more representative of normal exploratory behaviors, while a two-zone marble burying chamber better models anxiety behaviors as it allows for the avoidance of marbles. Nicolas et al. reported that C57BL/6J mice spend only 25% of time within the marble zone in the two-zone configuration [27]. However, Thomas et al. reported that 2- to 4-month-old male C57BL/6J mice do not avoid marbles, spend approximately equal time in both zones, and bury a similar number of marbles as in a one-zone configuration [21]. The inconsistent avoidance of marbles within the two-zone configuration furthers the evidence that marbles themselves are not inherently anxiogenic. Careful determination of which testing arena must be considered within a laboratory before conducting the MBT. If the research goal is to evaluate anxiety-like behaviors, a two-zone configuration is advantageous as it provides mice with the ability to avoid the marbles. However, if the research goal is to assess for changes in naturalistic behaviors or the presence of restrictive, repetitive behaviors, the one-zone MBT should be utilized.

6.6 Locomotion Testing

Concurrent locomotor activity with video tracking can be tested during the MBT [16, 27]. Locomotor activity is not associated with an increase or decrease in the number of marbles buried [16]. Additionally, measuring locomotion during the MBT can increase its predictive validity as a test for anxiety in pharmacological research [27].

6.7 Video-Based Analysis

The traditional MBT reports the number of marbles buried at the end of the task without further elaboration on the behaviors that led to the covering of marbles. As addressed previously, the marbles may be covered during marble-directed behaviors or "accidentally" during normal exploratory behaviors, as well as uncovered. There are other marble-directed behaviors, such as pushing around a marble with the snout, that do not cover marbles. The number of marbles buried has been found not to correlate with marble-directed behavior, but rather normal exploratory behaviors.

To accurately categorize marble-directed behavior, we propose using video-based behavioral analysis as described above. If using automated video analysis software, it is imperative to have the experimenter move their hand out of frame as quickly as possible, reduce glare, and ensure the rodent's color is sufficiently different from the coloring of the marbles. Any of the variables above can lead to errors

in tracking the mouse with software. While automation is helpful for time spent in certain zones and total movement during the task, marble-directed behaviors should be hand-scored to ensure accuracy.

7 Recommendations for Using the Marble Burying Task

- The marble burying apparatus should be the same size as the rodent's home cage. We suggest a 28 × 20 × 12 cm apparatus for mice.
- A fixed number of marbles and a standardized size of marbles should be adopted by researchers. We suggest twenty 1.42 cm marbles.
- A two-zone configuration of the marble burying task is ideal for characterizations of anxiety-like and obsessive-compulsive behaviors in the MBT [11]. When reporting data from the marble burying task, it is imperative to designate whether a one-zone or two-zone configuration was used.
- The marble burying task should be recorded and scored by two or more blinded observers. The video analysis should aim to differentiate and quantify the duration between marble-directed behavior and cage exploratory behaviors.
- Each report should contain a detailed behavioral ethogram and video examples of any novel, previously unreported behavior observed during the task.
- The singular usage of marbles buried at the end of the task should only be used as a screening test, rather than as a reportable metric. The number of marbles buried should be measured at different time points throughout the task. We suggest 5, 10, 15, and 20 min.
- If using the MBT as a screening test for anxiolytic and anti-compulsive pharmacological interventions, locomotor activity should be assessed. This can be accomplished with a separate cohort of mice or the same mice that will be tested in the MBT.
- For compulsive behavior analysis, researchers should consider repetitive trials of the MBT per animal to test for perseverance of behaviors, as well as other tasks to test for other repetitive behaviors (i.e., self-grooming).
- The marble burying task should be used in conjunction with other behavioral tasks known to measure anxiety-like behaviors, such as the open field test, elevated plus maze, elevated zero maze, light dark test, and social interaction test.
- If pharmacological agents are being tested, the marble burying task should be used in conjunction with behavioral tasks to measure locomotion, such as the open field test.

- When testing pharmacological compounds that have been clinically shown to be effective only after chronic administration, rodents should be dosed under a chronic administration protocol prior to the MBT, rather than a one-time dose 30 min before testing.

8 Notes

1. Using a larger or smaller cage may lead to changes in MBT data due to altered marble density. We recommend using the standard size mouse cage (28 × 20 × 12 cm mentioned above to increase external validity.
2. If using automated video analysis software, it is imperative to choose two colors with high contrast between both mouse coat and bedding color (for example, if using black mice, black marbles should not be used). If the mouse and marbles have similar coloring, automated software can incorrectly label a moving marble as the mouse.
3. To wash marbles adequately between testing rounds, an old pipette tip box with holes drilled for drainage can be used. Once the marbles have been cleaned and drained, the marbles should be spread out on an absorptive material to facilitate quick drying. Marbles must be thoroughly dried between tasks so that no bedding sticks to the surface of the marbles. Wet marbles will lead to an erroneously high number of marbles buried.
4. The handler should attempt to move their hand out of the testing apparatus as quickly as possible, as hand movement can disrupt automated software. The video clips should be analyzed starting after the handler's hand is outside the bounds of the apparatus on the tracking software.

9 Concluding Remarks

The marble burying task has rich roots in pharmacological testing and neurobehavioral models of anxiety, OCD, and ASD. However, multiple researchers have challenged the construct and predictive validity of the traditional format of the marble burying task and called for reform within the MBT [11–13, .21]. The traditional version of the MBT with the sole variable of marbles buried at the end of the task should no longer be reported in future studies as a metric of obsessive-compulsive, restricted-repetitive, and anxiety behaviors or anti-compulsive and anxiolytic effects of pharmacological

compounds, as its translational value is futile in this context. However, this traditional model still has validity as a screening method for new pharmacological compounds. If a difference in the number of marbles buried is observed on initial pharmacological screening or the MBT is being used in neurobehavioral models, video analysis should be used to characterize the effects on specific behaviors, including marble-directed digging, rearing, and exploration, in conjunction with other behavioral tests for a more thorough understanding of rodent behavior. By observing rodent behavior, it is possible to distinguish marble-directed and normal exploratory behaviors. This distinction will help contribute positively to the validity and translational value of this behavioral method. Future studies are needed to define strain variance and the effects of multiple test exposures to the arrays of behaviors observed within the modified MBT.

Competing Interests

The authors have no conflicts of interest to declare that are relevant to the content of this chapter.

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Chapter 6

Female Urine Sniffing Test to Study Natural Reward-Seeking Behavior in Rodents

Fernanda Daher and Manoela V. Fogaça

Abstract

Anhedonia, a prevalent manifestation in major depressive disorder (MDD), reflects the pathological reduction or absence of pleasure reactions and is frequently modeled in rodents to explore underlying neurobiological mechanisms. In these models, anhedonia can be measured as diminished motivation or responsiveness toward appetitive stimuli, providing a valuable window into cellular and circuit-level processes relevant to translational research. Here, we describe the female urine sniffing test (FUST), an ethologically based behavioral task to examine natural reward-seeking and positive valence responses in male mice or rats exposed to opposite-sex urine. Female urine, known to hold inherent motivational salience, is used to elicit investigatory behavior in male rodents, allowing researchers to evaluate the impact of chronic stress and the efficacy of rapid-acting antidepressants with distinct mechanisms of action. While FUST does not fully replicate the complexity of human anhedonia, it offers a simple, accessible, and low-cost approach to rapidly assess changes in positive valence and natural reward-seeking behavior. We aim to provide practical guidelines for implementing FUST and discuss its advantages, limitations, and potential for integration with modern circuit-level techniques to enhance its translational relevance.

Keywords Anhedonia, Odor, Urine, Reward-seeking behavior, Motivation, Ketamine, Scopolamine, *d*-Methadone, Stress

1 Introduction

"Markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day (as indicated by either subjective account or observation)." This is the definition of anhedonia, a core manifestation and diagnostic criterion for the depressive episode according to the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders, 5th edition, text revision [1]. Individuals with anhedonia may experience less interest in hobbies, social withdrawal, and a significant reduction in sex drive. Yet, the experience of at least one depressive episode characterizes major depressive disorder (MDD). The prevalence of depression is alarming: estimates in 2021 unveiled around 332 million cases of depressive

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disorders globally [2]; relevant literature reviewed between 2012 and 2015 indicated a high prevalence (38%–46%) of anhedonia documented in cross-national studies [3, 4]. With depression being one of the leading causes of societal burden worldwide, basic and preclinical research are essential to establish effective prevention, treatment, and the underlying causal biological pathways.

Unless laboratory testing or imaging is indicated by history or examination, diagnosis of depression is still made on the basis of behavioral malfunction [5]. Beyond behavioral assessments, rodent models can provide informative results to better understand the underlying mechanisms of MDD symptoms and evaluate potential therapeutics. In rodents, anhedonia-like behavior can be exemplified by reduced interest in and consumption of various natural rewards, including food, social interaction, sex, and exercise (e.g., voluntary wheel running) [6]. However, while rodent models have advanced our understanding of mood disorders, replicating the complexity of human emotional experiences and symptoms, such as anhedonia, remains a significant challenge. Human anhedonia encompasses nuanced cognitive and emotional processes, including diminished anticipation, motivation, effort, and subjective pleasure, which cannot be fully captured by simple behavioral readouts in mice. Behaviors like urine sniffing, sucrose consumption, or lever pressing, although informative, are only proxies for broader affective states and lack the richness of human subjective experience. Nevertheless, from an evolutionary perspective, mammals share the same basic sets of emotional systems, such as the reward-SEEKING system [7]. The term "SEEKING" (exploration/enthusiasm) refers to a positive affective subjective state that automatically informs animals they are on paths of survival and reproductive success. Indeed, reward-seeking systems (often referred to as brain reward networks) are evolutionarily conserved emotional circuits that underlie hedonic experiences (i.e., positive valence) and drive motivated behavior. These systems can generate hedonic responses in the absence of prior learning (unconditioned) while also integrating experience-dependent (conditioned) cues to guide reward pursuit, thereby supporting both innate and learned components of approach behavior and reinforcement [8]. Ultimately, the diminished activity of the human reward-seeking systems is thought to contribute to depression [7], and investigating the corresponding neural circuits in rodents can offer valuable insights into the mechanisms underlying these deficits and help inform the development of novel therapeutic strategies.

Seeking system-engendered emotions can be evaluated with rewarding effects of behavioral studies. Since Malkesman's original article was published, the female urine sniffing test (FUST) has utilized mice and rats as animal models to investigate natural reward-seeking behavior [9]. FUST is a modification of the olfactory habituation–dishabituation test [10], enabling experimenters

to quantify time spent sniffing a strong, rewarding odor. An aliquot of opposite-sex urine is a chemosensory cue generally attractive to rodents. The odorant located in the test cage elicits bouts of directed sniffing at the location of the odor by the test mouse [11]. With ethological relevance, sniffing of urinary molecules measures pheromone detection by the olfactory system of rodents in order to regulate endocrine status and signal social communication [12]. Female-derived urinary volatile and non-volatile molecules, such as isobutylamine and cortigynic acid, induce attraction and mounting behavior of a male breeding partner [13, 14]. In addition to eliciting innate reproductive responses, exposure to estrous female urine but not water significantly elevates levels of extracellular dopamine released in the mouse nucleus accumbens [9]. Pharmacological and optogenetic manipulations demonstrate that dopamine signaling in the nucleus accumbens is required for the control of pheromone-induced sexual preference of males for conspecific female mice [15].

Alongside other murine behavioral assays, the FUST holds advantages and limitations. It is a simple, accessible, and low-cost method. As with other olfactory tests, FUST is flexible for modifications: instead of using a cotton tip (see methods below), collected urine can be presented on a filter paper behind a mesh barrier, at one end of a three-chambered apparatus, or on a sponge [11]; bouts of male investigation of female urine can be scored manually or recorded with an optical beam-break detector [14]. Among paradigms suited to test reward-seeking behavior, FUST does not require mice to be food or water-restricted to drive behaviors, unlike operant tests involving natural rewards such as food and sucrose [6]. FUST does not rely on locomotor activity or learning/memory to assess spontaneous reward-seeking behavior [9], and can easily integrate into a battery of behavioral tests to provide multiple behavioral readouts. Additionally, FUST multiplexes with environmental, chemogenetic, optogenetic, and pharmacological manipulations that have been found to impair or facilitate reward-seeking behavior, such as chronic stress, designer receptors exclusively activated by designer drugs (DREADDs), channelrodopsins, and rapid-acting antidepressants [9, 16, 17]. What remains to be better explored is the use of FUST with female test mice, particularly after recent research demonstrating that the amygdala–accessory olfactory bulb circuit mediates attraction of female mice to male pheromone-containing urine [18]. Conversely, some limitations are associated with FUST. A major caveat is the use of a mouse strain with partial or total absence of the sense of smell (hyposmia and anosmia, respectively) or endocrine dysfunction. Because FUST is a rapid behavioral screen, it may not capture the range of behavioral domains affected by manipulations as well as the richness of a given domain. Comparing FUST outcomes with results from operant and effort-based tasks might help determine whether it uniquely assesses specific, ethologically relevant facets of anhedonia

or overlaps with broader reward deficits. Thus, more sophisticated paradigms in longitudinal studies should optimize translatability and better echo MDD as a heterogeneous entity [6].

Here, we describe FUST in all steps needed to elicit, record, and analyze seeking behavior for a natural reward in male rodents. This approach is particularly useful to rapidly identify factors that play a part in disturbed appetitive responses and to help therapeutic discovery.

2 Subjects and Materials

2.1 Animals

To investigate whether distinct classes of fast-acting antidepressants, including ketamine, *d*-methadone, and scopolamine, modulate reward-seeking behavior in rodents, the following animals are used:

- C57BL/6J mouse strain, either purchased from the Jackson Laboratory (common name: B6J, strain No.: 000664, Bar Harbor, Maine—USA) or obtained from in-house breeders.
- $M1^{f/+}Sst^{Cre-}$ littermates (wild-type, WT controls) of the conditional knockout $M1^{f/f}Sst^{Cre+}$ mouse strain. $M1^{f/+}Sst^{Cre-}$ mice are heterozygous for the floxed M1 allele (control) but do not carry the Cre-recombinase transgene, and therefore express subtype 1 of metabotropic acetylcholine muscarinic receptors (M1) normally in somatostatin (SST) interneurons [16].
- Sprague–Dawley rats were obtained from the Jackson Laboratory (New Haven, USA).

Test animals are male mice aged between 8 and 14 weeks and rats weighing 260–300 g at the onset of testing, thus after reaching sexual maturation. Female mice or rats from the same male strain are aged at least 8 or 9 weeks, respectively, at urine collection. All female rodents are sexually mature and naïve.

Male mice are housed in groups of three to five individuals per home cage (29 cm length × 18 cm width × 13 cm height). Male rats are single-housed 3–5 days prior to the drug treatments. All animals are housed in ventilated home cages within standard racks in the local animal facility, maintained on a 12/12 h light–dark cycle (lights: 6 AM to 6 PM), controlled temperature (22°C ± 1°C), with food and water *ad libitum*. FUST is conducted in a separate room between 11 AM and 3 PM.

All procedures were approved by the Institutional Animal Care and Use Committees (IACUC) and conducted per the guidelines for the care and use of laboratory animals of the National Institutes of Health (NIH).

2.2 Manipulation Studies: Chronic Unpredictable Stress and Rapid-Acting Antidepressants

To investigate the detrimental effects of a chronic stress paradigm during FUST, chronic unpredictable stress (CUS) is performed in male rodents for 21 days. Animals are exposed to a sequence of two random, unpredictable stressors per day as previously reported [19]. Diurnal stressors included: restraint (2 h), white noise (1 h), and cage shaking (30 min). Overnight stressors included: wet bedding, bedding removal, water deprivation, cage tilting, and peppermint odor. Inverted light–dark cycle and social isolation are stressors with a 24-h duration. Chronic unpredictable stress continues on the days between behavioral tests, but not on the days of treatments or prior to behavioral tests. Control animals (non-stressed) are not exposed to any stressors.

To explore the effects of rapid-acting antidepressants during FUST, separate cohorts of male animals are injected with either vehicle (saline) or a rapid-acting antidepressant:

- ketamine hydrochloride (Sigma-Aldrich, 10 mg/kg), a non-competitive antagonist of N-methyl-D-aspartate (NMDA) receptors, dissolved in 0.9% saline and administered a single time via intraperitoneal (IP) route;
- scopolamine (Sigma-Aldrich, 25 μg/kg), a non-selective muscarinic acetylcholine receptor antagonist, dissolved in 0.9% saline and administered every other day for 3 days via the IP route;
- *d*-methadone (Mallinckrodt, 20 mg/kg), an NMDA receptor antagonist and μ-opioid receptor agonist, dissolved in 0.9% saline and administered a single time via subcutaneous (SQ) route.

Doses are proven to induce antidepressant-like effects in mice or rats in basic, preclinical studies [16, 20–23]. Vehicle or drugs are injected at a volume of 10 ml/kg for mice and 1 ml/kg for rats. Because of the sustained effects of ketamine, scopolamine, or *d*-methadone on synaptic plasticity and behavior, behavioral testing usually starts at least 24 h after the single or last injection (Fig. 2).

2.3 Preparation of the Test Room

The test room should be maintained under dim lighting conditions, with an intensity of approximately 10 lux measured within the test cages. Light measurement can be done by using a digital light meter or mobile application. Experimental procedures may not be performed with female rodents in the same test room where male animals are subsequently tested. Importantly, the test room should be clean. Surfaces can be cleaned with 70% ethanol, and any source of odor should be removed from the test room to prevent confounding olfactory cues.

2.4 Equipment Setup

- Transparent standard home cages (mice: 29 cm length × 18 cm width × 13 cm height; rats: 41.2 cm length × 23.4 cm width × 30 cm height) with micro-barrier top;
- Clean bedding and new nesting material (two nestlets per test cage);
- Cotton-tip applicator sticks of 15.24 cm (cotton-tipped applicators);
- Wooden clothespin;
- Tape;
- Video camera for low light and a tripod;
- Single-channel pipette of 101–1000 μl;
- Pipette tip;
- 1.5 ml centrifuge tube;
- Stopwatch.

Each test cage consists of a standard home cage with bedding, nestlets, and a micro-barrier top. The cage should be transparent to enable clear observation and monitoring of the test rodent. Bedding and nestlets are included to replicate the familiar environment of a home cage. The micro-barrier top includes a filter paper into which we make a small hole to allow for the insertion of cotton-tipped applicators in the cage. To minimize distractions and standardize testing conditions, wire bar lids (or feeders), water bottles or water pouches, rodent chow, and enrichment devices should be removed from the test cage.

Test cages can be placed side by side on a shelf to allow for multiple testing sessions and simultaneous video recording. Four to five test cages can be organized per shelf. After mounting the video camera on the tripod, both the zoom function and tripod height are adjusted to capture rodent behavior across two shelves, covering eight to ten animals in total. The camera's field of view should cover the entire area containing test cages and animals while minimizing capture of surrounding space. After recording the first round of animals, the video camera and tripod are readjusted to capture rodent behavior from the last set of four to five cages on a single shelf.

For the female urine collection, females are gently restrained by scruffing (e.g., lifting the neck/back skin to hold them securely) and positioned over an empty home cage to allow for urination. After collecting a sufficient amount of urine from multiple females (8–12 animals), a single-channel pipette and pipette tip are used to aspirate urine from the cage floor and transfer it into a centrifuge tube for mixing.

3 Methods

After setting up the test room and equipment, the animals are transferred from the vivarium to the test room, where they remain undisturbed for at least 30 min before the onset of FUST (Fig. 1A). This acclimatization period is important to allow the animals to adjust to the room conditions. Importantly, disposable gloves are worn in every step of the experiment to avoid leaving olfactory cues. Gloves should be changed whenever necessary.

3.1 Habituation Phase

- Animals are placed in a freshly made individual test cage.
- A dry, sterile cotton-tipped applicator is inserted into each test cage through a small hole in the micro-barrier top (Fig. 1B).
- The cotton-tipped applicator is secured by a clothespin from the outside of the cage top. In turn, the clothespin is taped to the cage top to be steady (*see* **Note 1**).
- Animals spend 50 min to habituate to the test cage and the presence of the cotton-tipped applicator (*see* **Notes 2** and **4**).
- During this phase, the rodent (1) explores the test cage; (2) is introduced to the new cotton-tipped applicator by placing it into the test cage before the olfactory test to avoid neophobia.
- Subsequently, the cotton-tipped applicator is removed and disposed of.
- Animals remain in the individual test cages for 30 min.

3.2 Between-Phase Interval and Female Urine Collection

- During the interval between habituation and water sniffing phases, urine is collected from female conspecifics in the vivarium room (Fig. 1A).
- Each female rodent is grasped near the base of the tail and placed on the wire bar lid of a clean, empty home cage.
- Pulling back gently on the tail of the rodent causes it to pull forward on the wire bar lid. Next, the loose skin over the neck, shoulders, and back is scruffed for restriction.
- Caution must be used to avoid injuring the tail or toes of the animal.
- Restraint is an acute stressor to rodents; therefore, female animals may urinate spontaneously while being scruffed. If not, the animal can be lifted with its belly facing the cage floor, and gentle pressure can be applied to the lower abdomen to promote urination.
- Caution must be used not to injure the rodent.

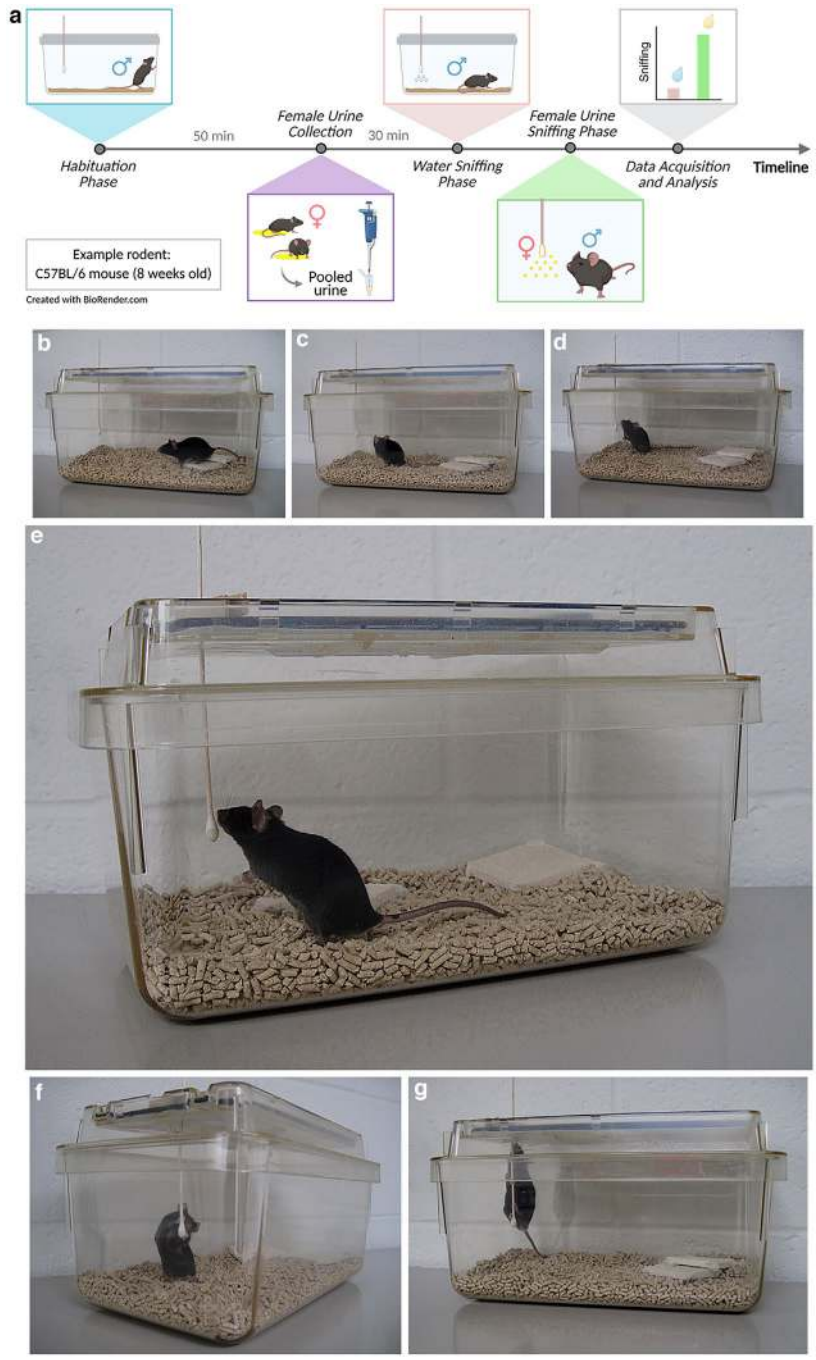


Fig. 1 Female urine-sniffing test. Experimental timeline (**a**) followed by a series of photos that sequentially illustrate the behavioral repertoire of a male C57BL/6 mouse upon the presentation of a cotton swab with female urine. The male mouse detects the urine-dipped swab inserted into the cage lid (**b**) and manifests motivation to approach (**c**) and (**d**) the source of female chemosignals. Next, the male mouse engages in extensive olfactory investigation (**e**) of the cotton swab dipped in female urine. At this moment, an observer with a stopwatch manually scores the time spent sniffing the cotton-tipped applicator. Notably, sniffing behavior accounts for the mouse's nose adjacent to and towards the cotton-tipped applicator, as depicted in (**e**). Examples of behaviors excluded from the analysis are time spent biting the applicator tip (**f**) and time spent climbing the applicator (**g**). (Photographs by the authors).

- With a single-channel pipette and pipette tip, urine drops and puddles are aspirated from the cage floor and wire bar lid, and the liquid is dispensed in a centrifuge tube.
- This procedure is repeated with multiple females housed in different groups, and their urine is pooled. The goal is to decrease random influences from the estrous cycle of any single group, as well as from male and female odors present in the room [12], and to increase the chances of collecting urine from different stages of the estrous cycle.
- Visual identification or cytology methods are not applied to verify stages of the rodent estrous cycle.
- Two cotton-tip applicator sticks are used for each male: one applicator for the water sniffing phase and another for the urine sniffing phase. Thus, two separate sets of cotton-tipped applicators are taken to the vivarium room.
- The first set of applicators is dipped in tap water and kept in a secure zipper-seal plastic bag. For the second set, a single drop of female urine is transferred from the centrifuge tube to the cotton tip of each applicator. Applicators dipped in urine are also kept in a separate zipper-seal plastic bag.

3.3 Water Sniffing Phase

- Thirty minutes after the habituation phase, the male rodents are exposed to the new cotton-tipped applicator dipped in tap water (Fig. 1A).
- The applicator is secured from the outside of the cage top as described in Sect. 3.1.
- Video camera is turned on immediately before inserting the applicators into the cages, and rodent behaviors are recorded for 5 min (*see* **Notes 3** and **4**).
- Applicator dipped in tap water is used as a control for disturbed phenotype in olfactory function.
- Finally, the water-soaked cotton-tipped applicator is removed and disposed of.

3.4 Female Urine Sniffing Phase

- Immediately after the water sniffing phase, a new cotton-tipped applicator with fresh female urine is inserted for an additional 5 min (Fig. 1A–E).
- The applicator is secured from the outside of the cage top as described in Sect. 3.1.
- Rodent behaviors are filmed as indicated in Sect. 3.3.
- At the end of this phase, the animal is returned to its home cage.

3.5 Timing in Steps (see Note 4)

Step 1, preparation of room and FUST equipment: 30 min.
Step 2, acclimation: at least 30 min.

Step 3, habituation phase: 50 min.
Step 4, between-phase interval, and female urine collection: 30 min.
Step 5, water sniffing phase: 5 min.
Step 6, female urine sniffing phase: 5 min.

3.6 Data Acquisition and Analysis

Adjustments in the display brightness of the PC or laptop may be necessary while watching the video material, as the dimly lit condition of the test room could impair accurate behavior scoring. Measurements of sniffing behavior during FUST should be conducted by trained experimenters (Fig. 1E; *see* **Note 5**). Latency to the first sniff and cumulative time spent sniffing the odorous applicator tip can be manually quantified with a stopwatch over 5 min by an observer blind to treatments. Latency for a male rodent to locate the cotton-tipped applicator with female urine is a measure of olfactory ability. Total time spent sniffing the attractive stimulus (female urine soaked into the cotton swab) indicates reward-seeking behavior, while the total time spent sniffing the neutral stimulus (water soaked into the cotton swab) is the control comparison. Importantly, exclusions from the analysis include time spent biting the applicator tip, cotton removal from the applicator tip, and time spent climbing the applicator (Fig. 1F–G).

Statistical analysis can be performed using SPSS statistics software (IBM, Version 30.0), and plotted graphs using GraphPad Prism (GraphPad Software, LLC, Version 10.5.0). All data sets are first tested for homogeneity of variances using Levene's test (*see* **Note 6**). In Sect. 4, sniffing duration is analyzed by two-way repeated measures ANOVA followed by the post-hoc Duncan when appropriate. Experimental groups (treatment or CUS) and odor condition (water- or urine-soaked applicator) serve as the between- and within-subject factors, respectively. Notably, in the rat experiment, since the non-stressed groups are not exposed to treatments, different groups within each condition (water or urine) were analyzed separately using one-way ANOVA followed by Duncan's test. Alternatively, nonparametric tests are applied whenever data sets are not assumed to follow a Gaussian bell-shaped distribution, namely Mann–Whitney *U* test (independent group comparison) and Wilcoxon matched-pairs test (odor condition comparison). The data are reported as mean ± SEM, and the statistical significance adopted is $P < 0.05$, two-tailed. Each experiment should be replicated at least two times.

4 Anticipated Results and Conclusions

Our group has tested different cohorts of male mice in two paradigms relevant to studying reward-seeking behavior in basic research. They were (1) 21 day chronic unpredictable stress (CUS) paradigm in $M1^{f/+}Sst^{Cre-}$ (WT) mice to impair reward-seeking behavior [16,

25]; (2) acute treatment of ketamine in naïve C57BL/6 mice or subchronic treatment of scopolamine in naïve $M1^{f/+}Sst^{Cre-}$ (WT) mice to facilitate this behavior [16, 26]. Test mice without experimental manipulations (negative controls) included: (1) non-stressed mice that remained group-housed and undisturbed in the vivarium, and (2) vehicle-injected mice that received saline. Of note, the olfactory stimuli—water and female urine—are presented sequentially rather than simultaneously due to the highly sensitive olfactory system of mice [9].

CUS is a chronic stress paradigm that replicates some of the circuit-level changes observed in MDD subjects and induces maladaptive behaviors, including anhedonia- and anxiogenic-like behaviors [25]. Notably, negative controls, such as the non-stressed group, will sniff more when presented with female urine on a cotton swab compared to a water stimulus, whereas chronically stressed mice typically do not display any preference. In accordance with this, our results show that CUS mice spent comparable time sniffing either water or female urine ($Z=-0.88$, $P=0.38$), whereas stress-naïve mice investigated urine significantly longer ($Z=-2.52$, $P=0.01$; Fig. 2A). During the female urine sniffing phase, CUS-exposed mice significantly spent less time sniffing female urine compared to the control mice ($U=10.00$, $Z=-2.89$, $P=0.003$; Fig. 2A), suggesting that the stress regimen impaired reward-seeking behavior in $M1^{f/+}Sst^{Cre-}$ (WT) mice. Conversely, a single injection of ketamine is shown to promote motivational behaviors and active coping in a rapid and sustained manner in stress-naïve mice, and to reverse the behavioral consequences of CUS [25, 26]. As shown in the experiment illustrated in Fig. 2B using non-stressed mice, both vehicle- and ketamine-treated groups sniffed more when presented with a female urine-dipped cotton swab compared to water (urine × water: $F_{\text{condition }1,19}=303.70$; $P<0.001$). In the female urine sniffing phase, male C57BL/6 mice injected with a single dose of ketamine (10 mg/kg) and tested 24 h later, sniffed female urine significantly longer than the vehicle-control group ($F_{\text{treatment }1,19}=8.67$; $P=0.008$; $F_{\text{interaction }1,19}=10.84$; $P=0.004$). Importantly, this effect is observed with different classes of rapid-acting antidepressants. In a separate experiment, mice were treated with either vehicle or scopolamine every other day for 5 days (three injections total, 25 μg/ml, IP). Both vehicle- and scopolamine-treated groups displayed higher sniffing duration toward female urine (vehicle: $Z=-2.31$, $P=0.02$; scopolamine: $Z=-3.06$, $P=0.002$), while the subchronic regimen of scopolamine increased sniffing time to female urine 24 h after the last injection ($U=32.50$, $Z=-2.28$, $P=0.02$; Fig. 2C). No effect was observed in group comparisons during the water sniffing phase of FUST across all three mouse experiments.

Rats are also commonly used in preclinical research to evaluate dopaminergic circuitry and natural reward-seeking behaviors.

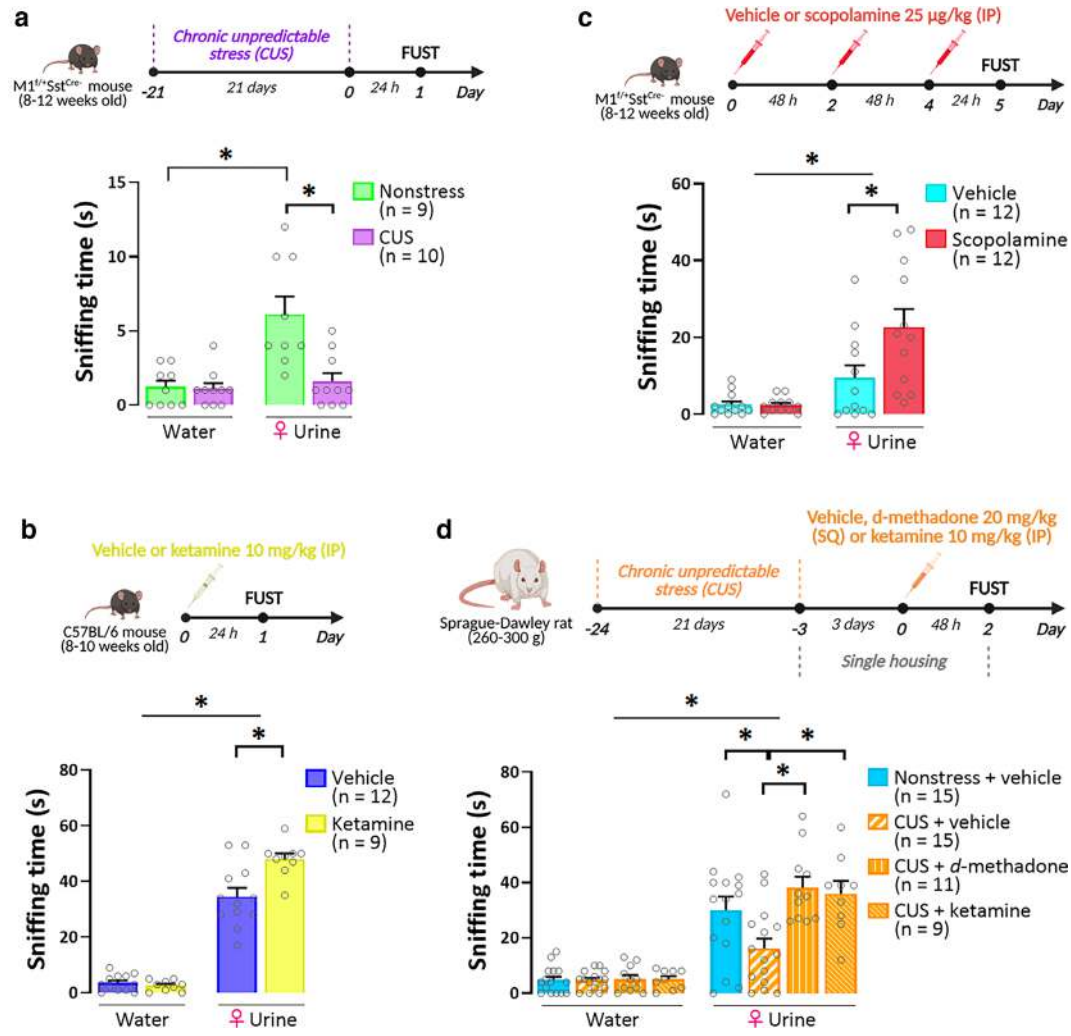


Fig. 2 Average time spent sniffing a cotton tip dipped in water (control) or female urine during FUST (**a**–**d**). Chronic unpredictable stress (CUS) throughout 21 days decreased the duration of sniffing the urine-dipped swab, while water sniffing remained unaffected in male mice (**a**) (modified from Fogaça et al. 2023). A single dose of ketamine at 10 mg/kg increased female urine sniffing time, while water sniffing remained unaffected in (**b**) (modified from Daher et al. 2024). Subchronic treatment with scopolamine at 0.25 µ g/kg increased female urine-sniffing time, while water sniffing remained unaffected in (**c**) (modified from Fogaça et al. 2023). In male Sprague–Dawley rats (**d**), 21 day CUS followed by single housing decreased sniffing time to female urine, whereas a single injection of d-methadone (20 mg/kg, SQ) or ketamine (10 mg/kg, IP) reversed this effect. Time spent sniffing a cotton tip dipped in water remained unchanged despite stress condition and drug dosing (modified from Fogaça et al. 2019). Data are plotted as mean ± SEM. *P < 0.05, Wilcoxon matched-pairs test and Mann–Whitney U test in (a and c), Two-way repeated measures ANOVA (**b**), or one-way ANOVA followed by Duncan test in (**d**). Created with BioRender.com.

We have demonstrated that FUST is a feasible behavioral method to assess female urine sniffing in rats. In the female urine sniffing phase, CUS rats injected with vehicle displayed lower sniffing

duration compared to non-stressed, vehicle-treated rats ($F_{3,46}=5.34$; $P=0.003$). This deficit was reversed by a single dose of *d*-methadone (20 mg/kg, SQ) or ketamine (10 mg/kg, IP) 48 h post-administration. All rat experimental groups were comparable during the water sniffing phase ($F_{3,46}=0.04$; $P=0.99$). Thus, our findings in mouse models were consistently replicated in rat experiments. Overall, the FUST is a responsive approach to stress exposure and/or rapid-acting antidepressants with distinct mechanisms of action, consistently revealing a facilitation of reward-seeking behavior following drug treatment.

We highlight that the results reported here concern C57BL/6, $M1^{f/+}Sst^{Cre-}$ (WT) mice, and Sprague–Dawley rats, which may differ from other strains and species. In fact, the average sniffing duration of female urine in control groups is comparable between experiments involving CUS and scopolamine alone, whereas both differ in magnitude from the control group in the ketamine experiment. One possibility for these differences is the distinct mouse strain tested. CUS and scopolamine experiments were performed with $M1^{f/+}Sst^{Cre-}$ (WT) littermates from a transgenic mouse line, which may display a lower baseline level of reward-seeking behavior during the FUST compared to the C57BL/6 mice in the ketamine experiment. Nevertheless, our findings demonstrate that the FUST is a reliable method for studying drug mechanisms and the effects of CUS in both standard and transgenic-derived mouse lines.

Among widely used reward-based assays, including the sucrose preference test and operant responding for food and other rewards, the FUST represents an effective and ethologically relevant tool for evaluating reward-seeking behavior in rodents, although specific circuits engaged during this task and its translational construct validity remain to be further elucidated. Measuring spontaneous sniffing responses of male mice or rats to chemosignals in female urine has proven sensitive to chronic stress paradigms and the effects of distinct fast-acting antidepressants. While it does not fully replicate the complexity of human anhedonia, the FUST stands out for being easy to implement, conceptually appealing, and requiring relatively minimal training for both rodents and experimenters.

5 Notes

1. During all phases of FUST, cotton-tipped applicators can be secured to the outside of the cage top using a clothespin, which may be additionally taped in place. Alternatively, cotton-tipped applicators can be secured inside the cage using tape if the rodent cage does not have a perforable micro-barrier filter top.
2. The subject's baseline state represents an independent variable affecting task performance, potentially confounding results if

not controlled. For testing a rodent strain with a heightened anxious-like response baseline, experimenters could consider increasing the time of the *Habituation Phase* and/or checking the experimental conditions that might exacerbate it, such as light intensity, noise, odors related to the room (e.g., rat odor while mice are being tested) or experimenter (e.g., cologne and perfume).

3. If the filming system fails during the task, experimenters should remove the cotton-tipped applicators from the test cages to avoid olfactory habituation. Having the video-recording system repaired, animals with skipped phases can be (re)tested at the end of the entire session or on the following day(s). Importantly, the *Female Urine Sniffing Phase* is subsequent to the *Water Sniffing Phase*. Cotton-tipped applicators should be replaced by fresh ones to avoid olfactory habituation and to be consistent with previous experiments/studies.
4. Variations of the FUST method with satisfactory results are described in the scientific literature. For instance, single-housed male mice are tested in their home cages, duration of the *Habituation Phase* is 30 min, and the *Water* and *Female Urine Sniffing Phases* are filmed and analyzed for 3 min each with a 45-min interval in between [27].
5. As an alternative to manual scoring of sniffing time and other measurements, deep-learning-based software packages such as DeepLabCut can assist researchers in quantifying animal behaviors by tracking multiple body parts of an animal across video frames [24].
6. Although standardized procedures are used to increase reproducibility, one cannot assume equivalent experiences during previous manipulations to FUST and/or inherent uniformity among subjects of the same species. Yet animals housed in the same cage are expected to influence each other on many important variables, including behavior. Therefore, FUST data can reflect the heterogeneity/variability of individual observations considerably. Experimenters may take into account effects at the subject level, adjustments in sample size when designing the experiment, and the use of measures of central tendency to describe the data set in the analysis.

Conflict of Interest

The authors have no conflicts of interest to declare that are relevant to the content of this chapter.

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Chapter 7

Acquisition and Extinction of Fear-Potentiated Startle in Rats

Ryan G. Parsons and Kehinde E. Cole

Abstract

The acoustic startle reflex can be potentiated by learned fear cues acquired through Pavlovian conditioning, commonly referred to as fear-potentiated startle (FPS). In a typical fear-potentiated startle experiment, rats are exposed to pairings of a cue (i.e., conditioned stimulus) that signals a foot shock (i.e., unconditioned stimulus) and later undergo testing in which startle stimuli are delivered in the presence or absence of the cue used to signal shock. Fear is operationally defined as the relative increase in the amplitude of the startle response in the presence of the cue that had signaled shock during conditioning versus startle responses in the absence of the cue. In this chapter, we provide detailed instructions on how to perform, analyze, and interpret the results of fear-potentiated startle experiments. We present detailed protocols for the study of both the acquisition of fear-potentiated startle and the extinction of fear-potentiated startle. We also discuss key considerations when it comes to experimental design and data analysis, and we offer a troubleshooting guide for commonly encountered problems.

Keywords Fear, Potentiated startle, Conditioning, Extinction, Protocol

1 Introduction

The acoustic startle reflex refers to a rapid contraction of facial and skeletal muscles in response to a loud, abrupt noise. This response is observed across many species and is thought to promote survival by helping organisms escape and avoid unexpected predatory attacks and to prepare them for future threats. Several features make the acoustic startle reflex particularly suitable for study in rodents. First, it can be reliably elicited by loud white noise bursts more than ~90 db [1]. Second, the amplitude of the startle response scales proportionally with stimulus intensity across a wide range of sound levels [2,3]. Third, the reflex occurs with a very short and highly stereotyped latency across animals [4]. Fourth, startle can be measured automatically by recording whole-body movements in specialized cages that convert cage movement into force. Finally, the essential neural circuits mediating the startle reflex have been largely identified [5], which

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has allowed for a detailed study of how circuits mediating emotional states interact with those mediating startle behavior.

The acoustic startle reflex can be modulated by innate and learned emotional states, such as anxiety and fear [6]. Fear potentiated startle (FPS) is one example of the emotional modulation of acoustic startle and refers to an increase in the amplitude of the acoustic startle reflex by cues that elicit fear because of their association with aversive stimuli during Pavlovian conditioning [7,8]. The potentiation of the acoustic startle reflex by conditioned fear cues in rodents was first reported by Brown, Kalish, and Farber [9], and has since been used extensively to study behavioral and neural mechanisms supporting Pavlovian fear conditioning [7,8]. Although the focus of this chapter is on the FPS paradigm, several other acoustic startle-based models have been developed in which certain biochemical manipulations or unlearned threat cues can potentiate the startle reflex [10–13]. Because startle behavior is evolutionally conserved across mammalian species, researchers have been able to adapt FPS and other models of the emotional modulation of startle to study various aspects of emotional behavior in humans and non-human primates [14–19]. Since these models engage emotional states that are dysregulated in individuals with fear- and anxiety-related psychopathologies [20,21], examining them across species represents a valuable approach for addressing translational research questions.

This chapter provides a detailed description of the methodology for performing and analyzing the acquisition and extinction of fear-potentiated startle in rats. We will also discuss issues related to experimental design and the interpretation of data obtained from FPS experiments and how to troubleshoot common problems.

2 Materials

2.1 Animals

Rats can be obtained from a commercial provider. The procedures described here are optimized for 2-month-old Sprague–Dawley rats obtained from Charles River Laboratories and have been used successfully with male and female animals in our laboratory [22,23]. Rats are housed in pairs and are left undisturbed for approximately 7 days following delivery from the vendor. The animals are housed under a 12:12 light–dark cycle with lights on at 7 AM and off at 7 PM, with all behavioral testing occurring during the light portion of the cycle. Food and water are provided ad libitum.

It is important to note that using mice requires a specific set of procedures, and there are sources which describe FPS protocols that have been used successfully [24].

2.2 Equipment

2.3 Acoustic Startle System

Commercial systems are available to conduct fear-potentiated startle from several different companies (e.g., Kinder Scientific, Med Associates, and San Diego Instruments). The protocol described here was developed using Kinder Scientific's StartleMonitor system. A standard setup might have two to eight individual boxes with components including a restrainer in which the rat is placed during startle testing, a shock chamber with a grid floor used during fear conditioning, and components for delivering noise, light, and shock. The restrainers sit atop load cells that convert the movement of the animal during and shortly after a startle trial into an electrical signal corresponding to force. These authors prefer using a system (Kinder Scientific) with load cell sensors given their superior accuracy and durability, although piezoelectric systems are used successfully in many laboratories. These restrainers and shock cages are housed in sound-attenuating chambers with exhaust fans to provide ventilation and background noise (~54 dB). Proper background noise is critical, as prior work has reported that the level of background noise can alter startle responses [25].

2.4 Additional Equipment

- Desktop computer (OptiPlex, Dell).
- Software capable of controlling the delivery of experimental stimuli (StartleMonitor, Kinder Scientific).
- Sound level meter (RadioShack, catalog number 33-2055).
- Light meter (VWR; catalog number 21800-014).

2.5 Calibration

The intensity of the startle stimulus should be calibrated with a sound level meter set to A weighting and fast response. The sensor of the meter should be in the center of the floor of the restrainer. The light stimulus is calibrated using a light meter with the sensor placed in the center of the sensing plate approximately 20 cm from the light source. The startle stimulus and the light should be calibrated at the start of each testing day. Prior to each experiment, the system must also be calibrated so that load cell output is scaled properly and set to convert to Force (N). Additional calibration of the mechanical and circuit offsets should be performed prior to each experiment. The details of these procedures can be found in the operator's manual. NOTE: Researchers using different startle systems will need to calibrate according to the manufacturers' recommendations.

3 Procedure

All procedures occur during the light portion of the light:dark cycle. Animals are handled for approximately 5 min per day for 7 days prior to behavioral testing. The first 3 days of handling occur in the colony room, during which the housing boxes are removed from the breeding racks and placed onto a utility cart. Each rat is gently removed from the box using the hand-under-method and held close to the experimenter's body. To acclimate the rats to being transported to the lab for behavioral testing, for the next 4 days rats are wheeled into the behavioral testing room and are handled for 5 min.

3.1 The Acquisition of Cued Fear-Potentiated Startle

3.2 Baseline Startle

Days 1 and 2: The purpose of this phase is to habituate the startle reflex prior to conditioning and, when possible, to match animals into experimental groups with equivalent average baseline startle responses. Rats are transported into the testing room in squads of four and are placed into the restrainers. After a 5-min stimulus-free period, they are exposed to 30 trials (30 s ISI) of a 50-ms, 95-dB (*see* **Note 2**) white noise burst. Rats are removed from the restrainers 30 s after the final startle trial. Between each session, the cages are cleaned with water and scented with a unique odorant (e.g., diluted ethanol, acetic acid, or ammonium hydroxide).

3.3 Fear Conditioning

Day 3: The purpose of this phase is for the rats to learn that a light cue (*see* **Notes 1 and 3**) predicts an aversive shock. Rats are placed into shock cages, and after a 5 min baseline period, ten pairings of a light CS (4 s; 82 lux) and co-terminating shock (UCS—0.5 s; 0.4 mA; *see* **Note 4**) are delivered with an intertrial interval of 4 min (*see* **Note 8**). Rats are removed from the shock cages 2 min after the final trial. Between each set of rats, the cages are cleaned and scented with an odorant different from that used during baseline startle.

3.4 Fear-Potentiated Startle Test

Day 4: The test phase for FPS is to determine the level of fear to the light cue that predicted shock during the conditioning phase. This test typically occurs 24 or 48 h after conditioning; however, longer intervals can be used as rats retain the memory over long periods of time. During this phase, rats are placed into the restrainers and are exposed to 30 trials (30 s ITI) of the startle stimulus after a 5-min baseline. An additional 40 startle trials (30 s ITI) are then delivered, with 10 of the startle trials occurring in the presence of the light CS. During the light-startle trials, the light CS is delivered for 4.0 s with the 50 ms startle stimulus delivered 3.5 s after the onset of the light. Each light-startle trial is followed by three startle-alone trials. Rats

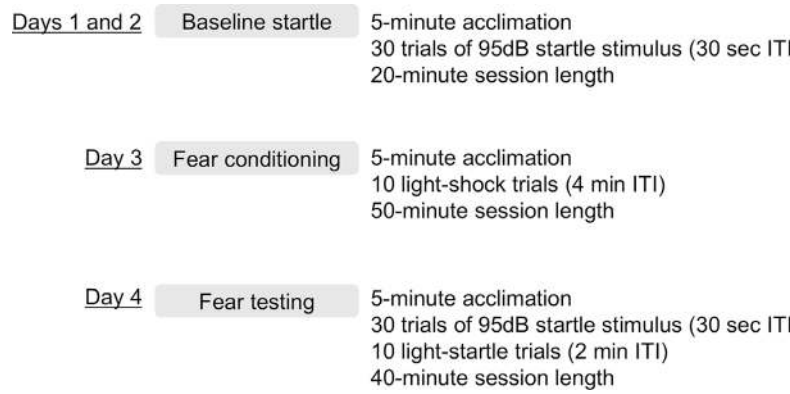


Fig. 1 Timeline of a fear-potentiated startle experiment

are removed from the restrainers after the final startle trial. Between each session, the cages are cleaned and scented with a novel scent. Figure 1 shows a summary of the basic behavioral protocol.

3.5 Data Analysis

3.6 Baseline Startle

The acoustic startle response is defined as the maximum peak amplitude during the 500 ms window following the presentation of the startle stimulus (*see* **Note 7**). The average startle response over the 30 startle trials is calculated for each rat for both days. Unless experimental manipulations have occurred prior to the behavioral testing, rats are assigned to groups so that the mean baseline startle amplitude is similar across all groups. When possible, the matching is performed so that each squad of animals has rats from each experimental condition so that order of testing and time of day are counterbalanced across groups. Group averages and standard error of the mean are then computed, and a *t*-test or analysis of variance is computed to confirm that the matching procedure was successful.

3.7 Fear Conditioning

The startle stimulus is not presented during the conditioning session, which precludes the measurement of FPS during the session. However, as a proxy for freezing behavior, fear of the context during the conditioning session can be observed indirectly by sampling the output of the load cell in the absence of any stimuli during the 5-min baseline and comparing it to the post-shock period. To analyze responses to the shock, load cell output during and shortly after the shock period can be captured and compared between groups [26]. This measurement can determine if any treatment performed prior to or during training affects sensory processing of the shock. Student's *t*-tests or analysis of variance are used to test differences between groups.

3.8 Fear-Potentiated Startle Test

The operational definition of FPS is the difference in startle amplitude during the CS from the startle amplitude in the absence of the CS. To calculate FPS, the average of the 10 light-startle trials and of the 30 startle-alone trials occurring between the light-startle trials is calculated for each rat. A difference score is then computed by subtracting the startle-alone trials from the light-startle trials, and this difference score is divided by the mean startle amplitude on startle-alone trials and multiplied by 100 to yield the percent fear-potentiated startle (*see* **Notes 5, 6, and 9**). In some circumstances it may be advantageous to calculate FPS relative to the leader trials (*see* **Note 6**). These values are tested statistically using a *t*-test or ANOVA. Table 1 depicts a sample data sheet showing the calculations for determining percentage FPS, while Fig. 2 portrays graphs of these data.

3.9 The Extinction of Fear Potentiated Startle

Fear extinction refers to the decrease in fear responses upon repeated presentations of the CS in the absence of the UCS. Extinction can refer to the within-session decrease in fear behavior while animals are presented with the CS alone during extinction training, and it can also refer to the retention of extinction learning when animals are presented with the CS at some interval after extinction training. Experimental studies of extinction often focus on the retention of extinction, which is operationally defined as the decrease in fear responses during extinction testing as compared to responses during extinction training. Because extinguished fear responses can re-emerge in some situations, extinction is thought to involve new learning rather than erasure or unlearning of the original association between the CS and UCS. The inability to extinguish fear is central to posttraumatic stress disorder, phobias, and anxiety disorders, and the standard treatment for these disorders, exposure therapy, is based on the principles of extinction learning [27–29]. Thus, there has been great interest in understanding extinction learning in the laboratory. Below we describe a basic extinction protocol using the fear-potentiated startle paradigm.

3.10 Baseline Startle and Fear Conditioning

Days 1–3: The baseline startle and fear conditioning sessions are identical to those described in the prior section.

3.11 Pre-Extinction Test

Day 4: The purpose of this phase is to test the level of FPS prior to extinction training. This test session is a shortened version of the "Fear-potentiated startle test" session described above, with three light-startle and three startle-alone trials, presented in a pseudorandom order. Fewer trials are presented so that this test does not itself produce extinction, and the test occurs in the chamber in which baseline startle was tested (*see* **Note 10**).

3.12 Extinction Training

Day 5: Rats are tested in startle chambers with several modifications to make the context unique. A floor consisting of coarse sandpaper

Table 1

Sample data sheet from a fear-potentiated startle experiment in eight male Sprague–Dawley rats

Measure	1	2	3	4	5	6	7	8	Mean	SEM
Baseline activity	0.148	0.107	0.297	0.284	0.19	0.371	0.153	0.185	0.217	0.033
Leaders	3.649	1.295	1.668	6.766	4.583	4.849	0.982	1.232	3.128	0.696
Startle alone	3.454	1.419	0.717	5.275	3.778	5.727	0.95	1.394	2.839	0.741
Light startle	4.935	3.746	2.258	6.811	4.878	8.883	1.853	3.574	4.617	0.997
Light-startle—startle alone	1.481	2.327	1.541	1.536	1.1	3.156	0.903	2.18	1.778	0.348
% potent	42.886	164.004	214.986	29.121	29.127	55.11	95.056	156.46	98.344	18.478
Startle alone—leaders	1.286	2.451	0.59	0.045	0.296	4.034	0.871	2.341	1.489	0.557
% potent (of leaders)	35.241	189.278	35.389	0.663	6.449	83.194	88.703	189.971	78.611	25.097

The numbered columns indicate individual rats and the calculation of group averages and standard error of the mean. The columns indicate data averaging within animals over trials or time and computations for determining the percentage of fear-potentiated startle

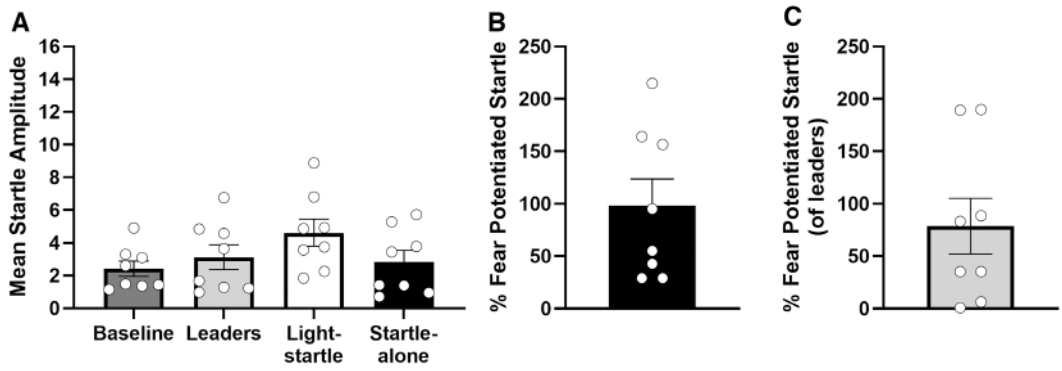


Fig. 2 Graphs from the data depicted in Table 1. (**A**) Mean startle amplitude during baseline startle, leader trials, light-startle trials, and during startle-alone trials between presentations of the light CS. The percentage of FPS during testing as computed relative to startle-alone (**B**) and leaders (**C**)

adhered to a plastic cutout is inserted; four Velcro strips are adhered to the walls of the chamber, and two ball chains are suspended from the ceiling of the chambers. During extinction training, rats receive 30 presentations of the light CS (30 s ITI) after a 5-min baseline period. These CS trials occur in the absence of shock and typically with no startle trials presented (*see* **Note 11**). Rats are removed 2 min after the final trial.

3.13 Post-extinction Test

Day 6: The purpose of this session is to test for the retention of extinction memory. Rats are returned to the chambers used during extinction training and given a test session like the pre-extinction test, but with 10 light-startle trials and 30 startle-alone trials. Rats are removed from the chambers 30 s following the final startle trial.

3.14 Optional Tests

Extinction learning is thought to involve new learning that competes with the memory for acquisition. This conclusion is based on observations that after extinction, responding to the CS can return in some circumstances. This includes the spontaneous recovery of fear due to the passage of time [30], the renewal of fear when the CS is presented outside the experimental context in which extinction occurred [31], and the reinstatement of fear to the CS following re-exposure to the UCS after extinction [32,33]. Each of these phenomena can be assessed using FPS, and the procedures are detailed below based on the procedures from a prior study [34].

Spontaneous recovery test: To test for spontaneous recovery of FPS, rats are given a post-extinction test 21 days after extinction training. The time point is chosen based on prior reports showing that spontaneous recovery of FPS has been observed when rats are tested 21 days after extinction as compared to rats tested 1 day after extinction learning.

Reinstatement: To assess reinstatement, rats are returned to the conditioning chambers and after 5 min they are exposed to five 0.4 mA foot shocks occurring with a 2-min intertrial interval. Rats are then removed from the chamber immediately after the final shock. The next day, rats should be administered another test session identical to the post-extinction test.

Renewal: To test for the renewal of fear after extinction, rats are given a post-extinction test with the same parameters as described above; however, the test session occurs in the same experimental context used during fear conditioning (*see* **Note 12**).

3.15 Data Analysis

Baseline startle and fear conditioning: The analyses for the baseline startle, fear conditioning, and shock reactivity data are identical to those described above.

Pre-extinction and post-extinction tests: The pre- and post-extinction test sessions are identical to the fear potentiated startle test described above and should be analyzed in the same manner (*see* **Note 13**). To analyze the retention of extinction, repeated measures ANOVA with test session as a within-subject factor can be used to determine if fear-potentiated startle differed from the pre- to post-extinction test, and between-subjects factors can be tested depending on the experimental details.

4 Notes

4.1 Fear Acquisition

1. When beginning FPS experiments in the lab, or when using a new stimulus to signal shock, it is advisable to test if the stimulus which will serve as the CS can alter startle responses by itself. This is because visual and especially auditory stimuli, which are commonly used as conditioned stimuli, can enhance startle if they are of sufficient intensity [13,25]. Generally, a one-off experiment testing if the to-be CS enhances startle when presented alone is sufficient. To do so, a test session can be given on a third day after the baseline startle sessions. This test session is typically identical to the fear potentiated startle test, except that fewer trials may be presented to prevent the latent inhibition if rats are to later undergo fear conditioning, e.g., [35].
2. Instead of a single intensity startle stimulus, many FPS experiments have been carried out with startle stimuli presented at different intensities, for example, at 95, 105, and 115 db. In this case, the baseline startle and FPS test sessions should be modified so that an equal number of each intensity of startle stimulus is presented, typically in a pseudorandom order. The advantage of using startle stimuli of multiple intensities is that

it accounts for variation in startle amplitudes across animals. For example, in rats with low-amplitude startle, presenting some higher-intensity startle trials can help avoid floor effects. Conversely, presenting some lower-intensity startle trials to rats with higher baseline startle responses can guard against ceiling effects.

3. Robust FPS has been observed with auditory, olfactory, and visual stimuli serving as conditioned stimuli [36–39]. Lights are used most often, although the intent and goals of the experiment should be considered. For example, if you are studying memory consolidation, conditioning the animals with the fewest trials necessary to support strong memory is generally advantageous so that the time window during which learning occurs is shorter. In this case, using olfactory cues to signal shock might be preferable, as studies have shown that FPS can be acquired with fewer trials when olfactory cues are used as CS's compared to other modality stimuli, e.g., [36]. Regardless of the sensory modality of the CS, the number of trials used during conditioning must also consider the expectation and goals of the experiment. For example, if testing a drug that is expected to disrupt learning, enough trials to produce robust learning in control animals should be delivered. Conversely, if testing a drug that is expected to enhance learning, using fewer trials is advantageous.
4. Many studies have reported that there is a non-monotonic relationship between the intensity of the shock used during conditioning and the expression of FPS [7,40,41]. Both low (e.g., 0.2 mA) and high (e.g., 1.6 mA) shock intensities result in low levels of FPS, while intermediate levels (e.g., 0.6 mA) support robust FPS [7]. The counterintuitive decrease in FPS with high-intensity shock is thought to occur because active escape behaviors are engaged, which interfere with expression of the startle response [42]. Thus, intermediate levels of shock should be employed during conditioning to yield strong FPS.
5. Fear-potentiated startle is typically measured by subtracting the amplitude of the startle-alone trials from the average of the CS-startle trials and expressing these values as a percentage. In many earlier studies, FPS was measured as the absolute difference between CS-startle and startle-alone trials [43,44]; however, it was subsequently reported that transforming the values into percent change scores is preferable, as this results in a more accurate measure of FPS across different levels of baseline startle [45].
6. There may be circumstances in which the percent FPS should be derived not from the percent change of CS-startle trials from startle-alone trials, but instead from the percent change relative

to the leader trials that preceded delivery of the CS-startle trials during testing. This is done when there is a significant increase in the startle responses during the startle alone trials after the CS has been presented, compared to the startle responses to the leader trials before the CS. In this case, a low percent FPS observed when calculating the percent change from the startle alone trials does not reflect low fear of the CS but instead might reflect residual fear that is maintained through the intertrial interval. Fear during the intertrial interval period during the light test session can be measured by subtracting the rat's average acoustic startle during the leaders from the startle trials during the ITI and expressing the difference as a percentage [46].

7. In some cases, there are small differences in precisely how the startle response is measured. With the equipment that we use in the laboratory, the startle response is quantified as the maximum positive amplitude during the 500 ms period after delivery of the startle stimulus. Alternatively, some systems quantify startle as maximum positive-to-maximum negative amplitude (i.e., peak-to-peak, peak-to-trough). In addition, the time window after the startle stimulus during which the startle response is measured can vary across systems, with values ranging from 200 to 500 ms being common.
8. The experiments performed in our lab have used a fixed 4 minute intertrial interval; however, many FPS experiments have used a variable intertrial interval with an average of 2- or 4 min. Using variable intervals theoretically reduces the possibility of temporal conditioning; however, to our knowledge, there have been comparative studies examining the effect of fixed versus variable intertrial intervals on FPS.
9. The percentage of FPS calculated relative to the noise-alone or leader trials usually falls within a range between 50% and 150% on average. The mean startle amplitude on the noise-alone test trials that occur in between CS presentations during testing is typically similar to the average startle amplitude during the leader trials. However, it sometimes is higher, perhaps because fear elicited by the light is partially maintained during the ITI. In this case, percent FPS using the noise-alone test trials may be lower than when using leaders. Either method is satisfactory if it is used across all comparison groups.

4.2 Fear Extinction

10. The pre-extinction test may alone produce some amount of extinction learning, although keeping the number of CS presentations low during the session helps guard against this outcome. Whether the pre-test produces extinction can be tested in pilot experiments in which FPS is assessed in rats that received the pre-test trials after conditioning are compared to

rats that did not. If there are no significant between-group differences during the test session, then it is likely that extinction learning did not occur.

11. In studies of the extinction of FPS, the extinction training session has most commonly been performed "off baseline" (i.e., in the absence of startle trials), which prevents the ability to measure FPS during the extinction training session. The reason for this is that there has been some indication that presenting the startle stimulus can alter extinction learning (personal communication, Mike Davis). However, to our knowledge, there has been no systematic test comparing extinction learning with and without the startle stimulus. While withholding the startle stimulus during extinction training is most common, there is at least one report published in which successful extinction occurred when the startle stimulus was delivered during extinction training [47].
12. The renewal of fear after extinction learning can be assessed as described above, where extinction training occurs in a novel context, and rats are tested to the extinguished CS in the context where conditioning occurred (i.e., ABA renewal). However, it can also be tested by conditioning and extinction occurring as described but with the renewal test occurring in a third novel context (i.e., ABC renewal), or it can be tested with conditioning and extinction occurring in the same context and the test occurring in a novel context (i.e., AAB renewal).
13. Levels of FPS during the pre-extinction test should range between 50 and 150 percent. For the post-extinction test, levels of FPS are commonly in the range of 0 and 80 percent [22,23,44,48]. The reemergence of fear responses after extinction is not often to the same level of responding prior to extinction [49,50]; therefore fear-potentiated startle values after spontaneous recovery, renewal, or reinstatement are most often lower than they were prior to extinction, but higher than they were at the end of extinction training or in comparison to animals that were not exposed to procedures that induce the recovery of fear after extinction.

5 Troubleshooting

The list below summarizes common problems encountered in fear-potentiated startle experiments, their potential causes, and suggested solutions. Many problems might occur because of faulty hardware or inadequate calibration, but this list assumes that the hardware is in working order and the setup was performed appropriately.

1. Problem: Low fear-potentiated startle (e.g., <25% potentiated startle).
   - Possible cause(s): Too low a shock intensity
   - Solution: Increase shock intensity

2. Problem: Low fear-potentiated startle (e.g., <20% potentiated startle).
   - Possible cause(s): Too high a shock intensity
   - Solution: Decrease shock intensity

3. Problem: Low fear-potentiated startle (e.g., <25% potentiated startle) relative to startle-alone trials and leader trials, but high-percent FPS relative to baseline startle.
   - Possible cause(s): Context fear obscuring FPS to the light.
   - Solution(s): Increase the discriminability of the context where testing occurs. Compute FPS relative to baseline startle.

4. Problem: Low fear-potentiated startle (e.g., <20% potentiated startle) relative to startle-alone trials, but high-percent FPS relative to baseline startle and leader trials.
   - Possible cause(s): Fear of the light maintained during the intertrial interval.
   - Solution: Lengthen the intertrial interval during testing. Compute FPS relative to baseline startle or relative to the leader trials.

5. Problem: Poor extinction learning.
   - Possible cause(s): Insufficient number of CS-alone trials during extinction training.
   - Solution: Increase the number of extinction trials. Add a second day of extinction training.

6. Problem: Poor extinction learning.
   - Possible cause(s): Extinction context is too similar to the conditioning context.
   - Solution: Alter more features of the contexts so that they are more easily distinguished.

6 Conclusion

In this chapter, we outlined protocols for both the acquisition and extinction of fear-potentiated startle. Fear-potentiated startle is a robust and reproducible model for studying the acquisition of learned fear, and it has been used to discover many of the neural mechanisms supporting fear learning. It has high face validity, as fear and anxiety in humans can be recognized in the form of an exaggerated startle response in the presence of threat. The model exhibits strong construct validity, supported by findings that pharmacological agents effective in reducing fear and anxiety in humans also reduce fear-potentiated startle in rats, and by the ability to dissociate FPS from general arousal by evaluating startle responses when fear is absent. Coupled with the fact that FPS can be examined across species, this makes it a powerful translational model for understanding fear reactions.

Conflict of Interest

The authors have no conflicts of interest to declare that are relevant to the content of this chapter.

Declarations All procedures need to be conducted with approval from the Institutional Animal Care and Use Committee and in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals.

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Chapter 8

The Rat Affective Bias Test and Reward Learning Assay

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Abstract

The affective bias test (ABT) and reward learning assay (RLA) were developed to provide translational methods to quantify affective biases in rats and are based on similar behavioral tasks used in human experimental medicine. Affective biases are neuropsychological processes through which emotional states modulate cognitive functions and behavioral responses. They have been found to influence decision-making, attention, and learning and memory. Negative affective biases are observed in patients with major depressive disorder and are directly modulated by antidepressant drugs. In rats, both the ABT and RLA are undertaken using naturalistic foraging and associative learning between a cue (a digging substrate) and a reward (food pellet). For the ABT, animals learn two cue-reward associations independently and under different treatment conditions, e.g., one in a negative affective state and one in a neutral state, with a subsequent choice test used to quantify the arising affective bias. The ABT can be used to quantify affective state-induced biases associated with new experiences, as well as generating a biased memory and then testing if interventions modify these if administered before the choice test. The RLA uses a similar method but with independent experiences of a high-value reward associated with one substrate and a low-value reward with the other. The animals remain in the same affective state throughout the test, and normal animals exhibit a reward-induced bias in the choice test, while animals in a putative depression-like state show impaired reward learning and a blunted reward-induced bias. Evidence from extensive pharmacological and psychosocial manipulations of affective state and studies in disease models suggests the ABT provides a translational method to study short-term changes in affective state, while the RLA is sensitive to core affective state and depression-related phenotypes.

Keywords Major depressive disorder, Neuropsychology, Psychopharmacology, Antidepressants, Rat

1 Introduction

Despite compelling evidence supporting the role of chronic stress in the development of depression-like states in rodents that can be quantified using traditional antidepressant screening assays, studies based on this combination of disease model and behavioral test have failed to translate to the clinic [1–9]. Chronic stress is not the only risk factor for MDD, and animal models of depression induced using other types of manipulation e.g., early life adversity (ELA) and chronic interferon-alpha treatment, do not generate reliable phenotypes

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in these tests [10–13]. This suggests these traditional tests may be relevant to stress-related mechanisms in rodents but with limitations in terms of translational validity [14].

In 2010, the NIMH developed the RDoC framework with the aim of breaking down traditional diagnostic boundaries and focusing on symptom domains. These domains may be transdiagnostic and are mapped onto different cognitive and affective behaviors and behavioral tasks which can be used in both human and non-human animals. In MDD, the primary domains of interest are positive and negative valences [15, 16]. Studies in MDD suggest patients and at-risk individuals exhibit negative affective biases which influence not only emotional behavior but also cognition [17–20]. Negative affective biases have been shown to influence attention, decision-making, learning and memory and motivation, and may contribute to the cause and perpetuation of MDD [17–20]. Direct modulation of affective biases is hypothesized to contribute to antidepressant efficacy. The neuropsychological hypothesis of antidepressant efficacy suggests the biochemical effects of conventional antidepressants interact with experience-dependent plasticity to generate more positively biased memories which build over time to generate a more positive mood state [21, 22]. Within this same model, rapid-acting antidepressants (RAADs) are thought to have more rapid and sustained effects due to their ability to modulate the affective biases associated with past experiences and enable their re-learning with a relatively more positive affective valence [22].

Although rodents cannot perform tasks directly related to the human emotional processing tasks used to quantify affective biases, these biases also influence learning and memory and decision-making behaviors, which can be adapted into tasks for rodents [23]. In 2004, Harding et al. [24] developed a judgment bias task (JBT) and showed that rats in a negative affective state were more likely to interpret an ambiguous cue as predicting a negative outcome. This was similar to findings in MDD patients performing comparable decision-making tasks [25–27]. Most studies using JBTs have addressed questions related to animal welfare [28], and in relation to MDD and antidepressant treatments, the data are mixed [29]. Acute pharmacological studies have found dissociable effects with conventional delayed-onset antidepressants vs. RAADs [30, 31]. Contrary to the findings in human decision-making tasks, acute doses of conventional antidepressants generally lacked specific effects; however, chronic treatment with fluoxetine or an acute low dose of the RAAD, ketamine induced more optimistic decision-making [31, 32]. Acute treatment with pro-dopaminergic drugs also induced more optimistic decision-making behaviors [32], suggesting effects may relate to changes in motivation and reward-related mechanisms. Studies using JBTs in animal models of depression are still limited, but more pessimistic choices have been observed in animals which

have experienced chronic environmental or social stress and ELA [33–35].

The affective bias test (ABT) was first developed as a task to predict antidepressant or pro-depressant effects with improved translational validity. Studies in healthy volunteers have consistently observed positive affective biases using emotional processing tasks following acute doses of different antidepressants [21, 36, 37]. These objective effects were dissociable from the subjective experience of mood and support a neuropsychological hypothesis of antidepressant efficacy [21, 22]. These findings suggest antidepressants induce acute effects on affective biases which can influence learning and memory and it is the interaction between these biological effects and experience-dependent plasticity that together generate efficacy [22, 38]. The ABT is an associative learning and memory task where specific, independent experiences (digging substrate + reward) are learnt under different affective states or drug treatments [39]. To quantify the arising affective bias, a choice test is used with animals presented with the two previously learnt cues. Using different pharmacological and psychosocial manipulations of affective state, the ABT demonstrates good predictive validity with positive or negative biases observed for drugs which are antidepressant or pro-depressant in humans, respectively [39–41]. Non-pharmacological manipulations of affective state (social play, restraint stress) also generate affective biases in directions consistent with predictions [40]. The effects of conventional antidepressants involve positive affective biases and new experiences, which may explain their delayed onset. In contrast, RAADs have been shown to attenuate negative affective biases associated with past experiences [38–40, 42]. Evidence from choice test data 24 h post-treatment with the RAADs ketamine and psilocybin suggests that both treatments facilitate the re-learning of affective biases associated with past experiences with a more positive affective valence [38]. The way conventional vs. RAADs interact with affective biases in the ABT could explain the differences in their onset of clinical effects. As well as the predictive validity of the ABT, evidence from mechanistic studies suggests similar neural circuits may modulate affective bias in humans and rats. FMRI studies in patients have shown increased activity in regions of the prefrontal cortex and amygdala and decreased activity in the nucleus accumbens [43]. Using targeted brain infusions (PFC) or excitotoxic lesions, similar brain areas have been shown to modulate affective biases in rats in the ABT [38, 42]. Further supporting the construct validity of the ABT is evidence that vulnerability to MDD involves negative affective biases [44, 45] and the early life adversity rat model of MDD exhibits increased sensitivity to corticosterone-induced negative affective biases [35].

The reward learning assay (RLA) and probabilistic reward task (PRT) were developed along the same principles as the ABT but

with a focus on a method to quantify reward learning impairments relevant to anhedonia in MDD. Most studies in animal models of depression use the SPT to quantify reward-related impairments [46]; however, studies in humans using a sweet taste test (STT) do not support this as a translational readout [47]. Patients with MDD did not show impairments in the STT, although studies in a healthy population of subjects with high or low anhedonia scores in the SHAPS (Snaith-Hamilton Anhedonia Scale) did find changes in this test [48]. Reward-related cognition, particularly learning and memory, has shown more consistent impairments in tasks such as probabilistic reward learning (PRT) and probabilistic reversal learning tasks (PRLT) [49–52]. Translational PRT and PRLT have been developed for rodents, with evidence of impairments observed in depression models [53, 54], although studies with antidepressants are still quite limited (for further details on these tasks, see [54–56]). The RLA does not have a direct equivalent in humans but may involve similar mechanisms to those modulating reward learning in the probabilistic reward task [52]. Like the ABT it is based on foraging and animal learning to associate different digging substrates with a food reward. In the RLA, each independent learning experience is paired with either a high- or low-value reward, with a choice test used to quantify a reward-induced bias. Animals remain in the same affective state throughout the learning sessions and memory test, and control animals develop a reward-induced bias. However, when animals are exposed to manipulations predicted to generate a depression-like phenotype, the reward-induced bias is blunted, suggesting an impairment in learning and memory for the cue-reward association [39]. Unlike studies in the FST and SPT, impairments in reward learning in the RLA have been observed in animal models of depression generated using early life adversity, chronic pro-inflammatory treatment, chronic pro-depressant drug treatment and chronic corticosterone and an animal model of chronic pain [57]. The animals used in these RLA studies were also tested in the SPT, and only the chronic corticosterone model also showed impairments in this test [10]. This confirms that reward-related impairments in the RLA and SPT do not involve the same mechanisms and changes in reward sensitivity may be particularly related to chronic stress manipulations.

2 Methods

2.1 Animals and Associated Husbandry

The following husbandry conditions are designed to ensure a stable positive affective state can be achieved, which is necessary for the successful running of the reward learning assay and affective bias test. For further guidance on improving laboratory rodent welfare, please visit the 3Hs Initiative website [58]. Before commencing

experiments, ensure that the correct ethical requirements have been met and approved by the relevant institutional/national animal ethical bodies.

Upon arrival, rats should be given at least 1 week to acclimatize to the animal unit and adjust to any changes in light cycle from the previous unit/facility. Given that rats are a social species [59], they should be housed in groups of at least two animals. Group size should not exceed four to prevent stress associated with overcrowding. As a minimum, the home cage should be enriched with bedding/nesting material (e.g., paper wool), an object that provides shelter (tunnel, red house) and an additional item to provide a tactile stimulus (e.g., rope, wooden block). Rats should be housed under a 12:12 reverse-lit lighting schedule, with all experimentation taking place in the active (dark) phase. Both training and experimentation phases require the rat to be under a mild food restriction. Rats should begin food restriction 3 days before onset of experimentation. Rats should be fed a fixed amount of food ~1 h post-testing. As a guidance, we provide ~18 g food per two male Lister Hooded rats. You may need to adjust the level of feeding depending on the strain, sex and age of the rat. There should be no excess pellets the following day, but rats should continue to maintain normal growth and be no less than ~90% of free-feeding weight. Animals managed at higher levels of food restriction may perform the task more quickly but are less likely to exhibit affective biases or reward-induced biases. Food restriction is helpful at the start of training to increase motivation but may not need to be maintained once animals are fully trained.

Before the onset of experimentation, rats should be habituated to handling (see 3Hs website for further details). A well-habituated rat should come to the front of the cage and be picked up easily by grasping around the shoulders. If pharmacological studies are planned, rats should additionally be habituated to our refined low-restraint method for intraperitoneal or subcutaneous injection. If the drug can be administered orally, the rat should be habituated to the consumption of a palatable solution [58, 60, 61].

If your experimental room is different to the animal holding room, the rat should be habituated to the method of transportation between rooms (e.g., travel bucket) to prevent overt change in affective state before the onset of testing.

Materials

The reward learning assay/affective bias test arena consists of a 40 cm × 40 cm × 20 cm arena with opaque Perspex sides to prevent distraction from external movement/objects. A lid can be used in initial training stages to prevent the rat from climbing out of the arena and further minimize distraction. The arena should be lined with a waterproof tray liner (LPS, UK), which allows for efficient changes between rats if the liner becomes dirty.

Three identical ceramic bowls (Ø10 cm) should be used to contain digging substrate. Digging substrate could be a range of different materials and textures. Example substrates are outlined in Table 1 and for more examples of substrates and combinations [39]. Ensure that the selected trio of substrates are sufficiently different from each other in texture but has a similar effort requirement for digging.

2.2 Bowl Digging and Discrimination Training

Initial Habituation

On the first day of habituation, rats are placed into the arena in their home cage pairs for 10 min. Two bowls are placed at the back of the arena with a 5-cm gap in between. The bowls should be empty except for reward pellets (45 mg, TestDiet UK). The number of fecal pellets and successful consumption of reward pellets should be noted. On the second day of habituation, the rats are placed into the arena alone under the same conditions. There should be no fecal pellets during this session, and rats should consume all the reward pellets. If these conditions are not met, the habituation session should be repeated the following day.

Shuttle Training

Following initial habituation, the rats should progress to shuttle training. In this session, two bowls are placed at the back of the arena as described above. A single reward pellet is placed in one bowl in either the 10 o'clock, 12 o'clock, or 2 o'clock position. The rat is placed near the bowls in the middle of the arena to prevent the formation of a side bias. Allow the rat to explore the bowls and consume the pellet for up to a maximum of 30 s. Once the rat has consumed the pellet, place a pellet in the left-hand corner of the arena and wait for the rat to find it. Once the rat has found this second pellet, gently remove the rat from the arena and hold them against your chest (or place it in a holding box). Put a new pellet in one of the three locations. Bait either the left or right bowl in a pseudorandom order. This should be repeated for 12 trials. If the rat does not find the pellet within 30 s, remove them from the arena and hold for 5 s before restarting the

Table 1
Example of the substrates routinely used

	Substrate "A"	Substrate "B"	Substrate "C"
Test 1	Cotton mix	Leather	Balloons
Test 2	Purple ribbon	Green raffia ribbon	Sparkly pompoms
Test 3	Dusters	Tissue paper balls	Yellow bath sponge
Test 4	Chubby wool	Shoe laces	Velcro
Test 5	Cellulose sponge	Corrugated paper	Perlite

trial. Completion of ten successful (non-omitted) trials is required for the rat to progress to the next stage of training.

Digging Training

In the first stage of digging training, complete the first trial as described above. In the second and third trials, place the pellet in the same location as before. This time, cover it with 1 cm of sawdust. Continue to leave the other bowl empty. Allow the rat to explore and find the pellet. If this does not occur within 30 s, record as an omission and remove the rat from the arena for 5 s. Repeat trials 2 + 3 until rats have successfully dug. Continue to place a pellet in the left-hand corner of the arena to promote shuttling behavior away from the bowls following a successful trial. From trial 4 onwards, continue to place the pellet in one of three locations, and make sure to randomize whether the pellet is placed in either the left or right bowl. This time, the rat is given a maximum of 15 s to successfully dig. If unsuccessful, this is recorded as an omission. Continue until 12 trials (excluding omissions) are completed (see **Note 1**).

The next day, run the first trial as described above. In the second trial, place the pellet in the same location as before and add 1 cm sawdust. This time, when the rat starts to dig, remove the other empty bowl. The rat may initially find this distracting, so allow extra time for the rat to reengage with the bowl and consume the pellet. Continue to place a pellet in the left-hand corner following a successful dig and collection of pellet. Allow a maximum of 30 s for a successful dig, and record as an omission if this limit is exceeded. In the third trial, repeat as before but this time add 2 cm of sawdust. This time, allow a maximum of 10 s before recording as an omission. Complete the remaining trials the same way. Continue until 12 trials (excluding omissions) are completed.

The next day, continue as described in session 2. However, you do not need to bait the left-hand corner of the arena. The rat should now reliably return to the corner of the arena following the completion of a digging trial. If not, additional trials with the corner baited may be necessary. Reduce the omission cut-off to 5 s. If the rat has three consecutive omissions, consider amending the latency cut-off. Do not progress the rat to the next stage of training until digging training is successfully completed.

Discrimination

In this final stage of training, the rat must successfully choose the baited substrate when presented with the option of two different substrates. Choose two different substrates ("A" and "B"). Choose whether "A" or "B" will be baited with one reward pellet. This is kept consistent throughout the session. Counterbalance this selection across your cohort of rats to ensure choice is not guided by a substrate bias. In each trial, make sure to position the baited substrate to the left or right in a pseudorandom order. In the first trial, allow the rat to explore both bowls. Do not remove the bowl once the rat has chosen. Do not use a cut-off of 30 s if the rat is exploring.

For the remaining trials, remove the unchosen bowl once the rat has made its choice by initiating digging in a bowl. Record the trial as "correct" (has chosen baited substrate), "incorrect" (has chosen non-baited substrate) or omission (has not made a choice by the 5 s cut-off time). Run until the rat has made six consecutive choices within a maximum of 20 trials. Once the rat has achieved this, it is considered trained and ready to undergo testing in the reward learning assay and affective bias test.

2.3 Reward Learning Assay

Following the successful completion of discrimination training, the rat can undergo the reward learning assay. This task tests whether the rat can successfully form a positive bias toward a high-value reward-paired substrate over a low-value reward-paired substrate. This assay can be used to ensure the task is working properly before proceeding onto the affective bias test. The task can also be used to detect pharmacological or phenotypic-mediated changes in core affective state, and also test whether acute drug administration has

Table 2
Overview of the effects in the reward learning assay in male rats

Reward Learning Assay: Control for general memory impairments		
Treatment at recall	**Dose**	**Outcome**
Ketamine	1.0 mg/kg	+[a]
	10.0 mg/kg	+[a]
	25.0 mg/kg	0[a]
Psilocybin COMP360	0.1 mg/kg	+[a]
	0.3 mg/kg	+[a]
	1.0 mg/kg	+[a]
Scopolamine	0.1 mg/kg	+[a]
Reward Learning Assay: The effects of chronic treatment on reward learning		
Treatment	**Dose**	**Outcome**
Chronic corticosterone	10.0 mg/kg	Reward learning deficit[b]
Chronic retinoic acid	10.0 mg/kg	Reward learning deficit[c]
Chronic IFN alpha	100.0 U/kg	Reward learning deficit[c]
Sub-chronic PCP	2.0 mg/kg	Reward learning deficit[d]
Chronic pain model (partial saphenous nerve injury)		Reward learning deficit[e]
Early life stress model		Reward learning deficit[f]

Animals when treated acutely prior to the choice test of RLA, showed two experimental outcomes: "+" reward-induced positive bias retained and "0" no reward-induced positive bias observed. In the effect of chronic treatments on reward learning, all models demonstrated significant reward learning deficits in the RLA [a–f][11, 35, 38, 41, 57, 67]

a non-specific effect on memory recall. See Table 2 for a summary of completed RLA manipulations.

The task consists of four pairing sessions which take place over four consecutive days. The fifth and final day is the choice test (Fig. 1A). In the first pairing session, the rat is presented with two bowls of substrate, similar to the setup in discrimination training described above, but with a different pair of substrates (see Fig. 1C). One substrate, substrate "A" is baited with either one (low value) or two (high value) reward pellets. This choice must be kept consistent across the session and should be counterbalanced across the cohort (see Table 3). The other bowl is filled with a different substrate but is not baited with a pellet; it is a "blank." As described in the discrimination training section, the rat must make six correct choices of the baited bowl within 20 trials to successfully complete the session. In the second pairing session, the rat is presented with a new substrate, substrate "B," which should be baited with the opposite number of pellets to substrate A (one or two). The other substrate is the "blank" used in the previous pairing session. Again, the rat must make six successful choices to complete the session. The third and fourth sessions are a repetition of the first and second pairing sessions. On the choice day, substrates "A" and "B" are presented at the same time, with no blank. This time, the rat must make a choice for 30 trials. To keep the rats engaged with the task, substrates "A" and "B" are randomly reinforced with a single reward pellet with a probability of one in three. This is to stop the rat from forming new associations within the session. A crushed pellet is mixed into A and B to stop the rat from sniffing out the pellet without making a decision based on previous association. The rat's latency to dig, substrate choice, direction of chosen bowl, and whether they found/ate a pellet is recorded.

Calculations

It is expected that a healthy, wild-type rat will form a positive bias toward the two-pellet paired substrate. To calculate the percentage bias relative to zero, the following calculation can be used: [(number of two-pellet paired substrate choices/30) × 100] − 50. A population-level substrate bias, pellet bias, and side bias can also be calculated. Example of the experimental data from RLA is illustrated in Fig. 1D.

3 Affective Bias Test

The ABT has been used to test a range of acute pharmacological or environmental manipulations on affective state-induced bias. Similar to the RLA, the ABT consists of four pairing sessions and a choice test. The ABT can be used to test the effect of a manipulation on new learning, or on its ability to modify an affective bias associated with past experiences (see Fig. 1B). For the new learning protocol,

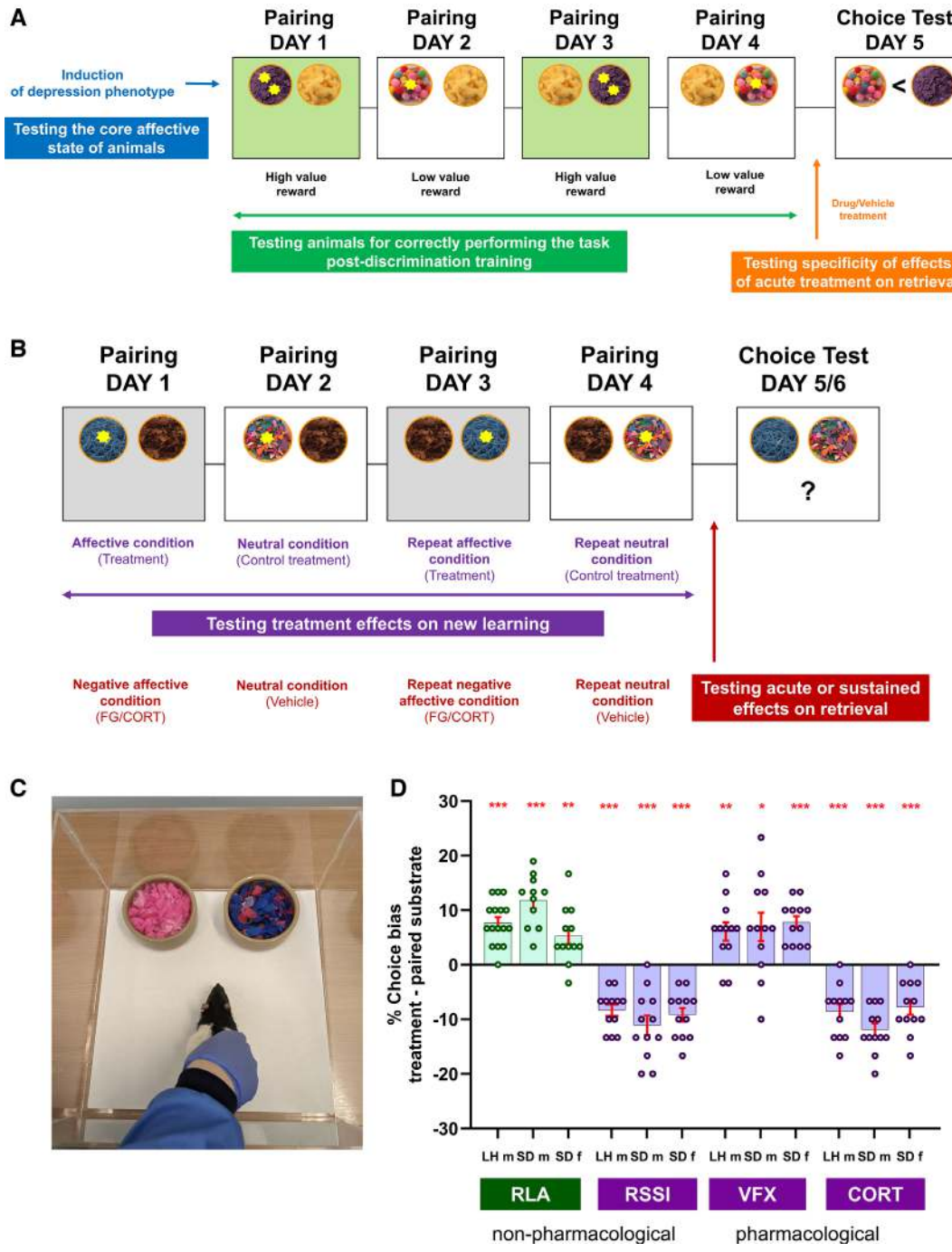


Fig. 1 Overview of the reward learning assay protocol to test if animals correctly perform the task post-discrimination training, to investigate the core affective state of animals, and to test for any acute, non-specific effects on memory following an acute drug treatment prior to the choice test (**A**). Overview of the affective bias test protocol to investigate the effects of positive or negative manipulations on new learning and acute or sustained effects of treatment on a negative affective bias (**B**). Photograph illustrates the ABT arena with bowls and rat position (**C**). Example of the data from the reward learning assay and the affective bias test (**D**). *LH: Lister Hooded, SD: Sprague Dawley, m: males, f: females, RLA: reward learning assay, RSSI: restraint stress and social isolation, VFX: venlafaxine, CORT: corticosterone, FG: FG7142 compound* (diagrams adapted from [38, 39])

Table 3
Example of standard experimental design testing in the reward learning assay

	Day 1	Day 2	Day 3	Day 4	Day 5
	Pairing 1	**Pairing 2**	**Pairing 3**	**Pairing 4**	**Choice test**
Group 1	A vs. C 1 pellet	B vs. C 2 pellets	A vs. C 1 pellet	B vs. C 2 pellets	A vs. B 30 trials
Group 2	B vs. C 1 pellet	A vs. C 2 pellets	B vs. C 1 pellet	A vs. C 2 pellets	A vs. B 30 trials
Group 3	A vs. C 2 pellets	B vs. C 1 pellet	A vs. C 2 pellets	B vs. C 1 pellet	A vs. B 30 trials
Group 4	B vs. C 2 pellets	A vs. C 1 pellet	B vs. C 2 pellets	A vs. C 1 pellet	A vs. B 30 trials

treatments (pharmacological, environmental or psychosocial) can be administered before or after the pairing session. To investigate effects on biased memories, an affective bias is first induced using a prototypical manipulation and then treatments are administered before the choice test. Please see Tables 4 and 5 for a summary of previously used manipulations.

3.1 New Learning

On the first pairing session, the rat is presented with a new substrate "A" and a new blank substrate. Substrate "A" is baited with one reward pellet. The rat is treated with a compound of interest or vehicle preceding the pairing session, taking into consideration the pharmacokinetics of the drug to determine the appropriate pretreatment time. The rat must successfully complete the criteria as previously described. On the second session, the rat is presented with a new substrate B and the same blank substrate. The reward value of both substrates "A" and "B" should be kept consistent across the sessions, and therefore substrate "B" is also baited with one reward pellet. This time, the rat is administered the opposite treatment choice to the preceding session (compound of interest or vehicle). Note, the pairing of substrate "A" and "B" with the manipulation of interest or vehicle should be counterbalanced across the cohort of rats (see Fig. 1B and Table 6). The third and fourth pairing sessions are a repetition of the first and second pairing sessions. The choice test is performed as described above in the RLA. This time, a bias toward or away from the compound of interest/environmental manipulation relative to 0 can be calculated. An example of the experimental data from ABT is illustrated in Fig. 1D.

3.2 Modulation of Negatively Biased Memories

In this version of the ABT, a compound known to induce a negative affective bias, such as FG1742 or corticosterone, is used as to generate a biased memory as described in the *new learning* protocol above. A compound of interest can then be administered acutely

Table 4

Overview of acute pharmacological and non-pharmacological manipulations in the ABT in male rats

Affective bias test: New learning studies					
Treatment	**Dose**	**Outcome**	**Treatment**	**Dose**	**Outcome**
Agomelatine	0.1 mg/kg	–[a]	Diazepam	0.3 mg/kg	0[a]
	0.3 mg/kg	–[a]		1.0 mg/kg	0[a]
	1.0 mg/kg	+[a]		3.0 mg/kg	0[a]
Amphetamine	0.3 mg/kg	0[a]	Duloxetine	1.0 mg/kg	+[b]
Aprepitant	0.1 mg/kg	0[a]		3.0 mg/kg	+[b]
	0.3 mg/kg	0[a]		10.0 mg/kg	+[b]
	1.0 mg/kg	0[a]	Ethanol	800.0 mg/kg	0[a]
	10.0 mg/kg	0[a]	FG7142	1.0 mg/kg	0[a]
	30.0 mg/kg	0[a]		3.0 mg/kg	–[a,d]
Bisphenol A	0.05 mg/kg	+[e]		5.0 mg/kg	–[a]
	0.5 mg/kg	+[e]		6.0 mg/kg	–[d]
Carbamazepine	3.0 mg/kg	0[c]	Fluoxetine	0.3 mg/kg	+[a]
	10.0 mg/kg	0[c]		1.0 mg/kg	+[a]
	30.0 mg/kg	0[c]		3.0 mg/kg	+[a]
Carbetocin	0 mg/kg	+[e]	Flutamide	1.0 mg/kg	0[e]
Citalopram	0.1 mg/kg	0[a]		10.0 mg/kg	+[e]
	0.3 mg/kg	0[a]	Formestane	1.0 mg/kg	–[e]
	1.0 mg/kg	+[a]		10.0 mg/kg	–[e]
	3.0 mg/kg	0[a]	Idazoxan	1.0 mg/kg	0[b]
Clomipramine	1.0 mg/kg	+[a]		3.0 mg/kg	+[b]

(continued)

Table 4 (continued)

Affective bias test: New learning studies					
Treatment	**Dose**	**Outcome**	**Treatment**	**Dose**	**Outcome**
Cocaine	3.0 mg/kg	0[a]		10.0 mg/kg	0[b]
Corticosterone	0.1 mg/kg	0[d]	Interferon alpha	10.0 U/kg	0[c]
	1.0 mg/kg	0[d]		100.0 U/kg	−[c]
	10.0 mg/kg	−[b,c]	Ketamine	1.0 mg/kg	0[i]
	30.0 mg/kg	−[c]		3.0 mg/kg	0[i]
Desmopressin	0 mg/kg	+[e]		10.0 mg/kg	0[i]
LPS	0.01 mg/kg	0[c]	Sertaline	1.0 mg/kg	+[d]
	0.1 mg/kg	0[c]		3.0 mg/kg	+[d]
	1.0 mg/kg	−[c]		10.0 mg/kg	+[d]
	10.0 mg/kg	−[c]	Testosterone	1.0 mg/kg	−[e]
Mirtazapine	0.3 mg/kg	+[a]		10.0 mg/kg	−[e]
Montelukast	1.0 mg/kg	0[c]	Tetrabenazine	0.1 mg/kg	0[c]
	3.0 mg/kg	0[c]		0.3 mg/kg	0[c]
Morphine	5.0 mg/kg	0[a]		1.0 mg/kg	−[c]
Nicotine	0.06 mg/kg	0[a]	Varenicline	0.03 mg/kg	0[c]
Oestradiol	1.0 mg/kg	+[e]		0.1 mg/kg	0[c]
	10.0 mg/kg	+[e]		0.3 mg/kg	0[c]
Progesterone	1.0 mg/kg	−[e]		1.0 mg/kg	0[c]
	10.0 mg/kg	0[e]	Venlafaxine	1.0 mg/kg	+[a]
Psilocybin COMP360	0.1 mg/kg	+[i]		3.0 mg/kg	+[a,d]

(continued)

Table 4 (continued)

Affective bias test: New learning studies					
Treatment	**Dose**	**Outcome**	**Treatment**	**Dose**	**Outcome**
	0.3 mg/kg	+[i]		10.0 mg/kg	+[a,d]
	1.0 mg/kg	−[i]	Vortioxetine	1.0 mg/kg	0[b]
Reboxetine	0.1 mg/kg	0[a]		3.0 mg/kg	+[b]
	0.3 mg/kg	+[a]		10.0 mg/kg	+[b]
	1.0 mg/kg	+[a]			
Retinoic acid	1.0 mg/kg	0[a]	**Manipulation**		**Outcome**
	3.0 mg/kg	−[a]	High-value reward		+[a,d]
	10.0 mg/kg	−[a]	Formalin injection in hind paw (acute pain model)		−[h]
Rimonabant	1.0 mg/kg	0[a]	Hot plate (acute pain model)		0[h]
	3.0 mg/kg	+[a]	Playpen		+[g]
	10.0 mg/kg	+[a]	Restraint stress and social isolation		−[a,d]
Scopolamine	0.03 mg/kg	0[i]	Social play		+[a,d]
	0.1 mg/kg	0[i]	Tickling		+[f]

Effect: positive bias "'+," no effect "0," negative bias "−" [a–i] [38, 40, 41, 68–72]

Table 5
Overview of acute and sustained modulation of a negative affective bias in the ABT in male rats

Affective bias test: Acute modulation of a negative affective bias				
Treatment to induce a negative affect	**Dose**	**Treatment at recall**	**Dose**	**Outcome**
FG7142	5.0 mg/kg	Bupivicaine (infusion)	0.75% w/v	No effect[a]
FG7142	5.0 mg/kg	Corticosterone (systemic)	10.0 mg/kg	No effect[b]
Corticosterone	10.0 mg/kg	DOI (mPFC infusion)	1.0 μg/μl	Attenuation of a negative bias[c]
FG7142	5.0 mg/kg	Ketamine (infusion)	1.0 μg/μl	Attenuation of a negative bias[a]
FG7142	3.0 mg/kg	Ketamine (systemic)	1.0 mg/kg	Attenuation of a negative bias[a]
FG7142	3.0 mg/kg	Ketamine (systemic)	10.0 mg/kg	Attenuation of a negative bias (nonspecific effects observed)[c]
FG7142	3.0 mg/kg	Ketamine (systemic)	25.0 mg/kg	Attenuation of a negative bias (nonspecific effects observed)[c]
FG7142	5.0 mg/kg	Muscimol (infusion)	0.1 μg/μl	Attenuation of a negative bias[a]
FG7142	3.0 mg/kg	Psilocybin COMP360 (systemic)	0.1 mg/kg	Attenuation of a negative bias[c]
FG7142	3.0 mg/kg	Psilocybin COMP360 (systemic)	0.3 mg/kg	Attenuation of a negative bias[c]
FG7142	3.0 mg/kg	Psilocybin COMP360 (systemic)	1.0 mg/kg	No effect[c]
Corticosterone	10.0 mg/kg	Scopolamine (systemic)	0.1 mg/kg	Attenuation of a negative bias[c]
FG7142	3.0 mg/kg	Venlafaxine (systemic)	3.0 mg/kg	No effect[a]
Affective bias test: Sustained modulation of a negative affective bias				
Treatment to induce a negative affect	**Dose**	**Treatment at recall**	**Dose**	**Outcome**
Corticosterone	10.0 mg/kg	Ketamine (infusion)	1.0 μg/μl	Inversion to a positive bias[c]
Corticosterone	10.0 mg/kg	Ketamine (systemic)	1.0 mg/kg	Inversion to a positive bias[c]

Table 5 (continued)

Affective bias test: Acute modulation of a negative affective bias				
Treatment to induce a negative affect	**Dose**	**Treatment at recall**	**Dose**	**Outcome**
FG7142	3.0 mg/kg	Ketamine (systemic)	10.0 mg/kg	Attenuation of a negative bias[c]
FG7142	3.0 mg/kg	Ketamine (systemic)	25.0 mg/kg	Attenuation of a negative bias[c]
FG7142	3.0 mg/kg	Psilocybin COMP360 (systemic)	0.1 mg/kg	Inversion to a positive bias[c]
FG7142	3.0 mg/kg	Psilocybin COMP360 (systemic)	0.3 mg/kg	Inversion to a positive bias[c]
FG7142	3.0 mg/kg	Psilocybin COMP360 (systemic)	1.0 mg/kg	Attenuation of a negative bias[c]
Corticosterone	10.0 mg/kg	Scopolamine (systemic)	0.1 mg/kg	Attenuation of a negative bias[c]

After induction of a negative affective bias with FG7142 or corticosterone, male rats are treated with a different compound, either 1 h (acute effects) or 24 h (sustained effects) prior to the choice test [a–c][11, 38, 42]

Table 6
Example of standard experimental design for testing manipulation-induced affective bias vs. control manipulation

	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
	Pairing 1	**Pairing 2**	**Pairing 3**	**Pairing 4**	**Choice test**
Group 1	A vs. C Vehicle or control	B vs. C Drug or manipulation	A vs. C Vehicle or control	B vs. C Drug or manipulation	A vs. B 30 trials
Group 2	B vs. C Vehicle or control	A vs. C Drug or manipulation	B vs. C Vehicle or control	A vs. C Drug or manipulation	A vs. B 30 trials
Group 3	A vs. C Drug or manipulation	B vs. C Vehicle or control	A vs. C Drug or manipulation	B vs. C Vehicle or control	A vs. B 30 trials
Group 4	B vs. C Drug or manipulation	A vs. C Vehicle or control	B vs. C Drug or manipulation	A vs. C Vehicle or control	A vs. B 30 trials

before the choice test or alternatively 24 h preceding the choice test to test sustained effects of the compound of interest. Its ability to modulate the induced negative bias is then determined statistically. Note, the use of a 24 -h pretreatment time requires the protocol to be extended to 6 days.

4 Conclusions

The ABT and RLA provide methods to quantify affective biases and reward learning impairments in rats, respectively. The results from studies to date suggest that these behavioral tasks offer improved translational validity when compared with conventional tests used to quantify depression-related behaviors in rats. The results from the ABT using pharmacological manipulations have achieved good alignment with findings in healthy volunteers performing emotional processing tasks following acute pharmacological manipulations [62–65], suggesting predictive validity. The targeted brain manipulation experiments also suggest construct validity and the potential to use the ABT to further elucidate the mechanisms underlying antidepressant-mediated affective bias modification. The breadth of treatments showing equivalent effects in human studies and the ABT suggests this method is not limited to certain classes of drugs and may be more appropriate than the FST for studying novel drug targets. Interestingly, the doses of antidepressants found to be effective in the ABT are similar to those used clinically and ~10 times lower than generally tested in the FST and SPT. As the ABT depends on an individual animal learning two different cue-reward associations under different conditions and a subsequent choice test to quantify the arising affective bias, it requires a within-subject design, limiting how it can be used in disease models. Studies in the early life adversity model compared the induction of a negative affective bias in response to an acute dose of corticosterone using a mixed study design with disease model as the between-subject factor and acute corticosterone as the within-subject factor. Using this design, it was possible to observe increased sensitivity to the corticosterone-induced negative bias in the early life adversity animals, consistent with increased vulnerability to negative emotional processing [35]. These early-life-adversity animals also exhibit blunted reward learning in the RLA, and studies using models associated with different risk factors for MDD suggest this is a consistent finding in rat models of depression and a more translational method to study etiological mechanisms.

Although the ABT and RLA have shown good translational validity in terms of the studies to date, both rely on manual foraging methods, which can be challenging to perform and achieve reliable and reproducible results. By their nature, the tasks are sensitive to affective state changes in the animals and stress associated with handling, and

they also require close interactions between the animal and handler when performing the tasks. They are also sensitive to changes in the animal's environment and stress associated with routine husbandry, so they require all those involved in managing the animals to be aware of the potential impacts of stress. Including positive control within the study design can be useful when investigating novel compounds in the ABT, and a control population should always be included in the RLA to ensure the predicted reward-induced positive bias is observed and the animals are performing the task correctly. Measures of trials to criterion, latencies and omissions are also recorded and used to check for any non-specific effects associated with either acute treatment in the ABT or phenotypic changes in the RLA. The RLA is also a useful control for studies investigating modulation of previously learnt biases in the ABT where attenuation of an affective bias would look similar for either a specific effect or when caused by amnesic effects. This control is performed in normal animals with a reward-induced bias generated and the treatment administered before the choice test. Where effects are specific to affective state-induced biases, no changes in reward-induced biases should be observed.

Overall, the ABT and RLA offer advantages over conventional tests for depression-related behaviors in terms of translational validity but also have their limitations. They are challenging tasks to run due to using foraging-based methods that require close interactions with the handler and are easily influenced by stress. The tasks have also only been reliably used in rat models. To date, attempts to automate the methods using equipment such as touchscreen or lever responses in traditional operant chambers have not been successful; however, we have been able to develop a semi-automated method to use in mice to run a reward bias assay similar to the bowl-digging reward learning assay [66]. The effect size observed for the ABT is ~1 [41] and similar to that seen in human experimental medicine studies, but this is smaller than would be expected for an FST experiment, meaning studies require larger sample sizes and may also be more susceptible to variation due to external factors, including stress. While this may limit the utility of the methods in terms of screening novel antidepressants, for mechanistic studies and understanding aetiological mechanisms, translational validity is critical.

5 Note

Some challenges may arise during the training and running of these assays that can be mitigated. Animals may not dig for rewards or reliably return to the handler after each trial. This may be because animals have not been habituated enough and are showing a stress response. To mitigate this, ensure that you do additional handling sessions to increase familiarity with the experimenter and experimental equipment. Ensure that the animals have enough time to

find the reward pellet in the corner of the arena. Continue this until the behavior is well established. Animals may develop a substrate or direction bias during the choice test, which confounds interpretation of choice test data. In this instance, repeat with a new set of substrates. You may use the example combinations detailed in Table 1. If the animal shows a direction bias, then the task may not have been learned correctly. Go back to the preceding training step to ensure that animals can discriminate effectively. Low performance during training or pairing sessions may be driven by low motivation to dig. Mildly increasing food restriction can increase motivation. Animals may consistently obtain over ten rewards in a choice test, suggesting that they are making decisions based on olfactory cues. To mitigate this, consider reducing the latency cutoff to speed up decision-making and reduce the chance of the rodent detecting the reward pellet via scent.

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Chapter 9

Modeling Innate Defensive Responses Using Looming Visual Stimuli in Mice

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Abstract

This chapter describes how looming visual stimuli can be used to study innate defensive behavior. Looming cues, such as a rapidly expanding overhead shadow, are evolutionarily conserved signals of approaching danger that can trigger instinctive avoidance responses across species, including humans. In rodents, overhead threat-like visual signals typically elicit either freezing or rapid escape to a shelter, and the response that occurs depends on stimulus features, perceived threat urgency, and environmental context. The chapter also contrasts looming with laterally moving "sweeping" stimuli, emphasizing that these different visual patterns bias distinct defensive strategies and reflect threat-dependent action selection rather than simple sensory reactivity. The chapter summarizes key circuit mechanisms proposed to support these behaviors, highlighting superior colliculus (SC)-centered pathways and downstream midbrain and brainstem networks that can drive freezing and escape. It outlines evidence for parallel SC output channels, including projections involving the parabigeminal nucleus (PBGN) and thalamic routes linked to the amygdala, as well as dorsal periaqueductal gray (PAG) circuits implicated in escape control. Finally, the chapter presents an example experimental workflow for implementing the looming assay and quantifying behavior, combining a controlled stimulus presentation setup with automated, deep-learning-based pose estimation (DeepLabCut) and data-driven behavioral parsing (Keypoint-MoSeq). This analysis framework enables the extraction of kinematic measures aligned with stimulus onset, the classification of flight vs. freezing responses, and identification of behavioral motifs that are expressed more frequently after stimulus onset.

Keywords Looming stimulus, Defensive behavior, Escape, Freezing, DeepLabCut, Keypoint-MoSeq, Animal model

1 Introduction

Animals share conserved neural circuits for detecting visual threats, and humans exhibit instinctive avoidance of looming objects. For instance, when a fast-moving object suddenly approaches, regardless of direction, humans instinctively engage in avoidance behaviors [1]. In high-threat environments such as combat zones, freezing can also occur as a strategy to reduce detection, particularly in response to overhead or incoming threats [2]. Even newborn human infants reflexively withdraw their heads and raise their hands when a dark

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object looms overhead [3]. In the wild, an overhead predator, such as a bird, casts a rapidly expanding shadow or visual profile as it descends. Such "looming" visual stimuli are potent, evolutionarily conserved danger cues that trigger instinctive defense behaviors across vertebrates. These behaviors align with ethological defense strategies and suggest that visual threat processing is evolutionarily conserved [4]. Looming is a visual cue defined by the expansion of an object's retinal image as it approaches, which provides information about collision risk and proximity.

In rodents, predator-like overhead cues typically trigger one of two innate responses: either freezing to avoid detection or rapid escape (to shelter) [5]. The action taken depends on factors such as the nature of the visual cue and the perceived immediacy of danger, as well as internal and external factors that bias action selection [6, 7]. On the circuit side, a looming stimulus engages retinal-to-superior colliculus-linked pathways and downstream midbrain/brainstem networks that can support both escape and freezing, which will be covered later. Internal state can shift defensive strategy selection, including neuroendocrine and motivational factors such as stress and arousal, as well as metabolic state (i.e., hunger) [8]. This is likely adaptive because the costs and benefits of freezing vs. escape change with physiological context and current goals. For example, oxytocin signaling in the central amygdala can suppress maternal freezing behavior, enabling mothers to protect their offspring instead [9].

In mice, the probability of freezing vs. flight depends on stimulus structure (e.g., collision-like expansion vs. other overhead motion cues), perceived urgency, and the animal's options in the environment. Vale et al. [6] demonstrated that mice nearly always run to a learned shelter when it is available, whereas in the absence of a known shelter, mice more often adopt a freezing strategy. These contexts suggest that looming evokes flexible defensive decision-making, rather than a fixed reflexive response to the stimulus.

Researchers have leveraged a simple experimental paradigm to mimic these conditions: an overhead black disk that quickly expands on a screen, simulating an approaching object [10]. This artificial looming stimulus elicits defensive reactions in animals without prior training, making it a powerful tool for studying the neural circuits of instinctive behaviors. Mice are well suited for this approach because looming drives a robust, quantifiable behavior (escape to shelter vs. freezing) in freely moving animals, and the underlying circuitry can be tested with cell-type and pathway-specific manipulations.

Mice can instinctively distinguish between an approaching threat and a harmless passing object based on visual cues. A fast-approaching, looming disk overhead (rapidly expanding in size) tends to provoke an escape/flight response toward shelter, whereas a small disk moving laterally overhead (a "sweeping" or cruising stimulus) more often causes the mice to freeze and remain motionless [11]. In

a study, mice responded differently to two purely visual threat cues. A rapidly expanding overhead disk (looming stimulus) triggered flight to a refuge in about 88% of presentations, and a slowly sweeping "sweeping" disk elicited freezing in 83.6% of trials (56/67 presentations across 38 mice). This pattern suggests that vision alone can drive instinctive action selection: an imminent, rapidly approaching threat biases animals toward immediate escape and shelter-seeking, while a slower, more distant-like threat favors freezing, potentially reducing detection by avoiding movement [5].

This pattern fits ethological theory: distant predators elicit freezing (to avoid detection), whereas imminent predators trigger flight. Mice can also flexibly switch defenses if the threat persists. Notably, mice often begin with one defense and then switch as needed. For instance, they might freeze briefly upon spotting a distant threat, then dash for cover if the threat continues to approach. This flexibility shows that even hardwired responses are modulated by context and ongoing risk assessment [12].

1.1 Neural Circuits for Looming vs. Sweeping Stimuli

Looming (rapid image expansion consistent with approach) and sweeping (lateral motion of a small overhead object) are both predator-relevant visual patterns, but they tend to bias different defensive strategies in mice [4] (Fig. 1). From the first step of visual processing, threat-selective computations can already shape which downstream circuits are recruited. Recent work suggests that synaptic filtering and cell-type-specific routing of threat signals through the retina into SC networks are responsible for transmission. For instance, synaptic filtering and cell-type-specific routing of threat signals in the retina contribute to looming selectivity [13]. The SC is a major hub where these visual signals are transformed into defensive commands via parallel output channels. Recent studies reveal that distinct superior colliculus (SC) outputs underlie the different behaviors evoked by looming and sweeping stimuli [14]. Shang et al. [15] found that parvalbumin (PV+) neurons in the superficial superior colliculus project to distinct targets, including the parabigeminal nucleus. A separate SC—pulvinar-like lateral posterior nucleus of thalamus (LP) pathway was later implicated in responses to looming stimuli. These pathways engage different downstream circuits [14]. Moreover, Shang et al. [7] found that activating the PV+ SC—parabigeminal nucleus (PBGN) pathway is sufficient to induce "fear" responses (robust freezing and aversion), whereas Wei et al. [10] identified an SC–LP–lateral amygdala route that is critical for looming-induced freezing. Neurons in the deeper layers of the SC that project to the dorsal periaqueductal gray (PAG) have been shown to integrate information about imminent visual threats. Specifically, a synaptic threshold mechanism at the SC–PAG synapse supports escape decisions, whereas excitatory and inhibitory networks in the PAG drive rapid escape (flight) behavior [16]. In summary, an SC–LP–amygdala

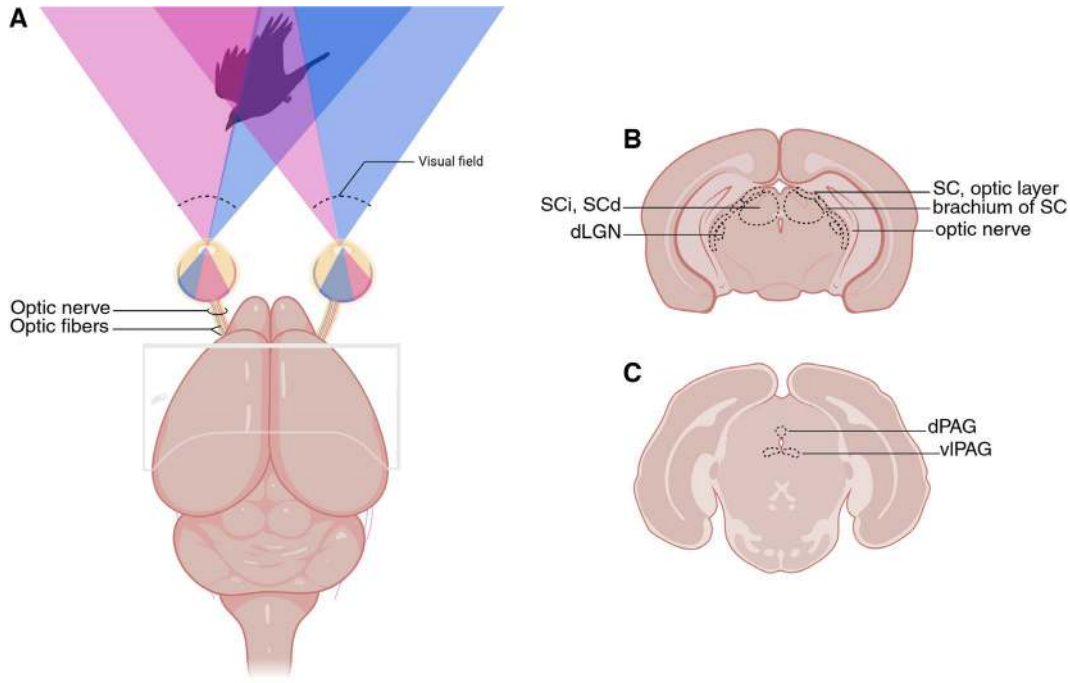


Fig. 1 Direct midbrain pathways supporting instinctive defensive responses to looming threat in mice. (**A**) Schematic of the mouse visual field illustrating detection of an overhead predator-like stimulus ("looming") by the retina. Retinal outputs travel via the optic nerve and optic tract to midbrain visual structures. (**B**) Common upstream route: retina → optic tract → SCs (superficial superior colliculus; visual layers), engaging SCi/SCd (intermediate/deep superior colliculus; premotor layers) that relay threat signals toward downstream defensive control nodes. (**C**) Downstream, signals from the SC converge on the periaqueductal gray, where different PAG subdivisions are linked to distinct defensive outputs. Pathways involving the dorsal PAG are associated with rapid activation of brainstem locomotor circuits (MLR/reticular formation) and escape/flight, whereas pathways involving the ventrolateral PAG are associated with recruitment of brainstem inhibitory/autonomic circuits and freezing/immobility. This figure depicts a simplified, direct SC-PAG-brainstem framework; amygdala- and thalamus-dependent pathways are not shown. Created in BioRender. O'Leary, A. (2026) https://BioRender.com/ejsptl0

circuit appears to underlie sustained freezing (sweeping-type stimuli), while SC–PBGN–amygdala (and SC–PAG) circuits are important in eliciting escape and then freezing in response to immediate threats.

Consistent with distinct SC outputs, optogenetic stimulation of the PV+ SC–PBGN pathway elicits a rapid escape followed by freezing, whereas stimulating the SC–LP pathway produces sustained freezing but no initial escape. In the same study, direct stimulation of PBGN neurons triggered an escape-then-freeze pattern, whereas stimulation of LP neurons induced only freezing [7]. Moreover, the same study showed that inhibiting the PBGN pathway biases animals toward freezing, while inhibiting the LP pathway promotes escape [7]. These data indicate competition between circuits: lateral overhead motion primarily engages the SC–LP–amygdala freeze pathway,

whereas looming stimuli additionally engage SC outputs (via PBGN and dorsal PAG) that induce flight, suggesting that one pathway's activity dominates and suppresses the other. For example, Wei et al. [10] mapped an SC–LP–lateral amygdala pathway that mediates looming-induced freezing. For directed escape, using imaging and optogenetic manipulations in freely moving animals, it has been shown that the dorsal PAG is sufficient for the initiation and execution of escape [16–20]. For example, stimulation of glutamatergic neurons and inhibition of tonically active GABAergic neurons in the PAG both elicit escape. Vice versa, inhibition of glutamatergic neurons and activation of GABAergic dorsal PAG neurons inhibit escape responses [16].

These findings suggest that an interconnected circuit of inhibitory interneurons and excitatory projection neurons in the dorsal PAG controls the probability of flight initiation, and its execution and termination [17]. Overall, the PAG acts as a central switch for instinctive defense behaviors, where inhibiting its output suppresses both freeze and flight responses to overhead threats.

1.2 Overview

To study how animals detect and respond to imminent threats requires a precise and objective quantification of rapid, multimodal defensive responses. This protocol integrates three complementary analytical frameworks: (1) controlled visual stimulus presentation mimicking predatory approach, (2) frame-by-frame pose estimation via deep learning, and (3) discovery of behavioral modules from pose trajectories using a hierarchical generative model. Together, these components enable characterization of threat assessment and defensive strategy selection.

The looming stimulus paradigm is adapted from previously established protocols with minor modifications detailed below [4, 5, 21].

2 Materials

2.1 Animals and Housing

- Mice (8–12 weeks old, both sexes).
- Group-housed; 12 h light/12 h dark cycle; food and water *ad libitum*.
- All procedures comply with institutional animal care guidelines and national regulations for the use of laboratory animals.

2.2 Behavioral Arena and Environment

- Arena: rectangular enclosure (45 cm × 50 cm × 33 cm), transparent non-reflective PVC panels, and housed inside a light-proof enclosure.
- Sound-attenuated cabinet to minimize auditory and visual distraction.
- Cleaning: 70% ethanol (between trials), air-dried.

2.3 Visual Stimulus Display and Refuge

- Monitor: LCD, 21 inch; 1920 × 1080 pixels; 60 Hz refresh rate; mounted horizontally at ceiling level.
- Background: gray (RGB: 128, 128, 128; ~50% luminance).
- Refuge (shelter-present trials): rectangular shelter (10 cm × 10 cm × 8 cm) with a single entrance facing arena center.

2.4 Video Acquisition and Software

1. Camera: monochrome CMOS; 1280 × 1024 pixels; 120 Hz frame rate; positioned above arena (~60 cm).
2. Synchronization: TTL trigger signals from the presentation software.
3. Stimulus presentation: PsychoPy Studio (v2026.1.0).
4. Pose estimation: DeepLabCut.
5. Behavioral motif analysis: Keypoint-MoSeq.

3 Methods

Each mouse is familiarized with the behavioral arena in daily 10 min sessions for at least 2 consecutive days prior to data collection [22]. During habituation, no visual threat stimuli are presented, and a gray background is displayed on the monitor to mimic baseline testing conditions. This acclimation period reduces novelty-related behavioral variability during experimental trials. Before behavioral testing, mice undergo a 3-day habituation protocol designed to minimize stress-induced behavioral variance (Fig. 2):

Day 1: handling by the experimenter and 10 min arena exposure.

Days 2–3: two separate 10 min sessions in the empty behavioral chamber with no visual stimuli presented.

This procedure promotes stable baseline exploratory behavior and reduces novelty-related confounds (*see* **Note 1**).

The testing chamber is a rectangular enclosure (45 cm length × 50 cm width × 33 cm height) constructed from transparent, non-reflective PVC panels, housed within a light-proof enclosure. Between trials, the floor is cleaned with 70% ethanol and air dried to eliminate residual olfactory cues. The chamber is housed within a sound-attenuated cabinet to minimize auditory distractions from laboratory activity.

A high-resolution LCD monitor (21 inch; 1920 × 1080 pixels; 60 Hz refresh rate) is mounted horizontally at the ceiling level of the chamber. This overhead placement creates a visual presentation similar to the bird's-eye perspective from which an aerial predator would approach. The monitor subtends approximately 90°–100° of the animal's visual field. The background is set to gray (RGB: 128, 128, 128; ~50% luminance), providing high visual contrast for

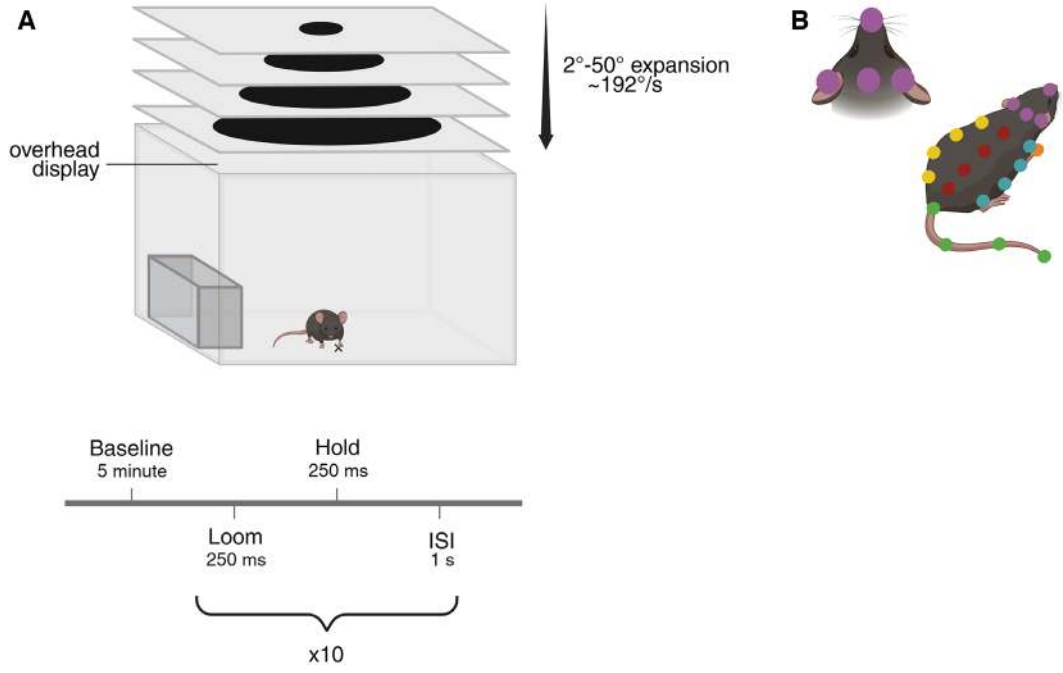


Fig. 2 Looming stimulus assay and example pose annotation for quantitative scoring. (**A**) Schematic of the behavioral setup and stimulus timing. A black disk is presented on an overhead display plane and expands from 2° to 50° (≈192°/s). Each trial includes a 5 min baseline period, followed by repeated looming presentations (250 ms expansion, 250 ms hold) separated by a 1-s inter-stimulus interval (ISI), delivered ×10 per trial. (**B**) Example DeepLabCut pose annotation frame illustrating the set of tracked body landmarks used to extract kinematic measures and classify defensive outcomes (e.g., escape-to-shelter vs. freezing). Created in BioRender. O'Leary, A. (2026) https://BioRender.com/ejsptl0

dark stimulus elements without inducing aversion. Custom scripts written in PsychoPy Studio (v2026.1.0) generate and render visual stimuli in real time.

In shelter-present trials, a rectangular refuge (10 cm width × 10 cm depth × 8 cm height) is positioned in one corner of the arena. The shelter features a single entrance facing the arena center, allowing unrestricted entry and exit (*see* **Note 2**). In no-shelter trials, the refuge is removed, creating an open-field configuration.

Behavioral sessions are recorded using a monochrome CMOS camera (1280 × 1024 pixels; 120 Hz frame rate) positioned directly above the arena at approximately 60 cm height. Video acquisition occurs at 120 frames/s and is synchronized with stimulus onset via TTL trigger signals from the presentation software. The 120 fps acquisition rate provides an 8.33 ms temporal resolution, sufficient to resolve rapid defensive movements, including escape initiation, postural transitions, and limb dynamics (*see* **Note 3**). Raw video is acquired in lossless AVI format and stored for offline analysis.

The looming stimulus consists of a filled black circular disk (RGB: 0, 0, 0) presented against a gray background, creating high visual contrast. The disk initially subtends 2° of visual angle and expands linearly from 2° to 50° over a 250-ms interval, corresponding to an angular expansion rate of approximately 192°/s. After reaching its maximum size of 50°, the disk remains static for an additional 250 ms before disappearing. Each trial contains ten successive looming presentations separated by 1 s inter-stimulus intervals (*see* **Notes 4** and **5**). The rapid half-second expansion sequence mimics the approach of an object on a direct collision trajectory and is ethologically relevant, as aerial predators can descend from several meters within comparable time scales.

The disk is centered on the display when it reaches maximum size. Prior to stimulus initiation, the mouse is positioned near the center of the arena to ensure it is outside the shelter and has unobstructed visual exposure to the expanding stimulus. In shelter-present trials, if the animal is inside the refuge at the scheduled onset time, stimulus presentation begins only after the mouse exits the shelter (*see* **Note 6**).

Each behavioral trial consists of three phases. In the initial phase, mice freely explore the arena while the display shows a continuous gray background (5 min, no stimulus). Baseline activity levels are recorded for comparison with stimulus-evoked behavior. In the second phase, a looming stimulus is presented as described above. Behavior is recorded continuously throughout the entire session, allowing capture of both immediate defensive responses to the stimulus and longer-term recovery toward baseline activity after stimulus offset.

Following behavioral recording, body position and postural dynamics are quantified using automated pose estimation. A set of ~32 anatomical landmarks is tracked from overhead video frames. The head region includes four landmarks (snout tip, head center, bilateral head points). The body midline is represented by five evenly spaced landmarks extending from the cervical to the sacral regions. Bilateral body surfaces are tracked using five landmarks per side positioned parallel to the midline. When limbs extend into the frame, four additional landmarks mark forelimb and hindlimb paws. The tail is represented by two landmarks: the base and the tip. The total landmark count ranges from 25 (when limbs are retracted) to 32 (when fully extended), with missing data handled through confidence-based interpolation (*see* **Note 7**).

Pose estimation is performed using DeepLabCut [23]. A ResNet-50 architecture is trained on approximately 800–1200 manually annotated frames, sampled across 15–25 recording sessions that represent diverse behavioral states. Training proceeds for 200,000–300,000 iterations using Adam optimization on a GPU-enabled workstation. After initial convergence, additional frames representing challenging postures are annotated and incorporated for refinement, followed by retraining for 50,000–100,000 additional iterations.

Network accuracy is evaluated using the percentage of correct keypoints (PCK), defined as the proportion of predicted landmarks within 10 pixels of manually annotated ground truth positions. In rodent pose estimation tasks, PCK values exceeding 95% are considered high accuracy [24] (*see* **Note 8**).

The trained network is applied to all videos in batch mode. For each frame (120 fps), the network outputs *XY* coordinates and confidence scores (0–1) for each landmark. Predictions with confidence >0.95 are retained as reliable, whereas those <0.75 are flagged for interpolation. Complete landmark trajectories are stored in HDF5 format with associated confidence values.

Frame-by-frame kinematic metrics are computed from extracted landmark coordinates. Body centroid position is calculated as the mean of all reliably detected landmarks. Instantaneous velocity is computed as frame-to-frame centroid displacement divided by the sampling interval (8.33 ms) and reported in cm/s (*see* **Note 9**). Acceleration is defined as the first derivative of velocity (*see* **Note 10**). Heading angle is calculated from the nose-to-thorax vector (0°–360°). Spinal curvature is derived from thorax, lower thorax, and sacral landmarks (0°–180°), where 180° indicates a straight axis and lower values reflect flexion; defensive crouching is typically associated with curvature <110° (*see* **Note 11**). Limb extension is quantified as the Euclidean distance from shoulder or hip joint to paw endpoints. Facial motion variance is calculated as variance in facial landmark displacement, capturing whisking or orienting micro-movements. All metrics are temporally aligned to stimulus onset ($t = 0$ ms) for cross-trial comparison (*see* **Note 5**).

DeepLabCut output is further processed using Keypoint MoSeq to identify recurring behavioral motifs [25]. The algorithm segments pose trajectories into discrete "syllables" representing stereotyped body configurations and movement sequences (*see* **Note 12**). Stimulus-responsive patterns are identified by comparing syllable occupancy and transition probabilities before vs. after stimulus presentation using non-parametric statistical comparisons ($p < 0.05$) (*see* **Note 13**). Syllables that increase in occupancy within 2 s of stimulus onset and exhibit elevated transition probabilities are classified as threat-related behaviors (*see* **Note 13**).

For each trial, behavioral category is assigned based on kinematic criteria. Flight is defined as centroid velocity exceeding 35–40 cm/s within 1–1.5 s of stimulus onset accompanied by directed movement toward the shelter (*see* **Note 2**). Freezing is defined as sustained velocity <5 cm/s within 2 s of stimulus onset together with reduced spinal curvature indicative of crouching (*see* **Note 11**). Trials not meeting these criteria are classified as no response. Automated classifications are verified by manual inspection, and only trials with ≥80% landmark tracking confidence are included in analysis.

4 Discussion

Looming stimuli provide a simple and ethologically relevant cue of impending collision, as rapidly expanding retinal images typically indicate that an object is approaching rapidly. Across vertebrates, such cues can trigger a rapid shift into defensive control, including immobility or active escape. In mice, the looming paradigm is useful because it links a well-defined visual input to quantifiable defensive outputs, while still allowing strategy selection to depend on context and state.

Responses to looming stimuli also offer a concrete entry point for thinking about trauma-related psychopathology. Interestingly, these hardwired responses can also emerge inappropriately in pathological contexts [26]. In individuals with anxiety disorders or post-traumatic stress disorder (PTSD), such responses may be triggered in the absence of an actual threat, reflecting a heightened sensitivity or unnecessary activation of innate defense circuits [27, 28]. Patients frequently describe disproportionate physiological arousal or a sense of imminent danger in response to benign stimuli, a phenomenon thought to arise from maladaptive engagement of survival-related neural pathways [29]. Importantly, defensive responses to looming stimuli are not purely reflexive but can be shaped by experience. Repeated presentation of non-threatening looming stimuli leads to habituation and reduced escape responses, demonstrating that animals learn to ignore stimuli that no longer predict danger [30, 31]. These findings indicate that looming paradigms are also useful for studying adaptive learning processes that suppress unnecessary defensive reactions.

Studying such phenomena directly in humans is challenging due to ethical and methodological constraints, particularly the inability to induce high-threat states under controlled conditions or to perform invasive molecular analyses. In contrast, rodents offer a valuable model because their defensive responses to looming stimuli are highly stereotyped and supported by well-characterized neural circuits [32]. These conserved, reproducible behaviors allow researchers to mimic certain aspects of human fear and anxiety disorders in the lab, enabling precise investigation into underlying mechanisms, symptom emergence, and potential treatment strategies.

Crucial to this investigation is the distinction between “looming” and “sweeping” visual stimuli, which recruit distinct neural circuits and defensive repertoires. A looming stimulus, typically a rapidly expanding dark disk, signals an object on a collision course and represents an imminent, proximal threat. From an ethological perspective, this mimics an attacking predator and triggers an explosive flight response mediated primarily by the superior colliculus and dorsal periaqueductal gray. In contrast, a sweeping stimulus such as a small spot moving across the overhead visual field at constant velocity

simulates a patrolling predator that has not yet initiated a strike. This "distal" threat elicits sustained freezing and risk-assessment behaviors, engaging forebrain structures involved in cognitive processing, anxiety, and uncertainty, such as the amygdala and bed nucleus of the stria terminalis, which can still be inhibited by the PAG [33].

This neuroethological divergence offers a framework for modelling the clinical heterogeneity of anxiety disorders. The response to looming stimuli may provide an ethological analogue for panic disorder, characterized by sudden, overwhelming surges of autonomic arousal and an urgent flight drive, often triggered by false alarms in the brain's proximity detection systems [34]. Conversely, the freezing and hypervigilance evoked by sweeping stimuli might align more closely with generalized anxiety disorder (GAD) or the "anxious apprehension" seen in PTSD, where the primary symptom is a sustained, inhibitory state of worry regarding potential, rather than immediate dangers [35, 36].

Pharmacological validation of these situations supports the Deakin and Graeff hypothesis, which postulates that panic and anticipatory anxiety are modulated by separate serotonergic systems [37]. Experimental evidence demonstrates that panicolytic agents, specifically selective serotonin reuptake inhibitors (SSRIs) like fluoxetine and tricyclic antidepressants (TCAs) like imipramine, significantly suppress the flight response to looming stimuli [38]. Importantly, this suppression is observed only after chronic administration, mirroring the delayed therapeutic onset in clinical panic disorder; acute administration often fails to reduce flight or may transiently exacerbate it.

Whether these drugs selectively bias responses to sweeping vs. looming visual threats remains to be tested directly. By studying these distinct visual paradigms that elicit evolutionarily conserved defensive behaviors in a context-dependent manner, researchers can move beyond generic measures of instinctive defensive behaviors to more accurately dissect the specific neural and molecular architectures underlying the heterogeneity of human anxiety disorders, ultimately improving the predictive validity of preclinical drug screening.

5 Notes

1. **(Habituation and baseline)**. If baseline exploration is highly variable (e.g., persistent wall-hugging, frequent pausing, or immediate shelter occupancy), extend habituation by additional daily sessions and keep handling and transport consistent across animals.
2. **(Shelter dependence)**. Flight probability depends strongly on shelter availability and the animal's knowledge of shelter location. If directed escapes are rare, confirm that (i) the

refuge is always in the same corner, (ii) mice have entered the refuge during habituation, and (iii) the refuge entrance faces the arena center.

3. **(Video blur during escape)**. If keypoint confidence drops specifically during rapid escape bouts, reduce camera exposure time and/or increase illumination (without changing stimulus/background settings). Motion blur is a common cause of low DeepLabCut likelihood values during fast locomotion.
4. **(Monitor alignment and clipping)**. Ensure the overhead monitor is centered and parallel to the arena floor. A stimulus that is clipped by screen edges or appears off-center can reduce salience and increase trial-to-trial variability.
5. **(Reflections and lighting)**. Reflections on PVC walls/floor can reduce contrast and create unintended motion cues. Use matte surfaces where possible and keep ambient lighting constant across sessions.
6. **(Stimulus onset timing)**. Present looming only when the mouse is outside the refuge and not already immobile. If the animal remains in the refuge at the scheduled onset, delay stimulus onset until it exits to ensure visual exposure.
7. **(Training set diversity for DeepLabCut)**. Include annotated frames spanning the full behavioral range (exploration, rearing, grooming, curled freezing postures, and fast escape). If errors cluster in one state (e.g., crouched freezing), add targeted frames from that state and retrain.
8. **(Confidence thresholds and interpolation)**. Thresholds for "reliable" vs. "low-confidence" keypoints should be tuned to video quality. Overly strict thresholds can cause excessive interpolation, whereas overly permissive thresholds introduce jitter that inflates velocity and curvature estimates.
9. **(Pixel-to-cm calibration)**. To report velocity in cm/s, calibrate pixel distance using a known-length reference object placed on the arena floor at the start of a session. Recalibrate if camera height, zoom, or arena placement changes.
10. **(Velocity smoothing)**. Frame-to-frame centroid velocity can be noisy due to subpixel jitter. If classifications are unstable, apply a short temporal smoothing window (e.g., a few frames) before thresholding, and report the smoothing method consistently across conditions.
11. **(Defining freezing)**. Freezing definitions based solely on low velocity can be confounded by brief pauses during exploration. Combining velocity with a postural index (e.g., spinal curvature consistent with crouching) improves specificity for defensive immobility.

12. **(Keypoint-MoSeq syllables are unlabeled by default)**. Keypoint-MoSeq returns discrete syllable IDs that must be interpreted post hoc. Assign user labels by inspecting exemplar clips for each syllable and using associated kinematic signatures (e.g., high velocity for escape-like syllables, low velocity + crouch for freeze-like syllables).
13. **(Comparing pre vs. post stimulus)**. When testing for stimulus-responsive syllables, use consistent analysis windows across trials (e.g., baseline vs. 0–3 s post-stimulus) and keep inclusion/exclusion criteria fixed (e.g., minimum tracking confidence).
14. **(Common "no response" failure mode)**. If many trials show no clear response, check (i) whether stimulus onset occurred while the mouse was visually oriented to the screen region, (ii) whether the animal was already immobile, and (iii) whether prior stressors (noise, recent handling) altered baseline state.

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Chapter 10

The Social Interaction Test *de modo* File in the Rat: Measurement of Anxiety and Sociability

Margus Kanarik and Jaanus Harro

Abstract

Several tests in rodents are based on social interaction. This chapter describes the original social interaction test as first described by Sandra E. File and J.R.G. Hyde, that was subsequently popularized and widely used as an anxiolytic drug screening test. For several objective reasons, this test, as originally conducted, has largely lost its former position in the measurement of anxiety, while social behavior continues to be exploited in animal tests. Nevertheless, this test possesses unique characteristics of value, and has more recently been applied to assess the expression of a basic personality trait, sociability, in the rat, and to study the underlying neurochemistry.

Keywords Social behavior, Anxiolytic drugs, Anxiogenic drugs, Individual differences, Sociability trait

1 Introduction

DSM-III established "anxiety disorders" for the scientific language in 1980, only three years after the demonstration of the molecular mechanism of action of benzodiazepine anxiolytics by the discovery of "benzodiazepine receptors" [1, 2]. The anxiolytic drug boom of 1970s was already fuelling the development of novel screening tests for drugs and anxiety models. Among these, the social interaction test was proposed by the group of Sandra E. File at King's College, London. In the field dominated by tests that were based on learning and involved food or water deprivation and/or electric shocks, the social interaction test was the first to apply ethologically relevant anxiety and to measure evolutionarily conserved natural behavior [3]. The first report on this test, published as a conference abstract, described the increasing effect of chlordiazepoxide and ethanol on partner-directed activity in a pair of adult (male) rats [4]. In a subsequent massive series of full papers (e.g., [5–9]for review), File and co-workers reported on effects of additional compounds and on

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the significance of a number of factors on social interaction. Many laboratories adopted this experimental design. Subsequently, several tests that rely on social behavior in the rat, some only variable by e.g., test arena size or testing time, and others rather different, have appeared under the name of social interaction test [10–12], social approach-avoidance test [13] or social motivation test [14]. Furthermore, there has been an explosion of method development for the assessment of social behaviors in mice [15–17]. The scope and purpose of the present chapter do not permit comparison of all these tests; instead, the original social interaction test with its advantages and weaknesses is described, including a novel extension of it for the study of basic temperament.

In the social interaction test, the time spent in active social interaction between two unfamiliar rats in a neutral arena is the principal, actually the sole measure. (It should be acknowledged that some investigators include or count any passive contact between the animals separately.) It is important to house rats individually for several days before the test [18]. Rendering rats anosmic did not reduce social interaction, but active social interaction is reduced by higher levels of light [6]. But whether light levels affect performance in the test depends on the light levels in the animal housing rooms, and the reduction of social activity observed in brightly lit arenas applies to rats maintained under low light levels. If the arena is unfamiliar, social behavior may be reduced, whereas this is not accounted for by increased exploration [3]. Instead, increases in behaviors like freezing, displacement activities, and defecation suggest that lower social activity relates to anxiety. Social interaction time is reduced by anxiogenic drugs such as FG 7142 [19] or yohimbine [20]. Drugs that promote dopaminergic activity can give false positive results in anxiolytic screening tests based on exploration of physical environment, but appear anxiogenic-like in the social interaction test [21].

With the advent of genetically modified mice in neuroscience, studies using rats diminished in number, and so did the use of behavioral tests that are more suitable for the rat. The social interaction test, as originally described, also has a number of drawbacks. Single-housing for seven to ten days before the test is necessary; this demands extra resources and raises the question of possible isolation stress or sensory deprivation effects. Animals are tested pairwise, and their behavior may be interdependent, posing challenges to the statistical analysis, and if only one rat is the target of measurement, or the mean score of a pair is the unit [9], the number of animals needed in the experiments increases. So other methods to quantitate social behavior, such as Crawley's three-chamber test [22], have largely replaced the social interaction test *de modo* File, but have been applied in many modifications and for a variety of purposes. Usually these tests attempt to measure social interest without focusing on

free physical interactions between the animals, while these provide stress buffering benefits that go beyond mere social proximity [23].

Sometimes higher social interest in these tests is labeled as sociability, but mostly this is estimated on the basis of single test, which is not sufficient to characterize trait-wise behavior.

Intriguingly, behavior of rats in the social interaction test has not been correlating well with their performance in other animal models of anxiety [24]. Similarly, panels of anxiolytic and anxiogenic drugs exhibit different profiles in the social interaction test as compared to other tests based on spontaneous behavior. Furthermore, psychostimulants such as amphetamine and caffeine increase locomotor activity and can sometimes lead to false positive results in exploration-dependent anxiety tests, but they reduce the time spent in active social interaction [25]. These discrepancies led us to the hypothesis that behavior in the social interaction test channels other important neural processes besides general anxiety production. Social behavior is the basis of one of the most generally accepted independent dimensions of human personality, separate from, e.g., emotional stability/neuroticism [26], and has its own genetic basis that is related to several psychiatric disorders [27]. Sociability is an individual's tendency or propensity to associate with other individuals, whereas the association is not driven by aggressive or reproductory urges [28]. The evolutionary continuity between humans and other animals suggests that basic dimensions of personality should be represented across species, especially in mammals [29, 30]. In animals, sociability can be expressed behaviorally as trait-wise proneness to initiate or actively accept social contact with a conspecific on neutral territory. Studies on prosocial behavior have shown that not all tested animals are prosocial [23].

If single-housed rats are submitted to the social interaction test repeatedly, each time with a new partner, the time they spend in social interaction varies test-wise; however, the social activity of rats in each test is in a relatively strong positive correlation with their average performance across multiple tests (Fig. 1), both in male and female rats [31–33]. This average level of social behavior or sociability follows normal distribution and is associated with susceptibility to chronic stress [32]. Individual differences in sociability have been found to correlate with serotonergic neurotransmission in several brain regions [31, 33] that are serving the regulation of social behavior [34, 35], and to be associated with the level of oxidative metabolism in brain regions such as the supraoptic and median preoptic nucleus, dorsal striatum, posterior paraventricular thalamus and median raphe [36]. Because disturbed social activity, either deficient or excessive, can be both a symptom and a risk factor of psychiatric disorders [37], sociability might serve as a valuable tool for studies of endophenotypes of psychiatric vulnerability.

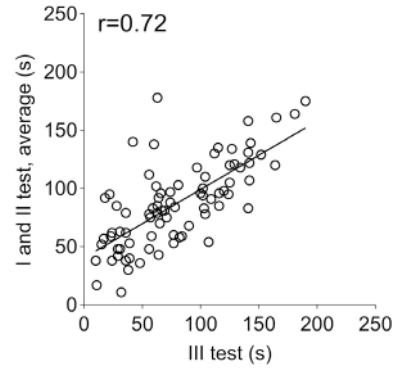


Fig. 1 The stability of sociability trait: in male Wistar rats, tested thrice with approximately one-week intervals, social interaction time in the third test correlated strongly with the average score of the other two tests. Data from separate experiments pooled, $n = 82$, Pearson's $r = 0.72$

2 Materials

2.1 Animals

Rats of a variety of strains have been used for drug screening in the social interaction test, but different batches of outbred rats from even the same supplier can significantly differ in the level of social activity [38]. The Fawn-Hooded strain has been found to present similar locomotor but relatively lower social activity in this test, as compared to other strains [39]. For the study of the sociability trait, we have successfully used male and female rats of Wistar and Sprague–Dawley strain. For testing, the animals must be weigh-matched, with body weight difference minimal, preferably not more than 5%. Little information is available on the optimal age of the rats, but a nonlinear relationship of active social interaction with age has been reported, with the highest values in young animals (2.5 months), a decrease at 4.5 months, and a slight increase in 6-month-old rats [40]. Because the reduction of social behavior in very advanced age is unlikely to indicate higher anxiety but rather is reflecting reduced social motivation [41], higher age of the animals may reduce the validity of the test as an anxiety measure. While the social interaction test can be reliably used in both male and female rats, some sex-wise differences have been reported [9] and should be considered while adapting the test in the laboratory. One report has suggested that special diets (soya and other phytoestrogen-free) can affect behavior in the social interaction test [42].

2.2 Housing

Single housing at least for a week, preferentially for 10 days is essential to increase social activity. Otherwise the conditions should follow the standard of the animal house. Light levels in the animal house

can affect performance in the test in case the arena is brightly lit and the rats are not accustomed to light.

2.3 Anxiolytic, Anxiogenic and Other Compounds

Drugs can be obtained from a number of providers. Pay attention to the molecular weight of salts and consider this for calculation and selection of the dose. Follow the suggested conditions for storage. Stabilities of the chemical compounds in solutions are variable, the safe option is to dissolve the drugs *ex tempore*. This may not be feasible owing to, e.g., very small quantity needed for a single experiment; in this occasion aliquots of the preparation can be stored frozen. In case the drug is not water soluble, emulsifiers and solubilizers such as polysorbates (e.g., different Tween products among others) can be efficient. Take care to administer proper vehicles to control animals, and keep in mind that some vehicles may produce inner cues.

2.4 Equipment for Behavioral Testing

The test chamber ($30 \times 30 \times 60$ cm) with transparent walls, floor covered with wood shavings. Keeping the area small facilitates social interaction.

2.5 Other

Lighting conditions should be adjusted to achieve the optimal level of social activity. Stopwatches and timers are needed, and a camera is recommended. Free behavioral coding programs like BORIS can be used for manually scoring behavior from video.

3 Methods

3.1 The Social Interaction Test

We place the partners simultaneously into a brightly-lit unfamiliar chamber. Observation time has been 10 min as in the original description. Social interaction time is either recorded directly be the observer or videotaped and measured later. The presence of an observer does not appear to affect exploratory and social behavior of the pair of rats, if no sudden movements or noise occurs. The total time spent in active social behavior (allogrooming, sniffing the partner, crawling under and over, following (see Fig. 2 for example) is recorded (*see* **Note 1**). While this rating may at the first glance appear as overtly subjective, in our experience inter-rater reliability has always been found to be good ($r > 0.90$) [31, 33].

The familiarity of the chamber and light levels can affect social interaction, and it has been suggested that four different test conditions should be distinguished: low light, familiar arena; low light, unfamiliar arena; high light, familiar arena; high light, unfamiliar arena [9]. One can expect the lowest level of anxiety in the low light, familiar arena condition and the highest in the high light, unfamiliar arena condition. This is important to consider in order to prevent floor and ceiling effects (*see* **Note 2**).

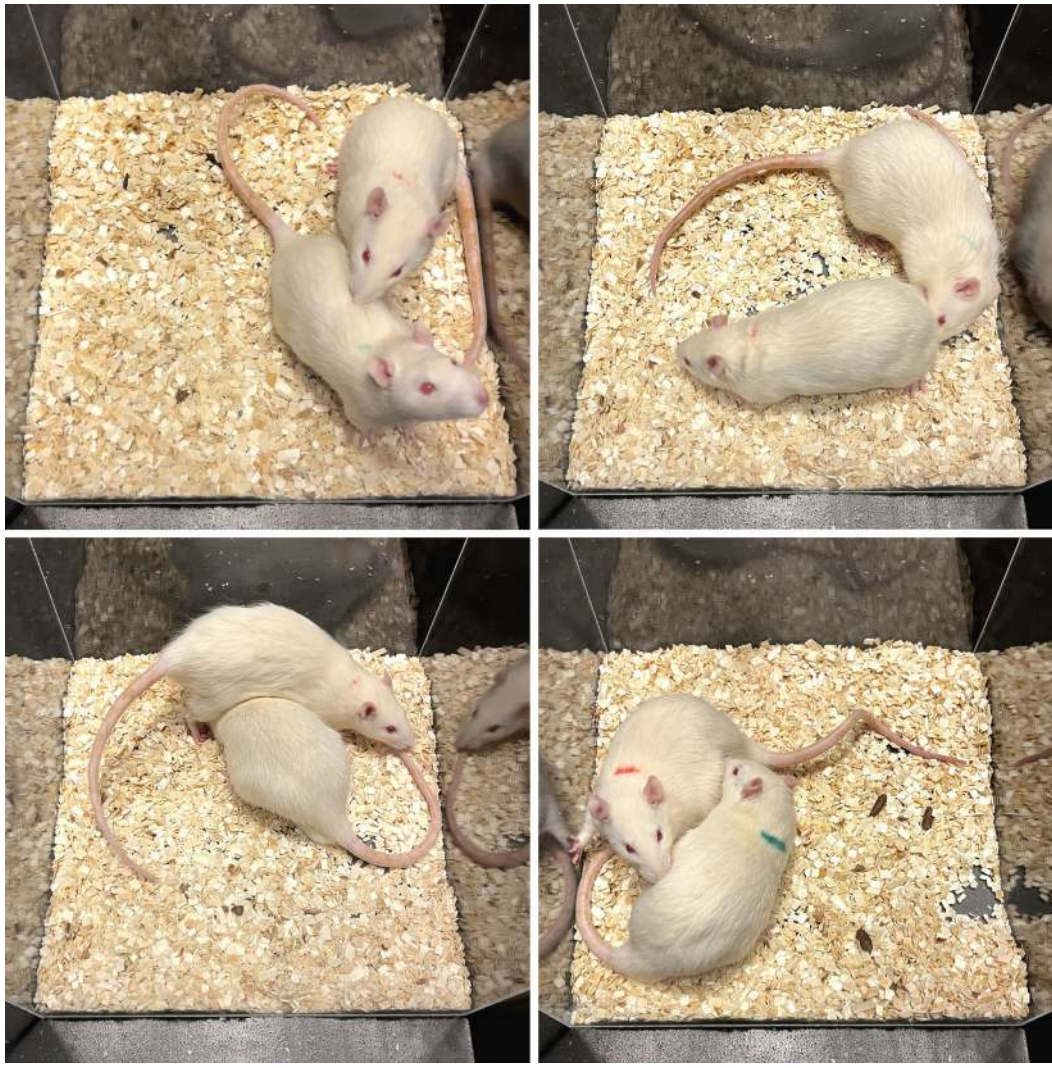


Fig. 2 Representative images illustrating rat behavior in the social interaction test. From upper left clockwise: dorsal sniffing; anogenital sniffing; both animals in active social interaction; crawling over or under the other animal

If a set of rats is repeatedly tested with novel partners, correlations of individual performances in two tests vary non-systematically (*see* **Note 3**). Behavior of the partner exerts a moderate influence: correlations coefficients are around 0.35. [31] (*see* **Note 4**).

Passive body contact, so-called "huddling" can be scored separately. It is not advised to sum passive and active social behavior scores, as the two react differently or even opposingly to stimuli [3], and the active social behaviors can also be separately scored, but not much use seems to have been made of such detailed assessment. We

consider it active social behavior if the rat is clearly showing interest in social contact, and record this for each animal separately.

3.2 Measurement of the Sociability Trait

In this instance, the social interaction test is performed three or four times, with new partners. Allow at least seven days between tests. The arithmetic average of social activity in all tests yields the estimate of sociability. Three tests should suffice, but in case of a validation experiment in new conditions, correlation of each test with the average of other tests should be calculated, lest the correlation be artificially inflated, and therefore four tests are necessary. The mean time spent in social interaction has been normally distributed in all of our studies [31–33, 36] (*see* **Note 5**).

4 Notes

1. Aggressive/agonistic encounters, if present, should not be included in the active social interaction behaviour score. In most conditions these are not observed, but if repeatedly observed, the conditions are suboptimal to study prosocial activity.
2. Experiments with genetically modified rats should be preceded by a validation study, because persistent neurochemical alterations can affect behavior in the social interaction, bearing importance for interpretation of social interaction time. Such is the case with lesioning of the serotonin system that reduces social interaction [43] and may confound anxiety-related behavior with impulsivity [44].
3. We have observed that correlations may be stronger between tests which directly follow each other.
4. These correlations represent the assumption of linear relationship but the mutual impact may be more complex. While the positive correlation suggests that mostly the social activity of one rat supports social behavior of the other, it can occasionally also be observed that overwhelming social interest is confining partner's social behavior.
5. The sociability measure can be used as a continuous variable or for grouping by the level of sociability. In neurochemical studies, we have mostly found the median split of the average social activity over all social interaction tests useful to classify the rats as low-sociability or high-sociability animals. Nevertheless, because the data follow normal distribution, it may be worthwhile to explore the fitting of the tertile split: In the study of regional levels of oxidative metabolism in the brain, a few brain regions exhibited nonlinear relationship with sociability, the high and low sociability groups being similar to each other

but having either higher of lower levels of metabolism than the medium sociability group [36].

Conflict of Interest

The authors have no conflicts of interest to declare that are relevant to the content of this chapter.

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Chapter 11

Prenatal Stress Exposure: Integrating Maternal Phenotypes and Offspring Behavioral Negative Outcomes

Floriana De Cillis, Tiziano Prodi and Annamaria Cattaneo

Abstract

The concept of early-life stress (ELS) encompasses both traumatic experiences occurring during childhood and adolescence, and adverse events taking place before conception and during the intrauterine period. Extensive evidence indicates that ELS is associated with an increased vulnerability to long-term mental health disorders. In this context, large epidemiological studies have highlighted the negative impact of prenatal stress (PNS) on offspring neurodevelopment. However, despite growing recognition that perinatal affective disturbances may interact with PNS-related mechanisms, the extent to which behavioral outcomes in mothers and offspring are jointly characterized remains limited. Experimental animal models provide a controlled framework to address this gap by enabling the parallel investigation of maternal and offspring behavioral phenotypes arising from prenatal adversity. In the following sections, we describe an animal model of PNS designed to investigate the impact of gestational stress on behavioral outcomes in both mothers and their offspring. Maternal behaviors assessed include self-directed and pup-directed behaviors, enabling the evaluation of PNS-related impairments in self-care and parenting. In parallel, offspring behavior is systematically examined across multiple domains. From a translational perspective, alterations in maternal self-directed behaviors may reflect anhedonia, whereas behavioral changes observed in the offspring may represent markers of anxiety- or depression-like phenotypes. Overall, this model provides a valuable tool for understanding how stress experienced during pregnancy can influence the mental well-being of both mother and offspring.

Keywords Prenatal stress, Early-life Stress, Maternal–offspring interaction, Behavioral phenotyping, Affective and cognitive outcomes, Translational animal models

1 Introduction

Early-life stress (ELS) is increasingly recognized as a critical determinant of vulnerability to mental health disorders later in life [1]. The definition of ELS is not limited to the traumatic experiences that occurred during childhood and adolescence. Still, it also includes the adverse events that happened during the intrauterine period and even before conception [2]. Accumulating evidence indicates that stressors acting during these sensitive developmental windows can induce long-term biological and neurodevelopmental alterations,

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thereby increasing the risk for a broad range of psychiatric outcomes, including anxiety disorders, mood disorders, neurodevelopmental disorders, and schizophrenia spectrum disorders [2–6]. Focusing on pregnancy, large epidemiological studies have reported the negative impact of prenatal stress (PNS) on offspring neurodevelopment [7, 8]. It has been proposed that perinatal depression might contribute to PNS-related processes, since it has been associated with disrupted attachment and long-term developmental consequences in the offspring [9]. Moreover, undertreated perinatal depression also increases maternal risks since it could be characterized by specific symptoms like persistent doubt in parenting abilities, difficulty bonding with the infant, and thoughts of death, suicide, self-harm, or harm to the infant, and it may be associated with limited engagement in care and impaired relationships [9, 10].

Regarding the impact of PNS on the offspring, its effects have been linked to alterations in the hypothalamic–pituitary–adrenal (HPA) axis and to sustained increases in glucocorticoid production. Prolonged exposure to elevated cortisol levels during gestation may be detrimental to fetal development, as it can interfere with the maturation and long-term regulation of the HPA axis in the offspring. Such dysregulation has been associated with enduring changes in stress responsivity and affective regulation, thereby increasing vulnerability to mood disorders, including depression, later in life [2, 11]. Furthermore, prenatal exposure to elevated glucocorticoid levels has been associated with epigenetic modifications, including alterations in DNA methylation patterns and changes in the expression of microRNAs (miRNAs) that regulate pathways involved in inflammation, immune function, and neurodevelopment [12–14]. These epigenetic changes may contribute to a pro-inflammatory status, which in turn can disrupt placental function. In particular, the upregulation of pro-inflammatory cytokines has been implicated in altered maternal–fetal signaling, with representative mechanisms including the capacity of interleukin (IL)-17A to cross the placental barrier and the tumor necrosis factor-α (TNF-α)–mediated downregulation of enzymes involved in estrogen biosynthesis [11, 15, 16].

These biological alterations, including the neuroendocrine and immune changes described above, together with maternal behavioral alterations, likely act as key mediators influencing offspring development, a hypothesis that can be systematically tested in preclinical models.

1.1 Animal Model of PNS

The development of an animal model of PNS is crucial for understanding how prenatal adversity can negatively shape both maternal behaviors and offspring developmental trajectories. PNS exerts its effects through two interconnected mechanisms: indirectly, by altering maternal behaviors such as caregiving and stress responsivity, which in turn influence offspring outcomes, and directly, by affecting

fetal development via neuroendocrine and immune pathways. For example, PNS reduces placental 11β-hydroxysteroid dehydrogenase type 2 expression, normally protective of the fetal brain against excess glucocorticoids, and leads to reduced birth weight in newborn pups [17].

Interestingly, longitudinal assessments of offspring behavior reveal a wide range of PNS-associated phenotypes, spanning from early postnatal life through adulthood. These include increased anxiety-like responses, impaired cognitive function, reduced social behavior, metabolic dysfunctions, and deficits in neuroplasticity [18–24].

Importantly, not all offspring exposed to PNS develop maladaptive outcomes [25]. Stratifying animals according to behavioral responses allows the identification of markers of susceptibility and resilience, providing insight into why some individuals maintain adaptive functioning despite PNS, while others become vulnerable. This approach, combined with observations of maternal behavior, highlights the interconnected contributions of maternal care, fetal programming, and individual variability in shaping offspring trajectories.

Perinatal adversity can be modeled experimentally using different paradigms. Among these, restraint stress applied during late gestation is commonly employed to mimic maternal adversity and to assess its biological and behavioral consequences in the offspring under controlled conditions. This design allows not only the confirmation of previously reported negative outcomes but also the investigation of novel endpoints and the linkage of maternal alterations to offspring trajectories in a comprehensive, mechanistic framework. The following section outlines the experimental materials and testing environments required to reproduce the behavioral paradigms used to investigate PNS for the assessment of maternal and offspring behavioral outcomes, including the Social Interaction (SI) Test, the Novel Object Recognition Test, the Splash Test, and the Sucrose Preference Test. Detailed procedural descriptions are provided in Sect. 3.

2 Materials

Adult Sprague–Dawley rats (*Rattus norvegicus*), including males (≈ 400 g) and nulliparous females (230–260 g), are used as the starting population for generating the PNS model through controlled mating. These animals allow for replication of behavioral assessments to investigate the effects of PNS on both dams and their offspring.

All animals are housed under standard laboratory conditions, with controlled temperature (21 °C ± 1 °C), relative humidity (55% ± 5%), and a 12:12 h light/dark cycle. Food and water are

available *ad libitum*, and animals are individually identified using non-toxic black marker markings.

Materials required for the induction of PNS include individual transparent plastic restrainers specifically designed for rodents. Each restrainer consists of a rigid cylindrical tube (20 cm length × 9 cm diameter × 9 cm height) with a single rear opening for animal insertion (Fig. 1). A small plastic insert immobilizes the animal inside the tube while allowing the tail to extend freely through a dedicated slot, ensuring normal respiration. Restraint sessions are conducted under a high-intensity light source (≈1500 lux) in a dedicated testing room with controlled environmental conditions.

Offspring behavioral assessment includes both arena-based and home cage-based assays. Arena-based behavioral tests are conducted in a square Plexiglas open-field arena (100 × 100 × 18 cm), which is adapted according to the specific experimental paradigm. For example, the SI test is performed in the arena by introducing a cylindrical gridded enclosure that allows olfactory interaction (sniffing) between the test animal and a conspecific. The arena is also used for the Novel Object Recognition Test, which requires the placement of three objects: two identical objects and one different object. Home cage-based behavioral tests are performed in individual transparent plastic cages appropriately sized for the animal. For the Splash Test, animals are placed directly in the cage, and a 10% sucrose solution is sprayed onto the dorsal coat to induce self-grooming behavior. For the Sucrose Preference Test, animals require two drinking bottles per cage. During habituation, both bottles contain water to familiarize the animals with the setup and minimize side bias. During the test, one bottle is filled with a 1% sucrose solution and the other with water.

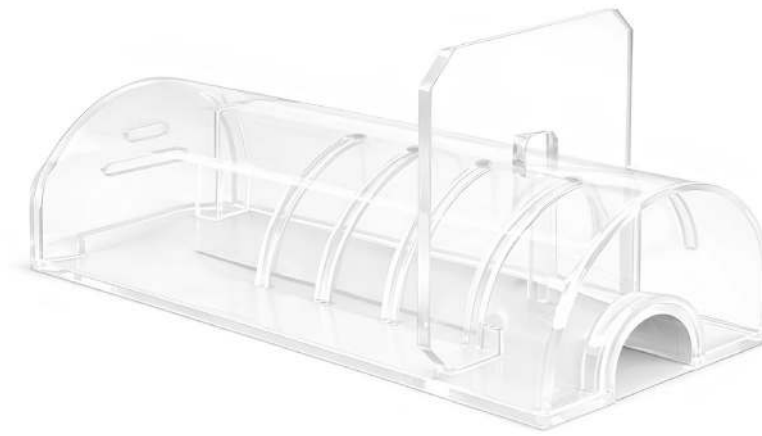


Fig. 1 Flat bottom rodent restrainers for experimental procedures. Representative image of the transparent plastic restrainer used for experimental procedures with rodents

All apparatuses are thoroughly cleaned with 70% ethanol between sessions to eliminate olfactory cues and prevent carry-over effects. Behavioral procedures are conducted in sound-attenuated, dimly illuminated rooms to minimize external interference. Behavior is recorded using a camera and timed with a dedicated digital timer.

3 Methods

3.1 Prenatal Stress Procedure and Maternal Behavior Assessment

After acclimatization and prior to PNS induction, females are paired with a male at a ratio of two females per male for 2 consecutive days. Pregnancy is then confirmed by weighing the females, and only pregnant dams are included in subsequent experiments (*see* **Note 1**).

The PNS paradigm is described as previously published [19, 22]. During the last week of gestation, pregnant dams are restrained for 45 min, three times per day, within predefined time windows from 09:00 a.m. to 05:00 p.m., with the timing of sessions varied across days to prevent habituation. Restraint is conducted under bright light conditions for 7 consecutive days, whereas control pregnant females remain undisturbed in their home cages. Cylindrical restrainers are thoroughly cleaned between sessions to minimize olfactory cues, and all procedures are performed under controlled environmental conditions.

To evaluate the outcomes of PNS exposure, a set of behavioral assays is employed. Maternal behavior is assessed on alternating days between postnatal day (PND) 1 and PND 9, for three 20 min sessions per day at approximately 09:00 a.m., 01:00 p.m., and 05:00 p.m. (± 30 min). Behaviors are scored every 2 min, resulting in 33 observations per day [26]. Recorded maternal behaviors (Fig. 2) include arched-back nursing (defined as the dam adopting a rigid, arched posture over the litter to facilitate active nursing), blanket or passive nursing (the dam lying over the pups without adopting an arched posture), and licking and grooming of the pups, which together are classified as pup-directed behaviors. Additional behaviors include off-nest activity (time spent away from the nest), eating and drinking, and self-grooming (cleaning of the dam's own fur), all classified as self-directed behaviors.

3.2 Behavioral Assessment in the Offspring

A set of behavioral assays is employed to evaluate the outcomes of PNS exposure, with the SI test, the Splash Test, the Novel Object Recognition Test, and the Sucrose Preference Test among the most used (*see* **Notes 2 and 3**). The SI test assesses social approach. Higher scores indicate greater social approach and lower anxiety-like behavior, whereas lower scores reflect social avoidance and increased stress susceptibility [27]. The Splash Test evaluates self-care and motivational behavior, particularly grooming activity [28]. Increased grooming is generally interpreted as an index of intact self-care and

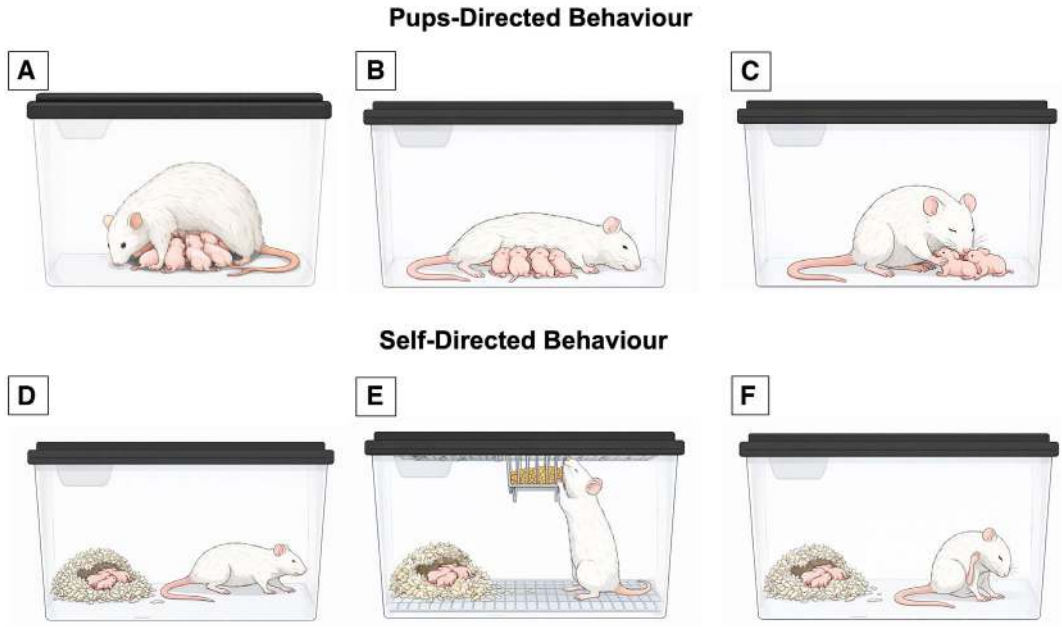


Fig. 2 Assessment of maternal behavior. Representative illustrations depicting the behavioral categories scored during the maternal behavior assessment. Pup-directed behaviors include (**A**) active nursing posture with arched back over the litter, (**B**) passive nursing in a prone position, and (**C**) pup-directed grooming. Self-directed behaviors include (**D**) exit from the nest, (**E**) non-pup-directed behavior, including eating and drinking, and (**F**) self-grooming

motivational drive, whereas reduced grooming is commonly associated with impaired self-care and depressive-like behavior. However, excessive or prolonged grooming can also be considered maladaptive, as it may reflect heightened stress reactivity, anxiety-like behavior, or compulsive-like patterns rather than normal self-maintenance [29]. The Novel Object Recognition Test measures recognition memory and cognitive performance based on differential exploration of novel versus familiar objects [30]. The Sucrose Preference Test assesses anhedonia, a core feature of depressive-like behavior. Increased consumption of sucrose solution indicates normal reward sensitivity, while decreased preference reflects reduced hedonic drive and anhedonia [31]. Detailed procedures for each test are provided below, and assessments can be performed at multiple developmental stages.

3.3 Social Interaction Test

The SI test is conducted in a square arena containing a grid enclosure that allows sensory and nose contact between animals while preventing physical interaction (Fig. 3A). The protocol consists of two consecutive 3 min phases: a habituation phase followed by a test phase. During habituation, the test rat is placed in the arena and freely explores both the open field and the empty enclosure. At the end of this period, the rat is temporarily returned to its home cage for 1 min,

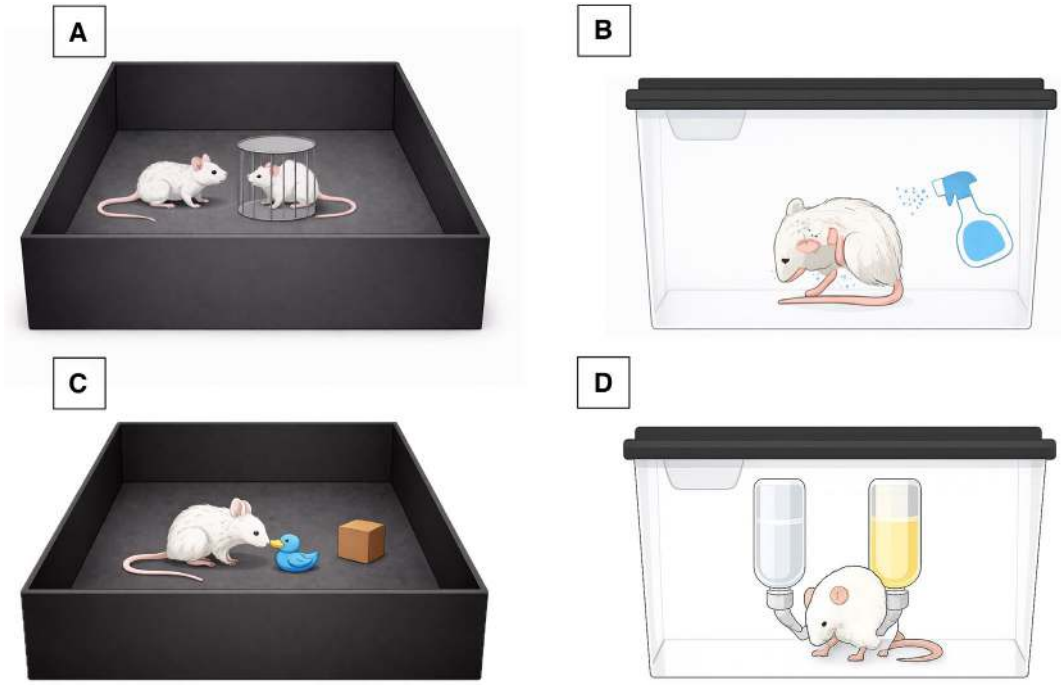


Fig. 3 Schematic representation of the behavioral tests used to evaluate the long-term effects of prenatal stress exposure. (**A**) Social Interaction Test, performed in an open-field arena containing a grid enclosure that allows sensory contact while preventing physical interaction; (**B**) Splash Test, in which a 10% sucrose solution is sprayed onto the dorsal coat of the animal; (**C**) Novel Object Recognition Test performed in an open arena with two identical objects or one familiar and one new; (**D**) Sucrose Preference Test, in which animals are given free access to a sucrose solution and water

while an unfamiliar control rat, matched for strain, age, and sex, is placed inside the grid enclosure. The test rat is then reintroduced for the test phase. During the test, social investigation behaviors are recorded, including approaches toward the conspecific, sniffing at the grid enclosure, and time spent in proximity to the conspecific [32]. Between testing of different animals, the arena is thoroughly cleaned with 70% ethanol to eliminate olfactory cues, and all animals are tested within the same time frame. All measures derived from the SI test are subjected to quantitative analysis as outlined in Sect. 4.

3.4 Novel Object Recognition Test

The Novel Object Recognition Test is conducted in a square open-field arena and consists of three phases: habituation, familiarization, and testing (Fig. 3C). The test exploits the natural tendency of rodents to explore novel objects more than familiar ones. On the day of testing, animals are first habituated to the experimental room. During the habituation phase, each rat freely explores the empty arena for 10 min. In the familiarization phase, two identical objects are placed equidistant from the walls, and the rat explores them starting from the center for 5 min before being returned to its home cage.

During the test phase, one familiar object is replaced by a novel object differing in shape, color, and texture, and the rat is reintroduced for 5 min in the arena. Exploration behavior is quantified by recording approaches toward each object, sniffing, and physical interaction (e.g., touching with the nose or forepaws). The experiment follows previously published protocols for Sprague–Dawley rats [33–35]. The arena and objects are cleaned between sessions to prevent olfactory cues from influencing behavior, and all animals are tested within the same time frame. Detailed procedures for behavioral scoring are described in the following sections.

3.5 Splash Test

The Splash Test is conducted in individual transparent cages by spraying a 10% sucrose solution onto the dorsal coat of the animals (Fig. 3B). The primary parameters assessed include total grooming duration, representing the cumulative time spent grooming, and grooming latency, defined as the time elapsed from sucrose application to the initiation of the first grooming bout. During grooming, discrete movements are scored, including face washing (using the forepaws to wipe or rub the face and head), body grooming (licking or rubbing of the flanks, shoulders, and dorsal coat using forepaws and mouth), and leg grooming (licking or cleaning of the hind limbs). All grooming behaviors are recorded for 5 min to provide a complete and detailed assessment of each subject. All animals are tested within the same experimental timeframe, and the procedure is performed individually in a clean cage to prevent social interference and minimize environmental confounds. Grooming behavior is recorded and quantified as outlined in the behavioral scoring and outcome measures section.

3.6 Sucrose Preference Test

Before testing, animals are habituated in their home cages to the presence of two identical drinking bottles containing water for 24 h. This habituation phase is essential to minimize side bias and to ensure that subsequent differences in fluid consumption reflect motivational preference rather than positional drinking habits. No food or water deprivation is applied at any stage of the procedure, allowing the assessment of spontaneous reward-driven behavior under baseline conditions. On the day of testing (Fig. 3D), rats are single-housed and provided with free access, for 3 h, to two bottles: one containing a sucrose solution and the other containing water [26]. During the test session, environmental noise and handling are kept to a minimum to prevent stress-induced alterations in drinking behavior. All drinking behaviors are monitored and quantified to provide a detailed evaluation of the behavior under baseline conditions. The Sucrose Preference Test is conducted within a consistent experimental timeframe for all animals, and each procedure is performed individually in a clean cage to prevent social interference and minimize environmental confounds. Anhedonic behavior is recorded and quantified as outlined in Sect. 4.

4 Behavioral Scoring and Outcome Measures

Behavioral outcomes in the offspring are quantified using a combination of manual observation and, when available, automated video-tracking approaches. All manual scoring is performed by operators blinded to experimental groups to minimize observational bias.

4.1 Quantification of Social Behavior

Social behavior is assessed using the SI Test, which provides a normalized index of social motivation by comparing interaction with an unfamiliar conspecific to exploration of an empty enclosure. The SI ratio is calculated as:

- SI Ratio = [Time spent interacting with the unfamiliar rat/(Time spent interacting with the unfamiliar rat + Time spent interacting with the empty enclosure)]

This ratio provides a normalized measure of social approach and preference [36]. Higher SI ratios indicate stronger social motivation and preference, whereas lower ratios reflect social avoidance and reduced social engagement.

4.2 Quantification of Cognitive Function via Novel Object Recognition Test

Cognitive and memory function is quantified by calculating a discrimination index (DI) based on the differential exploration of novel versus familiar objects during the test phase. Positive discrimination scores indicate intact recognition memory, whereas reduced or absent preference for the novel object suggests cognitive impairment [37]. For both sessions, object exploration time is measured, and the DI and the percent of total investigation time are calculated for each animal as follows [38]:

- DI: [(time spent with the novel object − time spent with the familiar object)/(time spent with the novel object + time spent with the familiar object)]
- Percent of total investigation time = [Time with novel object/(Time with novel object + Time with familiar object)] × 100

These measures quantify individual cognitive performance across subjects and allow standardized comparison between animals.

4.3 Behavioral Assessment of Motivation and Self-Care

Motivational and self-care behaviors are evaluated using the Splash Test. Grooming activity following sucrose application is analyzed in terms of both latency to initiate grooming and total grooming duration. Reduced frequency or duration of these behaviors, or increased latency to initiate grooming, is interpreted as decreased self-care and motivational drive, indicative of a depressive-like phenotype.

Total grooming duration represents the cumulative time spent engaging in grooming behavior during the test session. It can be expressed either directly as seconds of grooming or as a percentage of the total observation period (5 min), calculated as follows [39]:

- Total Grooming Duration (%) = (Time spent grooming (s)/300 s) × 100

Grooming latency is defined as the time elapsed between sucrose application and the onset of the first grooming bout.

4.4 Assessment of Hedonic and Motivational Responses Through Sucrose Consumption

Hedonic processing is examined using the Sucrose Preference Test. Sucrose intake is calculated as the proportion of sucrose consumption relative to total fluid intake. At the beginning and at the end of the test, consumption of both solutions is quantified by weighing the bottles and analyzed using the following formula [40]:

- Sucrose Preference (%) = [Sucrose intake/(Sucrose intake + Water intake)] × 100

Reduced sucrose preference is interpreted as an index of anhedonia and diminished reward sensitivity, whereas higher sucrose preference reflects intact hedonic processing and motivational drive.

4.5 Assessment of Susceptibility and Resilience Following Prenatal Stress

Behavioral outcomes across multiple domains can be used to stratify animals into resilient or vulnerable subgroups, providing a framework to assess individual variability in response to PNS [25]. Different approaches can be applied to identify resilient and vulnerable subjects. One commonly used method applied in the SI test involves calculating a standardized performance ratio for each animal relative to the control group, with classification based on the mean ± 1 standard deviation [41]. The following operational steps can be applied:

- Data collection: quantify the behavioral scores.
- Threshold determination: define resilient and vulnerable subgroups using the control mean − 1 standard deviation.

This framework allows for a quantitative and reproducible assessment of individual differences in social domain, facilitating subsequent biological analyses of factors associated with resilience or vulnerability in response to PNS exposure.

5 Translational Notes

The interpretation of behavioral modifications observed in animal models of PNS requires a translational perspective. Regarding maternal behavior, measures such as arched-back nursing, blanket or passive nursing, licking and grooming, off-nest activity, self-grooming, eating, drinking, and the number of nest exits can be considered indicators of both parental competence and maternal well-being. Particularly, self-directed behaviors may reflect alterations in affective state, including anhedonia or anxiety-related phenotypes [42], suggesting that exposure to PNS can negatively impact maternal

well-being during the postpartum period. In line with this interpretation, the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition, Text Revision reports that approximately 50% of postpartum major depressive episodes have their onset during pregnancy [43]. Conversely, pup-directed behaviors may be interpreted as expressions of parental caregiving abilities [44]. Therefore, alterations in these behaviors (e.g., a shift from active nursing patterns to more passive nursing strategies) may indicate that PNS also affects this critical domain of maternal functioning. Supporting this translational link, a recent systematic review reported that mothers who experienced anxiety and depressive symptoms during pregnancy were more likely to exhibit alterations in protection-related caregiving behaviors [45].

With respect to offspring behavior, assessments conducted at different PNDs allow for the evaluation of multiple behavioral domains across neurodevelopmental trajectories, capturing age-dependent changes that may parallel psychological constructs relevant to human psychopathology. For example, the SI test provides measures of social motivation, anxiety-related avoidance, and stress-related vulnerability, whereas the Splash Test is commonly used to assess self-care and motivational behavior [27, 46]. Similarly, the Novel Object Recognition Test evaluates recognition memory and cognitive performance, while the Sucrose Preference Test serves as an index of anhedonia [30, 47]. Taken together, these behavioral paradigms enable a multidimensional characterization of PNS-exposed animals, encompassing social functioning, affective state, and cognitive processes.

Beyond behavioral phenotypes, converging evidence indicates that PNS is associated with alterations in specific molecular and metabolic pathways within brain regions implicated in stress and emotion regulation, including the ventral hippocampus and the prefrontal cortex [3, 48]. Further investigation of these neurobiological alterations may strengthen the translational relevance of animal models, as structural and functional changes in the hippocampus, as well as impaired activation of the dorsolateral prefrontal cortex, have been observed in patients with depressive symptoms [49, 50].

In conclusion, the PNS model offers an experimental framework for investigating how early environmental adversity may contribute to vulnerability across generations. By integrating maternal and offspring behavioral phenotyping, this model facilitates the examination of associations between prenatal exposures and long-term alterations in affective, cognitive, and social functioning. Moreover, developmentally staged behavioral assessments provide insight into sensitive periods during which early perturbations may influence neurodevelopmental trajectories, with potential implications for future research in humans, particularly in the context of risk stratification and prevention.

6 Notes

1. To minimize stress associated with human handling and contact, and to avoid additional olfactory cues that may affect experimental outcomes and increase variability, several precautions are recommended. The same experimenter(s) should conduct all procedures consistently from the beginning to the end of the protocol. In addition, it is advisable to avoid the use of perfumes or other strong scents. All procedures should be conducted in accordance with institutional animal welfare regulations and national guidelines, and under the supervision of the animal welfare body and responsible authorities.
2. Careful consideration should be given to the order in which behavioral tests are administered within a test battery. As a general principle, testing should begin with the least stressful tasks and progress toward more stressful procedures, with the most aversive tests performed last. Assessments of anxiety-like and exploratory behavior should always be conducted first, as these measures are highly sensitive to prior experience and stress exposure. In contrast, tasks evaluating cognitive performance should be scheduled at later stages of the testing sequence. Although specific test sequences may vary depending on the experimental design, this general ordering principle is widely adopted to minimize stress-related confounds.
3. When female offspring are included in behavioral assessments, sex should be considered as a biological variable. Where appropriate, the estrous cycle stage should be monitored and balanced across experimental groups. If estrous cycle monitoring is not performed, this should be explicitly reported and justified, as hormonal fluctuations may influence behavioral outcomes and contribute to variability.

Conflict of Interest

The authors have no conflicts of interest to declare that are relevant to the content of this chapter.

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Chapter 12

Implementing Scarcity-Adversity Paradigms in Rodents to Study Disrupted Caregiver–Infant Interactions and Offspring Effects

Melissa Salazar , Jeffy Jackson and Millie Rincón-Cortés

Abstract

The scarcity-adversity paradigm, a variation of the limited bedding and nesting (LBN) model, is a well-validated rodent paradigm for studying the effects of postpartum adversity on maternal behavior and of early life adversity on neurobehavioral development in the offspring, respectively. This paradigm mimics postpartum resource scarcity by reducing bedding and nesting materials available to the dam during the early postpartum period. This impoverished home cage environment disrupts maternal caregiving patterns, leading to increased rough handling of pups and fragmented care. Thus, this manipulation recapitulates key features of low-resource/high-stress environments observed in human populations, making it highly relevant for investigating the neurobiological mechanisms underlying environmentally induced changes in maternal brain and behavior that can program long-lasting consequences in the offspring. This chapter provides a detailed, practical protocol for implementing the scarcity-adversity paradigm, equipping researchers with the methodological guidance needed to study its effects on maternal caregiving and offspring development.

Keywords Resource scarcity, Adversity, Maternal behavior, Development, Caregiver–infant interactions, Rodent models

1 Introduction

Early life adversity (ELA) is a potent risk factor for the development of affective, cognitive, and other stress-related disorders across the lifespan [1–4]. In both humans and animals, disruptions to the early caregiving environment can profoundly shape neural circuit development, stress physiology, and socioemotional behavior [5–11]Rodent models have been particularly valuable for mechanistic studies of ELA, as they allow precise experimental control over the timing, duration, and nature of environmental perturbations during sensitive developmental windows [12–14]

Rodent models that manipulate caregiving resources have been especially influential in advancing our understanding of how early

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environmental stressors alter maternal behavior and shape offspring development. Among these, the limited bedding and nesting (LBN) model has been widely adopted and has generated critical insights into the consequences of fragmented and unpredictable maternal care [15–17]. LBN involves restricting bedding and nesting materials and housing dams and pups on a wire mesh floor. This configuration reliably alters maternal behavior and impacts offspring stress physiology, neural development, and behavior across development [18–21].

The scarcity-adversity paradigm was developed as a complementary approach that emphasizes disruptions in maternal caregiving induced by resource scarcity while maintaining a more typical home cage physical environment. In contrast to LBN models that incorporate wire mesh flooring, scarcity-adversity procedures are conducted in standard solid-bottom cages, without mesh exposure [11, 16]. Resource limitation is achieved through a controlled reduction in bedding (and, in some implementations, the absence of nesting material), while preserving a familiar cage structure for both dam and pups. This impoverished home cage environment prevents the construction of an adequate nest, which in turn induces stress-related changes in maternal behavior [22]. These include increased nest-building attempts, rough or inefficient pup handling, stepping, shoving or dragging of pups, and a higher incidence of scattered litters [23–26]. Despite these disruptions, dams continue to provide nutrition, and pups typically maintain normal weight gain and physical development. This feature enables researchers to dissociate the effects of altered caregiving quality from those related to nutritional deprivation or overt physical stress.

By modeling resource scarcity at the level of the caregiver, rather than through maternal separation or direct physical manipulation of pups, the scarcity-adversity paradigm captures key aspects of poverty-related stress with high ecological validity. This approach more closely reflects real-world scenarios in which caregivers exposed to environmental stressors, including economic hardship, exhibit altered or inconsistent parenting behaviors, rather than complete absence of care [27–32]. In the sections that follow, we describe detailed methodological considerations for implementing the scarcity-adversity paradigm, including critical design parameters, commonly used assessments, expected outcomes, and troubleshooting recommendations.

2 Materials

2.1 Animals and Husbandry

To generate litters for scarcity-adversity studies, researchers have either mated male and female rats after 8 weeks of age using standard breeding procedures or used timed-pregnant female rats. If breeding in-house, mated female rats should be routinely monitored and

individually housed after pregnancy is confirmed (e.g., via the presence of a vaginal plug or significant weight gain, typically >40 g). This is done to ensure that the male has no contact with the pups, which reduces the risk of infanticide. Timed-pregnant females should be housed individually upon arrival and allowed to acclimate to the animal facility for at least 3–5 days before giving birth. Mated (i.e., pregnant) females are housed individually with additional bedding and nesting materials, a hutch, and ad libitum food and water access. Pregnant dams should be monitored daily (ideally twice a day) to determine the litter's date of birth. Signs that a rat dam is about to give birth include intense nest-building, restlessness, increased appetite, a markedly rounded abdomen, and small amounts of bloody vaginal discharge (i.e., spotting) around the bedding or cage. Parturition involves the dam stretching, hunching, and cleaning herself, and can last a couple of hours [33]. After the entire litter is born, the dam cleans, clusters, and begins nursing the pups [33]. We recommend leaving the dam undisturbed during this process, and if possible, for the first 24 h following parturition to avoid infanticide by the dam.

See Sects. 4.2–4.4 for guidance on strain selection, the use of multiparous animals and cross-fostering designs.

2.2 Housing Materials

To observe home-cage maternal behavior, animals should be housed in rat cages made from Plexiglas or an alternative material (e.g., polycarbonate, polypropylene) that allows a clear view of the dam and litter. Studies employing scarcity-adversity paradigms in rats have used aspen shavings or corncob bedding, with sufficient bedding to create a thin layer of bedding that covers the cage floor (100–1000 mL, depending on cage size). In one commonly used implementation, dams and litters assigned to the scarcity-adversity condition receive 500 mL of corncob bedding and no nestlet, whereas control dams receive 1800 mL of corncob bedding and a paper nestlet (~7 g) [34–37]. Bedding volumes are measured using graduated beakers (see Fig. 1 for bedding measurements and representative images of control and scarcity-adversity rat cages).

2.3 Observation and Assessment Tools

Accurate observation of maternal behavior in rat dams provides valuable data as it allows researchers to detect instances of sensitive and insensitive caregiving, as well as potential changes in self-directed behaviors, in control and scarcity-adversity dams. Previous studies have demonstrated that resource scarcity reliably increases insensitive pup-directed behaviors and stress-related behaviors in the rat dam [24–26, 34, 37]. Recommended equipment includes:

- Behavioral coding software or standardized observation sheets
- Video recording equipment (recommended for behavioral coding)
- Manual recording materials (e.g., clipboard, pen)
- Timer/stopwatch

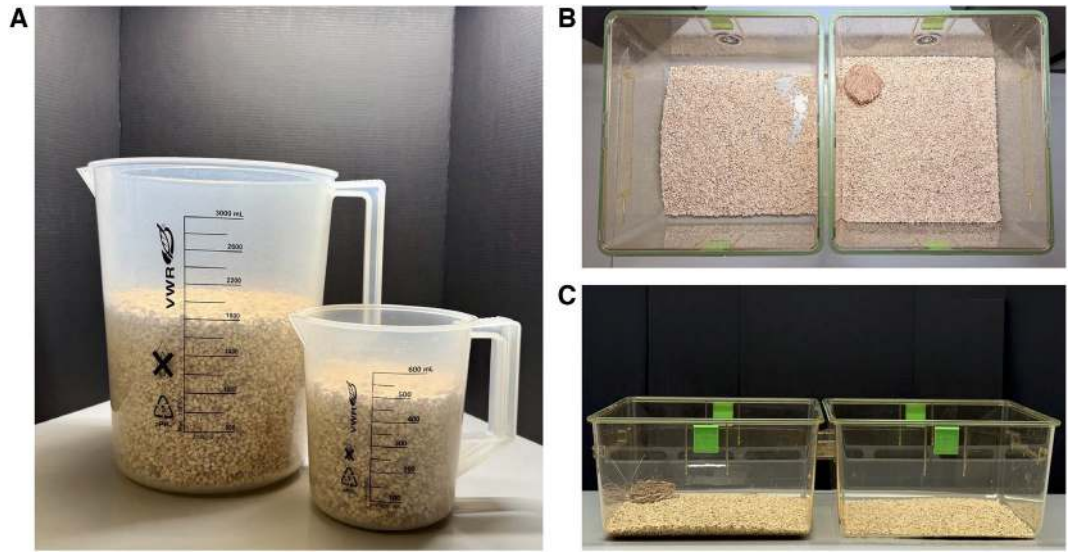


Fig. 1 *Creation of control and scarcity-adversity rat cages.* **A,** Use of graduated beakers to measure bedding amounts. **B,** A top (aerial) view of a scarcity-adversity cage (left) and control cage (right). **C,** A side view of a control cage (left) and a scarcity-adversity cage (right)

3 Methods

3.1 Preparation Prior to Implementation of Scarcity-Adversity (Pre-PD/PND 0)

1. Start breeding procedures or order timed-pregnant dams to arrive approximately 1 week before expected parturition.
2. House dams individually in standard housing conditions with 1800 mL bedding and one nestlet. Our group typically adds additional bedding (500 mL) and nesting (1 extra nestlet) material during this period.
3. Monitor daily for signs of parturition (nesting behavior, vaginal bleeding).
4. Check for births at least twice daily (morning and afternoon). The day of birth is designated as postpartum (PD) or postnatal (PND) 0. Our lab uses the PD designation in studies in which the dam is the focus of the study, and PND in studies in which the pups are the focus.

3.2 Cage Setup, Litter Culling, and Group Assignment (PD/PND 2)

1. Prepare control and scarcity-adversity cages. For the control cages, use a graduated beaker to measure out 1800 mL of corncob bedding into a clean, empty standard rat housing cage and add a paper nestlet. For the scarcity-adversity cages, measure out 500 mL of bedding into a clean, empty standard rat cage but do not add a nestlet (see Fig. 1).

2. Identify the sex of each pup using anogenital distance and cull litters to 10 pups (with equal pup sex distribution whenever possible) to standardize litter size.
3. Mark pups for individual identification if longitudinal tracking is required.
4. Weigh the dam and all pups to record baseline weights.
5. Randomly assign litters to control or scarcity-adversity conditions and place them in their respective cages, balancing for average pup weight.

Note: Ensure to transfer a small amount of the original home cage bedding to the new cage to maintain familiar scent markers.

See Sect. 4.1 for guidance on selecting an exposure window.

3.3 Maintenance of Scarcity-Adversity Conditions

1. Visually inspect cages as necessary for animal welfare monitoring and maternal observations. Otherwise, leave cages undisturbed.
2. Maintain the same cage and bedding conditions from PD/PND 2–9 (do not change bedding). If soiled bedding must be addressed for animal welfare reasons, this should be done only after the end of the maternal observation period (typically through PD/PND 6) and consist of quickly removing dirty bedding and adding a comparable amount of clean bedding, while preserving a pinch of the old bedding to maintain olfactory continuity.
    - **Note:** In the scarcity-adversity condition, some dams have been observed to hoard food pellets and use them to make a nest. When this behavior occurs, any food present on the cage floor should be promptly removed. If the removal of the cage from the rack is required to facilitate this procedure, an equivalent number of control cages should also be temporarily removed and returned to the rack to maintain comparable handling across conditions. Additionally, some scarcity-adversity dams also engage in manipulation of the Lixit water valve (i.e., water play), which can result in damp bedding. In such cases, bedding should be replaced with clean, dry bedding to maintain appropriate housing conditions. See Sect. 4.7 for additional troubleshooting tips.
3. Ensure unrestricted access to food and water and replenish if needed without disturbing the cage more than needed.

3.4 Maternal Behavior Observations (PND 2–6)

1. Conduct systematic observations to quantify maternal behavior differences between groups (see Table 1 for a list of common maternal behaviors to score). To do this, observe each dam two to three times per day (morning, afternoon, and, optionally, evening) for 30 min per session. If conducting

maternal observations during the dark cycle, use red light to minimize disturbance. Video recording is recommended for later detailed behavioral coding and reliability analysis.

2. *Manual (live) scoring*:
   (a) Fill out the header of maternal observation sheet listing experiment, date, time, observation day, litter number, pup age, room temperature and humidity, and other applicable details.
   (b) Start the stopwatch/timer to begin the first 5 min segment, recording data in row 1 (see below). Annotate instances of behaviors by adding a tally mark for behaviors that occur within this segment. Do this for each segment. If using a 30 min observation period, this will be broken down into six 5 min segments on your data sheet.

Table 1
Maternal behaviors scored in control and scarcity-adversity litters

Behaviors to Score	Definition
Dam in/out of nest	The nest is defined as the area in which most pups are located within the cage. In nest refers to the dam being located within the nest, in physical contact with the litter. Out of nest refers to the dam being positioned away from the nest/pups, not in contact with the litter
Sorties	The frequency of nest exits and entries the dam makes
Nursing	Dam is in nursing posture with pups attached to nipples. Note if arched-back, blanket posture, or side posture. Must observe pup latched onto dam's teat to confirm nursing
Licking/grooming	Dam actively licking or grooming pups. This includes pup paws, body or head
Anogenital licking	Dam actively licking anogenital region of a pup
Nest building	Dam gathering and/or arranging bedding material or attempting to construct/maintain nest
Stepping on pups	Dam stepping directly on pups while moving through cage. Non-examples include placing front paws on pup to position for anogenital licking
Dragging pups	Dam dragging pups across cage floor without proper retrieval behavior. Pups are typically nipple attached when this occurs
Transporting pups	Dam carrying pups in mouth from one location to another. Note that this behavior is distinct from retrieval, in which the dam transports a pup that is outside the nest back into the nest
Pushing/shoving	Dam actively pushing pups away with snout or paws
Scattered litter	Pups are dispersed throughout cage rather than huddled in nest area
Self-Care	Dam is eating, drinking, grooming (licking herself), resting or sleeping
Chase/Bite Tail	Dam is exhibiting circling behavior directed toward her tail or biting her tail
Other	This can include other behaviors such as exploring or wall climbing

i. Row 1 is for the :00–05 min mark
ii. Row 2 is for the :05–10 min mark
iii. Row 3 is for the :10–15 min mark
iv. Row 4 is for the :15–20 min mark
v. Row 5 is for the :20–25 min mark
vi. Row 6 is for the :25–30 min mark

Note: *It is important to score only behaviors that you can actually observe.* Bedding and cage orientations can partially obscure behavior and may lead to incorrect assumptions. For example, a visible view of a pup latched onto the dam's teat indicates nursing.

3. *Automated video scoring*: Use instantaneous sampling (record behavior at fixed intervals, e.g., every 30 s) or continuous recording with duration/frequency measures. Calculate percentage of time spent in each behavior or frequency counts per session. For additional resources on automated behavioral scoring, please see the following texts [38, 39].

3.5 Termination of Scarcity-Adversity Paradigm (PD/PND 9)

1. On the afternoon of PD/PND 9, transfer animals to clean, new cages with standard housing conditions (1800 mL bedding and a nestlet).
2. Maintain standard housing conditions until weaning (typically PND 21–23).

3.6 Post-Adversity Period and Weaning (PD/PND 10–23)

1. Continue to monitor pup development and maternal behavior if needed.
2. Change cages once per week.
3. Wean pups on PND 23 and house same-sex animals together (matched for condition, 2–3 per cage).

See Sect. 4.8 for research applications and downstream assessments and Sect. 4.12 for expected outcomes.

4 Notes

4.1 Timing and Developmental Windows

Most rodent studies typically implement scarcity-adversity paradigms within the first two postnatal weeks. Commonly used exposure windows include PD/PND 1–7, 2–9, and 8–12. These periods are based on extensive research indicating that early postnatal development represents a critical window for mother-infant attachment formation and that disruptions during this time can produce enduring neurobehavioral effects in the offspring [16]. Some studies have also implemented modifications of scarcity-adversity so that it occurs

intermittently outside the home cage for briefer periods per day (e.g., 60–90 minutes per day over a 5-day period) [40]. The selection of a specific exposure window should be guided by experimental aims, including whether the primary focus is on maternal outcomes, offspring outcomes, or their interaction.

4.2 Strain and Species

Most studies implementing scarcity-adversity paradigms in laboratory rats have used outbred strains, including Sprague–Dawley (SD) and Long–Evans (LE). Notably, strain differences in maternal behavior during the early postpartum have been reported, with LE rats exhibiting greater pup contact and increased nursing compared to SD or Wistar strains [41]. In addition, strain-dependent differences in behavioral and physiological indices of stress reactivity have been reported, although effects appear to be sex-dependent [42–44]. Taken together, these findings suggest that strain differences in maternal responsivity to resource scarcity may exist and could thereby influence experimental outcomes. Pilot studies are recommended when using new strains. Although less commonly used, the scarcity-adversity procedure has also been adapted for use in mice [45, 46].

4.3 Multiparity Effects

The number of reproductive experiences (i.e., multiparity) can influence how dams respond to stressors and dampen their neurobehavioral consequences [47]. Consistent with this, prior reproductive experience alters stress responsivity and maternal behaviors, suggesting that parity itself represents a critical biological variable shaping postpartum adaptations to stress [48]. As a result, the effects induced by scarcity-adversity may differ between first-time (i.e., primiparous) dams and dams with multiple reproductive experiences (i.e., multiparous). Additionally, there is evidence that elevated corticosterone levels during the first pregnancy can influence subsequent pregnancy outcomes and behaviors in the rat dam [49]. To control for these parity-related effects, we recommend the use of nulliparous (i.e., virgin) females undergoing their first pregnancy. If multiparous dams are used, experimental conditions should be kept constant across pregnancies and clearly reported [16].

4.4 Cross-fostering

Cross-fostering designs enable the disassociation of prenatal versus postnatal contributions to offspring outcomes and are commonly employed in scarcity-adversity paradigms, including those developed by the Roth lab [50–52]. In these designs, pups are fostered to a different (i.e., foster) dam prior to the onset of scarcity-adversity, typically within the first postnatal day. By fostering pups shortly after birth, the postnatal environment can be isolated from prenatal and genetic factors. The successful implementation of cross-fostering requires careful matching of litters and dams, including postpartum age, litter size, sex composition, and parity, as these variables can independently influence maternal behavior and offspring development. Notably, cross-fostering itself constitutes an experimental

manipulation and may introduce transient stress to both dams and pups. Therefore, appropriate control groups and consistent handling procedures are essential for accurate interpretation of outcomes.

4.5 Experimental Design Recommendations

(a) *Sample size*: For behavioral studies with moderate-to-large effect sizes, we recommend a minimum of six to eight litters. However, larger sample sizes ($n = 10$–12 litters/condition) are recommended for more subtle outcomes or when examining sex-specific outcomes.

(b) *Randomization*: We recommend randomly assigning litters to experimental conditions while balancing pup weight. Depending on the number of litters available at a given time, simple, block, or serial randomization methods may be used.

(c) *Blinding*: Observers analyzing maternal behaviors and conducting downstream assessments should be blinded whenever possible.

(d) *Litter effects*: Pups within the same litter share genetics, maternal care, and microenvironment [53]. To control for litter effects, we recommend using only one pup per sex (one male, one female) per litter per endpoint to avoid pseudoreplication [54]. Alternatively, the entire litter may be treated as the experimental unit if using multiple pups from the same litter [53].

(e) *Multiple cohorts*: Experiments should be conducted across multiple cohorts to assess reproducibility and control for potential batch effects.

4.6 Ethical Considerations

Scarcity-adversity procedures must receive Institutional Animal Care and Use Committee (IACUC) approval. Throughout the procedure, animal welfare should be the top priority. Daily monitoring for signs of distress or animal welfare concerns is essential. Any signs of severe distress, failure to thrive, or serious injury should prompt immediate veterinary consultation and potential early termination of the protocol for affected animals.

4.7 Troubleshooting Tips

As with any early life manipulation, unanticipated issues may arise during the implementation of the scarcity-adversity paradigm. A troubleshooting guide outlining common problems, potential causes, and recommended corrective actions is provided below to facilitate standardized decision-making and reproducibility (see Table 2).

4.8 Research Applications and Downstream Assessments

The scarcity-adversity paradigm has often been combined with various behavioral, hormonal, and neurobiological assessments to better understand the impact of this perturbation on both the dam and the offspring. However, most studies have focused on characterizing offspring effects [16, 23]. Below, we summarize commonly used downstream assessments and research applications, recognizing that specific choices should be guided by experimental aims.

4.9 Behavioral Assessments

- *Infant behavior*: Many studies have evaluated mother-pup interactions such as latency and duration of contact with dam and/or nipple attachment, sibling interactions, nest search and odor choice tests, and ultrasonic vocalizations [25, 26, 40, 55–61].
- *Anxiety-like behavior*: Tests such as the open field, elevated plus/zero maze, and novelty suppressed feeding have been used [52].
- *Responses to threat*: This includes olfactory threat conditioning and predator odor responses [52, 62, 63].
- *Alterations in reward-related behaviors*: These have included tests based on behavioral responses to natural rewards such as sucrose consumption and/or preference, palatable food consumption, social play, social preference and/or approach, and sexual behavior [26, 35, 55, 64–68].
- *Cognitive function*: These have included spontaneous alternation, reversal learning, rule shifting, object recognition, and spatial working memory tasks [52, 65].

Table 2
Troubleshooting guide

Problem	Possible cause	Solution
Pups showing poor weight gain	Excessive maternal stress; inadequate nursing; possible illness	Monitor closely. If weight loss continues to exceed 10% or pups appear weak or cold, consider early termination of procedure for that litter
Dam cannibalizing pups	Extreme stress response; possible pre-existing pathology	Immediately terminate procedure for that litter. Exclude data. Consider whether this occurs repeatedly with same supplier/strain and switch vendors if necessary
No observable differences in maternal behavior	Insufficient bedding reduction; inadequate observation periods; strain differences	Verify bedding amounts with measuring cups. If the amount is high, try decreasing the amount of bedding. Increase observation frequency and/or duration. Extend the observation window across multiple postnatal days. Consider strain-specific validation
High inter-observer variability in scoring	Unclear behavioral definitions; insufficient training	Conduct inter-rater reliability testing (aim for ≥ 0.80). Use video recordings for training. Refine behavioral definitions
Bedding becomes excessively soiled	Normal with reduced bedding; more concentrated waste	Expected and acceptable. Do not perform full cage changes during observation days. If necessary, clean the cage by removing dirty bedding and replacing with an equivalent amount of clean bedding to maintain bedding scarcity
Pups showing injuries	Excessive rough handling; possible aggression	Monitor closely. Minor scratches are expected. If severe injuries occur, consult veterinarian and consider early termination

- *Responses to inescapable stressors*: Multiple studies have used the forced swim test to assess stress-related behavioral coping strategies [26, 52, 66, 67].
- *Maternal behavior in F1 and F2 generations*: This has been done by assessing the adult female offspring (F1) of rat dams exposed to scarcity-adversity (F0). Behavioral assessments have included naturalistic home-cage maternal observations as well as pup retrieval and pup-baited T-maze tests [36, 69]. These assessments can be extended to include the F1 offspring (F2 generation) if transgenerational effects are of interest.

4.10 Hormonal Assessments

Studies have traditionally examined corticosterone (CORT) and/or adrenocorticotropic hormone (ACTH) levels in the dam or pups during and after scarcity-adversity exposure, including later in development (e.g., peripuberty and adolescence) [37, 56, 64, 70].

4.11 Neurobiological Assessments

Many studies have employed neurobiological assessments during or following scarcity-adversity exposure. In addition, several of these have incorporated assessments across development. These include:

- *Assessment of neural activity and/or molecular markers*: c-Fos expression, 2-deoxyglucose (2-DG) autoradiography, protein expression (i.e., immunoblotting), epigenetic alterations, altered methylation patterns, mRNA levels, and histone acetylation patterns [25, 55–58, 62–64, 66, 71–77].
- *Neurochemistry*: In vivo microdialysis, pharmacological manipulations to test causal relationships between hormone levels and/or brain activity patterns and behavioral outcomes [40, 55, 59, 64, 66, 70].
- *Structural analyses*: Dendritic morphology, cell density, region volume, neurogenesis, perineuronal nets, synaptic markers, and telomere length [56, 63, 77, 78].
- *Neuroimaging*: Resting-state functional magnetic resonance imaging, seed-based functional connectivity analyses [67].
- *Electrophysiology*: Single-unit extracellular recordings, whole-cell recordings, and local field potential (LFP) recordings [24, 26, 40, 55, 56, 59, 77, 79].
- *Circuit-level manipulations*: Optogenetics, chemogenetics [55, 77].

4.12 Expected Outcomes

Implementation of scarcity-adversity paradigms has been associated with reproducible alterations in maternal behavior and offspring outcomes across studies, although the magnitude and nature of these effects can vary depending on the timing of scarcity-adversity, age of assessment, and experimental design (e.g., presence of cross-fostering). These results are summarized below.

4.13 Maternal Behaviors

Scarcity-adversity dams have been reported to exhibit alterations across several maternal behaviors. These changes often include:

- Increased time spent nest-building
- Increased stepping on pups
- Increased dragging of pups
- Increased transport of pups
- Increased shoving of pups
- Increased nest entries/exits (sorties)
- Increased instances of pups outside of the nest (scattered litter)

These alterations have been most consistently reported when scarcity-adversity is implemented between PD/PND 2–9 [24, 34–37], with PD/PND 2–6 being the most robust period for detecting these changes [24, 34]. Day-by-day analyses of scarcity-adversity-induced changes in maternal behavior indicate that nest entries and exits are reliably affected, suggesting greater fragmentation/unpredictability of maternal behavior [34]. Stepping and dragging are also frequently observed across most observation days, highlighting persistent adverse pup-directed actions [34, 37]. With regard to sensitive caregiving behaviors, mixed effects have been reported from PD/PND 2–9, with studies reporting both increases in nursing [34, 36] and no change in nursing and pup grooming [35, 37].

Similar increases in adverse pup-directed caregiving behaviors have also been reported during PD/PND 8–12 [25, 26, 56]. However, these studies also report consistent reductions in sensitive caregiving behaviors, such as licking and nursing, which are not commonly observed during the PD/PND 2–9 period.

4.14 Pup Physical Development

In contrast to the LBN procedure, in which immediate and long-term reductions in pup weight gain are observed [21, 80], the majority of studies show that pups reared in scarcity-adversity conditions typically exhibit normal weight gain throughout the procedure and beyond [16, 26, 34, 36, 40, 56]. A small number of studies have observed modest reduction in weight prior to PND 10; however, these differences were transient and resolved by approximately PND 13 [36, 61]. With regard to developmental milestones, several measures appear to be impaired in scarcity-adversity pups compared to controls [34]. Specifically, male pups exposed to scarcity-adversity exhibit earlier emergence of the lower incisors compared to control males. Male and female pups exposed to scarcity-adversity display impaired surface righting and grasp reflexes compared to their control counterparts on PND 6 and PND 8, respectively [34]. Finally, female rat pups exposed to scarcity-adversity exhibit impaired cliff avoidance responses on PND 12, as indexed by longer latencies to turn away from the cliff edge.

4.15 Long-Term Behavioral and Neural Outcomes

Offspring reared under scarcity-adversity conditions typically exhibit a wide variety of long-lasting behavioral disruptions. Most studies have assessed depression-related phenotypes, which include social behavior assays, forced swim, and sucrose consumption during adolescence and/or adulthood [26, 35, 52, 55, 66–68]. Emerging evidence suggests that these outcomes may be age- and sex-specific [81], with a recent study showing that adolescent males engage in reduced social play behaviors whereas adolescent females display reduced sucrose preference [35]. Offspring outcomes may also depend on the type of experimental design used, as cross-fostered pups that experienced scarcity-adversity from PND 1–7 did not exhibit changes in these behaviors during adolescence, but displayed disrupted behavioral function during adulthood [52].

There is also evidence for enduring alterations in corticolimbic connectivity, amygdala reactivity and function, ventral tegmental area dopamine neuron activity as well as lateral habenula structure, function and connectivity [24, 26, 55, 66–68, 77].

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Chapter 13

Witness Social Stress in Rats: A Guide to the Use of an Inclusive Social Stress Paradigm in Behavioral Neuroscience

Margherita Barbetti , Andrea Sgoifo and Luca Carnevali

Abstract

The social defeat paradigm has been instrumental in forming our understanding of the neurobiology relevant to social stress-related disorders. However, the classic procedure is limited by the difficulty in distinguishing between the physical and psychological components of social defeat stress and its limited applicability to female rodents and different age-groups. In this chapter, we describe a refinement of this paradigm, the so-called "witness social stress" model, where the experimental rodent experiences the sensory and psychological components of aggression between two male conspecifics from the safety of an adjacent compartment within the same cage. This protocol variant not only mitigates the confounding variables associated with the physical component of social defeat, but also allows the inclusion of rodents of both sexes and different age-groups. In this chapter, we outline the methodology for implementing this paradigm in rats and highlight the utility of the witness social stress model for elucidating central and peripheral processes of social stress-related vulnerabilities in a more comprehensive way.

Keywords Witness stress, Rats, Social stress, Psychological stress, Sex differences

1 Introduction

Psychosocial stress is perceived as one of the most common and intense forms of environmental challenge encountered by people in daily life. Nowadays, there are numerous pieces of evidence linking psychosocial stress exposure to increased risk for psychopathologies, including depression and anxiety, as well as comorbid conditions such as cardiovascular diseases [1, 2]. However, existing studies regarding social stress-related vulnerabilities in humans tend to be more epidemiological than pathophysiological in nature. In this context, rodent models employing naturalistic social stress paradigms offer an indispensable means to explore biobehavioral mechanisms underlying social stress-related risk in a controlled setting. Among the various stress paradigms developed for this purpose, it is generally assumed that a focus on social stressors enhances the translational value of rodent models [3]. This perspective stems from the view that the

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social environment can serve as a significant source of stress for many animal species, including humans and rodents, whereas other laboratory stress procedures, such as restraint or electric shocks, are often criticized as being artificial [4]. A widely utilized social stressor in rodents is the social defeat model, which is based on the classical resident-intruder paradigm [5]. Initially designed for preclinical aggression research in a semi-natural laboratory setting, this paradigm has proven effective in investigating central and peripheral processes of social stress-related vulnerabilities, contributing to a substantial body of literature on the biobehavioral consequences of social stress exposure [6]. However, like any stress model, the social defeat paradigm has constraints that limit its translational value, such as the difficulty in discriminating between its physical and psychological components, and the limited applicability to females and different age-groups. Here, after providing an overview of the social defeat paradigm, we will discuss how its inherent limitations can be overcome by adding a witness component to the model. Then, we will describe the methodology for the use of the "witness social stress" model in rats. We will conclude by providing some examples of the utility of this model for a more comprehensive assessment of social stress-related processes in behavioral neuroscience.

1.1 From Social Defeat to Witness Social Stress

The social defeat paradigm exploits social conflict among individuals of the same species to induce a state of stress. Negative agonistic interactions are achieved by introducing the experimental animal (i.e., intruder) into the territory (i.e., home cage) of an older, aggressive, dominant male conspecific (i.e., resident). Social defeat of the intruder is the result of recurrent threatening behaviors and physical assaults by the resident, and is manifested through distinct behavioral patterns (e.g., submissive supine posture) that signify social subordination [7]. The experience of defeat extends beyond mere physical interaction, as it involves a subsequent psychological threat component induced by the presence of the aggressive resident in the vicinity of the intruder, but with physical separation between them, allowing for psychogenic exposure to the resident without physical harm [8, 9]. Despite variations in the adopted experimental protocol, as detailed in the review by Hollis and Kabbaj [9], consistent findings across several studies underscore the capacity of social defeat stress to elicit profound biological changes, ranging from behavior to molecules. For example, intruder animals exhibit depressive and anxiety-like behaviors [10, 11], alongside neuroendocrine alterations, such as increased corticosterone levels [12]. Additionally, they display a range of cardiovascular effects, such as increased heart rate [13] and blood pressure, impairments in cardiac autonomic circadian rhythms [14], and signs of cardiac hypertrophy [15]. The effects of chronic social defeat also encompass brain alterations, with socially defeated rodents displaying synaptic loss and damage in the medial prefrontal cortex [16], decreased hippocampal neurogenesis [17],

and neuroinflammation [18]. However, it is crucial to acknowledge certain inherent drawbacks of this paradigm. One important limitation regards the difficulty of applying this stress protocol to female rodents. Indeed, social defeat exploits a male-specific behavior—aggression—which is not typically expressed by female rodents during social interactions, except for specific circumstances, such as during the lactation period [19], or for specific strains or species (e.g., Syrian hamsters and California mice). Moreover, aggressive male rodents generally do not attack female intruders. Attempts to circumvent this latter limitation include the application of male urine onto females to induce male-female aggression [20], or mediobasal-hypothalamus lesions to induce female–female aggression [21]. However, these approaches, in addition to technical and logistical challenges, compromise the ethological validity of the social defeat paradigm. One of the main consequences of the limited application of social defeat to females is their widespread underrepresentation in preclinical social stress research. This is particularly concerning considering that women are twice as likely to experience depression as men [22], and recent research has demonstrated their heightened vulnerability to the adverse effects of psychosocial factors on cardiovascular disease [23]. Given the importance of sex in social stress-related vulnerabilities, it is important to address this gap by incorporating new sex-inclusive social paradigms in preclinical social stress research. This approach will not only enhance our understanding of sex differences in social stress-related central and peripheral processes, but also guide the development of preventive and therapeutic strategies to better target stress-related psychopathologies in a tailored sex-specific manner.

Also, it is essential to extend the focus beyond adult rodents and consider the significance of studying different age-groups. Childhood and adolescence are critical windows for the development of psychopathologies. Trauma or chronic stress exposure during these stages of life is linked to the onset of mood disorders either shortly thereafter or later in life [24]. Critically, the dependence of the social defeat paradigm on adult male territorial aggression limits its applicability to younger populations. Relatedly, older rodents are hardly studied in preclinical stress research. Therefore, there is a pressing need for new models of social stress that can be applied across the life span.

Lastly, even if the social defeat protocol encompasses a psychological threat component for the intruder, the effects of the physical interaction and sensory contact with the resident are intertwined. Therefore, even if the possibility of physical injury (and the resulting pain and inflammatory responses) is rare (especially in rats) and constantly monitored by the researchers, injury-related immune and inflammatory responses represent another potential confounding factor, challenging the possibility of generating information in rodents that can be translated into social stress-related processes in humans, which are generally rooted in psychological and emotional aspects.

To address these limitations, more recent studies have added a witness component to the model, in which a rodent (of either sex) experiences the sensory and psychological aspects of an aggressive interaction between two males from the safety of an adjacent compartment within the resident's cage [25]. The "witness social stress paradigm," also referred to as the "vicarious social defeat stress paradigm," was originally developed in mice to study the consequences of pure psychological stress exposure, modeling the experience of the so-called "vicarious trauma" [26, 27]. Unlike social defeat, with the intruder as the experimentally stressed rodent, in the witness social stress paradigm the physical interaction between the intruder and the resident is used as a source of stress for the witness rodent, which is placed behind a perforated partition. As such, the experimental (witness) rat is exposed only to sensory, but not physical, contact with the resident–intruder dyad, avoiding potential confounds related to physical interaction. Furthermore, this paradigm allows the inclusion of witness rodents of both sexes and different age-groups, enhancing its versatility. While the procedure of witness social stress in mice has been meticulously detailed by Sial and colleagues [26], a complete description of the same protocol in rats is lacking. In the following paragraphs, the procedure used for rats in our laboratory will be outlined.

2 Experimental Set-Up

2.1 Animals

For the witness social stress paradigm, several groups of rats are needed, which are listed below:

- *Witnesses*: male or female rats (*Rattus norvegicus*) of any age of the selected rat strain.
- *Intruders*: adult (from 75 to 90 days at the beginning of the protocol—*see* **Note 1**) male rats of the selected rat strain.
- *Residents*: adult (≥6 months old) male rats weighing ≥400 g, selected for their level of aggressiveness (see below for details). In principle, territorial aggression can be expressed by all rat strains. However, levels of aggressiveness vary among strains. In our laboratory, we use Wild-type Groningen rats, a laboratory-bred but originally feral strain of rats, which are characterized by a higher frequency of aggressive animals compared to other strains commonly used in rodent research (e.g., Wistar rats; for a comparison see [28]). Nevertheless, adult male retired breeder Long-Evans rats are also frequently used as residents [29, 30].
- *Partners*: oviduct-ligated female rats (≥2 months old). In the procedure used in our laboratory, female rats are anesthetized with isoflurane (2% in 100% oxygen). A lateral midline incision

is made on one flank to access the abdominal cavity; the ovary and the fallopian tube are then carefully exposed, and a ligature is tied between the uterine horn and the distal part of the fallopian tube. Afterwards, the ovary and fallopian tube are returned to the abdominal cavity and the skin incision is closed using surgical sutures. This procedure is then repeated on the other flank. This method preserves hormonal integrity and ensures regular receptivity of females. Once fully recovered from surgery (usually after 2 weeks), female partners are housed with male residents to enhance their territoriality.

2.2 Laboratory Environment

Several factors can influence the stress response of experimental rats. Thus, careful consideration of these factors is essential when planning and designing experiments to ensure the standardization and efficacy of witness social stress protocols.

- *Controlled laboratory environment:* rats should be kept in rooms with controlled temperature ($22 \pm 2\,^{\circ}C$) and humidity ($50\% \pm 10\%$). Furthermore, the witness social stress procedure should be conducted in a designated room to create an isolated stress-inducing context. This "stress room" should be protected against major interruptions, such as sudden noise bursts or inadvertent disturbances by people not involved in the research.
- *Circadian time of the stress procedure*: given that rodents are nocturnal species, it is recommended to house rats under a reversed light–dark cycle and to perform the stress procedure during the dark (active) phase.
- *Witness stress cages*: as for the "classic" social defeat procedure, territoriality represents a critical point for the witness social stress paradigm. Adult rats will establish territoriality when given sufficient living space and time to acclimate to the environment. Therefore, resident cages should offer sufficient space for the development of territorial behavior. Figure 1 illustrates a schematic representation of the cages employed in our laboratory, which should be designed with two compartments to allow the separation of the witness rat from the resident-intruder pair. These dimensions also allow the witness rat to have adequate space to freely behave while witnessing social defeat. A transparent Plexiglas partition with holes permits visual, auditory, and olfactory exposure to social defeat.
- *Resident aggressiveness*: as previously noted, territoriality is strongly enhanced in the presence of a female partner. Each resident should be paired with a female rat for at least one week before the start of the experiments. Screening of residents before the start of the experimental procedure is advisable to verify the presence of high levels of aggressive behavior. This can be done by introducing an unfamiliar male rat into the resident

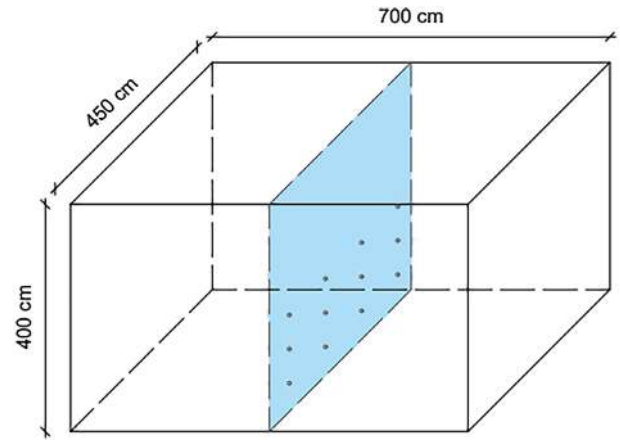


Fig. 1 *Witness stress cage*. Schematic representation of cages for the witness stress procedure employed in our laboratory

cage and evaluating the behavior of the resident animal. The screening procedure used in our laboratory lasts for 8 min, and it is repeated for three consecutive days. The number of attacks, as well as the latency to the first attack, are recorded. Typically, only resident rats with average latencies <180 s and displaying more than four attacks on average are selected for the witness stress procedure. Aggressive attacks are characterized by several key behaviors, such as the "offensive upright" posture (standing on hindlegs facing the opponent), "lateral threat" (moving slowly in a sideways direction toward the opponent), or "submission" (when the dominant rat is standing over the opponent, keeping it down by pushing against the cage floor or walls). Resident aggressiveness may vary over time and depending on environmental conditions (*see* **Notes 2–4**).

- *Experimenter presence*: The presence of one or more experimenters in the room is required to monitor the frequency and intensity of attacks. However, it is recommended that no more than two experimenters are present simultaneously. Moreover, efforts should be made to minimize odors (such as perfumes) and noise in the room.

3 Methods

3.1 Equipment

1. Resident rat housing polycarbonate cages ($L \times W \times H$: $70 \times 45 \times 40$ cm) with sanitized wood-particle bedding.
2. Polycarbonate cages for individual rat housing ($L \times W \times$ H: $48 \times 37.5 \times 21$ cm) with sanitized wood-particle bedding.

3. Plexiglas transparent partition with holes (diameter: 0.5–0.8 cm).
4. Digital stopwatch.
5. Notebook.

3.2 Procedure in Rats

1. Initially, experimental rats are randomly assigned to a witness social stress or control procedure.
2. Each witness rat is randomly assigned to the observation of an intruder rat throughout the experimental protocol (*see* **Note 5**).
3. Determine the resident-intruder pairs and record them in the notebook.
4. Remove any distracting elements from the resident's cage, including food pellets, water bottles, and environmental enrichment items.
5. Temporarily remove the female partner from the resident cage and house it in an individual cage with access to food and water during the stress procedure. Tags should be used to recognize each female partner and prevent their accidental reintroduction into a different resident cage.
6. Insert the partition into the resident cage to create two compartments, confining the resident rat in one compartment.
7. After 5 min, introduce the witness rat to the other compartment (*see* **Note 6**).
8. Immediately confine the intruder rat to the same compartment of the resident and start the stopwatch. The stress procedure adopted in our lab lasts 15 min. During the stress phase, record the number of attacks received by each intruder.
9. At the end of the stress phase, first remove the witness from its compartment and return it to its home-cage.
10. Wait for the physical separation of the intruder from the resident rat.
11. Remove the intruder from the cage and return it to its home-cage (*see* **Note 7**). Remove the partition from the cage.
12. Return the female partner to the resident's cage and restore water bottles, food pellets and enrichment items.
13. To ensure a high frequency of attacks over repeated episodes, expose intruders to different residents using a rotational design. Each witness rat should observe the social defeat of the same intruder throughout the entire experimental protocol.

3.3 Control Procedures

Data obtained in experimental rats exposed to the witness social stress paradigm can be compared with those obtained in rats exposed to different control conditions.

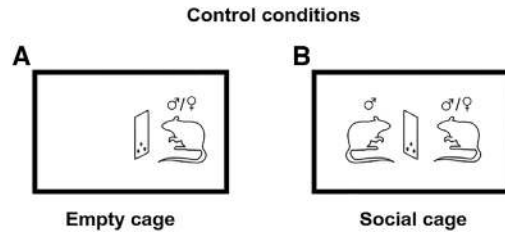


Fig. 2 *Control conditions for the witness stress protocol.* (**A**) In the empty cage condition, control rats are placed in one compartment of an empty cage, while in the "social cage condition" (**B**), rats are placed in a two-compartment cage with an unfamiliar non-aggressive male rat in the opposite compartment

- *"Empty cage" control condition* (Fig. 2A): control rats are placed in an empty cage, identical to the cage used for the witness social stress paradigm, for the same amount of time (e.g., 15 min). The empty cage is divided into two compartments by a Plexiglas partition as in the stress procedure, but, in this case, the control rat is confined to one of them, while the other one remains empty.
- *"Social cage" control condition* (Fig. 2B): control rats are introduced to one side of a two-compartment cage, identical to the cage used for the witness social stress paradigm. Immediately before, an unfamiliar adult male rat of the same strain is placed in the opposite compartment. The duration of this procedure matches that of the witness social stress paradigm (e.g., 15 min).

For both control procedures, employ cages with clean bedding. Additionally, ensure that these cages are thoroughly cleaned with alcohol before commencing a new procedure.

4 Conclusion

The witness social stress paradigm represents a valuable methodological alternative to the traditional social defeat model because of its applicability to understudied populations and its solely psychological nature (Fig. 3). Initial studies in mice comparing the effects of social defeat and witness social stress exposure revealed nearly identical changes in behavior, brain gene expression, and corticosterone levels [27, 31]. The procedure was then adapted for rats and similar results were obtained: both witness and intruder rats showed depressive- and anxiety-like behaviors alongside increased corticosterone levels [32, 33]. The responses were also similar at a cardiovascular level in rats, where witnesses displayed nearly identical tachycardic responses to those of socially defeated intruders [33]. Figure 4 reports an example of the stress-induced tachycardic response recorded in witness and intruder rats compared with control rats (placed in an empty cage) from our laboratory. Intriguingly, a recent study has identified a population of neurons in the ventromedial hypothalamus of



Fig. 3 *The witness stress procedure*. Photographs representing a male witness rat (on the right) observing the agonistic interactions between the intruder and resident rats (on the left). In the second frame, resident and intruder rats are displaying a typical "offensive upright" position

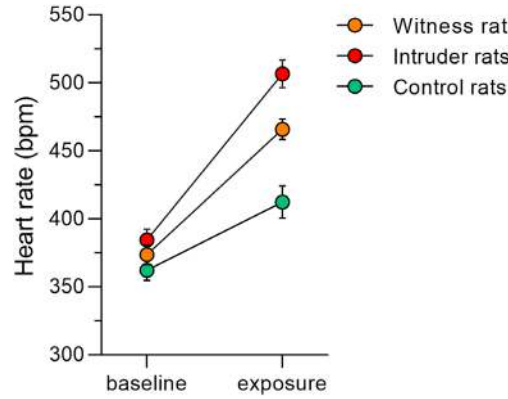


Fig. 4 Heart rate during an episode of social defeat or witness stress. Heart rate was recorded with implanted telemetry devices in male rats ($n = 6$/group) during a 15-min episode of social defeat (intruder rats), a 15 min episode of witness stress (witness rats), or during a 15-min empty cage exploration (control rats)

mice with specific mirroring properties, which activate when observing agonistic interactions between two conspecifics [34]. This discovery

provides valuable insights into the neural circuitry involved in vicarious stress processing and further validates the utility of the witness stress paradigm as a psychosocial stress protocol for rodents.

The versatility of the witness social stress paradigm is evidenced by various studies conducted on typically underrepresented populations, such as adolescent and female rodents. Of note, we recently applied the protocol described in this chapter to adult female rats and demonstrated the presence of adverse remodeling of the contractile properties of the heart upon repeated exposure to witness social stress [35]. Females exposed to witness stress were also found to exhibit behavioral, inflammatory, neuroendocrine, and cardiovascular changes [35–37], confirming the utility of this protocol to overcome the sex bias in preclinical stress research. Moreover, experiments employing this stress paradigm have also been conducted on adolescent rodents, which displayed behavioral alterations (e.g., decreased social behavior) alongside changes in brain gene expression and synaptic plasticity [31]. Intriguingly, this paradigm can be exploited to explore aspects often overlooked in the field of preclinical stress research, despite their extreme importance from a translational perspective. For example, in a recent study, the witness social stress protocol was applied to pregnant female mice to investigate whether psychological stress during gestation exerts adverse influences on offspring. Interestingly, this study has shown that witnessing the defeat of the mated partner during the gestation period induces anxiety-like behaviors in the male offspring, which are associated with several candidate genes related to offspring's abnormal behavior [38]. These findings underscore the efficacy of this paradigm in inducing several stress-related phenomena, ranging from molecular to behavioral changes. While social defeat remains a cornerstone model, the witness social stress paradigm offers distinct advantages, for example, the possibility to eliminate confounds related to the physical interaction with the resident animal and to include female and rodents of different age groups. From an ethical perspective, it should be acknowledged that a higher number of animals are required in witness social stress protocols compared to traditional social defeat paradigms. However, the above-discussed advantages of this model warrant its utilization in rodent research on social stress-related pathological risks. In embracing the witness social stress model, researchers can contribute to the identification of targets for treatment and prevention in a more inclusive and sex-aware manner, thus advancing our understanding of stress-related pathologies and facilitating the development of tailored interventions.

5 Notes

The materials and procedures outlined in the previous paragraphs reflect those employed in our laboratory with rats and serve as a general workflow. However, it is important to note that

both the materials (such as cage sizes) and procedures (including the length and frequency of the witness social stress paradigm and the choice of the control condition) are subject to variation and can be adapted to suit individual laboratory conditions and experimental requirements. This section is dedicated to sharing insights and suggestions for overcoming challenges and potential pitfalls encountered during the application of the witness stress protocol.

1. Intruders older than 4 months may become more difficult to submit because of their weight.
2. In our laboratory, we have observed that using the same resident rat more than once per day can lead to inconsistent levels of aggression. To ensure reliable results, we recommend limiting the use of each resident rat to once per day to maintain consistency in aggression levels across experimental trials.
3. Upon repeated exposures, residents may exhibit reduced attacks over time. In such cases, resident rats no longer displaying aggression towards intruders can be replaced.
4. It is important to avoid changing the bedding of resident rat cages in the 24 h before the stress procedure, as this may dampen aggression in residents, potentially impacting the stress response of the witness.
5. Intruders and witness rats may cohabitate together to amplify the consequences of witness social stress exposure. In case of opposite-sex pairs, consider housing them in cages equipped with a wire-mesh partition to separate the male intruder from the female witness rat to avoid the risk of pregnancy. It is important to note that this cohabitation can induce a phenomenon known as "social buffering," which may attenuate the stress response in both intruders and witnesses [39]. This phenomenon should be carefully considered when interpreting the results.
6. When simultaneously applying the witness social stress procedure to multiple animals (depending on the availability of dominant rats), it is recommended to confine the witness consistently to the same side (e.g., always on the right-hand side) to minimize the risk of inadvertently interchanging witness rats with other rats, including residents and intruders, which could lead to inaccurate data and compromised experimental integrity.
7. Social defeat in rats rarely results in severe injury to the intruder animals. In the rare event of severe wounds, the intruder should be promptly removed from the experiment and euthanized. If the intruder shows scratches, they can be cleaned with sterile saline and disinfected. Subsequently, the animal should be monitored daily.

Conflicts of Interest

The authors have no conflicts of interest to declare that are relevant to the content of this chapter.

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Chapter 14

A Predator-Based Psychosocial Stress Model of PTSD

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Abstract

Post-traumatic stress disorder (PTSD) is a complex psychiatric condition that can arise from exposure to a traumatic event and is characterized by symptoms such as heightened anxiety, hyperarousal, intrusive flashback memories, and impaired cognition. Although previous research has led to a basic understanding of the disorder, the specific neurobiological mechanisms underlying PTSD are still unclear. Preclinical models are essential to help elucidate the physiological and behavioral impacts of PTSD; they also provide a means to test the effectiveness of experimental pharmacological interventions. Here, we describe our predator-based psychosocial stress model of PTSD in rats, which combines two acute cat exposures and 31 days of social instability. This stress regimen leads to robust PTSD-like symptoms including, but not limited to, persistent anxiety-like behaviors, an exaggerated startle response, impaired cognition, and enhanced negative feedback of the hypothalamus-pituitary-adrenal (HPA) axis. Further testing has revealed neurobiological effects akin to those observed in PTSD patients, ranging from altered levels of small-molecule neurotransmitters to increased myocardial sensitivity to ischemic injury. Overall, our model encompasses key features of human PTSD and may serve as a valuable tool for investigating mechanisms of trauma-related psychopathology and testing the efficacy of novel therapeutic agents.

Keywords Animal model, Predator stress, PTSD, Social instability, Sex differences, Corticosterone
Authors KMK, MJS, and TRW contributed equally to this chapter.

1 Introduction

Post-traumatic stress disorder (PTSD) is a psychiatric condition that can develop following exposure to life-threatening experiences, such as sexual assault, wartime combat, or the unexpected death of a loved one [1]. In the United States, the lifetime prevalence of PTSD is estimated to be between 6.1% and 11.7% of the civilian population [2]. According to the *Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition* (DSM-5), there are four symptom clusters observed in PTSD patients: (a) intrusive memories; (b) persistent avoidance of stimuli associated with the traumatic event(s);

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(c) negative alterations in cognition and mood; and (d) increased arousal, reactivity, and hypervigilance [3]. Individuals who develop PTSD exhibit numerous physiological and behavioral symptoms that align with these clusters, such as heightened fear and anxiety, hyperarousal, re-experiencing of the traumatic event, impaired learning and memory, and enhanced negative feedback of the hypothalamus–pituitary–adrenal (HPA) axis [4].

Among the factors believed to increase the likelihood of developing PTSD are chronic stress, prior exposure to trauma, comorbid psychological conditions (e.g., depressive disorders, substance abuse disorders, anxiety disorders), environmental factors (e.g., adverse childhood experiences, low socioeconomic status), type of trauma, and neuroanatomical factors (e.g., smaller hippocampus) [5–13]. Heightened risk for developing PTSD can be epigenetically coded and affect susceptibility in subsequent generations [14–16]. In addition, studies have shown that women are twice as likely as men to develop PTSD [17–19], even when controlling for differences in the types of trauma experienced by each sex [20]. There are also protective factors that can decrease one's risk of developing PTSD. For instance, social support, through close relationships, increased social trust, greater connection with community, and a lack of social constraint or negative social reactions, significantly reduces the incidence of PTSD in trauma-exposed individuals [5, 12, 13, 21, 22].

Animal models of PTSD are useful for determining the safety and efficacy of possible treatments, providing rapid and cost-effective testing, and allowing for greater control and manipulation in studies that would be impractical or unethical to do with human participants [23, 24]. When modeling PTSD in non-human animals, studies can make use of either acute or chronic stress paradigms. In acute paradigms, the stressor is introduced only once, whereas in chronic models the stressor is introduced multiple times, often over a long period (potentially useful in modeling PTSD that results from repeated trauma). Research has shown that both acute and chronic paradigms can produce PTSD-like symptoms [25–29]. Among the most common stressors used in rodent PTSD models are restraint/immobilization, electrical shock, underwater trauma, predator or predator scent exposure, social defeat, social instability, and single prolonged stress [24, 26, 30–32]. Rodent PTSD models generally elicit physiological and behavioral changes, such as heightened anxiety, an exaggerated startle response, enhanced fear conditioning, resistance to fear extinction, reduced social interaction, and cognitive impairments. These models are effective at generating PTSD-like symptoms, but their effects, efficacy, and duration can vary between laboratories [25].

Yehuda and Antelman [33] developed criteria, which were later revised by Siegmund and Wotjak [34], for determining the validity of animal models of PTSD. These criteria include: (1) stressors should

produce biological and behavioral changes that resemble PTSD in humans, (2) stressors should produce PTSD-related sequelae in a dose-dependent manner, (3) stressors should produce biological changes that are long-lasting or strengthen over time, (4) stressors should produce biobehavioral changes that can be bidirectional [i.e., enhanced behaviors (intrusive reexperiencing) and reduced behaviors (avoidance and emotional numbing)], and (5) there is individual variability in responses to the stressors due to genetic and/or experience variables. The revisions added by Siegmund and Wotjak [34] included that the model should be able to assess associative and nonassociative trauma-related memories and that PTSD-like symptoms should be mitigated with chronic SSRI treatment.

In 2008, we developed a predator-based psychosocial stress model of PTSD in rats [29]. The development of this model was based, in large part, on previous work from our laboratory, in which we exposed rats to a natural predator, a cat, to examine its impact on learning, memory, and synaptic plasticity. Initial studies with such predator stress revealed that exposing rats to a cat impaired hippocampus-dependent spatial learning and memory in the radial-arm water maze (RAWM) [35–37], an effect that was associated with increased circulating corticosteroids and found to be unique to cat-induced activation of the amygdala [38, 39]. We also observed that cat exposure impaired synaptic plasticity in the hippocampus, while enhancing it in the amygdala [40, 41]. These neurological effects of cat exposure align with the presumed mechanisms (e.g., impaired hippocampal function; heightened amygdala activity) underlying the development of PTSD in traumatized individuals [42, 43]. Thus, cat exposure was used as a foundation for our model of the disorder.

The predator-based psychosocial stress model of PTSD, as developed by Zoladz et al. [29], consists primarily of two components. The first component is two exposures to a predator (while immobilized), and the second component is 31 days of social instability. Predator exposure (while immobilized) occurs on days 1 and 11, and social instability (randomly changing the rats' cage mates) occurs from days 1–31. Predator exposure was chosen as the primary stressor because it is an ecologically relevant stressor in rats that can mimic a traumatic experience that is the hallmark of PTSD [44–48]. It also reliably induces fear and avoidance behavior in rats [28, 44, 45, 49 ,50]. Thus, this component of the model is closely related to the DSM-5's symptom blocks b and d, as well as the core PTSD symptoms of fear, hyperarousal, and anxiety. Rats are immobilized while being exposed to a predator because a loss of control can exacerbate the stress response (e.g., [51–53]). Hypothetically, the immobilization would reduce the rodent's perception of control over the predator exposure, thereby increasing its physiological response to the stress experience. A second predator exposure occurs 10 days after the first to induce a re-experiencing of the trauma, similar to the

flashbacks that individuals with PTSD experience. Prior research has shown that an intense acute stress experience results in hypertrophy of neurons in the amygdala 10 days later [54]. As the amygdala is highly active during stress and integral to fear memory formation, the second exposure occurs 10 days following the first exposure to augment stress-related plasticity in this structure [23, 29]. The first predator exposure also occurs during the light cycle, while the second occurs during the dark cycle; this is done to create unpredictability as to when predator stress will occur.

The second component of the predator-based psychosocial stress model of PTSD is social instability. Throughout the experimental paradigm, each rat has its cage mate changed every day, with no two rats being housed together on 2 consecutive days. This was included in the model to mimic a lack of social support and stability, which increases the likelihood of developing PTSD [55–57]. Greater perceived social support, social network size and diversity, and community participation can increase resilience to stress and PTSD, while sex of the individual and friend vs. family support have been found to modulate these effects [21, 58–62]. In addition, preclinical work has shown that animals subjected to social instability stress (alternating between isolation and overcrowding) exhibit increased stress-related responses at both behavioral and molecular levels [63, 64]. Thus, removing the rodent's "social network" could lead to an increase in susceptibility to PTSD-like symptoms.

The predator-based psychosocial stress model of PTSD reliably induces myriad PTSD-like symptoms in rodents. The model generates both behavioral (an exaggerated startle response, increased fear and anxiety, impaired cognition) and biological (enhanced negative feedback of the HPA axis, neurotransmitter alterations) changes similar to those observed in individuals with PTSD. The use of an ecologically relevant predator exposure ensures a sufficient dose of stress to be outside the normal range of experience. The PTSD-like changes are also long-lasting (up to 4 months following predator exposure). Having both increased, or "enhanced," symptoms (e.g., an exaggerated startle response) and decreased, or "reduced," symptoms (decreased exploration of the open arms on an elevated plus maze) accounts for symptom bidirectionality. There is a lack of individual variability in PTSD-like symptoms in animals exposed to the model, but the model was developed to include factors known to increase a traumatized individual's susceptibility to developing PTSD and thus generate PTSD-related sequelae in all exposed animals. The predator-based psychosocial stress model of PTSD holds up well against the previously established criteria for animal models of PTSD [33,34] and is thus an effective tool to use for studying the neurological correlates of PTSD-related sequelae, as well as the ability of novel pharmacological agents to mitigate any observed effects.

2 Materials

2.1 Animals and Housing

2.2 Rodent Subjects

Most of the research with the predator-based psychosocial stress model of PTSD has utilized adult (8–12 weeks of age) Sprague–Dawley rats, including both males and females (depending on the study). The rats should be pair-housed in standard Plexiglas cages with free access to food and water on a 12-h light/dark schedule (lights on at 0700 h) in temperatures ranging from 64°F to 75 °F (45% ± 5% humidity). In previous work, the rats have been handled (2–3 min each) anywhere from 3 to 7 days prior to any manipulation to ensure comfortability with human interaction.

2.3 Predator (Cat)

An adult, gonadally intact female cat is used as the predator in this model, as female cats are known to be inherently more curious of the rats while also being notably less aggressive than their male counterparts. The female cat should be housed separately from any rodent subjects and under standard housing conditions (64°F–75 °F; 45% ± 5% humidity; 12-h light/dark schedule, lights on at 0700 h). Dry food and fresh water should be freely accessible to the cat, as well as a litter box that is cleaned daily to reduce odors and maintain a healthy environment. In addition to these necessities, the cat should be provided with items that support its physical and psychological well-being, such as various forms of enrichment (toys, climbing towers, scratching posts) and occasional positive human interaction (*see* **Note 1**).

2.4 Acute Cat Exposures: Equipment, Materials, and Setup

- Rats are first immobilized in plastic DecapiCones (Braintree Scientific; Braintree, MA). In order to secure the bag's opening, 1.5 inch binder clips are fastened around each rat's tail (*see* **Note 2**).
- A perforated, circular Plexiglas rat "pie" with wedge-shaped interior dividers (20 × 20 × 8 cm; Braintree Scientific; Braintree, MA) serves to prevent direct contact between the rats and the cat, while still allowing for full exposure of the rats to non-tactile sensory stimuli associated with the cat.
- A separate cage (made of metal or plastic) is needed to ensure that the rats are subject to adequate levels of cat exposure. In previous studies, a metal cage with the dimensions of 61 × 53 × 51 cm was used (e.g., [29, 65]).
- To entice the cat to explore the Plexiglas rat pie, wet cat food is smeared across the top of the pie.

2.5 Randomization of Cage Mates: Equipment, Materials, and Setup

- Tail markings are used so that rats can be accurately identified for randomization. Sharpies (Newell Brands; Atlanta, GA) have been found to be both long-lasting and easy to apply, with a broad spectrum of colors suitable for research needs.
- A cage mate log, or spreadsheet, is needed to effectively manage the daily reorganization of rat pairings. A randomization chart can be used as a helpful tool to ensure that all possible rat pairings are utilized, with minimized numbers of re-pairing (*see* **Note 3**).

3 Method (Fig. 1)

3.1 Acute Cat Exposure Procedure

The first acute cat exposure takes place during the 12-h light cycle, typically between 0800 and 1200 h, and following the procedures outlined below:

1. Ready the Plexiglas pie by loosening the top screw and removing the lid.
2. Place rats, nose first, into the DecapiCones, securing the open end around the base of the tail with a binder clip. Repeat until all rats are immobilized.
3. Place the rats into the triangular-shaped wedges within the Plexiglas pie and secure the lid with the screw.

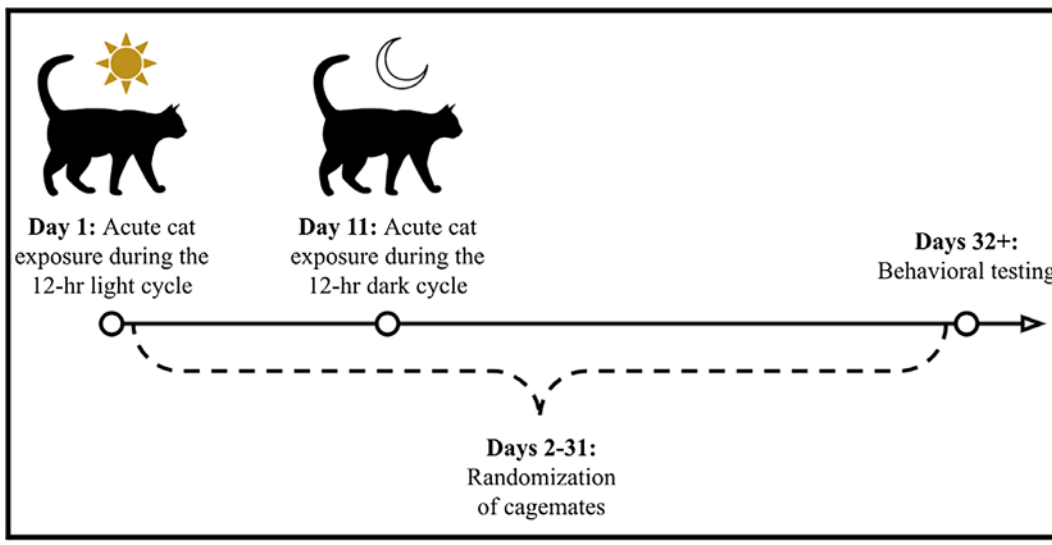


Fig. 1 A timeline of the procedures involved in the predator-based psychosocial stress model of PTSD

4. Once secured, smear wet cat food across the top of the Plexiglas pie.
5. Take the rat pie into the cat housing room and place it inside the metal cage.
6. Place the cat inside the metal cage, directly on top of the Plexiglas pie, and lock the cage. Allow the rats to be exposed to the cat for a period of 1 h.
   - Control rats remain in their home cages for the 1-h duration.
7. Re-apply wet cat food to the top of the Plexiglas pie approximately every 15 min throughout the 1-h cat exposure.

Ten days later, the second acute cat exposure takes place during the 12-h dark cycle, typically between 1900 and 2200 h. It follows the same procedure as the first exposure.

3.2 Randomization of Cage Mates

Tail Markings

1. Assign each rat a unique number.
2. According to the key provided (Fig. 2), use a Sharpie to mark the base of the tail with the correct color and line(s). All markings should correspond to the last digit of the number identifier (e.g., rat #391 would be marked with a single red line).
3. Ensure that each line of the marking is thick enough for easy identification and that it wraps almost entirely around the base of the tail.

Randomization Procedure

1. At the beginning of the experiment, house rats in numerical order pairs (i.e., XXX1 + XXX2, XXX3 + XXX4).
2. Every day, starting on the first day of acute cat exposure, rearrange the cohort pair combinations so that each rat has a different cage mate.
   - As unstable housing conditions are meant to invoke stress in the subjects, some monitoring may be necessary to ensure the health and safety of the rats and to prevent severe aggression or injury via their new cage mate.

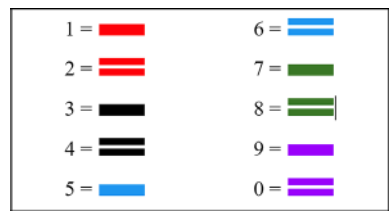


Fig. 2 Colors and number of lines used for tail marking individual rats

3. Label all of the new cages according to their new occupants, and repeat steps 2 and 3 daily, around the same time each day.
4. Once all possible cohort pair combinations have been exhausted, continue the randomization of cage mates by starting over with the initial numerical-order pairings. In previous studies, behavioral testing almost always occurred on day 32. While this means that some combinations will repeat during the 31-day-long stress period, the rats should never be housed with the same cage mate for 2 consecutive days.

    - Control rats remain with the same cohort pair combinations throughout the duration of the experiment.

4 Biological and Behavioral Endpoints

Previous work involving the predator-based psychosocial stress model has included various biological and behavioral endpoints, depending on the aim of the study. Researchers can choose to measure PTSD-like symptoms in a host of ways, some of which are described below.

4.1 Body/Organ Weight

Extensive research has demonstrated that chronic stress decreases body weight, largely due to changes in eating habits and hormone levels [66–68]. To measure this effect, we calculated growth rate (g/day) by taking the total body weight gained by the rat throughout the course of the experiment and dividing it by the length of the experiment (typically 31 days).

Preclinical research has shown that, due to the persistent increase in circulating corticosteroids, chronic stress results in adrenal gland hypertrophy and thymus atrophy [69–72]. With this in mind, we measured the weight of both glands to assess the physiological effects of stress in our rats.

4.2 Corticosterone and Cardiovascular Measurements

Corticosterone (CORT) is a hormone in rodents that is released by the adrenal glands during times of stress. In humans, abnormal cortisol responses to trauma, lower baseline levels of cortisol, and enhanced negative feedback of the HPA axis have all been linked to PTSD [73–79]. Thus, we measured CORT in rats after each acute cat exposure, at baseline at the end of the experimental paradigm, and following dexamethasone (DEX) administration (at the end of the experimental paradigm) to assess HPA axis function. To measure CORT, blood samples were collected via tail snips or saphenous venipuncture immediately after each acute cat exposure and during behavioral testing on day 32 (once immediately upon removal from the home cage, once following 20 min of immobilization stress, and once after a 1-h "recovery" period in the home cage) (*see* **Note 4**).

Heart rate (HR) and blood pressure (BP) are easily measurable indicators of sympathetic nervous system activity. As people with PTSD often exhibit elevated HR and BP [80], these measures were included in the study by Zoladz et al. [29]. HR and BP were assessed immediately following each acute cat exposure and at the conclusion of the experimental paradigm (following 20 min of immobilization stress in the blood sample timeline referenced above).

4.3 Heart Function

PTSD is associated with increased levels of pro-inflammatory cytokines (PICs), which may contribute to the higher risk of developing cardiovascular disease, a condition driven by inflammation [80, 81]. To examine cardiac vulnerability in our model, we removed rat hearts following behavioral testing and subjected them to 20 min ischemia and 2 h of reperfusion in a Langendorff isolated heart system [82]. Contractile function was measured throughout, and infarct size was assessed afterward.

4.4 Neurobiological Endpoints

In individuals with PTSD, there are numerous alterations in neurotransmitter levels. For instance, these individuals exhibit elevated norepinephrine (NE) and glutamate and decreased serotonin (5-HT) and gamma-aminobutyric acid (GABA). Additionally, dopamine (DA) has been found to be dysregulated. These changes correspond to symptoms such as hyperarousal [83], depression and irritability [84, 85], exaggerated fear, impaired cognitive function, and emotional dysregulation [86, 87]. The main areas impacted by these neurotransmitter changes are the amygdala, the prefrontal cortex (PFC), the hippocampus, and the ventral tegmental area (VTA). In previous research with the predator-based psychosocial stress model, the PFC and hippocampus were dissected on the last day of behavioral testing, and levels of NE, 5-HT, and DA were analyzed via high-performance liquid chromatography (HPLC) [88, 89].

In addition to significant neurotransmitter modulation, several types of inflammatory markers are elevated in both the blood and cerebrospinal fluid of patients with PTSD [90]. Inflammation in brain areas involved in fear, memory, and cognition likely contributes to symptom development. This creates a vicious cycle where symptoms fuel further inflammation, leading to chronic, debilitating changes in brain structure and function. Wilson and colleagues [91, 92] sought to explore PIC levels in rodents with PTSD-like symptoms. Real-time PCR and Western blotting were used on dissected PFC and hippocampal tissue to assess mRNA and protein levels of inflammatory markers, respectively.

4.5 "Traumatic" Memory

The foremost criterion for PTSD diagnosis is exposure to a traumatic event. Consequently, individuals with the disorder are often plagued with memories and/or dreams that activate stress responses even when no stressful stimulus is present. In an attempt to model a

traumatic memory, we placed psychosocially stressed rats in a distinct chamber immediately prior to each of the two cat exposures [93]. They remained in the chamber for 3 min, and a tone was played during the last 30 s. During behavioral testing, the rats were tested for their freezing responses to the chamber (context) and the tone (cue) (*see* **Note 5**).

4.6 Elevated Plus Maze

The elevated plus maze (EPM) is a behavioral test used in rodents to assess anxiety-like behavior [94]. As its name suggests, the EPM is a plus-shaped apparatus with four arms that extend from a central point. Two of the arms are surrounded by high walls (closed arms), and two of the arms are not (open arms); the entire platform is elevated several feet off the ground to make venturing into the open arms a "risky" decision. The most commonly used dependent measures in the EPM are the time spent in each area of the maze and the number of entries made into each area of the maze. In theory, less time spent in the open arms and fewer entries into the open arms equate to heightened anxiety in the rats. In our model, we tested rats on the EPM for 5 min, typically on the first day of behavioral testing (i.e., day 32).

4.7 Acoustic Startle Response

As a consequence of elevated NE and glutamate in the body, hyperarousal and exaggerated startle responses are key features of PTSD [95–99]. The acoustic startle response (ASR) test measures reflexive behaviors in rodents in response to a loud and unexpected noise [100]. In our PTSD model, we assessed ASR in response to several noise intensities, typically after EPM exposure on the first day of behavioral testing.

4.8 Radial-Arm Water Maze (RAWM)

The RAWM is a behavioral apparatus used to examine spatial learning and memory in rodents. It was originally developed by Diamond and colleagues, who reported that cat exposure impaired hippocampus-dependent learning and memory in the RAWM [35, 101]. The RAWM consists of a circular pool divided into six arms, with a hidden platform submerged at the end of one "goal" arm. Rats are placed in the maze and given 1 min to explore and find the platform. The number of incorrect arm entries is used to quantify performance. In our model, we exposed rats to twelve acquisition trials, followed by a single "short-term"/retention memory trial 1 h later. A single long-term memory trial was completed the next day.

4.9 Novel Object Recognition

The novel object recognition (NOR) test serves to assess recognition memory. It consists of two major phases: the training phase, in which two identical objects are presented, and the testing phase, where one of the familiar objects is replaced with a new one. For each trial, rats are given 5 min to explore the testing arena. Naturally, rats are inclined to investigate novelty. However, it has been

found that factors like stress, anxiety, and damage to certain areas of the brain can result in neophobia, or avoidance of the novel object in favor of the familiar one [102–104]. In light of the disruptions to hippocampal function and avoidance behaviors in individuals with PTSD, we hypothesized that psychosocially stressed rats would exhibit a preference for familiarity.

4.10 Pharmacological Manipulations

The predator stress model has been used to test the efficacy of several pharmacological agents. Typically, the first administration of these agents occurred 24 h after the first acute cat exposure and continued for the duration of the experiment [65, 105, 106]. Alternatively, some researchers have waited until the completion of the stress regimen before administering drug treatment [89, 91]. Regardless of timing, post-stress treatment aligns with real-world therapeutic use. Furthermore, as many drugs geared toward PTSD treatment are not always efficacious, research on the therapeutic potential of novel agents, and not their preventative abilities, is crucial.

5 Observed Biological and Behavioral Effects

Unless otherwise stated, it should be assumed that the following results were obtained from studies involving *male* rats.

5.1 Body/Organ Weights and Physiological Stress Responses

Rats exposed to the predator-based psychosocial stress model have consistently displayed reduced growth rate, adrenal gland hypertrophy, and thymic atrophy [29, 93, 105–108]. Following the first and second cat exposures, psychosocially stressed rats exhibited a significant increase in CORT [29]. By day 32, psychosocially stressed rats demonstrated reduced baseline CORT levels compared to controls (Fig. 3a) and a greater suppression of CORT following the administration of dexamethasone (Figs. 3b and 3c); these findings are suggestive of enhanced negative feedback of the HPA axis in psychosocially stressed rats. Individuals with PTSD consistently exhibit lower baseline cortisol levels and exaggerated cortisol suppression following administration of dexamethasone, reflecting enhanced glucocorticoid receptor feedback sensitivity within the HPA axis [109–111].

Psychosocially stressed rats also exhibited elevated HR and increased systolic and diastolic BP following each cat exposure [29]. After the 31-day paradigm, psychosocially stressed rats exhibited greater immobilization stress-induced increases in systolic and diastolic BP, but a blunted increase in HR, relative to controls [29]. The findings suggest overall autonomic imbalance and greater sympathetic tone in psychosocially stressed rats. Humans experiencing PTSD exhibit chronically elevated HR, BP, and decreased HR in response to novel stressors [112–114].

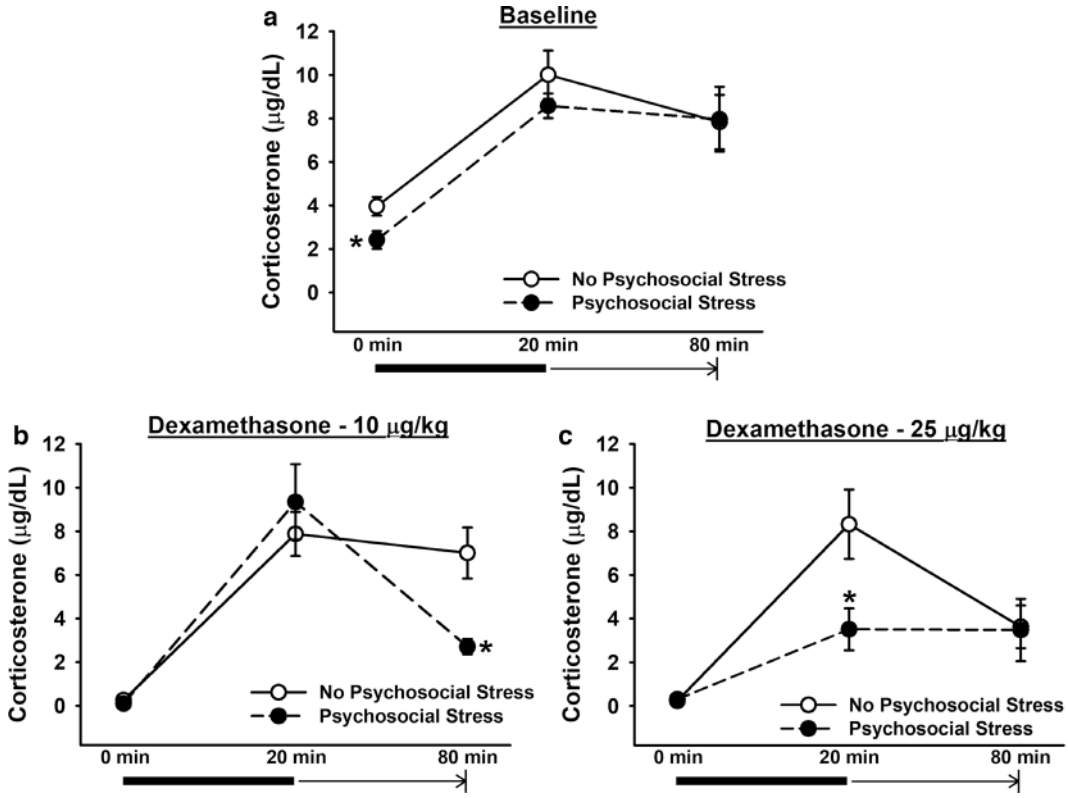


Fig. 3 Following the 31-day paradigm, a baseline blood sample was collected from male rats immediately upon removal from their home cage. The rats were then immobilized for 20 min, after which a second blood sample was collected. The rats were returned to their home cages for 60 min, and a final blood sample was collected. Psychosocially stressed rats exhibited lower baseline corticosterone (CORT) levels than controls (**a**). On the day of blood sampling, rats were treated with dexamethasone to assess whether psychosocially stressed rats would exhibit enhanced negative feedback of the HPA axis. Following administration of 10 μg/kg dexamethasone, psychosocially stressed rats displayed a greater decline in CORT levels than controls after the 20 min of acute immobilization (**b**). Administration of 25 μg/kg blocked the acute immobilization-induced increase in CORT levels in psychosocially stressed rats (**c**). Data are presented as means ± SEM. *$p < 0.05$ relative to no psychosocial stress (Figure is reprinted from Zoladz et al. [93] with permission from the publisher.)

5.2 Susceptibility to Myocardial Ischemic Injury, Genetic Expression in Heart Tissue

Following 20 min of myocardial ischemia and 2 h of reperfusion, psychosocially stressed male rats had larger infarcts and diminished recovery of contractile function than controls (Fig. 4). In contrast, psychosocially stressed females exhibited no significant differences in infarct size or functional recovery following ischemic insult [82]. Further histopathological investigation confirmed that hearts from psychosocially stressed males displayed multifocal ventricular lesions characterized by tissue necrosis, infiltration of mononuclear immune cells, and collagen deposition, a crucial player in myocardial fibrosis [108]. These changes were accompanied by shifts in gene expression associated with endothelial-to-mesenchymal transition and extracellular matrix remodeling, supporting increased vulnerability to ischemic

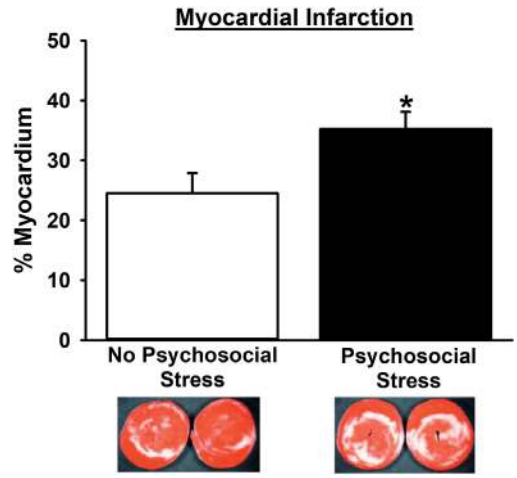


Fig. 4 Male rats exposed to the predator-based psychosocial stress paradigm exhibited larger infarcts than controls as a result of ischemic injury. Representative images of heart tissue reveal a greater amount of infarct (white areas of tissue) in psychosocially stressed rats. Data are presented as means ± SEM. *$p < 0.05$ relative to no psychosocial stress (Figure is reprinted from Rorabaugh et al. [105] with permission from the publisher)

injury [108, 115, 116]. In addition, there was an upregulation of genes involved in angiogenesis and myofibroblast activation. Specifically, alpha-smooth muscle actin (ACTA2) and T-cadherin (encoded by CDH13) were elevated in hearts of psychosocially stressed rats, but not controls. ACTA2 is a hallmark of myofibroblast differentiation and contributes to myocardial remodeling and repair processes [117]. T-cadherin, a cell adhesion molecule, promotes endothelial cell migration, survival, and vascular cell proliferation, key features of adaptive tissue remodeling and angiogenesis (both crucial in myocardial recovery following injury) [118, 119].

Individuals with PTSD are at significantly higher risk for hypertension and cardiovascular disease, including atherosclerosis, myocardial infarction, and heart failure [112, 120]. Chronic stress related to PTSD often leads to endothelial dysfunction and myofibrosis-related biomarker elevations. Upregulation of ACTA2 in humans contributes to scar formation on the injured heart, whereas the upregulation of T-cadherin is associated with angiogenesis and vascular remodeling following injury (like atherosclerosis) [108]. Because the predator-based psychosocial stress model replicated PTSD-linked cardiovascular alterations, such as hypertension, myocardial fibrosis, and gene-expression changes, it provides a reliable translational framework for investigating hypertension, cardiac remodeling, and molecular factors of cardiovascular risk in humans with PTSD.

5.3 Neurotransmitter Alterations

Psychosocially stressed rats displayed elevated levels of NE and DA in the PFC and elevated NE in the hippocampus [88]. 5-HT and its metabolite, homovanillic acid (HVA), were lower in these brain areas in psychosocially stressed animals. Western blotting confirmed these findings, showing that, in psychosocially stressed rats, tyrosine hydroxylase (TH), the rate-limiting enzyme for NE and DA, was upregulated and tryptophan hydroxylase (TPH), the rate-limiting enzyme for 5-HT, was downregulated in both the PFC and hippocampus. These neurochemical alterations exhibited by psychosocially stressed rats are similar to those observed in individuals with PTSD. For example, elevated urinary and plasma NE, DA dysregulation, and decreased 5-HT transmission are linked to PTSD-related symptoms, such as hyperarousal, intrusive memories, anhedonia, anxiety, irritability, and emotional dysregulation [29, 84, 88, 93, 121]. These neurotransmitter abnormalities are particularly detrimental in the PFC and hippocampus, areas crucial for working memory, fear extinction, and emotional regulation [84, 98]. PFC and hippocampal impairments disrupt memory integration, adaptive stress responses, and overall emotional regulation prevalent in those with PTSD [84, 122].

5.4 Inflammatory, Oxidative Stress, DNA Methylation

Chronic stress impairs immune function and drives an overproduction of PICs, such as interleukin-1 beta (IL-1B) and components of inflammasomes (such as NALP3) [92]. This surge in cytokine production triggers a self-sustaining positive feedback loop, increasing nitric oxide (NO) and reactive oxygen species (ROS), which inflict tissue damage, inflammation, and cell death. Superoxide, an ROS byproduct of oxidative metabolism, is linked to neuroinflammation and neuronal cell death when elevated [123]. Psychosocially stressed rats exhibited a significant increase in superoxide and total ROS in the hippocampus, PFC, and adrenal glands, suggestive of oxidative damage in PTSD-relevant brain regions [92]. Additionally, whole-blood superoxide levels increased progressively throughout the 31-day stress regimen (days 0, 12, and 32), confirming systemic, time-dependent escalation of oxidative stress. mRNA levels of IL-1B and NALP3 were significantly elevated in the hippocampus, PFC, and amygdala in psychosocially stressed rats, while protein expression for both markers was significantly increased in the hippocampus and PFC relative to controls [91, 92]. Consistent with pro-inflammatory findings, the levels of anti-inflammatory cytokines (AICs) IL-4 and IL-10 were significantly reduced in both the hippocampus and PFC of psychosocially stressed rats, suggesting a failure to neutralize chronic neuroinflammation [124, 125].

Epigenetic regulation of brain-derived neurotrophic factor (BDNF), a key mediator of synaptic plasticity and cognitive function, was altered following exposure to the psychosocial stress paradigm, consistent with alterations found in psychological disorders such as PTSD [126, 127]. Psychosocial stress led to hypermethylation of *bdnf* in the dorsal CA1 region, yet no significant changes were

observed in the PFC or amygdala [107]. These molecular changes may underlie hippocampal dysfunction, memory deficits, and anxiety-like behavior.

5.5 "Traumatic" Memory of Cat Exposures

On day 32, psychosocially stressed rats exhibited robust freezing behavior upon exposure to the context (chamber) and cue (tone) that were previously associated with the cat exposures (Fig. 5a, b) [93, 106, 128]. This freezing response reflects core PTSD symptomatology in humans, where environmental stimuli, such as specific sights, sounds, smells, or places, provoke intense fear responses and a re-experiencing of the traumatic event(s). Our measure of the rats' memory for the acute cat exposures makes this model a useful tool to examine the mechanisms that underlie traumatic memory formation in people with PTSD.

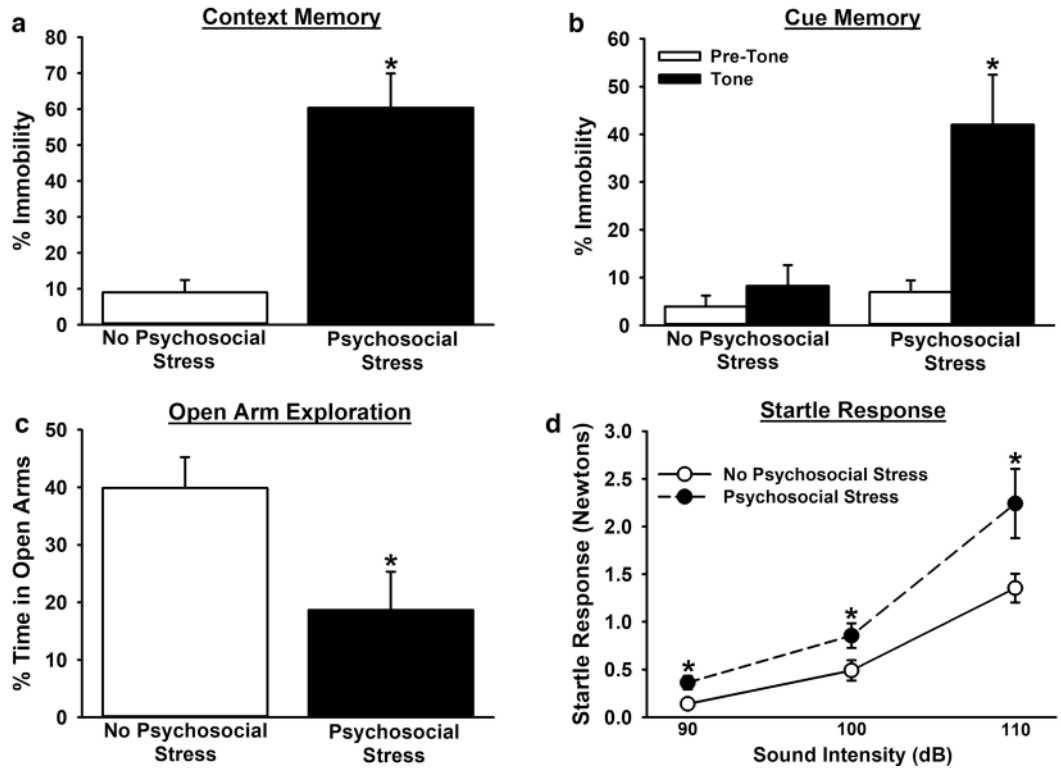


Fig. 5 Immediately prior to each of the acute cat exposures, male rats were placed in a fear conditioning chamber (no shock was administered) for 3 min; a 30-s tone was played at the end of the chamber exposure. At the end of the 31-day paradigm, psychosocially stressed rats exhibited greater immobility than controls upon exposure to the chamber previously associated with the cat exposures (**a**) and upon exposure to the tone previously associated with the cat exposures (**b**). Psychosocially stressed rats also spent less time than controls in the open arms of an elevated plus maze, indicative of heightened anxiety (**c**). Psychosocially stressed rats exhibited greater startle responses than controls to noise bursts of three different intensities (**d**). Data are presented as means ± SEM. *$p < 0.05$ relative to no psychosocial stress (Figures a–c are reprinted from Zoladz et al. [93] with permission from the publisher; Figure d is reprinted from Zoladz et al. [106] with permission from the publisher)

5.6 Anxiety-Like Behavior and Hyperarousal

Psychosocially stressed rats spent less time in the open arms of the EPM, without a reduction in total arm entries (i.e., overall locomotion), suggestive of heightened anxiety (Fig. 4c) [29, 89, 93]. Psychosocially stressed rats also exhibited exaggerated startle responses to 90, 100, and 110 dB noise bursts, revealing robust hyperarousal (Fig. 4d) [29, 106]. Clinically, individuals with PTSD demonstrate elevated anxiety and hyperarousal, even in settings absent of immediate threats [129, 130].

5.7 Cognitive Function

Zoladz et al. [29] found no effect of psychosocial stress on RAWM performance. In contrast, psychosocially stressed rats exhibited impaired recognition memory in the NOR assessment, as evidenced by equivalent exploration times for novel and familiar objects [29, 128]. The authors reasoned that psychosocially stressed rats were not impaired in the RAWM because it is an emotionally arousing task. Indeed, individuals with PTSD frequently exhibit enhanced attention and memory for emotionally arousing information [131–135], and preclinical work has shown that stress exposure can enhance performance on emotionally arousing tasks, such as fear conditioning [136–138]. The emotionally neutral nature of the NOR task enabled a cognitive deficit to be observed in psychosocially stressed rats, which is consistent with the types of cognitive deficits observed in humans with PTSD (e.g., for word lists) [139, 140], as well as in chronically stressed rats in preclinical work (e.g., Y maze) [141, 142].

5.8 Alcohol

Alcohol is commonly used by individuals with PTSD to cope with hyperarousal and intrusive memories [143]. Zoladz et al. [144] reported that, following the 31-day stress paradigm, psychosocially stressed rats exhibited greater ethanol preference and consumption than controls. Importantly, for these effects to be observed, the rats had to have been exposed to ethanol prior to the onset of the stress paradigm. These findings suggest that alcohol seeking and intake for alleviation of PTSD symptoms may be correlated with previous alcohol exposure and consumption [143].

5.9 Female Focus and Potential Sex Differences

Initial work involving female rats demonstrated that the 31-day psychosocial stress paradigm exerted no anxiogenic effect on the EPM in psychosocially stressed females [82]. However, this null result could have been due to greater behavioral variability in female rats, as a result of the rapid-cycling nature of the female estrous cycle. In a subsequent study, Zoladz et al. [145] found that, when using a very large sample of female rats ($n=71$) and controlling for stage of estrous, psychosocially stressed females did exhibit heightened anxiety on the EPM (Fig. 6a) and in the open field, thus replicating the effects repeatedly observed in psychosocially stressed males. Moreover, ovariectomy had no significant impact on the observed effects. The consistency in effects across male and female rats resonates with some of our past research showing that acute cat exposure

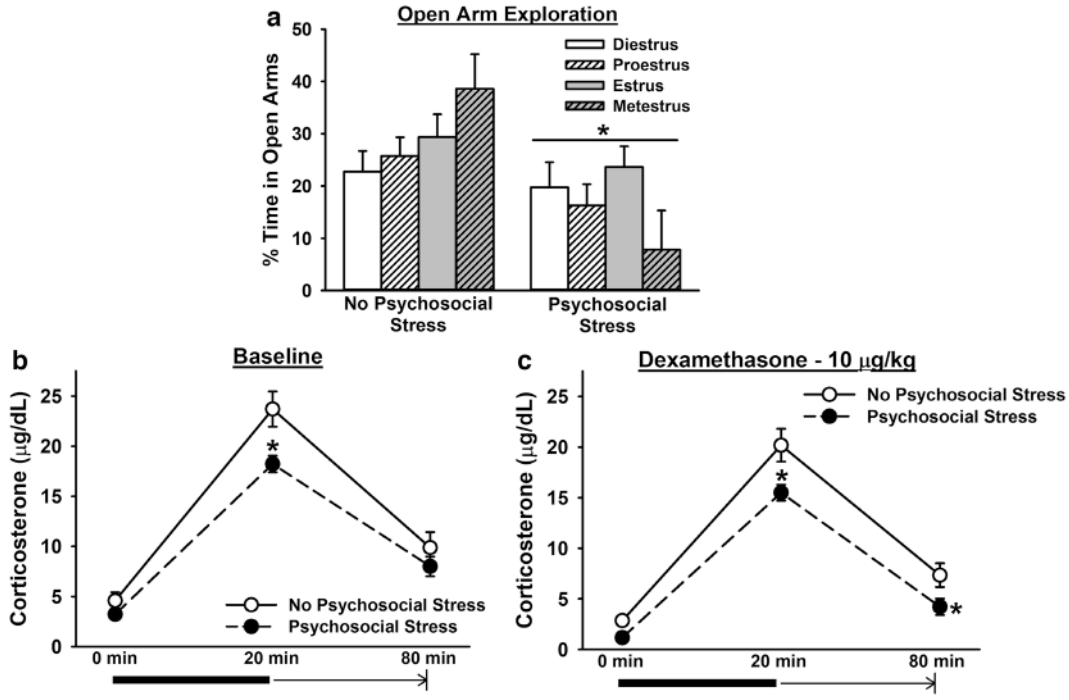


Fig. 6 In female rats, exposure to the 31-day stress paradigm resulted in heightened anxiety on the elevated plus maze, indicated by these rats spending less time in the open arms than controls (**a**). Psychosocially stressed females also exhibited a blunted increase in corticosterone (CORT) levels after acute immobilization stress (**b**). After treatment with 10 μg/kg dexamethasone, psychosocially stressed females displayed lower CORT levels than controls following 20 min of acute immobilization and following 60 min in their home cage (**c**). Data are presented as means ± SEM. *$p < 0.05$ relative to no psychosocial stress (Figure a is reprinted from Zoladz et al. [145] with permission from the publisher; Figures b and c are reprinted from Zoladz et al. [147])

produced a largely equivalent impairment of spatial memory in both sexes [146]. In another study, psychosocially stressed females exhibited a smaller acute stress-induced increase in CORT levels (Fig. 6b) and a greater suppression of CORT following dexamethasone administration (Fig. 6c), reflecting enhanced negative feedback of the HPA axis [147]. However, researchers have been unable to replicate an exaggerated startle response in psychosocially stressed females, which is consistent with some other animal models of PTSD [148] and possibly PTSD symptom expression in humans (e.g., [149]). In addition, unlike males, psychosocially stressed females fail to exhibit enhanced myocardial sensitivity to ischemic injury. Collectively, the findings in psychosocially stressed females suggest important sex differences in the development of PTSD-like sequelae do exist and warrant further investigation.

5.10 Pharmacology

Several pharmacological agents have been evaluated for their ability to mitigate PTSD-like outcomes in the predator-based psychosocial stress model. A 7-day regimen of sertraline, which began at

the conclusion of the 31-day stress paradigm, normalized 5-HT levels in the hippocampus and PFC of psychosocially stressed rats, but no anxiolytic effect of sertraline was observed [89]. Instead, sertraline administration produced a significant increase in NE in the cerebrospinal fluid (CSF) and an increase in blood plasma of psychosocially stressed and non-stressed controls. In terms of inflammation, sertraline decreased the levels of PICs IL-1B and TLR4, while upregulating AICs IL-4 and IL-10 in the hippocampus and PFC of psychosocially stressed rats. Overall, these findings suggest sertraline has a more complex role in the treatment of PTSD-like symptoms, as it normalizes 5-HT levels and inflammatory markers within the brain, but elevates NE levels (associated with hyperarousal) and exhibits no anxiolytic effect in psychosocially stressed rats.

Valproic acid (VA), a histone deacetylase inhibitor, reduces oxidative stress, inflammation, and neurotransmitter imbalances in the predator-based psychosocial stress model [91]. Chronic VA treatment (which began at the conclusion of the stress paradigm and lasted for 30 days) reduced the elevated levels of ROS (superoxide) and PIC (TLR4, NALP3, IL-1B, and IL-18) mRNA and protein in the hippocampus and PFC. VA also normalized dysregulated neurotransmitter profiles associated with psychosocial stress by lowering NE and restoring 5-HT levels, making these profiles comparable to non-stressed controls. Behaviorally, VA exerted an anxiolytic effect on the EPM in psychosocially stressed rats.

Tianeptine, an atypical tricyclic antidepressant with mood-stabilizing properties, attenuated almost all of the PTSD-like symptoms exhibited in psychosocially stressed animals. Specifically, tianeptine decreased the strength of context and cue fear memories (Fig. 7a, b), led to less anxiety-like behavior on the EPM (Fig. 7c), and reduced startle responses in psychosocially stressed rats (Fig. 7d). Tianeptine also normalized adrenal and thymus weights and stabilized cardiovascular activity, including HR and systolic/diastolic BP, indicating strong efficacy in regard to therapeutic effects in the model [106].

Clonidine, an alpha-2 adrenergic agonist, did not reduce the strength of context or cue fear memories. However, it did decrease anxiety-like behavior on the EPM and reduced startle responses; it also prevented chronic stress-induced cardiovascular changes, including HR and systolic/diastolic BP, in psychosocially stressed rats [106]. Clonidine failed to prevent adrenal gland hypertrophy, thymus atrophy, or a reduction in growth rate. Furthermore, clonidine did not protect against myocardial hypersensitivity to ischemic injury, measured by infarct size and post-ischemic contractile recovery [105]. Propranolol, a commonly used beta blocker, did not reverse anxiogenic behavior on EPM, nor did it protect against myocardial hypersensitivity to ischemic injury in psychosocially stressed rats [105].

The tricyclic antidepressant amitriptyline reduced the strength of context and cue fear memories, decreased anxiety-like behavior on the EPM, and normalized startle responses [106]. However,

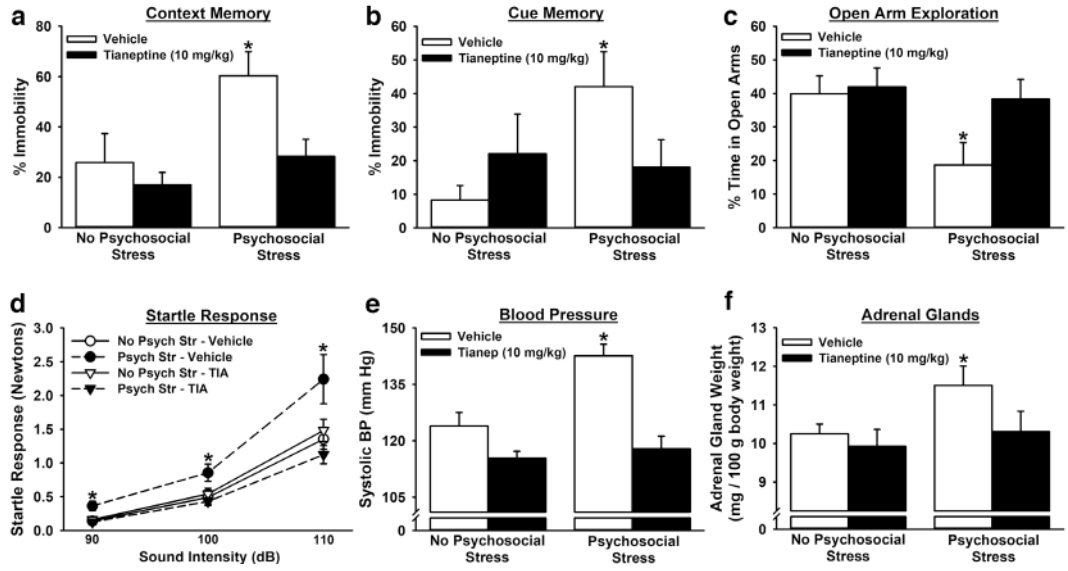


Fig. 7 In psychopharmacology work with the predator-based psychosocial stress paradigm, male rats were treated with tianeptine or vehicle beginning one day after the first cat exposure and continuing throughout the remainder of the paradigm (including days of behavioral testing). Tianeptine led to reduced immobility upon exposure to the chamber (**a**) and tone (**b**) previously associated with the cat exposures. Tianeptine also blocked the effects of psychosocial stress on anxiety (**c**), acoustic startle response (**d**), systolic blood pressure (**e**), and adrenal gland size (**f**). Data are presented as means ± SEM. *$p < 0.05$ relative to psychosocial stress + tianeptine (Figure is reprinted from Zoladz et al. [106] with permission from the publisher)

amitriptyline did not prevent adrenal gland hypertrophy, thymus atrophy, reduced growth rate, or damaging cardiovascular effects (increased HR and systolic/diastolic BP). Although amitriptyline exhibits promising alleviation of PTSD-like symptoms, its side effect profile may limit its suitability for PTSD treatment, particularly given the increased cardiovascular vulnerability in the PTSD population.

Ketamine, a non-competitive NMDA receptor antagonist, demonstrated dose- and sex-dependent effects in rats exposed to the predator-based psychosocial model [65]. In psychosocially stressed rats, 10 mg/kg ketamine prevented decreased growth rate in both males and females, exerted anxiolytic effects on the EPM in males, normalized startle responses in females, and led to anxiolytic effects in the open field in males and females; most of these effects were not observed for the 15 mg/kg dose of ketamine.

6 Conclusions

The predator-based psychosocial stress model was developed based on factors known to increase a traumatized individual's susceptibility for developing PTSD. It involves two acute exposures to an ecologically

relevant stressor [a predator (cat)], combined with daily social instability. It also includes the elements of stressor uncontrollability and unpredictability to augment the stress responses in rats. The model results in myriad biological and behavioral symptoms that mirror those observed in people with PTSD, including a robust memory for the acute cat exposures, heightened anxiety, an exaggerated startle response, impaired cognition, enhanced negative feedback of the HPA axis, greater myocardial sensitivity to ischemic injury, elevated NE, decreased 5-HT, and increased inflammation, to name a few. Because of its ability to produce such a diverse set of symptoms of such a complex psychiatric condition, this model should be extremely useful to researchers who aim to understand the mechanisms underlying the development and maintenance of PTSD.

7 Notes

1. For this model of stress, it is important to use an adult, gonadally intact female cat that is energetic and interested in the rats. To maximize the stress response in the rats, the cat needs to direct its attention to the rats during the 1-h stress procedure. Over time, laboratory cats can become more and more disinterested in the rats, which can weaken the effects of the stress paradigm on various endpoints. Thus, it is possible that new laboratory cats may need to be acquired to replace those that have been used extensively as stressors in the model.
2. In earlier research performed by Zoladz et al. [29], psychosocially stressed rats were immobilized in the DecapiCones for 15 min prior to being exposed to the cat for 45 min. Other work by Zoladz and colleagues revealed that the 15-min immobilization period was unnecessary and that the rats could simply be placed with the cat, immediately following immobilization, for a full hour.
3. In most previous work with the predator-based psychosocial stress model, rats exposed to the psychosocial stress paradigm were run in groups of at least ten rats. This seemed to provide enough variability in the cohort pair combinations that the stress of social instability was maximized. Running psychosocial stress groups with fewer than ten rats could result in decreased stress responses in the rats, possibly due to the reduced number of cohort pairings.
4. If investigators examine the impact of this stress model on baseline HPA axis function, it is vital that the first blood sample be obtained no more than 2 min following removal of the rats from their home cages. If obtaining the baseline

sample requires more time, it could be confounded by an acute stress-induced increase in circulating CORT levels.

5. When researchers use our model to study the resulting traumatic memories, exposure of the rats to the fear conditioning chamber and tone must be as close in time as possible to the immobilization and cat exposure. Rats in the psychosocial stress groups must be immobilized immediately upon removal from the chamber and quickly placed in the Plexiglas pie with the cat. This ensures that the conditioned stimuli (i.e., chamber and tone) are temporally linked with the unconditioned stimuli (immobilization and cat).

Conflict of Interest

The authors have no conflicts of interest to declare that are relevant to the content of this chapter.

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Chapter 15

Modeling Neuroinflammation in Depression: Acute, Sub-chronic, and Chronic Lipopolysaccharide (LPS) Protocols

Danielle S. Macedo

Abstract

Mounting evidence supports an immune-driven subtype of major depressive disorder (MDD), characterized by low-grade inflammation, prominent motivational/energy symptoms, and distinct biological trajectories. In rodents, systemic administration of lipopolysaccharide (LPS), a robust activator of innate immunity, enables controlled study of immune-to-brain signaling relevant to these phenotypes. However, the interpretative validity of LPS-based depression paradigms depends on temporal precision, dosing strategy, and rigorous separation of adaptive sickness behavior (an acute, protective response to inflammation) from depressive-like phenotypes (maladaptive changes that persist beyond sickness). This chapter provides a methodological framework for acute, sub-chronic, and chronic intermittent ("hit-and-run") LPS protocols, focusing on rationale, implementation, validation endpoints, and translational scope. Acute paradigms are presented as tools to interrogate early immune-to-brain signaling after sickness resolution; sub-chronic paradigms exploit peripheral endotoxin tolerance to model persistent depressive-like behavior with sustained central neuroimmune activation; and chronic intermittent paradigms model relapse vulnerability, immune sensitization, and cumulative neurobiological burden across repeated inflammatory episodes. Across protocols, we highlight common pitfalls, interpretative boundaries, and methodological safeguards, particularly the need for central endpoints beyond plasma cytokines, to support mechanistic inference in inflammation-associated depressive phenotypes within stratified and precision psychiatry frameworks.

Keywords Lipopolysaccharide (LPS), Neuroinflammation, Depression models, Sickness behavior, Immunopsychiatry, Endotoxin tolerance, Microglial activation, Oxidative stress, Immune–brain communication, Neuroprogression

1 Introduction: LPS Models as Tools to Probe Immune-Driven Depressive Phenotypes

Major depressive disorder (MDD) is biologically heterogeneous and cannot be fully explained by the classical monoaminergic hypothesis alone [1]. While altered serotonin, norepinephrine, and dopamine signaling remains central to current treatments, a substantial fraction of patients show partial or no response, underscoring the need to consider additional mechanisms [2].

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Over the past three decades, converging evidence has identified immune–brain interactions as contributors to depressive pathophysiology. The cytokine theory of depression proposes that peripheral immune activation can induce central neurobiological changes that overlap with depressive symptom dimensions [3, 4]. Within immunopsychiatry, pro-inflammatory cytokines, particularly interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor (TNF), are conceptualized as signals that can engage brain pathways involved in motivation, energy balance, and affective regulation [5–7].

Importantly, MDD is increasingly viewed as a set of partially overlapping biological subtypes. Among these, an immune-driven phenotype has substantial empirical support and is often characterized by low-grade systemic inflammation, energy/motivation symptoms, comorbidity with metabolic/inflammatory disease, poorer outcomes, and differential antidepressant responsiveness [8]. This perspective motivates biomarker-informed stratification and emphasizes that LPS paradigms should not be treated as "models of MDD as a whole," but as experimental tools targeting inflammation-associated symptom dimensions and trajectories.

Acute sickness behavior is evolutionarily conserved and adaptive, whereas persistent or dysregulated inflammatory signaling can lead to longer-lasting behavioral and neurobiological changes that resemble aspects of depression [9]. Longitudinal human data further suggest that systemic inflammation can precede depressive symptoms in at least a subset of individuals [10–12].

Experimentally, lipopolysaccharide (LPS), a component of Gram-negative bacterial membranes, is widely used to model immune-to-brain communication in rodents. As a pathogen-associated molecular pattern (PAMP), LPS activates Toll-like receptor 4 (TLR4) [13], triggering MyD88- and TRIF-dependent signaling and downstream NF-κB activation with robust cytokine induction [14–18]. This chapter focuses on how to use LPS protocols with sufficient temporal control to dissociate sickness from depressive-like phenotypes, and how to validate each protocol with appropriately timed central endpoints.

1.1 Methodological Considerations for Temporal Dissociation: Sickness vs. Depressive-Like Phenotypes

A core challenge in inflammation-based depression models is separating transient sickness behavior from depressive-like phenotypes. Acute immune activation produces a conserved syndrome: reduced exploration and locomotion, hypophagia, fever, and social withdrawal, that can reduce motivated behavior but is not equivalent to core depressive features such as anhedonia, passive coping, or cognitive inflexibility.

Misclassification is common: reduced activity during the acute inflammatory phase can be incorrectly interpreted as "depression-like behavior." To reduce this risk, acute LPS paradigms require strict temporal separation between the sickness phase and affect-related behavioral testing.

In rodents, sickness behavior typically peaks ~2–6 h after systemic LPS and largely resolves by ~24 h [19–21]. In contrast, central neuroimmune processes can outlast overt sickness, including microglial activation, altered cytokine signaling in limbic regions, and changes in tryptophan metabolism via the indoleamine-2,3-dioxygenase (IDO) pathway [22]. Therefore, tests intended to index depressive-like phenotypes (e.g., sucrose preference, forced swim, social interaction) should be conducted only after sickness has resolved, typically no earlier than 24 h post-LPS [21]. This increases the likelihood that behavioral changes reflect affective/motivational dysfunction rather than nonspecific malaise or motor suppression.

Acute LPS designs are useful for studying early immune-to-brain signaling, but they do not mimic the sustained or fluctuating inflammatory contexts seen in chronic medical comorbidities (e.g., obesity, autoimmune disease). To address this, extended paradigms have been developed to model persistence, immune adaptation, and cumulative neurobiological burden. Two common approaches are (i) a sub-chronic repeated-administration model and (ii) a chronic intermittent ("hit-and-run") model. These probe different facets of immune–brain dynamics and require different safeguards and interpretative boundaries. The temporal structure and key evaluation windows of the acute, sub-chronic, and chronic intermittent LPS protocols are summarized in Fig. 1.

2 Materials

The reproducibility and interpretability of LPS-based models of depression are highly sensitive to experimental variables that are often underestimated in standard pharmacological studies. These include the bacterial serotype and batch of the endotoxin, the genetic background of the animals, housing and handling conditions, and procedural factors related to dosing and tissue collection. The specifications below are intended to minimize batch effects, reduce biological noise, and ensure a robust and interpretable neuroinflammatory response (*see* **Notes 1** and **11**).

2.1 Animals: Strain and Housing Considerations

Genetic background critically shapes both peripheral immune responsiveness and central behavioral outcomes following LPS administration. *C57BL/6J mice* are the most widely used strain in neuroinflammation research due to their high sensitivity to LPS-induced sickness behavior and subsequent depressive-like phenotypes. They exhibit a robust Th1-biased immune response and provide maximal comparability with the existing literature. *BALB/c mice* display higher baseline anxiety-like behavior and a Th2-skewed cytokine profile. Although suitable for comparative immune studies, their distinct affective baseline complicates direct comparison with standard LPS depression paradigms. *Outbred stocks* (e.g., Swiss mice and

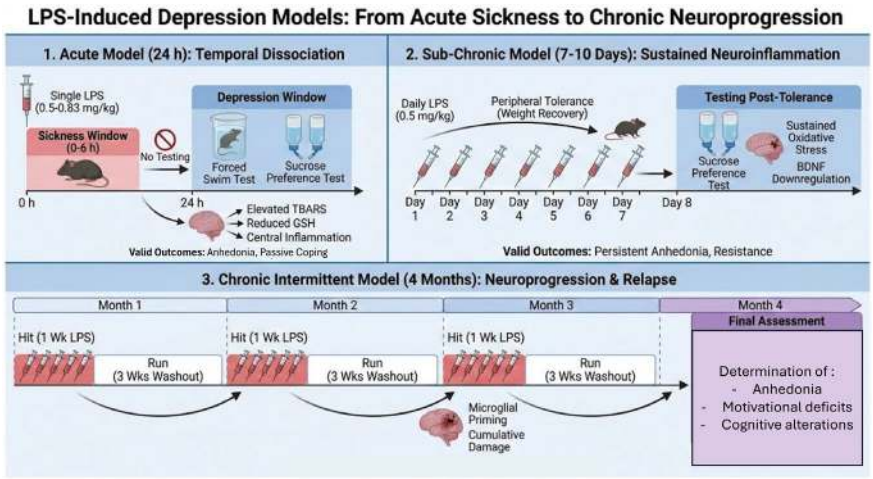


Fig. 1 *Overview of acute, sub-chronic, and chronic intermittent LPS protocols for modeling depression and neuroprogression*. This schematic summarizes the three distinct methodological approaches detailed in the chapter, highlighting their specific timelines, evaluation windows, and expected validated outcomes. **1.** Acute Model (24 h): illustrates the critical temporal dissociation following a single high-dose LPS injection. A "Sickness Window" (0–6 h, red shaded area) characterized by somatic malaise invalidates behavioral testing. The "Depression Window" (blue shaded area) emerges at 24 h, allowing for valid assessment of anhedonia (Sucrose Preference Test) and immobility due to passive coping (Forced Swim Test), correlating with central oxidative markers (elevated TBARS, reduced GSH). **2.** Sub-chronic Model (7–10 Days): depicts repeated daily administration of low-dose LPS. While peripheral tolerance develops (indicated by weight recovery), central neuroinflammation is sustained. Testing 24 h after the final dose exposes persistent anhedonia, oxidative stress, and BDNF downregulation. **3.** Chronic Intermittent Model (4 Months): summarizes the "Hit-and-Run" paradigm consisting of four repeated cycles of 1 week exposure ("Hit") followed by 3 week washout ("Run"). This long-term protocol drives cumulative microglial priming and neuroprogression, resulting in late-stage outcomes such as cognitive rigidity (Morris Water Maze performance), mitochondrial dysfunction, and cumulative neuroimmune burden

Wistar rats) are commonly used in pharmacological screening but exhibit substantial inter-individual variability in immune sensitivity and behavioral baselines. Studies employing outbred animals typically require larger sample sizes to achieve adequate statistical power and should explicitly report variability metrics.

For the protocols described in this chapter, male C57BL/6 J mice aged 8–12 weeks (20–25 g) are recommended to minimize biological variability and maximize reproducibility.

Animals should be acclimatized to the housing facility for a minimum of 7–10 days before experimentation. Because LPS interacts synergistically with psychological stress, minimizing handling, noise, and cage disruption during this period is essential to avoid ceiling effects in inflammatory and behavioral readouts (*see* **Notes 9** and **10**). Animals should be group-housed (three to five per cage) unless experimental endpoints require social isolation. All behavioral testing should be conducted during the light phase (typically 09:00–14:00)

to control for circadian modulation of corticosterone and cytokine signaling (*see* **Note 3**).

2.2 Reagents and Solutions

The biological activity of LPS varies markedly according to bacterial strain, purification method, and batch. LPS derived from *Escherichia coli* serotypes O127:B8 or O111:B4 is recommended, as these preparations consistently elicit robust peripheral and central cytokine responses in rodents.

A single serotype and lot number must be used throughout a study. Batch-to-batch variability, even within the same supplier, can substantially alter effective potency (expressed in endotoxin units, EU) (*see* **Note 1**).

LPS is supplied as a lyophilized powder and should be dissolved in sterile, endotoxin-free 0.9% saline (PBS is acceptable, but saline is preferred for intraperitoneal administration). Prepare a concentrated stock (e.g., 1 mg/mL) using silanized glass tubes. LPS is amphiphilic and readily adsorbs to untreated plastic surfaces, leading to unpredictable loss of material and dosing inaccuracies. Vortex vigorously for at least 15 min to ensure complete solubilization, as LPS tends to form micellar aggregates. Aliquot the stock solution into small volumes using silanized glass vials or verified low-binding tubes. Store at −20 °C and avoid repeated freeze–thaw cycles. On the day of injection, dilute the stock solution to the desired concentration using sterile, pyrogen-free saline.

Control animals must receive sterile, certified pyrogen-free 0.9% saline at the same injection volume (typically 10 mL/kg). Even trace endotoxin contamination in the vehicle can elevate baseline inflammatory markers and compromise group comparisons (*see* **Note 2**).

2.3 Euthanasia and Tissue Collection

The method of euthanasia is not a neutral procedural detail in neuroinflammation studies; it can alter molecular readouts and therefore should be selected with attention to both ethical requirements and molecular fidelity [23, 24]. Terminal anesthetics used for overdose euthanasia (e.g., ketamine/xylazine, isoflurane, pentobarbital) can rapidly affect neurotransmission, cerebral metabolism, and intracellular signaling. Ketamine is a fast-acting antidepressant via NMDA receptor antagonism, and volatile anesthetics can change brain metabolism within seconds, effects that may confound cytokines, neurotransmitters, and phosphorylation-dependent signaling cascades [25].

When permitted by institutional and national guidelines, rapid decapitation without prior anesthesia can provide high fidelity for neurochemical and signaling measurements. If anesthesia is required, it should be standardized across all groups, and its potential confounding effects explicitly acknowledged (*see* **Note 14**).

2.4 Equipment and Disposables

The use of 1 mL tuberculin syringes fitted with 27G–30 G needles is recommended for injection. Smaller-gauge needles reduce tissue trauma and injection-associated stress.

A balance accurate to at least 0.1 g is required. In sub-chronic and chronic protocols, daily body weight monitoring serves as a primary index of sickness behavior and animal welfare.

LPS is a potent respiratory and dermal hazard. Powder handling must be performed in a chemical fume hood while wearing gloves and a face mask to prevent inhalation and environmental contamination.

3 Methods

3.1 Protocol I: The Acute Challenge (The "Two-Phase" Temporal Dissociation)

The acute LPS protocol is designed to dissociate transient sickness behavior from inflammation-driven depressive-like phenotypes through strict temporal separation of immune activation and behavioral assessment [20, 26, 27]. When properly implemented, this model enables the investigation of early neuroimmune mechanisms underlying affective dysfunction while minimizing confounds related to motor suppression or generalized malaise (*see* **Note 15**).

In C57BL/6 J mice, effective doses typically range from 0.5 to 0.83 mg/kg administered intraperitoneally (i.p.). Doses ≥1.0 mg/kg substantially increase the risk of severe systemic illness and mortality and are therefore not recommended for behavioral studies aimed at modeling depression rather than sepsis [28].

Because LPS potency varies across batches, doses should be adjusted based on the reported endotoxin activity (EU/mg) when available. Pilot testing is strongly recommended for each new lot to confirm induction of sickness behavior without excessive morbidity (*see* **Note 1**).

LPS should be administered during the early light phase (typically between 09:00 and 10:00). Immune responses are under circadian regulation, and injections performed during the dark phase may yield attenuated cytokine induction and increased variability (*see* **Note 3**). A standardized volume of 10 mL/kg should be used for both LPS and vehicle injections (*see* **Note 4**). Control animals must receive an equivalent volume of sterile, pyrogen-free 0.9% saline under identical handling conditions (*see* **Note 9**).

Immediately following systemic LPS administration, animals enter a well-defined sickness phase characterized by reduced locomotor activity, piloerection, ptosis, social withdrawal, and transient hypophagia. This phase typically peaks between 2 and 6 h post-injection and reflects an adaptive host response to immune activation rather than a depressive-like state (*see* **Note 5**). Observation of reduced spontaneous locomotion or exploratory behavior during this window serves as confirmation that the immune stimulus was effective. Optional short open-field assessments (≤5 min) may be used solely for validation purposes. Motivation-dependent or stress-based behavioral assays (e.g., forced swim test, tail suspension test, sucrose preference) must not be performed during this window.

Reduced performance at this stage reflects motor suppression and sickness rather than immobility due to passive coping and will result in false-positive outcomes [29, 30].

Behavioral assessment of depressive-like phenotypes should be conducted no earlier than 24 h after LPS administration. At this time point, overt somatic sickness responses, such as fever, hypolocomotion, and anorexia, have largely resolved, whereas central neuroimmune alterations may persist. These include sustained microglial activation, altered cytokine signaling within limbic regions, oxidative imbalance, and shifts in tryptophan–kynurenine metabolism [31] (*see* **Note 12**).

This delayed assessment window is essential to attribute observed behavioral changes to affective and motivational dysfunction rather than residual sickness.

For behavioral endpoints, the Sucrose Preference Test (SPT) is used to assess anhedonia. LPS-treated mice typically exhibit a reduction in sucrose preference at 24 h post-injection. Procedure: animals should be habituated to a two-bottle choice paradigm (1% sucrose vs. water) for at least 48 h before LPS administration. Preference can be assessed over a 1 h or overnight period beginning at 24 h post-injection [32]. The Forced Swim Test (FST) is employed to assess stress-coping strategy. Procedure: mice are placed in a cylinder filled with water maintained at 23–25 °C for 6 min. Immobility is quantified during the final 4 min of the test. Scoring should be performed blind to group assignment (*see* **Note 15**). Immobility is commonly interpreted as a passive coping-like response (reduced active coping) and should not be described as "behavioral despair". An alternative scoring approach includes a 1 min habituation period followed by 5 min of immobility recording [26]. Locomotor activity must be evaluated (e.g., open-field test) to confirm recovery from sickness. Increased immobility in the absence of locomotor deficits supports interpretation as a depressive-like phenotype rather than residual lethargy [33].

Molecular validation should be performed immediately after behavioral testing to verify the persistence of neuroimmune alterations. Central pro-inflammatory indices, such as IL-1β and TNF, or markers of microglial activation, are typically elevated at 24 h, even when circulating cytokines have normalized [20]. Acute LPS challenge is also associated with decreased reduced glutathione (GSH) and increased lipid peroxidation (e.g., TBARS) in the hippocampus and prefrontal cortex [20]. Together, these molecular signatures support the interpretation that behavioral alterations reflect sustained central inflammatory and redox imbalance rather than residual peripheral sickness.

Because behavioral testing itself can act as an acute stressor and modify immune signaling, cytokine and glial readouts should not be treated as a purely "post-behavior" validation step. In systemic LPS paradigms, circulating cytokines often peak within hours and

may return toward baseline by ~24 h, whereas central inflammatory indices can remain detectable beyond this window in a region- and context-dependent manner [34]. Accordingly, molecular endpoints should be anchored to predefined post-injection time windows, and interpretation should explicitly distinguish (i) LPS-driven neuroimmune activation from (ii) behavioral testing–evoked stress effects [35] (*see* **Note 7**).

3.2 Protocol II: The Sub-chronic Repeated Administration (7–10 Days)—Modeling Persistent Neuroinflammation with Peripheral Tolerance

The sub-chronic LPS protocol is designed to model sustained inflammation-associated depressive-like behavior while minimizing the confounding effects of acute sickness. This paradigm exploits the phenomenon of endotoxin tolerance, in which repeated systemic exposure to LPS leads to attenuation of peripheral immune responses, whereas central neuroimmune activation may persist [36, 37].

Repeated daily administration of LPS induces a progressive reduction in peripheral cytokine release and somatic sickness responses, including hypolocomotion and body-weight loss [38]. Importantly, this peripheral adaptation does not necessarily reflect resolution of central immune signaling.

This dissociation renders the sub-chronic protocol particularly suitable for studying failures of neuroimmune resolution and for evaluating interventions aimed at restoring central homeostasis rather than suppressing acute inflammation.

For this protocol, LPS is administered intraperitoneally at a fixed dose of 0.5 mg/kg once daily for 7 consecutive days or 0.83 mg/kg in rats [37]. This dose reliably induces an initial sickness response followed by the development of peripheral tolerance without excessive morbidity. Escalating-dose regimens (e.g., 0.5 → 0.75 → 1.0 mg/kg) have been proposed to counteract tolerance. However, these approaches increase variability and the risk of severe systemic effects. For modeling persistent depressive-like behavior, a constant dose is generally preferable [39]. Injections should be performed at the same time each day (preferably during the early light phase) to minimize circadian variability in immune responsiveness. To reduce local peritoneal irritation and fibrosis, injection sites should be alternated daily between the left and right lower abdominal quadrants (*see* **Note 9**).

Body weight must be recorded daily before each injection. Early weight loss (days 1–3) is expected and serves as an index of acute sickness. Stabilization or partial recovery of body weight by days 4–5 indicates the emergence of peripheral tolerance (*see* **Note 8**). Animals exhibiting weight loss exceeding 20% of baseline, persistent hypothermia, or severe lethargy should be removed from the study in accordance with ethical guidelines. Qualitative assessment of grooming, posture, and spontaneous activity should be performed daily to detect delayed or cumulative distress not captured by body weight alone.

Behavioral testing should be conducted 24 h after the final LPS injection (typically on day 8 or 11, depending on the protocol

Box 1. Comparison between acute and sub-chronic LPS protocols

Dimension	Acute LPS protocol (Sect. 3.1)	Sub-chronic LPS protocol (Sect. 3.2)
Primary objective	Dissociate transient sickness behavior from early inflammation-driven depressive-like phenotypes	Model persistence of depressive-like behavior under conditions of peripheral immune tolerance
Dosing regimen	Single intraperitoneal injection	Daily intraperitoneal injections for 7–10 consecutive days
Typical dose	0.5–0.83 mg/kg	0.5 mg/kg (constant dose preferred)
Peripheral immune response	Robust and transient cytokine surge	Progressive attenuation due to endotoxin tolerance
Sickness behavior	Prominent at 2–6 h; largely resolved by 24 h	Present during early days (1–3), diminishes with repeated exposure
Behavioral testing window	≥24 h after injection	24 h after final injection
Risk of sickness confounds	High if temporal dissociation is not respected	Low if testing is delayed until after tolerance develops
Central neuroinflammation	Sustained despite resolution of somatic sickness	Persistent or enhanced despite peripheral immune adaptation
Oxidative stress profile	Acute depletion of antioxidants and transient lipid peroxidation	Chronic redox imbalance with sustained glutathione depletion
Primary behavioral endpoints	Anhedonia, stress-coping strategy	Anhedonia, stress-coping strategy, social withdrawal
Translational relevance	Screening of rapid-acting antidepressants; early immune-to-brain signaling	Modeling failure of neuroimmune resolution and persistence of symptoms
Main limitation	Vulnerable to misinterpretation if sickness is not controlled	Does not fully model long-term neuroprogression or relapse

duration) (*see* **Note 12**). Testing during the active dosing phase is discouraged, as residual sickness responses may confound behavioral outcomes. This post-treatment window allows for confirmation that depressive-like behaviors persist despite resolution of overt somatic

illness, thereby strengthening attribution to sustained central neuroimmune alterations (*see* **Note 15**).

The detection of behavioral changes follows the recommendations of Protocol I since sub-chronically treated animals typically exhibit robust anhedonia, increased immobility in stress-based assays, and social withdrawal, without significant suppression of basal locomotor activity. Compared with the acute protocol, repeated LPS

Box 2. Common pitfalls and misinterpretations in acute and sub-chronic LPS models

Pitfall 1: Interpreting acute sickness as depression *Issue*: Behavioral suppression observed within the first hours after LPS administration is frequently misclassified as depressive-like behavior. *Correction*: Sickness behavior (0–6 h post-injection) reflects an adaptive immune response and must be temporally dissociated from affective testing. Depressive-like phenotypes should only be assessed ≥24 h after LPS administration.

Pitfall 2: Testing behavior during the active dosing phase in sub-chronic protocols *Issue:* Behavioral testing conducted during days 1–6 of repeated LPS administration is confounded by residual sickness and ongoing peripheral inflammation. *Correction:* Behavioral assessments should be restricted to at least 24 h after the final injection, when peripheral tolerance has developed, and somatic symptoms have subsided.

Pitfall 3: Assuming plasma cytokines reflect central neuroinflammation *Issue*: Normalization of circulating cytokines is often misinterpreted as resolution of brain inflammation. *Correction*: Peripheral immune tolerance does not imply central immune quiescence. Validation of the model requires direct assessment of central markers (e.g., brain cytokines, microglial activation, and redox imbalance).

Pitfall 4: Overdosing LPS to "strengthen" the phenotype *Issue*: Increasing LPS dose beyond recommended ranges can induce systemic toxicity, sepsis-like states, and mortality, undermining behavioral interpretation. *Correction*: Use the lowest dose that reliably induces the desired phenotype (typically 0.5 mg/kg in C57BL/6 J mice) and avoid doses ≥1.0 mg/kg in behavioral studies.

Pitfall 5: Ignoring circadian modulation of immune responses *Issue*: Variability in injection timing introduces uncontrolled circadian effects on cytokine release and behavioral outcomes. *Correction*: Standardize all injections and behavioral testing to the same light-phase window, preferably early in the light cycle.

Pitfall 6: Using locomotion-dependent tests without motor control validation *Issue*: Increased immobility in stress-based assays may reflect residual lethargy rather than immobility due to passive coping. *Correction*: Always confirm recovery of basal locomotion (e.g., open-field test) before interpreting immobility-based outcomes.

Pitfall 7: Treating sub-chronic LPS as a model of neurodegeneration *Issue*: Persistent behavioral deficits are sometimes overinterpreted as evidence of irreversible neurodegeneration. *Correction*: The sub-chronic protocol models incomplete neuroimmune resolution, not frank neurodegeneration. Claims of neuroprogression require longitudinal structural, synaptic, or cognitive validation.

Pitfall 8: Neglecting batch, serotype, and handling variability *Issue*: Differences in LPS serotype, lot number, housing stress, or handling procedures introduce major reproducibility issues. *Correction*: Use a single serotype and lot per study, minimize stressors, and explicitly report housing and handling conditions.

administration is associated with sustained depletion of reduced glutathione and chronic elevation of lipid peroxidation products, particularly in the hippocampus and striatum [37].

In the case of evaluation of neuroinflammatory alterations, while circulating cytokine levels may normalize, central inflammatory markers and microglial activation states frequently remain elevated, supporting the concept of a peripheral–central dissociation [36].

Although the sub-chronic LPS protocol models the persistence of depressive-like behavior in the absence of overt sickness, it does not recapitulate all features of chronic human depression. Rather, it should be viewed as a model of incomplete neuroimmune resolution following repeated inflammatory insults.

Conclusions regarding neuroprogression or neurodegeneration should therefore be drawn cautiously and supported by convergent molecular and structural endpoints.

3.3 Protocol III: Chronic Intermittent ("Hit-and-Run") LPS Administration—Modeling Relapsing Inflammation and Neuroprogressive Trajectories

The chronic intermittent "hit-and-run" protocol is designed to model recurrent inflammatory insults separated by recovery periods, thereby approximating the relapsing–remitting inflammatory exposure observed in several chronic medical conditions associated with depression. By interleaving brief LPS challenges with extended washout phases, this paradigm limits complete endotoxin tolerance while promoting cumulative central adaptations that persist beyond peripheral immune resolution [40, 41].

A single or continuously repeated immune challenge inadequately captures the episodic nature of inflammation in clinical settings. The "hit-and-run" approach operationalizes the hypothesis that each inflammatory episode ("hit") induces lasting microglial priming, oxidative stress, and synaptic vulnerability that continue to evolve during apparent remission ("run") [40].

The inclusion of washout phases allows partial normalization of peripheral cytokines, thereby re-sensitizing the immune system to subsequent challenges and enabling the study of sensitization, relapse liability, and cumulative neural burden. This design is particularly suited for interrogating transitions from reversible affective disturbances to persistent cognitive and circuit-level dysfunction [41].

The chronic protocol spans 16 weeks (4 months) and is organized into four identical cycles: (i) **Active phase (1 week):** LPS is administered intraperitoneally once daily for five consecutive days (e.g., Monday–Friday); (ii) **Washout phase (3 weeks):** No injections (or saline vehicle for control groups) are administered for 23 days to allow resolution of acute sickness and partial recovery of peripheral immune markers, and (iii) **Repetition**. The 4 week cycle (1 week on/3 weeks off) is repeated four times.

LPS doses between 0.5 and 0.75 mg/kg (i.p.) are typically sufficient to elicit robust immune activation during each active phase without excessive mortality.

The extended washout periods substantially mitigate tachyphylaxis. However, if pilot data indicate diminishing behavioral or molecular responses in later cycles, a modest dose escalation (e.g., $0.5 \rightarrow 0.75$ mg/kg) may be considered with caution.

All injections should be performed at the same circadian time point across cycles to minimize temporal variability in immune responsiveness (*see* **Note 3**).

For longitudinal monitoring and welfare, during active phases, body weight should be monitored daily; during washout phases, weekly monitoring is sufficient. Persistent weight loss, dehydration, or signs of systemic distress necessitate removal from the study. Qualitative assessment of coat condition (e.g., piloerection, ungroomed fur) provides a sensitive longitudinal index of chronic stress and self-care deficits that is distinct from acute sickness behavior. Given the prolonged duration of the protocol, objective criteria for experimental discontinuation and exclusion must be defined a priori and applied uniformly across groups (*see* **Notes 6, 8,** and **10**).

Behavioral assessment should include inter-episode testing during the washout phase, scheduled in the latter half of the washout window (e.g., weeks 3–4 of each cycle). This timing is intended to detect residual effects and stable baseline shifts in affective state after recovery from acute sickness. Persistence of anhedonia or motivational deficits weeks after the last LPS exposure suggests incomplete recovery and may indicate increased vulnerability to relapse. A final assessment should then be conducted at month 4. After the fourth cycle, the behavioral focus should shift toward cognitive domains, prioritizing hippocampal- and prefrontal-dependent tasks (e.g., novel object recognition and spatial memory paradigms), which are not consistently altered by acute or sub-chronic protocols (*see* **Notes 13** and **15**).

Chronic intermittent LPS exposure is expected to produce enduring central neuroimmune changes that may persist even when peripheral cytokines have returned to baseline. For this reason, neuroinflammation in this model is best conceptualized as a shift in microglial activation state and inflammatory signaling tone, rather than as sustained systemic cytokinemia. Compared with acute or sub-chronic paradigms, the hit-and-run design is also well suited to detect longer-lasting redox disturbances, including sustained oxidative and nitrosative stress and emerging mitochondrial dysfunction across cortico-limbic circuits. Because structural and synaptic effects are plausibly cumulative, endpoints such as dendritic complexity, synaptic protein expression, interneuron vulnerability, and regional volume changes are most appropriately prioritized at the final time point, when integrated effects are expected to be maximal.

Box 3. Comparison between sub-chronic and chronic intermittent LPS protocols

Dimension	Sub-chronic LPS protocol (3.2)	Chronic intermittent "Hit-and-Run" protocol (3.3)
Primary objective	Model persistence of depressive-like behavior under peripheral immune tolerance	Model relapse, sensitization, and cumulative neuroimmune burden
Conceptual framework	Incomplete resolution of central inflammation	Recurrent inflammatory "hits" with incomplete recovery
Overall duration	7–10 days	~16 weeks (4 months)
Injection structure	Daily injections without interruption	Cyclic exposure: 1 week on/3 weeks off
Typical dose range	0.5 mg/kg (constant dose)	0.5–0.75 mg/kg
Dosing strategy	Fixed dose to allow development of endotoxin tolerance	Stable dose across cycles; optional minimal escalation in later cycles
Tolerance management	Tolerance is an intrinsic feature of the model	Washout phases prevent full tolerance and re-sensitize immune response
Peripheral immune profile	Blunted cytokine response over time	Recurrent cytokine induction with partial normalization between cycles
Central immune state	Persistent microglial activation despite peripheral tolerance	Progressive microglial priming and immune sensitization
Sickness behavior	Present early (days 1–3), resolves by mid-protocol	Restricted to active phases; absent during washouts
Behavioral testing window	24 after final injection	During washout (inter-episode) and at the final endpoint
Primary behavioral domains	Anhedonia, passive coping, social withdrawal	Anhedonia, relapse vulnerability, cognitive impairment
Cognitive endpoints	Not reliably affected	Robustly affected at late stages
Oxidative/redox profile	Sustained but moderate imbalance	Accumulating oxidative and nitrosative stress
Structural outcomes	Limited synaptic vulnerability	Dendritic, interneuronal, and volumetric alterations
Translational relevance	Chronic low-grade inflammation, treatment resistance	Neuroprogression, relapse, inflammatory comorbidity
Main limitation	Does not model relapse or long-term progression	Methodologically demanding; lower replication density

The chronic intermittent LPS protocol should be interpreted as a model of risk accumulation and neuroimmune sensitization rather than a direct surrogate for established neurodegenerative disease. Replication across laboratories remains more limited than for acute and sub-chronic paradigms, and strong claims of irreversible degeneration or proteinopathy are not warranted without convergent evidence spanning structural, molecular, and behavioral levels. Nevertheless, when implemented with rigorous longitudinal control, this protocol provides a distinctive experimental window into how recurrent inflammatory episodes can progressively compromise affective and cognitive resilience over time.

4 Conclusion and Future Perspectives

Lipopolysaccharide-based paradigms remain among the most robust and versatile experimental tools for interrogating immune-to-brain communication in rodents [5]. When applied with strict temporal and methodological control, these models enable the dissociation of adaptive sickness responses from maladaptive affective and cognitive dysfunctions driven by sustained neuroimmune signaling.

As detailed in this chapter, the translational validity of LPS-induced depression models depends less on the magnitude of the immune challenge than on protocol selection, temporal alignment of behavioral assessments, and appropriate validation of central endpoints. Acute protocols are best suited to capture early neuroimmune mechanisms and to screen rapid-acting antidepressant strategies, provided that behavioral testing is delayed beyond the sickness window. Sub-chronic paradigms allow the study of persistent depressive-like behavior under conditions of peripheral immune tolerance, offering a framework to examine failures of neuroimmune resolution. Chronic intermittent "hit-and-run" designs extend this logic by modeling relapse vulnerability and cumulative neurobiological burden resulting from recurrent inflammatory insults.

Across all protocols, a recurring methodological principle emerges: peripheral immune readouts alone are insufficient to define model validity. Direct assessment of central inflammatory, oxidative, and synaptic markers is essential to support behavioral interpretation and to avoid conflating systemic immune adaptation with recovery of brain homeostasis.

From a forward-looking perspective, the utility of LPS models will increasingly depend on their integration with higher-resolution analytical approaches. Circuit-specific manipulations, including optogenetic and chemogenetic strategies, may help disentangle how inflammation within discrete neural networks contributes to specific symptom dimensions such as anhedonia, motivational deficits, or cognitive rigidity. Similarly, the application of single-cell and spatial transcriptomic techniques offers the opportunity to resolve microglial and astrocytic

heterogeneity across acute, sub-chronic, and chronic inflammatory states, testing whether proposed concepts such as immune priming correspond to stable and identifiable cellular signatures.

Importantly, extended and intermittent LPS paradigms also provide a platform to investigate mechanisms of treatment resistance and loss of antidepressant efficacy over time, phenomena that are difficult to model using single-hit inflammatory challenges. However, claims regarding neurodegeneration or irreversible pathology must remain conservative and supported by convergent longitudinal, structural, and functional evidence.

In conclusion, when executed with methodological rigor and interpretative restraint, LPS-based protocols offer a powerful and flexible framework for studying the temporal dynamics of inflammation-associated depression. Their greatest strength lies not in reproducing major depressive disorder in its entirety, but in enabling precise experimental dissection of how immune challenges, when unresolved or recurrent, progressively compromise affective and cognitive resilience.

5 Notes

1. *Reagent identity and consistency*: Report the LPS source, strain/serotype, catalog number, lot, and stated potency (*EU/mg*) when available. Avoid mixing different lots within a single experiment to ensure reproducible immune activation.
2. *Endotoxin contamination control*: Use certified endotoxin-free saline, tips, and tubes for all dilutions. Prepare fresh working solutions to reduce artifacts related to adsorption or aggregation.
3. *Circadian standardization*: Standardize injection and behavioral testing to a fixed time of day (and light–dark cycle) because circadian rhythms strongly modulate immune and HPA axis responses.
4. *Route and volume precision*: Maintain a consistent injection route and volume (typically 10 mL/kg) across all animals. Small deviations can significantly alter the kinetics and severity of the inflammatory response.
5. *Dissociating sickness behavior*: Acute LPS produces transient sickness-like effects (e.g., lethargy, ptosis) that dominate locomotor readouts. Avoid interpreting behavioral immobility within the peak sickness window (0–6 h) unless locomotion is explicitly controlled.
6. *Humane endpoints and welfare*: Predefine clinical scoring and humane endpoints (e.g., weight loss thresholds, coat condition, posture) for systemic inflammation. Document all exclusions transparently.

7. *Behavior-naïve cohorts*: Including animals that undergo euthanasia without behavioral testing allows for the dissociation of LPS-driven neuroimmune activation from stress effects evoked by the testing procedures themselves.
8. *Habituation and tolerance tracking*: Track body weight, food intake, and baseline locomotion longitudinally. Stabilization or recovery of body weight typically indicates the emergence of peripheral endotoxin tolerance.
9. *Handling stress*: Repeated injections and handling are potent stressors. Match handling across control groups and consider sham injections where appropriate to isolate the effects of the immune challenge.
10. *Randomization and batch control*: Randomize cage positions and testing orders. In multi-month protocols, include internal controls to quantify and correct for potential day-to-day drift or plate-based assay effects.
11. *Sex and age as modifiers*: Chronic immune activation often exhibits sex- and age-dependent effects. Report sex explicitly and avoid pooling data unless the study is sufficiently powered or the pooling is scientifically justified.
12. *Timepoint discipline*: Predefine the exact interval between the final LPS exposure, the last behavioral task, and euthanasia. Differences as small as 12–24 h can significantly shift cytokine and redox markers.
13. *Behavioral battery and cognitive confounds*: Fix the order and spacing between tests to avoid high-stress tasks immediately preceding cognitive assessments. Ensure locomotor capacity is not a limiting factor in cognitive readouts.
14. *Anesthesia and terminal procedures*: Use the same anesthetic regimen for all groups, as various anesthetics can alter inflammatory signaling and phosphorylation states. Keep dissection times short and comparable across groups to reduce pre-analytical variance.
15. *Blinding and sampling*: Behavioral scoring and endpoint quantification must be blinded to the experimental group. If feasible, collect both plasma and specific brain regions to distinguish vascular from parenchymal immune contributions.

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Chapter 16

Infrared Thermography (IRT) as a Tool for Study of Animal Emotion

Shigeru Watanabe

Abstract

Infrared thermography (IRT) is an effective, non-invasive, and animal-friendly method for studying emotional responses in animals. As a non-contact technique, IRT enables simultaneous measurement of multiple subjects without causing stress or interference. It has been widely used to monitor temperature changes in hairless regions—such as the eyes and noses of primates and dogs, and the dorsal surfaces of rodents—particularly in research on social aversion. This review examines current applications of IRT and explores its potential for assessing complex social emotions, including inequality aversion. Although IRT provides reliable indices of emotional intensity, its capacity to distinguish between qualitatively different emotional states remains limited. Finally, the review outlines a protocol for the design and implementation of an IRT-based study.

Keywords Infrared thermography, Emotion, Inequality aversion, Social stress

1 Introduction

Measurement of body temperature using infrared thermography (IRT) is widely employed across various research fields. Kastberger and Stachl [1] reviewed the broad applicability of IRT in biological studies, including veterinary science, physiology, pharmacology, psychology, animal welfare, and conservation. IRT has been utilized for animals in laboratory settings [2] as well as for wildlife [3]. Notably, IRT has proven to be an effective tool for investigating energy utilization in farm animals [4, 5] and is applicable even to large animals like elephants [6].

Vianna and Carrivie [7] were among the first to use IRT to study emotions in rats, revealing a decrease in tail and paw temperature in fear-conditioned animal experiments. This marked a significant advancement in using IRT to explore emotional states in animals. The conditioned fear-induced hyperthermia observed was not attributed to shivering thermoregulation; however, the mechanisms of

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non-shivering heat production remain unclear [8]. Anxiety in rodents has been behaviorally assessed using the open-field test and elevated plus-maze. IRT applied in these contexts demonstrated an increase in body temperature, showing a strong correlation between thermal imaging and behavioral measures of anxiety [9]. Today, IRT is increasingly recognized as a powerful tool for experimental studies of animal behavior, particularly in investigating emotional responses.

1.1 Mechanism of Infrared Radiation Thermography (IRT)

Infrared radiation was first discovered by Wilhelm Herschel (1738–1822). In an experiment, he placed a thermometer just outside the visible spectrum of sunlight and observed an increase in temperature [10], detecting energy emitted as infrared radiation. Infrared radiation has wavelengths ranging from approximately 0.72 microns to 1.0 mm. Any object with a temperature above absolute zero (0 K) emits infrared radiation, with the intensity directly proportional to the object's temperature. By measuring this radiation, an object's temperature can be determined.

Infrared devices convert this radiation into electronic signals, and arrays of sensors produce infrared images. These images are color-coded to enhance interpretability in a process called thermography. Since the COVID-19 pandemic, infrared thermography has been widely adopted for temperature screening in public spaces, making thermal imaging a common tool in daily life.

The relationship between the energy of infrared thermal radiation and an object's surface temperature is described by the Stefan-Boltzmann law, which applies to an idealized object known as a blackbody. A blackbody has a radiation ratio of 1.0. However, as animals are not perfect blackbodies, it is necessary to estimate their radiation ratio to obtain accurate temperature measurements. This is typically done by comparing infrared thermography (IRT) readings with those from contact thermometers. For example, Watanabe [11] estimated the radiation ratio for the body surface of mice to be 0.98. Adjustments like this ensure accurate temperature assessments when using IRT on animals.

1.2 Advantages of Infrared Radiation Thermography (IRT)

IRT offers numerous advantages for measuring the temperature of animals in research settings:

1. **Non-invasive**: Unlike implanted transducers or rectal temperature recordings, IRT is entirely non-invasive, reducing stress on the animals. This feature allows for repeated assessments of the same animal, making it especially valuable in longitudinal or developmental studies [12].
2. **Non-contact measurement**: IRT is a non-contact method, ensuring that the animal's behavior remains undisturbed. This makes it suitable for both experimental and natural environments where minimizing interference is critical.

3. **Integration with other sensors**: IRT can be combined with other physiological monitoring tools, such as electrocardiography (ECG), breathing monitors, nasal thermistors, or galvanic skin response (GSR) devices. This integration enhances the reliability of autonomic response measurements and provides a more comprehensive understanding of the animal's physiological state [13].
4. **Monitoring multiple animals simultaneously**: IRT facilitates the simultaneous recording of temperature data from multiple animals. This capability is crucial for studying social behaviors and interactions in group settings.
5. **Operability in darkness**: Since IRT does not require visible light, it can measure temperatures in complete darkness. This feature is particularly advantageous for studying nocturnal animals or conducting experiments in low-light conditions.

Additionally, specialized software, such as *ThermoLabAnimal* [14], simplifies data analysis and interpretation, further enhancing the practicality of IRT in animal research.

2 Measurement of Emotion Using Infrared Radiation Thermography (IRT)

Body temperature can be categorized into surface temperature and core temperature. The body surface serves as the interface between the internal environment and external surroundings, making surface temperature highly susceptible to environmental factors. Consequently, surface temperature (often measured at the skin) can fluctuate based on these external influences. In contrast, core temperature is a more reliable indicator of an animal's metabolic rate and internal physiological state [15].

Despite these differences, many studies have reported a correlation between surface temperature, measured by IRT, and core temperature. For example, Saegusa and Tabata [16] found that the eye and back surface temperatures of mice exhibited patterns of change similar to those observed in cloacal temperature, a common proxy for core temperature. Similarly, significant correlations have been noted between eye temperature and cloacal temperature in dogs [17, 18] and horses [19]. In my observations, there was a clear correlation between the dorsal surface temperature of ICR mice measured using IRT and rectal temperature (11, supplementary experiment).

2.1 Event-Related Infrared Radiation Thermography (IRT)

Thermoregulation in animals is influenced by various factors, including environmental conditions (e.g., cold or heat), infection, hunger, and emotional states such as fear, stress, or pleasure. Emotional states, in particular, can significantly impact body temperature regulation.

Using IRT after an emotion-inducing event enables the detection of these changes, a method referred to as "event-related IRT." For example, Wongsaengchana et al. [20] demonstrated that acute stress leads to increases in tail and ocular temperature in rats, highlighting that emotional stress induces measurable changes in surface temperature.

Emotions are generally categorized into two broad types: positive and negative. Both types trigger autonomic responses involving the sympathetic and parasympathetic nervous systems. Sympathetic responses increase body temperature to prepare the body for action, facilitating muscle activity needed for "fight or flight" reactions. Conversely, parasympathetic responses reduce body temperature, helping to mitigate harm, such as excessive bleeding, in stressful situations. Consequently, autonomic responses to negative emotions often involve contradictory processes, where both increases and decreases in body temperature may occur.

Event-related IRT focuses on temperature changes in response to specific events, such as emotional stimuli. Importantly, it is the relative change in temperature, rather than the absolute temperature value, that is most significant. The baseline temperature—measured prior to the emotional event—serves as a critical reference point. The difference between the baseline and post-event temperature provides key data for analyzing emotional responses using IRT.

2.2 Regions of Interest (ROI)

IRT can capture temperature data from any body surface, but selecting the appropriate region of interest (ROI) is essential for obtaining accurate and meaningful measurements. Several physiological factors must be considered when selecting an ROI, including the presence of hair, skin color, skin thickness, and capillary density [3]. Hair itself does not generate heat but serves as an insulating layer, potentially affecting temperature measurements. To mitigate this, researchers often choose hairless regions for IRT. One particularly promising ROI is the ocular region, as it is hairless and highly vascularized, making it sensitive to physiological changes.

Blenkuš et al. [21] reported discrepancies between body surface temperature (which increased) and tail temperature (which decreased) following mild stressors such as handling, tail pinching, or tunnel handling in mice. Similarly, Chotard et al. [22] observed distinct temperature changes in four different facial regions of monkeys in response to various emotional stimuli. These findings suggest that different ROIs may exhibit unique temperature responses to specific physiological or emotional states.

In human studies, the choice of ROI is equally important. Nummenmaa et al. [23] conducted a study using a topographical self-report method to map bodily sensations associated with various emotions in a large population ($n = 701$). The study demonstrated that emotions are not only consciously perceived but also correspond

to distinct somatosensory experiences across the body. Each emotion produced a unique map of sensory responses, providing evidence that emotions have measurable physiological correlates that can be spatially mapped. These findings underscore the importance of ROI selection in emotional research, as different emotions may induce specific thermoregulatory responses across various body regions.

In rodent studies, the dorsal surface is commonly used for temperature measurements due to its accessibility and relevance to thermoregulation. However, because this area is often covered by fur, researchers may shave the fur or use hairless (nude) rodent strains for more accurate measurements [24]. The dorsal region also contains brown adipose tissue (BAT), which generates heat and plays a crucial role in thermoregulation, especially under stress.

Given the complexity of the relationship between stress and thermoregulation, it is crucial to clarify the anatomical pathways through which stress affects temperature in specific ROIs. Improved understanding of these pathways will enhance the interpretation of IRT data and facilitate the linkage between physiological changes, emotional states, and behavioral responses.

2.3 The Nose and Eyes as Windows to Emotion

The nose is often regarded as a key indicator of emotional responses in primates. Infrared radiation thermography (IRT) has been widely employed to detect these emotional responses [25, 26], with the nose serving as a common region of interest (ROI) in such studies. Experimental protocols typically involve three phases: a pre-stimulus (baseline) phase, a stimulus presentation phase, and a post-stimulus phase. IRT data from the baseline and post-stimulus phases are frequently analyzed, as the stimulus presentation phase often includes substantial behavioral responses that could interfere with accurate temperature measurements. These behavioral responses might mask the underlying thermoregulatory changes linked to emotional states. However, it is important to recognize that temperature changes observed at the nose may not exclusively result from emotional responses. Other factors, such as airflow patterns or blood flow changes related to muscular activity, can also influence nose temperature. For example, in infant studies, Nakanishi and Imai-Matsumura [27] noted that factors unrelated to emotional stimuli, such as respiration or physical movements, can impact nasal temperature measurements.

Nose IRT and Negative Emotions in Primates

Early studies on emotional responses in primates often used fixed-head setups. Nakayama et al. [28] were among the first to document a decrease in nasal temperature in rhesus monkeys immobilized in a monkey chair and exposed to a "treating person." The monkeys displayed behavioral signs of negative emotions, such as bared-teeth expressions, staring with an open mouth, and lip-smacking. These behaviors were linked to a decrease in nasal temperature, suggesting

that nasal temperature reduction may be an indicator of negative emotional states. Similarly, Kuraoka and Nakamura [13] reported a decrease in nasal temperature in rhesus monkeys when shown video clips of aggressive conspecifics. While neutral vocalizations like cooing or screaming caused no significant effect, combining aggressive videos with vocalizations amplified the temperature decrease, further highlighting the role of visual and auditory stimuli in eliciting emotional responses.

Chimpanzees also exhibit decreases in nasal temperature in response to emotional stimuli. Kano et al. [29] found that playback sounds or videos of conspecifics fighting caused a significant decrease in nasal temperature in chimpanzees. In contrast, videos of orangutan fights only slightly reduced nasal temperature. Behavioral signs of excitement, changes in heart rate, and the absence of elevated cortisol levels suggested the involvement of the autonomic nervous system rather than a stress-related endocrine response. The magnitude of decrease in the nasal temperature correlated with the intensity of the emotional stimulus, with conspecific fights evoking the strongest responses compared to non-conspecific fights or neutral sounds.

Advances in IRT methodologies have shifted from fixed-head setups, like monkey chairs, to more naturalistic conditions. For instance, researchers have used small food items to control head position in free-moving animals. Dezecache et al. [30] measured nasal and ear temperatures in wild, free-moving chimpanzees, observing a decrease in nasal temperature and a corresponding increase in ear temperature following exposure to conspecific vocalizations. Ross et al. [31] used IRT in a sanctuary setting to monitor facial temperatures in 29 chimpanzees, noting distinct temperature patterns between active and inactive animals. These studies demonstrate the potential of IRT to provide insights into emotional responses in naturalistic environments, despite challenges such as environmental factors (e.g., atmospheric conditions, humidity, and camera angle) that can influence readings.

Chotard et al. [22] examined four facial areas—the peri-orbital region, nose bridge, nose tip, and upper lip—in several primate species, including common marmosets, white-throated capuchins, rhesus macaques, Bornean gibbons, and western lowland gorillas. Their findings showed that negative emotions, such as those evoked by food delays and teasing, were associated with decreased nasal tip temperature and increased upper lip temperature. Ermatinger et al. [32] found that male common marmosets exhibited a decrease in nasal temperature when exposed to playback of aggressive vocalizations.

Notably, individual variation significantly influences thermal responses to negative emotions. Bhattacharjee et al. [33] categorized coping behaviors in long-tailed macaques as either aggression-based or non-aggression-based. They found that aggression-based individuals exhibited an increase in nasal temperature when exposed to a

predator, whereas non-aggression-based individuals showed no such change. In contrast, Mazzamuto et al. [34] reported no personality-related differences in thermal responses—measured at the eyes, nose, and ears—in squirrels under stress.

Overall, studies using IRT to measure emotional responses in primates predominantly report hypothermic responses, where blood is redistributed from peripheral tissues to vital organs. This physiological response likely functions as a protective mechanism against potential blood loss during stressful situations.

Nose IRT and Positive Emotions in Primates

Positive emotions, such as pleasure or calmness, can induce hyperthermia through sympathetic activation. This temperature increase may result from physiological arousal or thermoregulatory responses. However, positive emotions may also lead to a reduction in body temperature, particularly during deep relaxation or calm states, as the overall metabolic rate decreases and the body becomes more prone to heat loss, especially in cooler environments.

Unlike negative emotions, the relationship between positive emotions and body temperature changes is less consistent. For example, Heintz et al. [35] used IRT to study three western lowland gorillas interacting with familiar humans. Two gorillas exhibited a decrease in nasal temperature, while the third showed an increase, illustrating the variability of thermal responses to positive stimuli. The authors highlighted the limited validity of IRT in these contexts, noting that thermal responses may depend on the type of stimulus and the situational context.

In a broader investigation, Chotard et al. [22] examined thermal responses to both positive and negative emotional stimuli across several primate species. Positive stimuli—such as access to preferred food, playback of food-related vocalizations, and affiliative social interactions (e.g., play or tickling)—were associated with decreased nasal tip temperature and increased peri-orbital temperature. Expanding on this, Brügger et al. [36] focused on the thermal responses of non-breeding marmosets to auditory social cues. Female marmosets exhibited a decrease in nasal temperature when exposed to playbacks of positive interactions. In contrast, non-breeding males showed an increase in nasal temperature in response to negative interaction playbacks, food calls, or chatter calls.

While IRT holds great promise for studying animal emotions [37], challenges remain. The hypothermic response associated with negative emotions is relatively straightforward to interpret, but the same response in the context of positive emotions is more complex and requires further investigation.

In summary, emotional stimuli, whether social or non-social, and whether positive or negative, often cause a decrease in nasal temperature in primates. However, IRT cannot distinguish between positive and negative emotions or between social and non-social

stimuli. Instead, it detects the intensity or magnitude of an emotional response without identifying the specific quality of the emotion. Non-primate studies, such as those conducted in cows, also suggest that nasal IRT can measure emotional magnitude but cannot discriminate between different emotions [38].

Eye IRT and Emotion

The surface temperature of the eye is an ideal region for IRT because the eye remains hairless, even in species with fur. As a result, eye IRT is frequently used in studies of non-primate mammals and birds. Ikkatai and Watanabe [39] recorded eye surface temperatures in budgerigars, observing an increase following handling stress. Similarly, Moe et al. [40] reported eye temperature increases in chicks after restraint-induced stress, indicative of hyperthermic responses. The effects of handling on surface temperature depend on the region of interest (ROI). For example, Edgar et al. [41] found that, in hens, comb temperature decreased significantly, while eye temperature displayed an initial decrease followed by an increase, and head temperature rose steadily over 20 min post-handling. The greatest temperature changes were observed in the comb, leading the authors to recommend the comb as a primary ROI, despite its unclear function in domestic chickens. Herbon et al. [42] further investigated comb and wattle temperature in hens, finding that stress intensity predicted the degree of initial cooling and the delayed warming of these regions. These two phases of temperature change provided valuable metrics for quantifying stress.

Dogs are a commonly studied species in eye IRT research. Elias et al. [43] measured eye temperature at seven positions in 465 greyhounds before and after races. Eye temperature increased post-race, reflecting stress and arousal. Interestingly, eye temperature also rose as the race number approached, with higher pre-race eye temperatures correlating with poorer race outcomes [44]. Social interactions also influence canine eye temperature. Riemer et al. [45] reported a decrease in eye temperature when dogs were separated from their owners (negative emotion) and an increase upon reunion (positive emotion). Similarly, Travain et al. [17] observed increased eye temperatures when dogs received food from their owners (positive stimulus). However, they also noted similar increases when dogs visited a veterinarian (negative stimulus). Flint et al. [46] compared canine eye and nose temperatures across six scenarios designed to elicit either positive or negative emotional valence. They found that surface temperatures were significantly lower for high-arousal negative scenarios than for high-arousal positive scenarios, highlighting the impact of environmental differences on IRT results.

In animals other than dogs, Stewart et al. [47] observed a rapid drop in eye temperature in calves during the first 5 min following disbudding, followed by a rise above baseline levels for the remainder of the observation period. Similarly, Redaelli et al. [48] found that

horses' eye temperature increased following training. In sheep, the temperature of the lacrimal caruncle was significantly elevated during restraint and remained high after a veterinary assessment and administration (VAA) compared to baseline levels before restraint [49].

These studies highlight that eye temperature reflects emotion-induced changes across a variety of species. However, while eye temperature is sensitive to emotional states, it cannot reliably distinguish between positive and negative emotions.

3 Stress-induced Hyperthermia (SIH)

Stress triggers a variety of autonomic responses, including changes in heart rate, blood pressure, and respiration rate. One key physiological response to stress is an increase in body temperature, known as stress-induced hyperthermia (SIH) [50]. SIH has been observed in the core body temperature of various rodent species, such as rats [51, 52], mice [53], and rabbits [54]. A similar phenomenon, termed "psychological fever," has been reported in humans [55].

Traditionally, rectal temperature has been used to measure core body temperature (e.g., [56]). However, infrared thermography (IRT) has become a preferred non-invasive alternative for measuring body temperature [57–60].

3.1 Neural Mechanisms of SIH

The neural mechanisms underlying SIH have been extensively studied [61, 62]. Figure 1 schematically illustrates these mechanisms. The preoptic area of the hypothalamus (POA) integrates thermo-sensory information and sends it to the dorsomedial hypothalamus (DMH). The DMH projects to the rostral medullary raphe nucleus (RMR) in the medulla, which activates brown adipose tissue (BAT) via the ventral horn. Mitochondria in BAT regulate thermogenesis and contribute to heat production.

Stress activates the corticolimbic system, relaying input to the ventromedial prefrontal cortex (VMP), including the dorsal peduncular cortex and dorsal tenia tecta. The VMP transmits this information to the DMH-RMR system, which leads to BAT activation. Optogenetic stimulation of the DMH-RMR pathway induces heat production in BAT, whereas optogenetic inhibition prevents it [64].

Beyond BAT regulation, the DMH-RMR system controls skin blood vessels, skeletal muscles, and heat production in muscles, all of which contribute to thermoregulation. However, studies on knockout mice lacking uncoupling protein-1 (UCP-1)—a protein that activates mitochondria in BAT to produce heat—show that SIH induced by cage exchange is not abolished by UCP-1 deficiency. This suggests that SIH may not solely depend on UCP-1-driven BAT activation [65].

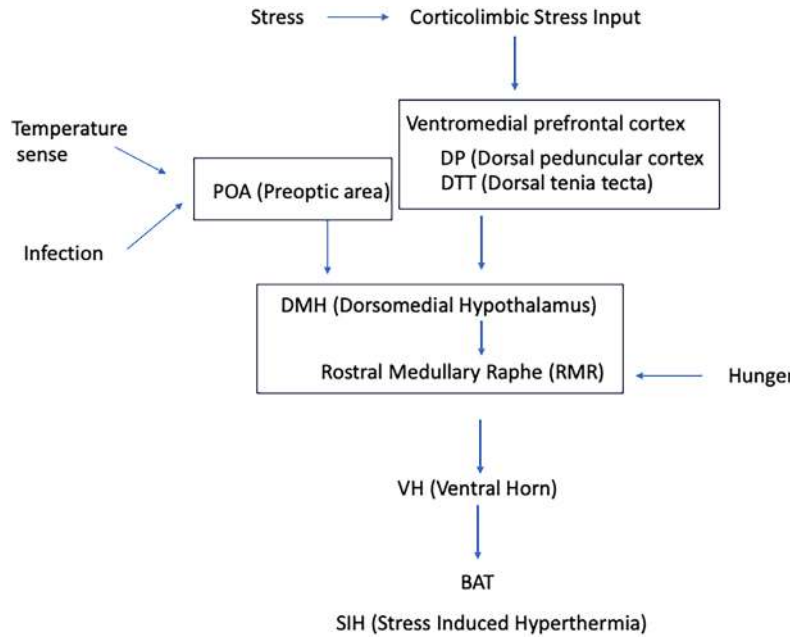


Fig. 1 Neural mechanisms of SIH. Schematic illustration of the neural mechanisms underlying stress-induced hyperthermia (SIH). Stress activates the ventromedial prefrontal cortex, which transmits signals to the dorsomedial hypothalamus (DMH). Subsequently, brown adipose tissue (BAT) in the dorsal surface area is activated via the Raphe-VH. Adapted from Nakamura [63]

Other factors influencing thermoregulation include thermo-sensory input (e.g., skin temperature), infection, and starvation. Peripheral thermossensors, including those in the skin, send temperature information to the POA. Infection activates the POA via immune signaling, while hunger directly activates the DMH-RMR system.

The transient receptor potential vanilloid 1 (TRPV1) is a peripheral thermo-sensor involved in feedback regulation of SIH [66]. Desensitization of TRPV1 through resiniferatoxin injection enhances SIH [67], indicating TRPV1's role in modulating this thermoregulatory response.

3.2 Socially Induced SIH

Various stressors induce hyperthermia in mice, including exposure to a novel cage [68], physical restraint [57, 69, 70], and small cage housing, restraint, and encircling handling in rats [20]. Fear conditioning also induces hyperthermia [8], although the role of brown adipose tissue (BAT) in this context remains unsupported. Additional stressors include open-field and plus-maze tests in rats [71], exposure to predator odors such as methylthiazoline in female mice [72], as well as anesthesia, scruffing, and intraperitoneal saline injections [73].

Social stress specifically induces stress-induced hyperthermia (SIH) in both core and surface body temperatures. A single episode

of social defeat causes SIH lasting several hours, whereas repeated social defeats result in long-term SIH that can persist for up to 8 days [74]. Social stress is associated with elevated corticosterone levels [75] and increased body temperature in rats [76]. The oxytocin system also plays a crucial role in thermoregulation during social stress. Oxytocin system inactivation impairs thermoregulation in mice [77, 78]. Harshaw et al. [79] demonstrated that antagonism of the oxytocin receptor elevates SIH triggered by exposure to unfamiliar conspecifics, highlighting oxytocin's importance in regulating SIH induced by social stress.

4 Higher-order Social Cognition Detected by IRT

Social inequality stress, a more complex form of social stress, can emerge from disparities in social circumstances, and this stress induces hyperthermia.

4.1 Inequality Aversion in Positive Situation

The social inequality stress is exemplified when one individual experiences greater happiness or rewards than another, potentially eliciting negative emotions such as envy or reversed empathy in the less advantaged individual [80]. This emotional response to inequality is not exclusive to humans; nonhuman animals also exhibit aversive reactions to social inequality, a phenomenon known as inequality aversion.

Inequality aversion encompasses two primary dimensions: behavioral and emotional. The behavioral aspect involves actions such as refusing rewards in inequitable situations or demonstrating a preference for equal over unequal distributions of resources. Research in this area has predominantly focused on behavioral data, particularly in primates [81–84]. For example, capuchin monkeys have been observed to reject cucumbers when other monkeys receive more desirable grapes [81]. This behavior, though seemingly counterintuitive—since receiving any food is better than none—suggests an emotional response to perceived unfairness. Similar behaviors indicating inequality aversion have been observed in other primates [85].

Inequality aversion has been documented in non-primate species. For instance, Range et al. [86] trained dogs to "give the paw" and then extinguished the behavior. Dogs displayed faster extinction when observing another dog receiving rewards compared to when trained alone without rewards [86, 87]. Behavioral inequality aversion has also been reported in mice [88]. Oberliessen et al. [89] examined inequality aversion in rats using a T-maze paradigm. In this setup, one option provided food solely for the subject, while the other provided food for both the subject and a cage mate. The rats consistently preferred the option that led to an equal food distribution, indicating a preference for equality. Despite these findings,

there remains significant debate about the prevalence and extent of inequality aversion in nonhuman animals [82, 84]. A meta-analysis by Ritov et al. [90], which reviewed 18 species, concluded that there is no conclusive evidence supporting inequality aversion across species. Watanabe [91] used SIH as an index of inequality aversion in mice and found that food-deprived mice surrounded by cage mates consuming food exhibited SIH (right panel of Fig. 2). These findings suggest that mice display inequality aversion at the level of autonomic responses. Such aversion may be induced by the perceived risk of food depletion.

4.2 Inequality Aversion in Negative Situation

Most research on inequality aversion has focused on appetitive situations, while aversion in unpleasant contexts, such as restraint stress, has been less frequently studied [11, 91, 92]. Restraint stress, induced by placing animals in cylindrical restraint holders, can trigger SIH. Studies have shown that mice restrained in holders while their cage mates are freely moving (the social inequality condition) exhibit higher SIH levels compared to other conditions [11]. These findings suggest that social inequality amplifies stress in restrained mice. This conclusion is supported by several stress indicators [91]. First, corticosterone levels were highest in restrained mice when their cage mates were free to move. Second, the memory-enhancing effect of stress was more pronounced under the social inequality condition than in the single-stress condition (where a mouse was restrained without observing free-moving counterparts). Together, these observations—elevated corticosterone, enhanced memory effects, and increased SIH—provide strong evidence that social inequality exacerbates stress.

The influence of social conditions on stress responses may be explained by anti-predatory behavior. A restrained animal surrounded by freely moving cage mates might experience heightened stress

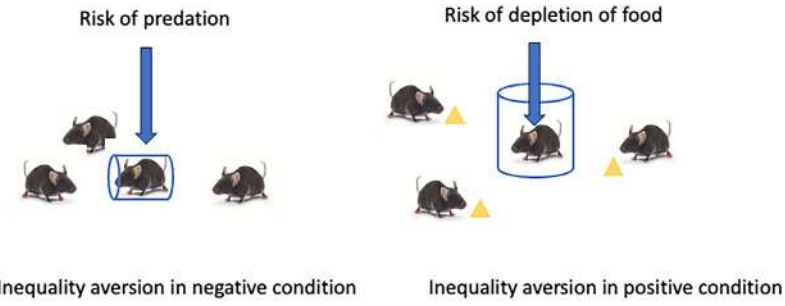


Fig. 2 Schematic representation of inequality aversion. **Left**: A mouse restrained in a holder and surrounded by freely moving cage mates experiences inequality aversion due to an increased perceived risk of predation. **Right**: A hungry mouse observing cage mates eating experiences inequality aversion due to the perceived risk of food resource depletion

due to a perceived increased risk of predation. In contrast, when all animals are restrained, the perceived threat decreases due to the dilution effect, wherein a larger group reduces the likelihood of any single individual being preyed upon.

Another explanation for inequality aversion under restraint stress relates to social threat. Restrained mice may feel vulnerable and unable to defend themselves or escape from their freely moving cage mates. Observations support this explanation: in the social inequality condition, free-moving mice often climbed onto the restraint holders, but the narrow slits prevented physical biting or harm to the restrained mice. This suggests that the stress experienced in the inequality condition is unlikely to be caused by physical attacks or social defeat. To test the role of cage mates' movement in the stress-enhancing effect, Watanabe [11] examined the social inequality condition using anesthetized cage mates. In this modified scenario, cage mates were not restrained but were immobile. The results showed that anesthetized cage mates did not produce the same stress-enhancing effects as freely moving ones. This finding supports the explanation that anti-predatory behavior plays a critical role in the stress experienced under social inequality (left panel of Fig. 2).

4.3 Two Types of Inequality Aversion

Inequality can be categorized into two types: disadvantageous inequality and advantageous inequality. Disadvantageous inequality occurs when an individual receives less than others, while advantageous inequality arises when an individual receives more than others. Humans typically experience stronger negative emotions in situations of disadvantageous inequality, where they are at a disadvantage relative to others. However, research on advantageous inequality aversion in animals remains limited [93]. Even in humans, advantageous inequality is generally less aversive than disadvantageous inequality.

As described earlier, mice that were restrained while their cage mates were free to move (the disadvantageous inequality condition) exhibited SIH. In contrast, free mice paired with restrained cage mates (the advantageous inequality condition) did not exhibit SIH [92]. These findings suggest that disadvantageous inequality is more stressful than advantageous inequality.

Pre-feeding Alters Inequality Aversion in Food Delivery Scenarios

Inequitable food delivery has also been used to study SIH in mice [94]. Food-deprived mice surrounded by cage mates consuming food (disadvantageous inequality condition) exhibited SIH. This response can be attributed to the perceived risk of food resource depletion. In contrast, mice that received food while their cage mates were food-deprived (advantageous inequality condition) showed a slight increase in body temperature, but this increase was not significantly different from the equality condition, where all mice received food. Interestingly, pre-feeding the test mice mitigated SIH in the disadvantageous inequality condition. Satiated mice did not exhibit

aversion to cage mates consuming food, suggesting that feeling of "hunger" is a critical factor in disadvantageous inequality aversion. When test subjects were pre-fed and no longer hungry, the behavior of cage mates eating food did not elicit a stress response, as food depletion was no longer relevant to them. These findings emphasize that the relationship between an individual's internal state and the states of others is key to understanding inequality aversion in food-related scenarios.

Discrepancy of Behavioral Aversion and Emotional Aversion

In a previous experiment [94], the body temperature of mice was measured in a chamber with two compartments: one containing inaccessible cheese and the other containing a cage mate eating cheese. Hungry mice displayed significantly higher body temperatures in the compartment with the cheese-eating cage mate compared to the compartment with inaccessible cheese. This finding indicates that the cage mate's eating behavior caused SIH in the hungry mice.

Interestingly, behavioral tests in a similar setting revealed a preference for inequality rather than aversion. The test mice spent more time in the compartment containing the cheese-eating cage mate than in the compartments containing either inaccessible cheese or a cage mate alone. These results highlight a contradiction: SIH, indicative of stress or aversion, coexists with a behavioral preference for the aversive condition. Because infrared thermography is a non-contact method, it enables simultaneous recording of SIH and behavioral preference [94]. In subsequent experiments, in which both behavioral preference and body surface temperature were measured simultaneously (see Fig. 3), mice spent more time in the compartment with the cheese-eating cage mate, even though this condition induced SIH. This paradoxical behavior suggests that while social inequality induces stress, it also drives approach behavior. The attraction may

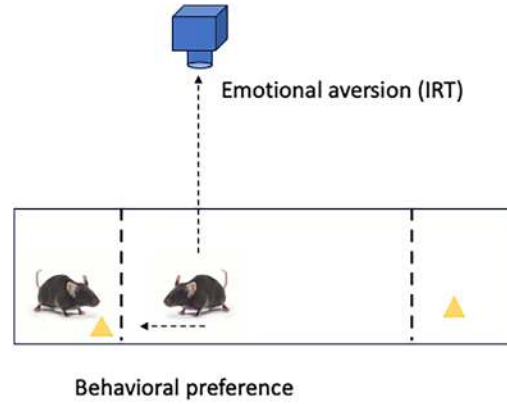


Fig. 3 Simultaneous recording of behavioral preference and SIH. An eating cage mate attracts the mouse (indicating behavioral preference) but also triggers stress-induced hyperthermia

stem from the informative value provided by observing the cage mate's eating behavior.

The Dual Nature of Aversive Stimuli

The presence of a cage mate eating food has an aversive effect, as evidenced by SIH, but test mice still spent more time in this condition, exhibiting seemingly contradictory behavior. A similar paradox has been observed in studies involving operant behavior maintained by aversive stimuli. For instance, Morse et al. [95] showed that monkeys, under a fixed-interval schedule of electric shocks, displayed behavior patterns similar to those maintained by food reinforcement. While this behavior required prior exposure to shocks to establish the response pattern, the monkeys voluntarily performed actions to produce the aversive stimulus.

One possible explanation for this paradox is the informative value of aversive stimuli. Exposure to an aversive stimulus may provide critical environmental information. In humans, this concept is reflected in preferences for bad news or curiosity about adverse scenarios, such as horror movies or learning about wealthy individuals, even if these evoke negative emotions. Rogers-Carter et al. [96] reported approach behavior in female rats toward stressed conspecifics subjected to electric shocks in social preference tests. Langford et al. [97] found that mice approached conspecifics in pain, further supporting the idea of information-seeking behavior.

The distress of conspecifics may attract others, despite its aversive nature. Watanabe [98] examined behavioral preferences for a conspecific injected with formalin in a hind paw. Mice spent more time in a compartment with the formalin-injected cage mate than in a compartment with an uninjected mate, suggesting a preference for the distressed conspecific. However, conditioned place preference experiments revealed that compartments associated with formalin-injected conspecifics induced conditioned aversion rather than preference [98].

This discrepancy indicates that while the distress of a conspecific may elicit approach behavior, it also triggers adverse emotional effects, leading to conditioned aversion. These findings demonstrate the complex interplay between behavioral attraction and emotional aversion in response to distressing stimuli.

5 Protocol of IRT Study of Higher Order Social Cgnition

The following are examples of IRM experiment, basically from [99].

5.1 Animals

Male C57BL6/J mice are used in this study. They are housed in groups of four per cage ($30 \times 20 \times 13$ cm) with ad libitum access to food and water. The room temperature is maintained at 24°C, and the mice are kept under reversed 12-h light/dark cycles. All animals

are handled for approximately 5 min per day for 7 days prior to the start of the experiment. The dorsal hair of the mice is shaved under anesthesia. The exposed skin was then cleaned using a hair removal cream.

5.2 Apparatus

1. *Restraint holder*: a mouse holder designed for blood sampling (ASONE, To-12B-508-02, Japan) is used to restrain each animal (see Fig. 4). The holder is cylindrical (diameter: 30 mm; length: 100 mm), made of transparent acrylic, and features a slit (5 × 50 mm) through which the dorsal body surface temperature was measured.
2. *Infrared thermal imaging camera:* an infrared thermal imaging camera (G100, NEC Avio) is utilized. To determine the radiation ratio, the mouse's body surface temperature measured by the infrared thermometer is calibrated against the temperature obtained using a contact thermometer (CSK 1260, SATO Keiryoki MFG Co. Ltd., with a probe diameter of 1.0 mm). The radiation ratio is consistently around 0.98.
3. *Testing chamber:* a transparent cage (30 × 20 × 13 cm) served as the experimental chamber.

5.3 Procedure

1. *Experimental design*: in this experiment, single SIH and SIH under social inequality conditions are compared. The subject mouse is placed in a restraint holder and positioned within the testing chamber. Under the social inequality condition, three cage mates are also placed in the testing chamber. The experiment is conducted under three conditions to evaluate physical contact: light (120 lx), dark, and light without social contact. In the latter condition, the restrained mouse is placed inside a transparent tube (a cylindrical acrylic tube, diameter: 10 cm,

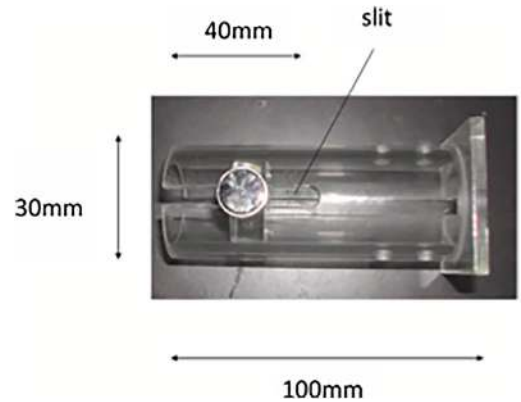


Fig. 4 A mouse holder designed for blood sampling is repurposed as a restraint holder. The upper slit allows for the recording of body temperature

height: 20 cm) within the experimental chamber to prevent physical interaction with the three cage mates (*see* **Note 1**).

2. *Recording:* thermal images of the mice are captured using an infrared thermal imaging camera positioned 60 cm away. Body surface temperatures are calculated from the thermal images using the software package InfRecAnalyzer NS9500 Lite (NS9500LT, NEC Avio). The region of interest (ROI) is defined by drawing a rectangle around the slit of the restraint holder on the thermal image, and the highest temperature within this region is recorded. The room temperature is maintained at 23°C, with humidity levels between 60% and 75%. (*see* **Note 2**).
3. *Baseline*: the subject mouse is housed overnight in a single-animal cage (20 × 15 × 13 (height) cm) prior to testing to avoid stress from being handled directly after removal from a group cage. The mouse is then placed in the experimental chamber, where its body surface temperature is measured in a free-moving state. Thermal images of the body surface are recorded twice while the mouse is motionless and flat. The first recording is taken approximately 5 min after the mouse is placed in the chamber, although the exact timing depends on its movements. The second recording is taken about 1 min after the first, with timing again dependent on the mouse's behavior. The average of these two measurements is recorded as the baseline body temperature.
4. *Testing:* after the subject mouse is placed in the holder, the lid is secured, and the holder is placed in the testing chamber under the specified test conditions. This experiment assesses the body temperature at a steady state rather than tracking changes over time. Body temperature measurements are taken twice, 20 min after the mouse is secured in the holder. For the inequality condition, cage mates are introduced into the testing chamber immediately after the subject mouse is positioned. At the end of the testing period, the subject mouse is removed from the holder and returned to its group-living cage.

5.4 Results

Figure 5 illustrates the example of results. The inequality condition resulted in significantly greater SIH (mean = 1.72°C, SD = 0.76) compared to the single condition (mean = 0.78°C, SD = 1.29) in the light room. Similarly, in the dark room, the inequality condition produces greater SIH (mean = 2.0°C, SD = 0.80) than the single condition (mean = 0.99°C, SD = 1.10). However, this SIH-enhancing effect is not observed in the no physical contact condition, where the inequality condition (mean = 1.16°C, SD = 0.63) and the single condition (mean = 1.34°C, SD = 1.09) show comparable results.

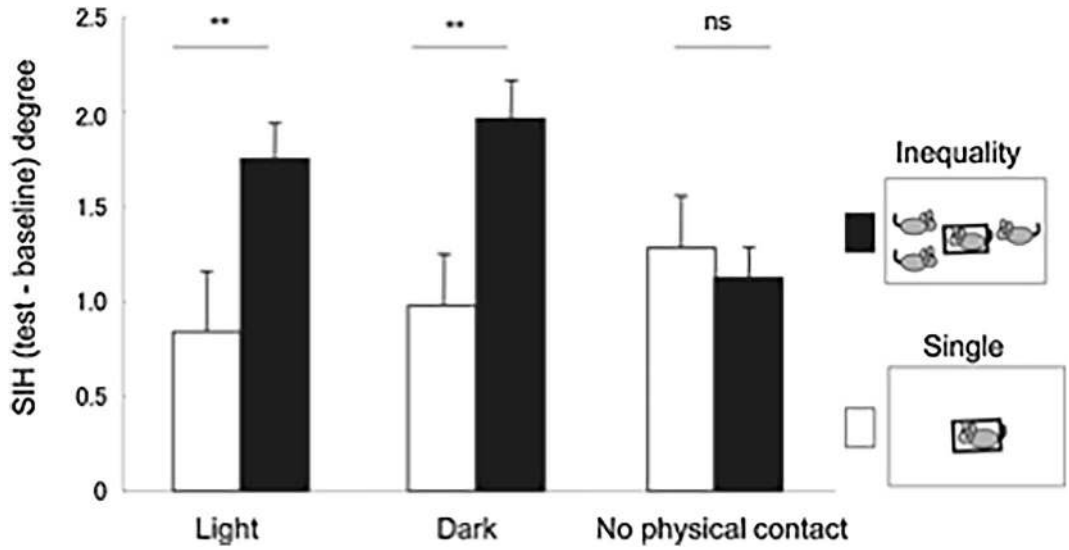


Fig. 5 Comparison of single stress and social inequality aversion. The vertical axis represents the change in body temperature from baseline. Inequality increases stress-induced hyperthermia (SIH) under light and dark conditions but not in the absence of physical contact [99]

The light condition includes visual, olfactory, auditory, and tactile cues, while the dark condition lacks visual cues but retains the others. In the no physical contact condition, tactile cues are absent, but visual cues are present. Olfactory and auditory cues are limited but not entirely eliminated in the no physical contact condition.

5.5 Notes

1. *Experimental setting and baseline:* in infrared thermography (IRT) studies, the primary data is not the absolute body temperature but the deviation from the baseline (control condition). Some studies analyze time-locked temporal changes in temperature relative to the baseline (event-related IRT), while others calculate the difference in average temperature between the test and baseline conditions. A critical aspect of these approaches is isolating the effect of the independent variable. The experimental design must ensure that only one variable is manipulated, with all other variables remaining consistent between the control and test conditions. Even minor environmental changes during the test condition can influence body temperature measurements. Therefore, it is essential to maintain identical conditions in the test chamber for both test and control phases, with the independent variable being the only difference. Since the key data relies on temperature differences from the baseline, careful measurements during the baseline phase are crucial. Temperature can drift over time, making appropriate sampling intervals essential for accuracy. Additionally, when subjects are housed in groups,

the testing order may influence results due to potential order effects (e.g., [21]).

2. *Camera angle and distance:* electromagnetic radiation travels in straight lines, and measuring thermal radiation at acute angles reduces the detected radiation, making the object appear cooler than its actual temperature. Although this effect is minor for uneven surfaces, the optimal angle for measuring electromagnetic radiation is perpendicular to the emitting source. When capturing thermal images of the dorsal surface of rodents, ensure the animal is lying flat and avoid taking measurements during rearing behavior to ensure consistency.

Foster and Ichiji [100] demonstrated that IRT images taken at a 90° angle to the eye, nasal plane, and sagittal plane on horses' faces yielded significantly different recorded values. To identify the optimal measurement point for assessing arousal, stress, and pain, they exposed horses to a novel object as a stressful event. Their findings revealed a significant positive correlation between eye temperature measured at a 90° angle to the sagittal plane and heart rate variability. Consequently, they suggested this position as optimal for such studies. The intensity of thermal radiation is also influenced by the distance between the camera and the subject. To ensure accurate and comparable measurements, the distance between the camera and the subject must remain consistent during both the baseline and experimental phases.

Conflict of Interest

There are no conflicts of interest. This research was supported by a Grant-in-Aid for Scientific Research 18H01104 and W01KB24010.

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Chapter 17

Decoding Anxiety Circuits by Integrating Behavioral Assays with Neural Recordings

Joeri Bordes, Daria Ricci, Leona Stont, Thibault Bittar and Anna Beyeler

Abstract

Anxiety disorders are among the most prevalent psychiatric conditions, and preclinical rodent models are essential for dissecting the neurobiological mechanisms underlying their physiological and behavioral symptoms. Anxiety-related behaviors in mice emerge from neural circuits that integrate external threats with internal state. This chapter provides a practical guide to combining canonical anxiety assays with modern in vivo neural recording tools to dissect neural circuits controlling anxiety, which are molecularly and anatomically defined (cell types and projections, respectively). We first summarize widely used behavioral paradigms to evaluate anxiety-related behaviors, and discuss their validity and limitations for modeling human levels of anxiety. We then outline experimental strategies for monitoring neural population activity, focusing on optical sensor imaging and electrophysiology, and describe analysis frameworks based on event-locked, space-based, correlation, and latent-space approaches to extract anxiety-related neural metrics. Finally, we highlight recent advances in multimodal longitudinal recordings and discuss how their integration may enable multiscale, mechanistic models of anxiety with improved translational relevance.

Keywords Mouse models of anxiety, Neural circuitry, Imaging, Electrophysiology, Optogenetics

1 Introduction

Anxiety is an evolutionary and adaptive mechanism present in animals, including humans, allowing them to respond to anticipated dangers. It is manifested via a complex interplay of physiological responses, such as activation of the stress response system, and behavioral responses, such as avoidance and risk-assessment behaviors. However, anxiety can become a persistent and excessive response that is no longer matched to environmental demands, ultimately developing into pathological anxiety. Anxiety disorders are the most prevalent class of psychiatric conditions, with yearly prevalence estimates up to 18% [1]. These disorders carry a high personal and societal cost, including impaired quality of life, increased risk for

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comorbid depression and substance use, and significant economic burden. Yet, our understanding of the neurobiological mechanisms that generate their physiological and behavioral symptoms remains far from complete. Although it is impossible to recreate the exact nature of human anxiety disorders in a preclinical model, mouse models play a crucial role in anxiety research. Preclinical mouse models provide a controlled environment, where symptoms of anxiety disorders can be investigated, including physiological and behavioral alterations, to unveil the underlying neurocircuitry down to the single-cell resolution. Understanding how anxiety is implemented at the level of neural circuits is essential for moving beyond symptom-based descriptions toward mechanism-based treatments. Converging clinical and preclinical evidence points to distributed networks involving the amygdala, hippocampus, prefrontal and insular cortices, and extended limbic and midbrain structures. Yet, the precise patterns of activity, connectivity, and neuromodulation that distinguish adaptive from pathological anxiety remain poorly understood. Dissecting these pathways at the resolution of defined cell types, projections, and neuromodulatory systems is therefore critical for identifying novel therapeutic targets. This chapter focuses on the experimental strategies that make such circuit-level dissection possible in mouse models of anxiety. We first review canonical behavioral assays used to quantify anxiety-like behaviors in rodents. We then describe in vivo recording approaches that leverage genetically targeted imaging strategies and electrophysiological techniques to monitor defined neuronal populations during these tasks. Finally, we outline how different analytical frameworks can extract neural metrics of anxiety that link activity patterns to specific behaviors, resulting in an analytical toolbox for systematically connecting observable anxiety-related phenotypes to their underlying circuit mechanisms.

2 Canonical Behavioral Assays for Anxiety in Mice

2.1 Conceptual Foundations of Anxiety Assessment in Mice

Anxiety-like behavior in mice is commonly operationalized using approach-avoidance conflict paradigms [2–5]. These paradigms rely on the natural tendency of mice to explore novel environments while simultaneously avoiding conditions associated with increased predation risk, such as height, openness, or bright illumination. Anxiety is inferred from the suppression of exploration or goal-directed behavior under these conflicting motivational pressures. A defining feature of most anxiety assays is their dependence on novelty. Behavioral responses are strongest during the first exposure to the test environment, when uncertainty and risk assessment are maximal [6]. As animals gain experience, behavioral output is progressively shaped by habituation and learning, rather than anxiety alone. Consequently, most canonical anxiety assays in mice exhibit limited

test-retest reliability and are poorly suited for repeated-measures or longitudinal designs [7, 8]. This novelty dependence represents both a strength—allowing robust detection of acute anxiety-like responses—and a major limitation for repeatability and interpretation.

The interpretation of behavioral assays in animal models critically depends on their validity with respect to the human condition they are intended to model. In anxiety disorder research, validity is commonly discussed in terms of face validity, construct validity, and predictive validity [6, 9]. Face validity refers to the extent to which observable behaviors, such as avoidance of open, elevated, or brightly illuminated spaces, parallel core features of human anxiety. Predictive validity reflects the ability of a model to detect the effects of clinically effective anxiolytic compounds, particularly benzodiazepines and related agents [10, 11]. Construct validity concerns whether the neural circuits, neurochemical systems, and genetic factors engaged by the assay are relevant to anxiety-related processes. Because no single behavioral assay fully captures all dimensions of anxiety disorders, anxiety-like behavior in mice is best evaluated using a battery of complementary tests and interpreted with careful consideration of confounding variables such as novelty, locomotor activity, and prior experience [12].

Another important conceptual distinction is between state and trait anxiety. State anxiety refers to transient, situational fluctuations in anxiety level, such as the acute response to entering an elevated open arm, that are strongly influenced by immediate context and novelty. Conversely, trait anxiety describes relatively stable, enduring differences between individuals in expressing anxiety-like responses across situations and time. Most classical tests (e.g., EPM, OFT, LDB) are primarily sensitive to state anxiety during the first exposure, but can reveal trait-like differences when behavioral measures are aggregated across tasks or related to independent indices such as physiological reactivity or neural activity in anxiety-related circuits. Interpreting results, therefore, requires considering whether a given assay is being used to probe instance state anxiety, underlying trait anxiety, or an interaction between the two.

2.2 Elevated Plus and Zero Mazes (EPM and EZM)

The Elevated Plus Maze (EPM) is a cross-shaped apparatus consisting of two open arms and two closed arms, elevated above the floor (Fig. 1). Anxiety-like behavior is inferred from avoidance of the open arms, which combine elevation and lack of protective walls. Mice must resolve a conflict between exploratory drive and aversion to open, elevated spaces [13]. The Elevated Zero Maze (EZM) is a circular variant of the EPM in which open and closed quadrants alternate along a continuous ring (Fig. 1). By eliminating the central square present in the EPM, the EZM reduces ambiguity in arm entries and transitions [14]. Commonly reported parameters include the time spent in and number of entries into, exposed or anxiogenic compartments, as well as the latency to first entry into these areas.

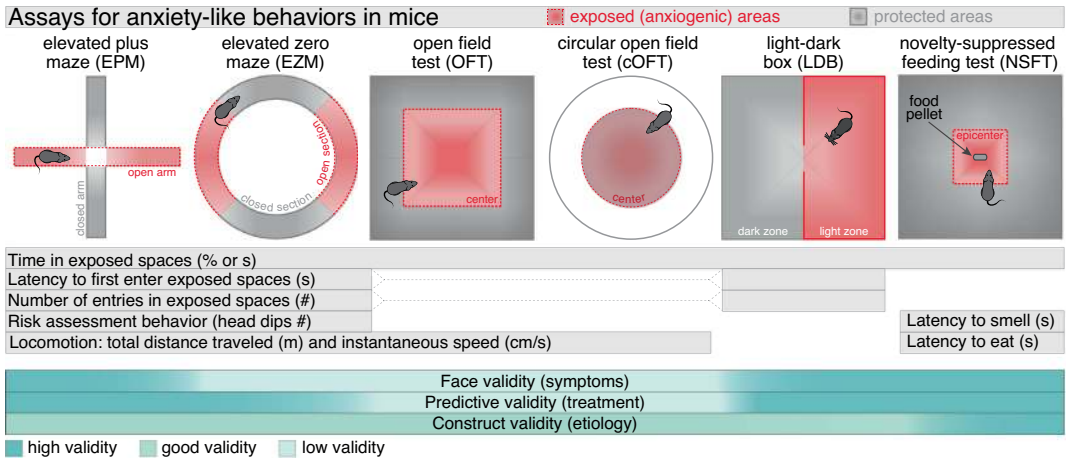


Fig. 1 Canonical behavioral assays for anxiety in mice. Schematic representations of commonly used assays to assess anxiety-like behavior in mice (top views): elevated plus maze (EPM), elevated zero maze (EZM), open field test (OFT), circular open field test (cOFT), light-dark box (LDB), and novelty-suppressed feeding test (NSFT). Below each test, the main different measures classically taken are listed. At the bottom, the strength of validity features, including face, predictive, and construct validity, is represented

In addition, ethologically derived risk-assessment behaviors, such as stretch-attend postures and head-dipping, are frequently quantified (Fig. 1). Collectively, these measures are interpreted as indices of acute anxiety-like behavior, capturing the animal's conflict between the motivation to explore and the avoidance of open spaces [15]. Measures of overall locomotor activity, including total distance traveled and instantaneous speed (defined as movement velocity during periods of non-immobility), are also routinely assessed to control for potential confounding effects of hypo- or hyperactivity on anxiety-related readouts and to facilitate more accurate interpretation of behavioral outcomes [6, 10].

The EPM and EZM are widely used assays of anxiety-like behavior in rodents, supported by strong face and predictive (pharmacological) validity. Avoidance of open, elevated spaces parallels innate threat-avoidance behavior, while predictive validity is demonstrated by robust sensitivity to clinically effective anxiolytics, particularly benzodiazepines [4, 5]. Construct validity is more limited, as performance is strongly influenced by novelty, arousal, prior experience, and locomotor activity, complicating the interpretation as a stable measure of trait anxiety [6, 12]. The widespread use of the EPM and EZM is closely linked to the development of classical anxiolytics. Early compounds such as barbiturates produced anxiolysis but were confounded by sedative effects. The introduction of benzodiazepines marked a major advance, providing rapid anxiolytic effects via positive allosteric modulation of $GABA_A$ receptors with relatively preserved locomotion at appropriate doses. Benzodiazepines reliably increase open-arm or open-segment exploration in both tasks, establishing

them as benchmark assays for detecting acute, GABAergic anxiolysis [4, 16]. In contrast, anxiolytics requiring chronic administration, such as selective serotonin reuptake inhibitors, often show weak or inconsistent effects after acute dosing, indicating that these paradigms primarily assess acute anxiety-like states [17, 18]. Both assays are also highly sensitive to habituation and learning effects. Repeated exposure reduces open-arm exploration, a phenomenon termed one-trial tolerance [19], which occurs even in untreated animals and reflects prior experience rather than enduring changes in anxiety. Repeated testing also reduces sensitivity to anxiolytic drugs [20]. Accordingly, the EPM and EZM are typically administered only once per animal, and repeated testing should be avoided or interpreted with caution. In test-retest designs, alternating between the EPM and EZM has been proposed as a partial mitigation strategy, as the EZM appears less sensitive to habituation [21].

2.3 Open Field Test (OFT) and Circular Open Field Test (cOFT)

The Open Field Test (OFT) consists of a large, enclosed square arena in which mice are allowed to freely explore (Fig. 1). Anxiety-like behavior is inferred from avoidance of the central area of the arena, a behavior termed thigmotaxis [22, 23]. A variation of this test is the circular Open Field test (cOFT), allowing retesting of the same mice with a novel environment to overcome habituation. It is essential to distinguish between the general locomotor activity, indexed by total distance traveled, and anxiety-related behavior, indexed by center-based measures [24]. Anxiety-related parameters include time spent in the center and distance traveled in the center (Fig. 1). Total distance traveled and instantaneous speed should be reported separately as a control for locomotor effects [25]. The OFT is simple to implement and allows simultaneous assessment of locomotion and anxiety-like behavior. However, center avoidance is a relatively weak anxiogenic stimulus and is highly sensitive to environmental variables such as lighting intensity and arena size. As with other novelty-based assays, repeated exposure results in rapid habituation and diminished anxiety-like responses. Therefore, OFT center measures are best interpreted during initial exposure and in conjunction with other anxiety assays [6]. The OFT and cOFT exhibit moderate face validity, with center avoidance reflecting approach-avoidance conflict in an open environment, and acceptable predictive validity under well-controlled conditions [23]. However, construct validity is constrained by the strong influence of locomotor activity, exploratory drive, and environmental parameters, calling for cautious interpretation of center-based measures as indices of anxiety [25].

2.4 Light-Dark Box (LDB)

The Light-Dark Box (LDB) consists of two compartments: a dark, enclosed chamber and a brightly illuminated open chamber connected by a small opening [26]. The test exploits the innate preference of mice for dark environments while preserving exploratory motivation (Fig. 1). Common measures include the time spent in

the light compartment, latency to first enter the light compartment, and number of entries. Increased exploration of the light compartment is interpreted as reduced anxiety-like behavior (Fig. 1). The LDB has strong ethological relevance and robust pharmacological sensitivity to anxiolytic compounds [26, 27]. Compared with the OFT, it often provides clearer separation between locomotor and anxiety-related effects. Nevertheless, the LDB is also novelty-dependent. Repeated testing leads to reduced avoidance of the light compartment, reflecting habituation rather than sustained changes in anxiety [28]. The LDB demonstrates good face validity, as it capitalizes on the ethologically relevant conflict between exploration and light avoidance, and strong predictive validity, particularly for acute anxiolytic drugs [26, 27]. As with other novelty-based paradigms, however, construct validity is limited by habituation and learning effects that emerge with repeated exposure, reducing its suitability for longitudinal assessments of anxiety [21].

2.5 Novelty-Suppressed Feeding Test (NSFT)

The Novelty-Suppressed Feeding Test (NSFT) measures the suppression of feeding behavior in a novel environment [29]. Following food deprivation, mice are placed in an unfamiliar arena containing a food pellet, and the primary outcome measure is the latency to initiate feeding. In some protocols, additional measures such as latency to smell the food or time spent in the epicenter are also recorded. This assay integrates anxiety-like behavior with motivational and decision-making processes. NSFT shows high construct validity and is particularly sensitive to chronic antidepressant treatment, distinguishing it from tests that primarily detect acute anxiolytic effects [29, 30]. However, it is influenced by hunger, metabolism, and learning, animals rapidly learn the task contingencies. Therefore, NSFT is not well-suited for repeated testing within the same subjects, limiting its use in longitudinal designs.

NSFT is often considered to have relatively high face and construct validity, as it engages anxiety-relevant decision-making processes under motivational conflict and recruits neural circuits implicated in stress and affect regulation [30]. Its predictive validity is strongest for chronic antidepressant treatment rather than acute anxiolytics, highlighting that it probes a partially distinct dimension of anxiety-related behavior [10, 29].

2.6 General Considerations: Novelty, Habituation, and Repeatability

A central limitation of many anxiety assays in mice is their strong reliance on novelty-induced approach-avoidance conflict. As the novelty of the testing environment diminishes with repeated exposure, behavioral responses progressively shift from anxiety-driven avoidance to experience- and learning-based strategies. This transition leads to several well-recognized limitations, including:

1. Reduced sensitivity to anxiolytic manipulations, particularly those effective only under high-novelty conditions
2. Poor test-retest reliability, as behavioral measures are strongly influenced by prior experience

3. Limited suitability for longitudinal designs, where repeated assessment of the same individuals is required.

Accordingly, best-practice guidelines recommend the use of experimentally naïve animals, avoidance of repeated exposure to the same apparatus, and the combination of multiple complementary behavioral assays to obtain convergent evidence for anxiety-like phenotypes [6, 10, 31]. Taken together, these assays vary substantially in their balance of face, construct, and predictive validity, reinforcing the need to interpret anxiety-like behavior in mice using convergent evidence from multiple tests rather than reliance on any single paradigm.

In addition to task novelty, pre-test handling procedures represent a critical but often underappreciated source of variability. Repetitive gentle handling, as well as the method of contact between the experimenter and the mouse prior to testing, can substantially influence anxiety-like behavior, particularly in assays such as the elevated plus maze. Mice subjected to regular gentle handling or non-aversive handling methods (for example, tunnel handling or cupping) typically display increased open-arm exploration and reduced avoidance compared with non-handled or tail-handled mice, consistent with lower apparent anxiety levels [32–34]. Failure to standardize handling procedures can therefore introduce systematic differences between experimental groups and across laboratories, undermining reproducibility and cross-study comparisons.

3 In Vivo Imaging Leveraging Genetically Targeted Strategies

Given the broad range of behavioral assays discussed in the previous sections, our understanding of anxiety-like behavior is becoming increasingly rich at the level of observable actions. However, these approaches alone do not capture how specific neural circuits generate and modulate these behavioral states. To bridge this gap, the next step is to monitor activity in defined neuronal and other cell populations with high spatial and temporal precision. The next section describes the use of genetically targeted imaging approaches that couple molecular sensors to different recording strategies.

3.1 How to Express Molecular Sensors in Target Neural Populations

Transduction

Transduction is a natural mechanism for the transfer of genetic material between organisms via a viral vector. Since the 1970s, many viral vectors such as lentiviruses (LV), adeno-associated viruses (AAV), or rabies virus (RV) have been rendered replication-deficient through genetic manipulation and have thus become essential tools in molecular biology and neuroscience. In neuroscience, they make it possible

to express genes of interest in populations of neurons or glial cells of a living organism (in vivo) or on cells maintained in culture (in vitro, Fig. 2A) [35]. The expression of the gene of interest can be restricted to certain cell types through the insertion of a promoter in the vector, such as synapsin (neuronal promoter), CaMKIIα (calcium/calmodulin-dependent protein kinase 2α, glutamatergic neuron-enriched promoter), GFAP (astrocytic promoter), and many others. For some vectors, the addition of recombination sites for the Cre recombinase (Cre) enzyme may add an additional level of specificity to the expression of the gene of interest (Fig. 2A). While under natural conditions, transduction relies on the integration of viral DNA into the host genome, contemporary viral transduction technologies often allow robust episomal expression of genes of interest without stable integration into the genome, especially in the case of AAV, whereas LV typically integrates. This has a considerable advantage in terms of expression levels and flexibility, since many copies of the vector can be expressed within the same cell, but also requires attention to the dose-dependent toxicity of some effectors or recombinases [36]. However, each viral vector has a specific tropism for a given cell type. Indeed, each vector has a different probability of binding and being endocytosed by a cell type, depending on the adhesion molecules present on the two transduction partners and on the particular capsid or envelope used. Tropism is therefore also dependent on the target species and on serotype choice, as illustrated by the broad use of engineered AAV capsids (e.g., AAV1/2/8/9 or AAV2-retro) to preferentially label distinct neuronal populations or projection pathways [37]. Currently, viral transduction of nerve cells has been performed in different mammals, ranging from mice to humans, with varying efficiencies and safety profiles. Despite numerous studies, the mechanisms of molecular recognition and transduction of the different vectors and their serotypes remain only partially understood, which motivates continued capsid engineering efforts [38].

Transgenic Lines

The use of transgenic lines to express a gene of interest reduces the variability due to the cell type and tropism of the viral vector. The number of transgenic mouse lines continues to grow exponentially, as proteins are created to manipulate and visualize neuronal activity. Knowing that the development of a mouse line remains laborious, the current standard is to produce mouse lines expressing Cre recombinase in a cell population, then to target the molecular tool of visualization or manipulation through the use of a floxed viral vector or a floxed reporter allele inserted at a permissive locus (Fig. 2B).

Viral Strategy in Transgenic Lines

To further refine cell-type and circuit specificity, viral vectors are frequently combined with conditional expression cassettes based on site-specific recombination systems. The most widely used system in neuroscience is the Cre/loxP system, in which the bacterial Cre

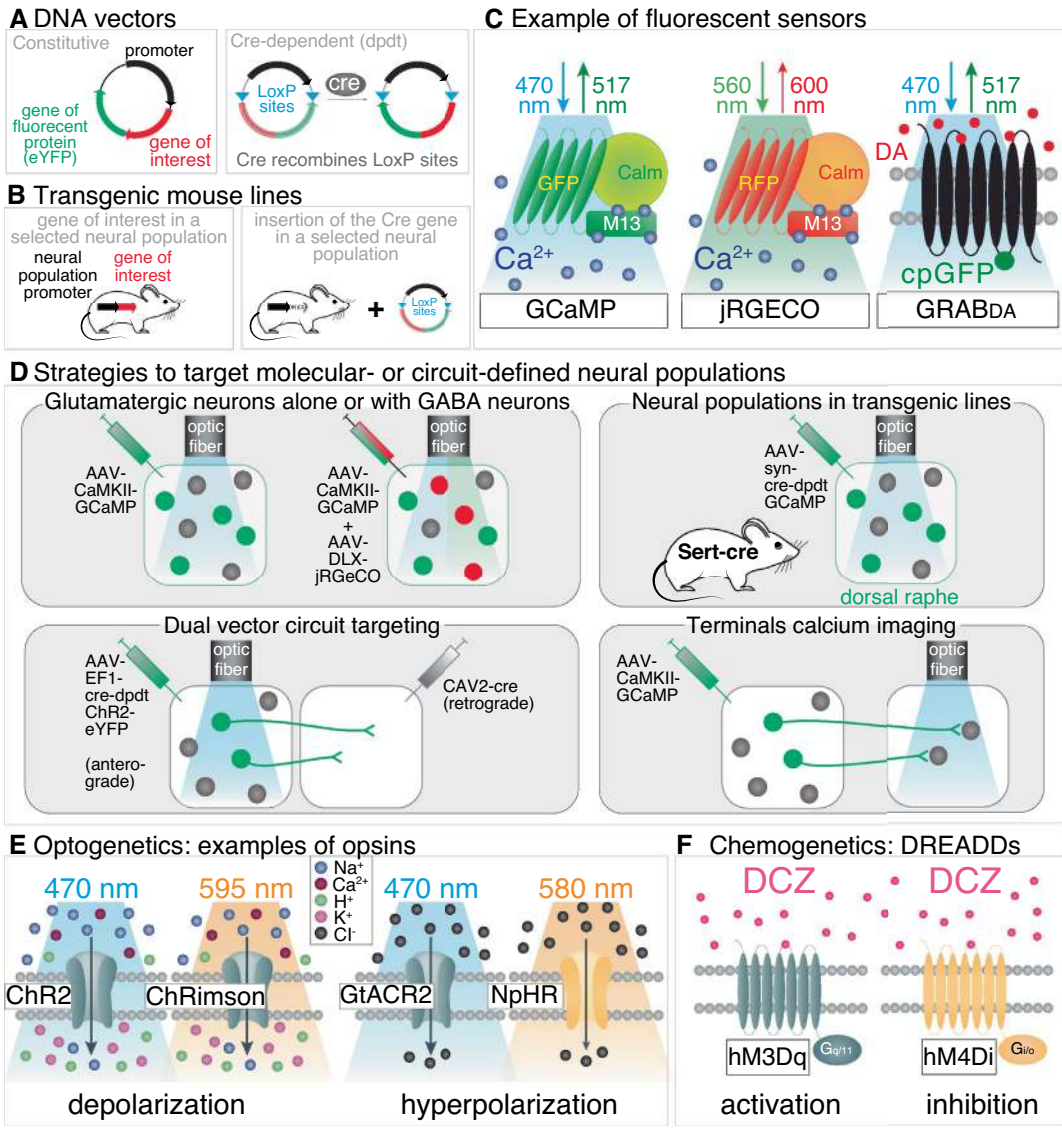


Fig. 2 Molecular tools to image and modulate neural populations and neural circuits. (**A**) Viral vectors with a selective promoter allow the expression of a gene of interest specifically in cells that express genes under the same promoter as the vector. Expression can also be made dependent on a Cre recombinase. In this case, expression of the gene of interest requires the presence of the Cre enzyme to recombine loxP sites on the vector. (**B**) Genetically modified lines can constitutively express a gene of interest in a specific cell type. This gene of interest can be Cre, thereby allowing the expression of any transgene encoded by a Cre-dependent virus. (**C**) Examples of fluorescent sensors for imaging calcium dynamics, such as GCaMP or jRGECO, or sensors for neuromodulators such as dopamine (DA). (**D**) Examples of strategies to target molecular- and circuit-defined neural populations, using a dual-color (top left), or single-color strategies. (**E**) Examples of opsins that enable activation or inhibition of neurons expressing them at their membrane in the presence of blue or amber light: ChR2: channelrhodopsin-2, GtACR2: Guillardia theta anion channelrhodopsin-2 or NpHR: halorhodopsin. (**F**) Examples of G protein–coupled receptors modified to respond exclusively to an exogenous compound, DCZ (Deschloroclozapine), to induce activation or inhibition of cells expressing these DREADDs (Designer Receptors Exclusively Activated by Designer Drugs). hM3Dq and hM4Di: modified human muscarinic receptors

recombinase recognizes short loxP sequences and mediates recombination between them. In a typical "floxed" (flanked by loxP) expression cassette, a transcriptional STOP sequence or the coding sequence of interest is placed between two loxP sites so that Cre activity either removes a STOP (turning expression ON) or excises a gene (turning expression OFF), depending on the cassette design [39]. In viral vectors, this logic is exploited in double-floxed inverted open-reading frame (DIO or FLEX) designs, where the gene of interest is inserted in an inverted orientation between pairs of antiparallel loxP and lox2272 sites [40]. In the absence of Cre, the gene remains non-functional, whereas Cre expression in a defined neuronal population flips and locks the cassette in the correct orientation, resulting in robust, cell-type-specific expression of the molecular sensor or effector. By combining such viral strategies with Cre-driver mouse lines, brain-region- and cell-type-specific expression of sensors or actuators can be achieved with high reproducibility. In addition to the Cre/loxP system, alternative recombinases such as Flp/FRT and Dre/Rox expand the possibilities for expressing multiple sensors in distinct cell types. Using recombinases in parallel increases the flexibility of circuit tracing and functional imaging, enabling the exploration of several neuronal populations or modalities at the same time with minimal interference between expression cassettes.

3.2 Sensor Imaging

In the 1980s, Roger Tsien's laboratory at the University of Cambridge developed the first fluorescent probes sensitive to the concentration of calcium, such as Fura-2 [41]. These tools made it possible to visualize the activity of in vivo and ex vivo sets of neurons in superficial cortical layers. In 1997, Tsien's laboratory used the first genetically encoded calcium indicators (GECI) and revealed the activity of neurons in targeted populations [42]. Since then, a wide range of genetically encoded reporters has been developed to monitor neural activity with cellular and subcellular resolution. These molecular tools convert changes in intracellular ions, membrane voltage, or neuromodulator levels into optical signals that can be captured during anxiety-related behavior [40]. In the context of anxiety-related tasks, they enable the dissection of how defined neuronal populations and circuits encode anxiogenic and protected contexts, approach-avoidance decisions, and internal states over time [43]. The most widely used class of activity reporters is GECIs, such as the GCaMP family, which translate transient increases in intracellular calcium associated with action potentials into changes in fluorescence (Fig. 2C). GCaMP-type sensors consist of a circularly permuted fluorescent protein fused to calmodulin and the CaM-binding M13 peptide. When intracellular calcium rises, calmodulin binds the analyte (calcium, Ca^{2+}) and engages the M13 peptide, which induces a conformational change that reshapes the fluorescent protein, thereby increasing fluorescence. Newer GCaMP variants, now in their eighth

generation, have been engineered to greatly enhance sensitivity, dynamic range, and kinetics, resulting in markedly improved signal-to-noise ratios in vivo. Although calcium imaging is crucial for the understanding of neural activity patterns, calcium signals are intrinsically slower than changes in membrane voltage, especially the brief action potentials that occur on the millisecond timescale. The GECIs integrate activity over tens to hundreds of milliseconds rather than resolving individual spikes with high precision. For decades, only electrophysiological recordings have allowed to analyze precise action potential discharge patterns.

More recently, protein-engineering strategies have enabled the development of genetically encoded voltage indicators (GEVIs), which couple fast, voltage-dependent conformational changes to fluorescence readouts [44]. Compared to calcium imaging, voltage imaging directly reports both suprathreshold and subthreshold membrane potential fluctuations, including hyperpolarizing events that are largely invisible to GECIs, but achieving this at scale requires careful management of fundamental trade-offs between imaging speed, spatial resolution, and signal-to-noise in the optical design. Therefore, these voltage-sensitive probes can follow synaptic potentials and action potentials with much higher temporal resolution than GECIs, approaching the millisecond timescale, however, these probes do typically suffer from a lower signal-to-noise ratio and greater sensitivity to motion and phototoxicity in vivo, underscoring the continued importance of in vivo electrophysiology. In parallel, advances in high-speed optical microscopy (e.g., 1- and 2-photon designs) are increasingly critical, because they determine how well GEVIs can capture subthreshold events and fast population dynamics across large fields of view [45].

In parallel, an expanding family of biosensors for neuromodulators and peptides (e.g., dopamine, serotonin, norepinephrine, corticotropin-releasing factor) reports the engagement of specific signaling pathways during anxiety-related behaviors (Fig. 2C). Anxiety-related behaviors often rely on a complex combination of multiple neuromodulators and peptides, together with activity-dependent changes in defined neuronal populations. The development of red-shifted calcium indicators such as jRGECO-family sensors [46] (Fig. 2C) and more recent far-red indicators [47, 48] now enables multiplexed imaging of several modulators and activity reporters in parallel, improving our understanding of the temporal relationships with which these different signals shape anxiety-related behavioral states. In addition to spectral multiplexing, many of these sensors have been optimized for particular imaging modalities (e.g., 1- vs. 2-photon) and expression strategies (e.g., soma-restricted vs. cytosolic), providing flexibility for studying specific anxiety circuits. For all these sensor families, practical performance during behavior depends on properties such as dynamic range, on- and off-kinetics,

baseline brightness, and photostability, as well as on subcellular targeting strategies (e.g., soma-restricted versus pan-neuronal), which together determine the interpretable temporal and spatial resolution of circuit activity during anxiety-related tasks.

A critical consideration when using biosensor strategies is the phenomenon of buffering, in which biosensors bind their analyte (e.g., calcium, dopamine) and temporarily remove it from the free pool. If the sensor is expressed at high concentration or has a very high affinity, this binding can significantly alter local concentration dynamics, which may distort the kinetics and amplitude of physiological signals. In the case of calcium indicators, this can directly influence neuronal excitability or synaptic transmission. Therefore, appropriate biosensor design and experimental use should balance sensitivity (sufficient binding to detect the signal) with minimal perturbation (limited additional buffering), which depends on factors such as affinity, expression level, subcellular targeting, and the timescale of the behavior being studied.

3.3 Recording Approaches: Increasing Resolution from Neural Population to Single-Cell Resolution

Neural circuit activity underlying anxiety spans multiple spatial and temporal scales, from synchronized population dynamics across brain regions and thousands of neurons to precisely timed firing in individual cells. Different recording approaches have evolved to bridge these scales, starting from global population-level readouts that capture bulk signaling in deep brain structures using fiber photometry, through mesoscopic imaging of cellular ensembles in freely behaving animals using miniscopes, to high-resolution cellular and subcellular imaging in constrained setups using 2-photon microscopy. This progression, from averaging over large populations to resolving single-neuron identity and fine structure, allows researchers to link circuit-level coordination with cellular mechanisms of anxiety processing.

Measurements of Neural Population Signal Using Fiber Photometry

After the development of the first GECIs around the 2000s, a need for freely-moving, affordable, and easily accessible tools for in vivo fluorescence imaging was becoming apparent. In 2005, Adelsberger et al. showed a simple single-fiber laser setup to monitor calcium signals in the neonatal mouse cortex in vivo [49]. This established the basic principle that an implanted optical fiber could both deliver excitation light and collect population fluorescence. In broad terms, fiber photometry measures bulk fluorescence changes from a population of labeled neurons through an implanted optical fiber in the brain structures of interest (Fig. 2D). A critical refinement has been the use of excitation near the isosbestic point of GCaMP, where fluorescence is largely calcium-independent, to generate a reference channel that can be used to correct for motion and photobleaching artifacts in a 470/405 nm photometry set-up [50]. Using such a dual-wavelength approach in anxiety research, for instance, Nicolas

et al. showed that the anterior insular cortex was activated during the exploration of exposed, anxiogenic spaces, but not in protected, safe spaces [43].

Another key breakthrough was the application of fiber photometry to projection-specific GCaMP recordings in behaving mice by Günaydin et al., who used fiber photometry to assess ventral tegmental area to nucleus accumbens activity during freely-moving social behavior [51] (Fig. 2D). In the context of anxiety, this enabled the exploration of specific neurocircuits, such as the anterior insular cortex neurons that project to the basolateral amygdala (BLA), which were shown to increase their activity during the exploration of exposed, anxiogenic spaces [43]. Similarly, another study using circuit-specific fiber photometry of the neurons of the ventral tegmental area (VTA) projecting to the BLA demonstrated that activity in this midbrain–amygdala pathway is tightly linked to the expression of anxiety-like behavior after chronic social defeat stress [52].

The state-of-the-art fiber photometry systems now have multi-wavelength and multi-channel systems, enabling simultaneous readout of spectrally distinct sensors as well as multi-fiber site recordings. Using seven fibers on distinct brain regions, Kim et al. simultaneously recorded GCaMP signals in freely moving mice and showed that the onset of social interaction with a novel mouse produced not only time-locked activity increases in each area but also a significant, brain-wide increase in pairwise correlations across the network, indicating enhanced functional coupling during social behavior [53]. By combining multi-fiber recording with projection-specific labeling of VTA dopamine (DA) neurons, Kim et al. further demonstrated that VTA DA somata and their projections to the nucleus accumbens (NAc), BLA, and prefrontal cortex (PFC) exhibit distinct, simultaneously measured response patterns to reward and aversive stimuli, showing a target-specific coding within a single neuromodulatory system. Furthermore, these photometry approaches were also extended to dual-color recordings by simultaneously monitoring DA and non-DA VTA neurons with red and green calcium indicators, revealing distinct population-specific patterns to reward and shock in the same circuit [53]. Together, these multi-region and multi-wavelength fiber-photometry strategies open new avenues for anxiety research by enabling phase-locked monitoring of activity across distributed circuits while simultaneously resolving the contributions of defined cell populations and neuromodulatory or neuropeptide signals to anxiety-related states.

One-Photon Microscopy

The modern 1-photon miniscope concept emerged with the integrated, head-mounted fluorescence microscope developed by Ghosh et al., which used a lightweight (1.9 g) device with an onboard LED and CMOS sensor coupled to a GRIN lens to record large populations of neurons in freely behaving mice, establishing the basic

architecture adopted by most current miniscopes [54]. These head-mounted one-photon miniscopes enable cellular- and sometimes subcellular-resolution imaging in freely moving animals through a GRIN lens implanted above the region of interest.

Commercial miniscope systems (e.g., Inscopix nVista) provide an out-of-the-box solution with optimized optics, acquisition hardware, and analysis pipelines, which greatly lowers the barrier to entry and supports high-throughput studies, but at a relatively high financial cost. In parallel, substantially more affordable open-source systems have been developed, such as CHEndoscope [55] and the UCLA Miniscope family, which offer comparable signal quality and expanding fields of view at the price of greater hands-on involvement in assembly, calibration, and maintenance. Regardless of the chosen system, miniscopes allow recording from hundreds of neurons simultaneously during freely moving behaviors, including anxiety paradigms, across both cross-sectional and longitudinal experiments. Miniscope studies have already yielded important insights into anxiety-related circuits. For example, recent work using GRIN-lens imaging of vCA1 pyramidal neurons identified largely non-overlapping subpopulations of neurons that preferentially encode anxiety- versus fear-related behaviors, and linked these ensembles to distinct inhibitory microcircuits, highlighting how longitudinal single-cell imaging can reveal functional specialization within a homologous brain region [56].

Compared to fiber photometry, miniscopes provide single-cellular resolution and access to the spatial organization of activity patterns, but they require more invasive optical implants, heavier head-borne hardware, and more complex data processing for segmentation, motion correction, and spike inference. Spike inference is a computational technique used to reconstruct the precise timing of neural action potentials ("spikes") from the slower and indirect calcium imaging recordings. Furthermore, long-term recordings remain challenging with miniscopes due to tethering, connector wear, and progressive photobleaching or photo-toxicity, although newer designs with lighter housings, wireless options, and improved CMOS sensors (e.g., the UCLA MiniXL, see [57]) are gradually mitigating these limitations. Multi-region miniscope imaging is, in principle, possible using multiple GRIN implants or relay-lens configurations, but is technically demanding because of space constraints on the skull and cumulative weight, and multi-wavelength operation is typically limited to two channels (e.g., green and red) in LED-based designs, whereas the fiber photometry laser-based benchtop systems can more easily cover green, red, and far-red bands.

By bridging cellular resolution with freely moving behavior, one-photon miniscopes occupy a crucial intermediate scale between bulk photometry and high-resolution two-photon imaging, enabling circuit-level dissection of anxiety-related ensembles over ethologically relevant timescales.

Two-Photon Microscopy

The concept of using a two-photon fluorescence microscopy was introduced by Denk, Strickler, and Webb, using a laser-scanning approach that achieved intrinsic optical sectioning with reduced out-of-focus photodamage [58]. Within a decade, this technique was adapted for deep tissue imaging in the rodent neocortex and hippocampus, where it enabled high-resolution visualization of single neurons, dendrites, and spines in vivo through cranial windows. Two-photon laser-scanning microscopy offers high spatial resolution and optical sectioning with reduced scattering, making it the method of choice for imaging fine structures (e.g., dendrites, spines) and densely labeled networks in superficial cortical areas or, via GRIN lenses, in deeper brain structures [59].

Usually, in order to perform 2-photon photometry, animals were head-fixed to enable precise mapping of activity in identified neurons and subcellular compartments during virtual-reality or constrained anxiety paradigms, such as head-fixed elevated mazes or learned threat tasks that probe avoidance and approach behavior. Compared to one-photon miniscopes, these two-photon systems provide superior axial resolution, better control over excitation volume, and deeper penetration. However, their implementation typically requires large, vibration-isolated benches, high-power pulsed lasers, and precise alignment, making them substantially more complex and expensive than 1-photon or fiber-based approaches.

Recent technological developments aim to bring two-photon imaging into more naturalistic conditions. Freely moving 2 P systems, such as fiber-based endoscopic designs (e.g., 2P-FEnDo from the Emiliani group), place the scanning head and laser off the animal and relay the excitation and emission through a flexible imaging fiber bundle, allowing cellular-resolution two-photon imaging during self-paced behavior at the cost of more complex optics and typically reduced fields of view. Complementary efforts have produced head-mounted or semi-mobile two-photon devices (including recent UCLA 2P-miniscope developments) that miniaturize the scanning module and couple it to GRIN lenses, enabling deeper imaging with better axial sectioning than 1-photon miniscopes, but still with higher weight and stronger constraints on behavior compared to LED-based systems [60]. From the perspective of anxiety research, two-photon imaging is particularly powerful when fine spatial precision is essential, for example, to distinguish dendritic integration in layer-specific microcircuits of medial prefrontal or ventral hippocampal regions that differentially control anxiety versus learned fear. Nevertheless, its main limitations for studying anxiety-related behavior remain the constraints on naturalistic movement imposed by head-fixation or heavy headgear, the technical complexity, and, in many setups, a reduced field of view compared to modern large-FOV miniscopes. Together, fiber photometry, 1-photon miniscopes, and 2-photon microscopy provide a continuum from global population signals to single-cell and subcellular readouts, allowing researchers to match

spatial and temporal resolution to specific questions about how anxiety-related circuits are organized and modulated.

3.4 Combination with Modulation of Neural Activity (Optogenetics, Chemogenetics)

Optogenetics

Optogenetics is a technology born from the combination of the genetic approaches described above with optical methods to control neuronal activity. At the interface of these two worlds, the discovery of light-sensitive molecules capable of regulating neuronal activity initiated a new field of research (Fig. 2E). In 2002, Hegemann, Nagel, and collaborators expressed the gene coding for an algal protein, channelrhodopsin-2 (ChR2) [61], a blue-light-sensitive cation channel that depolarizes cells expressing this membrane protein. Three years later, Boyden et al. expressed ChR2 in mouse hippocampal neurons and showed that brief blue light pulses could reliably control neuronal firing *in vitro* [62]. Finally, in 2007, Adamantidis et al. genetically targeted ChR2 expression in hypocretin neurons of the hypothalamus and demonstrated a causal link between the activity of these neurons and the induction of wakefulness during sleep [63]. Since then, a multitude of viral vectors and transgenic mouse lines have been used to express ChR2 in different neuronal populations and have demonstrated the involvement of these populations in various emotional and cognitive functions in vivo, including anxiety-related behaviors [64].

Nowadays, there is a long list of opsins that allow activation and inhibition of neuronal populations with different wavelengths and kinetics (Fig. 2E). Red-shifted excitatory opsins such as ChRmine, ReaChR, and ChRimson extend activation into the red spectrum, where light scatters less and penetrates deeper, which is particularly useful in combination with green sensors or for targeting of deeper anxiety-related brain structures.

The two most common proteins used to inhibit neuronal activity are halorhodopsin (NpHR3.0) and archaerhodopsin T (ArchT3.0), both derived from archaebacteria. NpHR3.0 is permeable to chloride ions in the presence of amber light (around 590 nm), whereas ArchT3.0 is a proton pump activated by green light (around 530 nm). A new modified ChR2 has also been developed to be permeable to chloride ions in the presence of blue light (GtACR2). All these inhibitory opsins cause hyperpolarization of the cells expressing them during illumination (Fig. 2E).

Like any new technology, optogenetics has generated great enthusiasm, but it is important not to overlook its limitations. One frequently discussed issue is the potential effect of light stimulation *per se*, either locally in the brain (heating) or via the visual system

(light leakage stimulating the retina). It is therefore essential to compare effects in opsin-expressing animals with control groups expressing only a fluorescent protein (e.g., eYFP) but receiving identical light stimulation. Similarly, high levels of exogenous protein expression can impair cellular functions, which again calls for appropriate control groups. Another major limitation is the spatial restriction of stimulation due to limited light penetration, especially for blue light. New optical fibers, such as tapered or "cut-in-tip" designs, have been developed to allow more homogeneous light diffusion within the targeted region, and red-shifted opsins improve penetration in tissue. Many studies have also moved away from categorical statements that a population is strictly "necessary" or "sufficient" for a behavior, and instead describe optogenetic manipulations in terms of gain-of-function or loss-of-function, since behavioral effects are often partial modulations rather than all-or-none switches. Finally, optogenetic activation typically drives synchronous firing in many cells, which is not physiological and can obscure native spike timing and coding. Despite these limitations, optogenetics remains a first-choice approach thanks to its unique temporal resolution and cellular specificity. Importantly for this chapter, it is often combined with recording techniques described in Sect. 3.3, for example, by monitoring calcium signals with fiber photometry or miniscopes while optogenetically manipulating specific inputs or cell types in anxiety circuits.

Chemogenetics

Chemogenetics is also based on genetic modification of proteins, but in this case, they are engineered to interact selectively with exogenous ligands rather than light. Different classes of proteins have been created, including modified kinases, enzymes, G-protein-coupled receptors, and ligand-gated ion channels. The most widely used chemogenetic tools are DREADDs (Designer Receptors Exclusively Activated by Designer Drugs), developed in the Roth laboratory from human muscarinic receptors (hM) that were mutated to lose sensitivity to acetylcholine and gain sensitivity to synthetic ligands [65] (Fig. 2F). The two most common DREADDs are hM3Dq, a Gq-coupled receptor that induces cellular activation (in part via increased intracellular calcium), and hM4Di, a Gi-coupled receptor that induces cellular inhibition. Initially, these receptors were activated by clozapine-N-oxide (CNO), but it was later shown that CNO can be converted to clozapine in vivo and exert off-target effects, especially at high or chronic doses. Consequently, more specific and higher-affinity ligands such as compound 21 or perlapine have been developed [66]. As with optogenetics, it is essential to include control groups expressing only a fluorescent protein but receiving the same ligand injections, to control for drug effects. Unlike optogenetics, chemogenetic activation and inhibition are not limited by light spread: all cells expressing DREADDs are modulated following systemic or local injection of the ligand. Chemogenetics, therefore,

enables manipulation of distributed neuronal populations with good cellular specificity but relatively low temporal resolution, as effects typically build up over minutes and last tens of minutes to hours. This is well-suited for probing how sustained changes in activity of a given region or projection alter anxiety-like behavior over an entire session (e.g., in elevated plus maze or open field tests), and can be combined with calcium imaging or electrophysiology to measure circuit-level consequences. However, chemogenetic manipulations remain artificial interventions and do not themselves reveal how information is encoded by spike timing and patterns in the targeted neurons. To identify these coding mechanisms, it remains essential to record electrical or optical activity at the population and single-unit level, and to interpret optogenetic or chemogenetic perturbations in the context of the natural dynamics.

4 Electrophysiological Recordings

Electrophysiology traces back to Luigi Galvani's late-18th-century frog experiments and was formalized by Emil du Bois-Reymond's 19th-century quantification of rapid electrical changes in nerves and muscles. The shift to in vivo brain recording accelerated with Hans Berger's first human EEG in 1924 [67] and, by the late 1950s, extracellular single-unit recordings enabling receptive-field mapping [68, 69]. Since then, arrays, chronic implants, and high-density probes have scaled in vivo electrophysiology to multi-site population recordings in freely-moving animals (Fig. 3A).

In vivo electrophysiology provides millisecond-precision, direct measurement of action potentials and local field potentials (LFPs), summed population activity, from intact, behaving brains (Fig. 3B). It reaches deep structures inaccessible to optical methods and, with high-density probes, reveals population coding and network dynamics invisible from single neurons [70]. Recordings in awake animals link activity directly to perception, decisions, and movement [71], and LFP oscillations translate naturally to human EEG and intracranial recordings [72]. However, in vivo recording is invasive; tissue damage and foreign-body responses degrade signal quality over time. It samples only a small fraction of neurons, is vulnerable to drift and noise, and precise anatomical localization remains challenging in long-term experiments.

4.1 Types of Electrodes

In vivo electrophysiology uses extracellular electrodes varying in shape, channel count, and sampling scale. This choice determines whether you record single units, LFPs, or both, which structures you reach, and recording stability over time. The table below summarizes main electrode families and their typical uses in rodents and primates (Table 1, Fig. 3A).

Table 1

Properties of electrode types to perform in vivo electrophysiology

Electrode type	Channels	Spatial scale	Advantages	Disadvantages	Typical application
Microwire, microwire array	1–32	Very local cluster (tens– hundreds of μm)	Flexible, relatively low damage	Limited channels, fixed geometry	Deep nuclei/cortex ensembles in rodents and primates
Tetrode	4 (one each wire)	Very local (few nearby cells)	Excellent single-unit isolation, movable in drives	Labor-intensive fabrication, small coverage, rodent-focused,	Hippocampal and cortical single units in freely moving rodents
Silicon probe (Michigan, NeuroNex, multi-shank)	16–128	1D laminar column (hundreds of μm–mm along depth)	Across-layer spikes + LFP, precise geometry	Rigid, more damage, fixed after implant	Laminar cortex/hippocampus (acute or semi-chronic) in rodents
Penetrating MEAs (4 × 4, Utah-type grids)	16–128	2D cortical patch (few mm^2)	High local density, 2D map	Shallow depth, rigid, higher insertion trauma	Motor/sensory cortex ensembles, BMIs, population coding, peripheral nerve
Neuropixels (1.0/2.0)	384–1536	1D depth profile, multi-region	Hundreds of units, standardized large-scale	Expensive, single-use, huge data, difficult to combine with optogenetics	Large-scale multi-area rodent/primate recordings
Surface electrodes (ECoG, flexible arrays)	8–64	2D cortical surface patch (mm–cm), LFP only	Very low invasiveness, broad chronic coverage	No single-unit spikes, poor depth resolution, limited to the cortex	Cortical oscillations, brain states, chronic LFP in rodents/primates

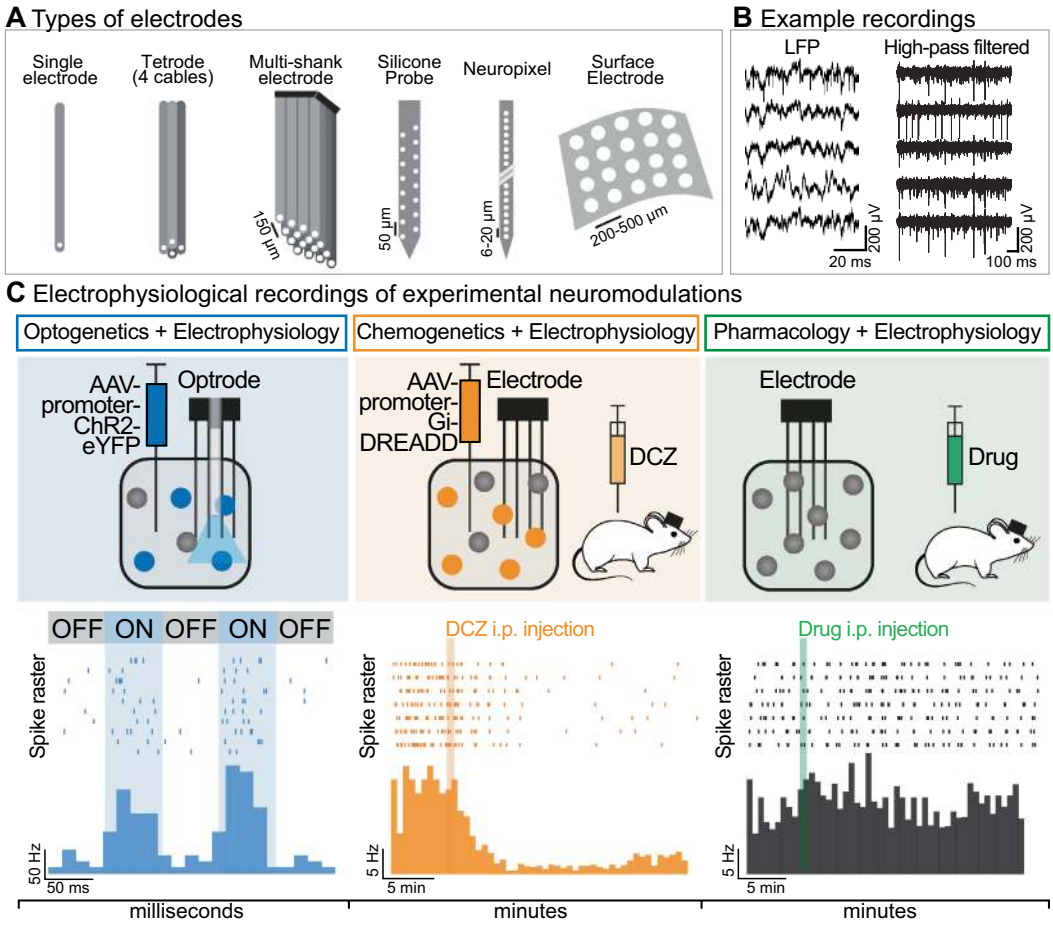


Fig. 3 In vivo electrophysiology approaches: electrode types, signal properties, and integration with neuromodulation techniques. (**A**) Types of electrodes used for in vivo extracellular recordings. From left to right: single electrode, tetrode (4 twisted microwires), multi-shank electrode, silicon probe (e.g., Michigan-type), Neuropixel probe, and surface electrodes (ECoG/µECoG). (**B**) LFP traces (left) represent summed synaptic activity from local neural populations, while single-unit traces (right) show high-frequency action potentials from individual neurons. (**C**) Integration of electrophysiology with neuromodulation approaches. Electrophysiological recordings can be combined with three major neuromodulation strategies to establish causal links between neural activity and circuit function. (1) Optogenetics + electrophysiology (blue): A viral vector (AAV-promoter-ChR2-eYFP) drives light-sensitive channelrhodopsin expression in a genetically defined cell population. Paired optrode recordings capture light-evoked spiking with millisecond precision, as shown in the spike raster and peri-stimulus time histogram (PSTH) aligned to ON/OFF light epochs (scale: 50 Hz, 50 ms). (2) Chemogenetics + electrophysiology (orange): an inhibitory DREADD (AAV-promoter-Gi-DREADD) is activated by systemic injection of deschloroclozapine (DCZ, i.p.) in a freely moving mouse. The resulting suppression of firing is captured in the spike raster and firing rate histogram over minutes (scale: 5 Hz, 5 min), illustrating the slower temporal dynamics of chemogenetic modulation compared to optogenetics. (3) Pharmacology + electrophysiology (green): systemic drug injection (i.p.) produces a modulatory effect on neural firing captured across a comparable timescale (minutes), providing a drug-circuit interaction profile without the need for genetic manipulation. The bottom axis illustrates the relative temporal resolution of each approach, from milliseconds to hours. ChR2 channelrhodopsin-2, DREADD designer receptor exclusively activated by designer drug, DCZ deschloroclozapine, i.p. intraperitoneal

4.2 Local Field Potentials (LFPs)

LFPs are low-frequency voltage fluctuations that reflect the summed synaptic and membrane currents of local neuronal populations, rather than the action potentials of single cells. They arise when synchronized inputs to aligned dendrites create current sinks and sources, generating extracellular fields that can be measured with microelectrodes. This population-level signal is rich in oscillations across multiple frequency bands: Delta (~1–4 Hz), Theta (~4–12 Hz), Beta (~13–30 Hz), and Gamma (~30–120 Hz). These rhythms provide a temporal framework for organizing spiking activity and coordinating communication between brain regions [73–77]. Within anxiety circuits, Joshua Gordon's lab treated LFP synchrony, especially shared theta rhythms between regions, as a quantitative measure of long-range functional coupling between brain areas. In mice exploring anxiogenic environments, they reported increased theta-range synchrony between ventral hippocampus (vHPC) and medial prefrontal cortex (mPFC) [78]. Crucially, they went beyond correlation: optogenetic inhibition of vHPC terminals in mPFC reduced anxiety-like behavior and selectively decreased vHPC-mPFC theta synchrony, supporting a functional role for this pathway in organizing anxious state dynamics [79]. In parallel, their work in basolateral amygdala (BLA) showed that fear versus safety is associated with distinct theta–gamma coupling patterns, including switching between slow gamma (40–70 Hz) and fast gamma (70–120 Hz) nested within theta [80]. Overall, these results indicate that LFP synchrony can reflect, and help drive, anxiety-related circuit coupling.

LFPs encode rich information about neural population activity, spanning oscillatory dynamics, cross-frequency interactions, and aperiodic broadband structure. Extracting this information requires analytical approaches that range from well-established spectral methods to emerging AI-based frameworks.

LFP analysis is often organized around three widely used, largely "standardized" approaches: estimating power across frequencies (spectral power), separating oscillatory peaks from the broadband aperiodic background (spectral parameterization), and measuring interactions between frequency bands (cross-frequency coupling).

- 1.Power spectral density (PSD) is the basic entry point and aims to summarize how signal power is distributed across frequencies and to support comparisons of band-limited activity across conditions or brain states. Starting from a pre-processed LFP, the signal is split into overlapping time segments. Each segment is tapered with a window function and Fourier-transformed to obtain a periodogram; these periodograms are then averaged to produce a smoother PSD estimate (Welch's method). A key limitation is interpretability: the PSD mixes true narrowband oscillatory peaks with the aperiodic "1/f-like" background, so band-power changes can reflect shifts in either component.

- 2.Spectral parameterization (Fitting Oscillation and One Over Frequency (FOOOF), now distributed as specparam Python library) explicitly targets this interpretability issue: its aim is to disentangle the aperiodic background from true oscillatory peaks, so that oscillation-related effects are not confounded with broadband shifts, and to provide compact, physiologically interpretable parameters. Starting from a PSD, a frequency range is chosen. The aperiodic component is fit, and then residual peaks above that fit are detected and modeled (commonly with Gaussians) to extract peak center frequency, bandwidth, and amplitude [81].
- 3.Phase–amplitude coupling (PAC) adds a cross-frequency interaction view: its aim is to quantify whether the phase of a slower rhythm systematically modulates the amplitude of a faster rhythm, as a proxy for hierarchical coordination across timescales. The LFP is band-pass filtered into a low-frequency "phase" band and a high-frequency "amplitude" band; the Hilbert transform yields instantaneous phase and the amplitude envelope; then a coupling statistic (e.g., mean-vector-length–based measures) is computed, often across many frequency-pair combinations to form a comodulogram (2D-heatmap). Because classical PAC estimates can be sensitive to filter choices, sharp transients, and non-sinusoidal waveforms, state-space/generative PAC approaches shift the aim toward more artifact-resistant inference by explicitly modeling latent oscillatory components and estimating coupling parameters in that model space, often enabling time-varying coupling estimates with better control of confounds.

Machine-learning methods increasingly complement these classical tools and are typically applied when the objective is detection, classification, or representation learning.

- 1.One-dimensional convolutional neural networks (1D-CNNs) learn temporal filters directly from labeled LFP segments and are frequently used for event detection or brain-state classification.
- 2.Transformers extend this framework through self-attention, which learns to weight informative time points and channels, supporting multichannel decoding and capturing longer-range temporal dependencies. For a given time point, the model compares it to many other time points and learns to give higher weight to those that help the prediction.
- 3.autoencoders, based on unsupervised analysis, aim to compress LFP events into low-dimensional latent representations that can organize waveform diversity (and sometimes denoise through reconstruction)

- 4.LFADS (Latent Factor Analysis via Dynamical Systems), although also unsupervised, aim to infer latent dynamical structure underlying neural time series and produce denoised reconstructions by modeling data as arising from a low-dimensional dynamical system. [5] LSTMs (Long Short-Term Memory networks) and related recurrent networks aim to model sequential dependencies for temporally structured classification (e.g., predicting or labeling epochs) [82–84].

Taken together, classical and AI-based approaches are best viewed as complementary: classical methods prioritize interpretable, hypothesis-linked descriptors, while deep learning prioritizes flexible representations that can scale to high-dimensional, multi-region recordings and optimize decoding performance. In practice, a common workflow is to extract interpretable spectral and coupling features and then test whether adding learned representations from raw LFP segments improves sensitivity or generalization for the specific experimental question.

4.3 Single Units

Complementary to LFP recordings, extracellular single-unit recordings isolate the activity of individual neurons rather than the pooled activity of a local population. When an electrode is positioned in brain tissue, it can detect the rapid extracellular voltage transient generated when a nearby neuron fires, producing a brief (~1 ms), stereotyped deflection known as a spike (action potential). These spikes are "all-or-none" events: each one has a characteristic shape and amplitude for that neuron, rather than varying continuously like LFP amplitude [85]. Because a single electrode often picks up spikes from several nearby neurons, computational spike-sorting methods are used to cluster waveforms by shape and timing, assigning them to putative single units and yielding separate spike trains for each cell [86, 87]. In behaving animals, such single-unit recordings allow researchers to link the firing patterns of identified neurons to specific sensory features, movements, or cognitive variables, providing a cell-level view that complements the population-level perspective offered by LFPs.

Single-unit analysis begins with spike sorting, assigning each detected extracellular action potential to a putative neuron. In the classical pipeline, the high-pass filtered trace is scanned for threshold crossings, a short waveform snippet is extracted around each event, features are computed, and a clustering algorithm, such as a Gaussian mixture model or manual cluster cutting, groups waveforms into distinct units. Once clean spike trains are available, the most basic characterization is the peri-stimulus time histogram (PSTH), which bins spikes across repeated trials aligned to a stimulus or behavioral event to produce a trial-averaged firing-rate profile. To understand what a neuron encodes, firing rates are plotted against a stimulus or behavioral parameter (direction, position, frequency) to build a tuning curve, typically fit with a parametric model to extract preferred

stimulus, bandwidth, and gain, while cross-correlograms and joint PSTHs probe interactions between neurons by quantifying spike–spike timing structure, and spike–field coherence links single-unit firing to ongoing LFP oscillations [88].

A common follow-up is to classify units as putative excitatory or inhibitory neurons using extracellular signatures and, where available, ground truth. In cortex and hippocampus, excitatory pyramidal cells tend to have broader spikes with longer trough-to-peak durations and lower average firing rates, whereas fast-spiking inhibitory interneurons show narrow, sharp waveforms and higher baseline firing. Quantitative features such as trough-to-peak time, peak-to-trough ratio, waveform asymmetry, recovery slope, autocorrelogram shape, and burstiness can be combined with simple thresholds or machine-learning classifiers to assign units to "narrow interneuron" versus "wide pyramidal" classes [89].

AI-based methods increasingly complement these classical analyses at multiple levels. Modern spike sorters such as Kilosort 4 already blend learned template matching, graph-based clustering, and automatic drift correction to handle thousands of channels, and fully deep-learning pipelines using CNNs or autoencoders can perform detection, feature extraction, and clustering within a single network. For decoding, deep architectures (CNNs, LSTMs, Transformers) can learn direct mappings from population spike trains to behavioral variables such as position, velocity, or choice, often outperforming linear or GLM-based (Generalized Linear Model) decoders when neural-behavioral relationships are nonlinear. At the population-dynamics level, latent-variable models such as LFADS (Latent Factor Analysis via Dynamical Systems, a variational autoencoder with a recurrent generator) infer de-noised, single-trial latent trajectories from spiking data, while contrastive-learning methods like CEBRA (Consistent EmBeddings of high-dimensional Recordings using Auxiliary variables) learn low-dimensional embeddings that jointly capture neural structure and behavior and generalize across subjects and even recording modalities, going beyond what PCA can reveal [90–92]. Together, these AI-driven approaches extend classical single-unit tools by discovering latent structure in large populations, scaling to high-dimensional datasets, and enabling powerful end-to-end decoding, while classical methods remain essential for interpretable, hypothesis-driven readouts of single-neuron coding.

4.4 Combination with Neuronal Modulations

In vivo electrophysiology becomes substantially more powerful when combined with techniques that allow selective manipulation of neuronal activity. These integrative approaches allow perturbing specific cell types, pathways, or neuromodulatory systems while simultaneously monitoring the resulting changes in LFPs, single-unit firing, and network dynamics. Key integrative approaches include combining electrophysiology with optogenetics (light-based control

of defined cell types), chemogenetics (DREADD-mediated control via designer ligands), and targeted pharmacology (systemic or local administration of receptor-specific drugs).

Specialized "optrodes" integrate optical fibers with microelectrode arrays to deliver light and record electrical signals from the same brain region, often within a single implant device. Combining optogenetics with electrophysiology has proven particularly valuable for dissecting oscillatory mechanisms, for example, selectively activating parvalbumin-positive interneurons at gamma frequencies can entrain or disrupt local gamma oscillations, directly testing the necessity of specific interneuron subtypes for rhythm generation [93–95].

Unlike optogenetics, which provides millisecond-scale temporal control, chemogenetic modulation operates on slower timescales (minutes to hours) and does not require implanted light sources, making it suitable for chronic or repeated manipulations. Combining DREADDs with in vivo electrophysiology allows monitoring of how pathway-specific activation or inhibition delivered via systemic injection of the actuating ligand alters firing rates, LFP oscillations, coherence patterns, and behavioral states over extended experimental sessions. For instance, chemogenetic inhibition of a specific projection pathway can reveal whether that pathway is necessary for maintaining particular network oscillations or behavioral states, while electrophysiological readouts provide a continuous, quantitative measure of the circuit response [96, 97].

Finally, acute pharmacological interventions, such as systemic or local drug infusions targeting specific neurotransmitter receptors, can probe the contribution of neuromodulatory systems to circuit activity in a temporally controlled manner. Pharmacological manipulations typically act on receptor subtypes distributed across multiple cell classes and projections, thereby revealing how neuromodulators globally reshape firing rates, oscillatory power, and cross-regional coupling without requiring genetic access to specific neuronal populations. When combined with in vivo electrophysiology, such approaches allow experimenters to monitor, in real time, how the onset, plateau, and washout of a given compound translate into changes in single-unit responses, LFP signatures, and behavioral state, and to relate these dynamics to clinically relevant drug effects such as anxiolysis, sedation, or psychostimulation. These paradigms are particularly useful for dissecting the circuit actions of therapeutic agents, identifying dose-dependent transitions in network regimes, and mapping which oscillatory or firing-rate patterns track beneficial versus adverse behavioral outcomes.

A recent study by Couderc and colleagues exemplifies how combining electrophysiology with cell-type-specific optogenetic modulation and dopaminergic pharmacology can dissect neuromodulatory control of anxiety circuits in the anterior insula. In this work, the authors expressed ChR2 in D1-receptor-expressing neurons of the

anterior insular cortex and performed single-unit recordings with an optrode while mice explored the elevated plus maze, allowing them to directly link light-evoked changes in D1-aIC firing to moment-to-moment fluctuations in anxiety-related behavior. Optogenetic activation of D1-aIC neurons produced robust, time-locked increases in firing in a subset of units and shifted maze exploration toward closed arms, indicating that transient increases in D1-insular output are sufficient to bias behavior toward an anxiety-like state. In parallel, intra-insula and systemic manipulations of D1 receptors with SKF38393 and SCH23390 revealed that dopaminergic signaling reorganizes the balance of excited and inhibited units and alters how insular neurons encode open versus closed arm entries, consistent with the changes in unit categories and excitation/inhibition ratios [98].

Such approaches can dissect the circuit-level mechanisms of clinically relevant drugs and identify the specific oscillatory or firing-rate changes that mediate therapeutic or pathological effects. Taken together, in vivo electrophysiology offers several key advantages over fiber photometry and miniscope-based calcium imaging: it provides millisecond-scale sampling and direct detection of action potentials, preserving precise spike timing, bursts, and fast oscillations that are strongly blurred by the slow calcium transients measured with GECI-based fiber or miniscope recordings. Because it measures extracellular voltage rather than fluorescence, in vivo electrophysiology avoids photobleaching and phototoxicity entirely, and high-density silicon probes (e.g., Neuropixels or laminar arrays) can sample many sites across cortical layers or deep structures from a single implant, yielding access to both single-unit/multi-unit spikes and local field potentials without requiring genetic reporters or clear optical access. Conversely, compared with these imaging approaches, it cannot directly report neuromodulator or peptide levels (for which fiber/miniscope imaging with GRAB, dLight or other GPCR-based sensors is well suited), is more vulnerable to motion- and interface-related noise and spike-sorting errors, makes it difficult to unambiguously track the same neuron over days or weeks, and only infers cell type indirectly from waveform and firing properties, whereas fiber/miniscope imaging can combine Cre-LoxP-based genetic labeling, structural information, and genetically encoded sensors to achieve robust cell-type-specific readout of activity and neuromodulatory signaling [99, 100].

5 Neural Metrics of Anxiety and Anxiety-Related Behaviors

The previous sections have focused on how to record neural activity during anxiety-related tasks, using genetically encoded sensors with fiber photometry, miniscopes, two-photon imaging, as well as using in vivo electrophysiology (Fig. 4A). These methods yield

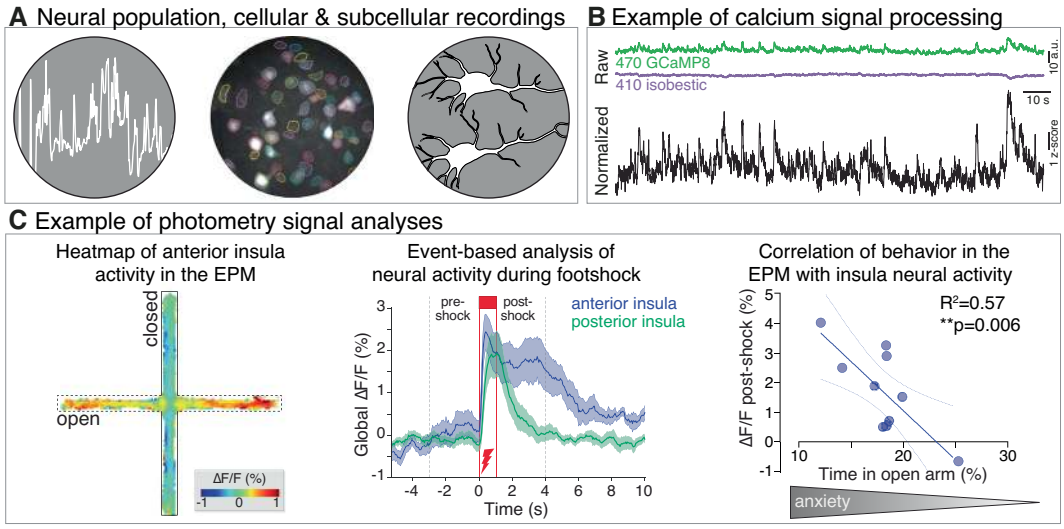


Fig. 4 Analysis strategies for neural imaging. (**A**) Schematic example of neural recordings at different spatial resolutions, illustrating the progression from a global signal to cellular and subcellular read-outs. (**B**) Example of a typical preprocessing pipeline for fluorescence signals, showing conversion of the raw trace to a normalized signal suitable for quantitative analysis. (**C**) Examples of three complementary analysis approaches applied to anxiety-related assays. Left, space-based analysis: spatial heatmap of $\Delta F/F$ across the elevated plus maze, highlighting differences in neural activity between open and closed arms. Middle, event-based analysis: peri-event time histograms of global insular activity aligned to the onset of mild foot shocks, illustrating transient post-shock responses in anterior and posterior insula. Right, correlation analysis: example of a negative correlation between post-shock $\Delta F/F$ and time spent in the open arms of the elevated plus maze, linking neural activity to individual differences in anxiety-like behavior. Data is coming from Nicolas et al. [43]

continuous activity traces from defined brain regions, cell types, and projections, but by themselves they do not specify which aspects of this activity are most informative about anxiety states. Neural metrics of anxiety aim to quantify how these signals relate to discrete events (e.g., entries into anxiogenic spaces, drinking bouts), spatial context (e.g., open versus closed arms), and individual differences in state and trait anxiety across animals and paradigms. At the single-region or single-cell level, event-locked averages and space-binned activity maps link neural responses to well-defined behaviors or locations. At the multi-region level, correlation matrices and low-dimensional embeddings summarize how distributed circuits co-activate during anxiety-like states. More recently, unsupervised and representation-learning methods have started to extract latent behavioral axes directly from joint neural-behavioral data, complementing classical supervised analyses.

5.1 Pre-Processing and Normalization of Neural Signals

As described in Chap. 2, tools such as fiber photometry and miniscopes provide access to different levels of neuronal activity, from intracellular calcium and extracellular neurotransmitters to membrane voltage and second messengers. In particular, bulk fiber-photometry

fluorescence reflects a mixture of sensor dynamics, optical properties of tissue, movement artifacts, photobleaching, hemodynamic fluctuations, and biological noise, leaving researchers with the task of carefully preprocessing and analyzing the signal to reach meaningful biological interpretations [99]. Choices regarding baseline correction, isosbestic control fitting, $\Delta F/F$ computation, z-scoring, filtering, window selection, and statistical approach can significantly influence the results, especially when circuit activity is integrated with behavior, where the signal is aligned to extracted behavioral timestamps and intervals in a millisecond timescale. While custom processing pipelines and scripts remain a common approach, several open-source platforms now provide integrated preprocessing, event alignment, and visualization pipelines, such as GuPPy [101], pMAT [102], and Pyfiber [103].

Working with neural activity data, therefore, typically involves two major stages: preprocessing of the raw fluorescence signal and subsequent data analysis. Preprocessing aims to transform the raw signal into a form that more accurately reflects the underlying physiological process of interest. Because the signal consists of many components, including indicator fluorescence, autofluorescence from optical components, excitation bleed-through, movement artifacts, photobleaching, and hardware noise, careful signal conditioning is required. Preprocessing generally consists of four sequential steps: filtering, bleaching correction, movement correction, and normalization.

A common first step is temporal filtering. In most fiber photometry experiments, the sampling rate of the equipment (often 100–1000 Hz) exceeds the effective temporal bandwidth of the indicator; the biological signal is typically confined to lower frequencies (e.g., 2–10 Hz for GCaMP6f or dLight), whereas noise is spread across the spectrum. Improving the signal-to-noise ratio is therefore commonly achieved using low-pass filtering to remove high-frequency noise while preserving the slower dynamics of calcium or neuromodulator signals [99].

The second step is to correct for photobleaching. Photobleaching produces a slow decline in fluorescence intensity over time. Because bleaching evolves on a slower timescale than most physiological signals, its time course can be estimated by fitting a smooth function to the signal, commonly a double exponential, and then correcting the trace by subtracting or dividing by this fitted curve. In dual-wavelength photometry, a calcium-independent control channel (for example, 405 nm excitation near the isosbestic point of GCaMP) is often used instead of, or in addition to, explicit exponential fitting: the control channel is linearly fit to the activity-dependent channel and then used to correct for bleaching and motion in a single regression-based operation, which can be carried out during the normalization step.

After filtering and bleaching correction, the next step is normalization (Fig. 4B). Normalization transforms the corrected fluorescence signal into a dimensionless metric suitable for comparison across sessions, animals, and recording conditions. The standard approach expresses fluorescence change relative to a baseline, $\Delta F/F$, obtained by subtracting a baseline fluorescence value (F0) from the signal at each time point and dividing by F0. F0 can be defined in several ways, such as a pre-event window, a running low percentile, or using the isosbestic control signal as a reference. The aim is to express activity as a fractional change that is comparable across sessions, animals, and brain regions. An alternative or additional normalization is z-scoring, where each trace is mean-centered and divided by its standard deviation, often after fitting and subtracting the control channel. Z-scoring places activity in units of baseline variability and is particularly useful for defining objective thresholds (e.g., $z > 2$) for event detection or for comparing sensors with different absolute amplitudes, although it depends on the chosen reference window and can be biased by large artifacts.

A related challenge is the presence of large, non-stationary transients or artifacts, such as rare movement-induced spikes or extremely large sensor events, that can bias averages and correlations. Several recent workflows therefore detect and remove outlier segments before baseline fitting and normalization, for example, by thresholding the signal or its derivative and excising or interpolating the affected points. More integrated pipelines combine artifact detection, isosbestic correction, and normalization, and explicitly recommend trimming large outliers before computing trial-averaged metrics or fitting statistical models to event-locked responses. Together, these preprocessing steps, filtering, bleaching correction, artifact handling, and appropriate normalization are crucial to ensure that downstream event-based, space-based, and unsupervised analyses faithfully reflect anxiety-related neural dynamics rather than technical confounds.

5.2 Event-Based Analysis

A central goal of fiber photometry in behavioral neuroscience is to determine whether neural or molecular activity exhibits systematic modulation around defined events (Fig. 4C). In anxiety research, such events can be externally scheduled (e.g., cue onset, reward or foot-shock delivery), generated by the animal (e.g., drinking, food consumption, social approach or escape), or defined by state transitions such as entering an anxiogenic zone in a maze. Event-based analysis provides an intuitive way to visualize and quantify how the signal increases or decreases relative to these moments in time.

After preprocessing and normalization (e.g., $\Delta F/F$ or z-scored signals), event-based analysis typically proceeds in three steps: event extraction, temporal alignment, and quantification. First, each behavioral event is identified and time-stamped, and an event window is defined that includes a pre-event baseline period and a post-event

response period. The length of this window should match the kinetics of the sensor related to the event and region of interest: for calcium indicators, a common window length is in the order of −5 to +5 to 10 s, in order to capture the full wave, whereas faster neuromodulator sensors may require shorter windows to preserve temporal specificity [104]. Second, fluorescence segments from all occurrences of the same event type are aligned to event onset, producing a set of event-centered traces. Finally, these traces are summarized, often by averaging across trials, to obtain a mean event-related response, and by computing metrics such as peak amplitude, area under the curve, or response latency for statistical comparison between conditions or groups [105]. Even if global normalization has already been applied, event-based analysis usually includes an additional local baseline step. A common approach is to estimate a baseline from the pre-event portion of each trial and express the event-related change relative to this local baseline, either as event-based $\Delta F/F$ or as a local z-score. This helps account for slow drifts across the session and ensures that modulation is quantified relative to the immediate pre-event state rather than a distant reference period. Careful definition of the event window and baseline interval before looking at the data is important to avoid biased selection of windows that exaggerate or minimize apparent effects. Event-based strategies have been widely used to probe anxiety-related circuits. For consummatory behaviors, Nicolas et al. aligned fiber-photometry calcium signals in insula and in insula → amygdala projections, to individual licking bouts during sucrose (appetitive) and quinine (aversive) consumption, and showed that event-locked activity in these pathways differentiates positive from negative ingestive valence in an anxiety-relevant insula–amygdala circuit [43]. In a related study, Zhao et al. aligned insular calcium signals to the first lick of each water-drinking episode and found transient increases in activity at bout initiation, with responses during early licking significantly elevated relative to pre-lick baseline, illustrating how peri-event alignment can isolate neural responses specifically linked to the onset of drinking [106]. Beyond consummatory behaviors, event-based analyses have been used for sensory cues and aversive stimuli. Nicolas et al. aligned insular activity signals to the onset of mild foot-shocks, demonstrating increased anterior insula activity immediately after shock and showing how these ensembles integrate information during negative valence [43]. In the basolateral amygdala, Sengupta et al. analyzed calcium activity relative to shock delivery during fear conditioning and found that peri-shock responses in basolateral amygdala neurons are critical for maintaining the salience of conditioned stimuli that precede the aversive outcome, thereby supporting the formation and persistence of fear memories [107]. During anxiety assays, Couderc et al. used the onset of entries into anxiogenic spaces as event markers, utilizing the transition into the open arms of the elevated plus maze [98]. By aligning dopaminergic

signals in the anterior insular cortex to these entry points, they observed transient increases in dopamine activity specifically at open-arm entry, indicating that dopaminergic signaling in this region is tightly coupled to the decision to explore anxiogenic zones. Finally, in social paradigms, Bordes et al. combined machine-learning-based behavioral segmentation (DeepOF), aligning noradrenergic signals in the basolateral amygdala to the initiation of social interactions and revealing blunted event-locked noradrenergic responses in mice lacking the stress-risk gene fkbp5 [108].

Together, these studies illustrate how event-based metrics, built on carefully preprocessed and normalized signals, provide a flexible framework to link temporally precise components of anxiety-related drinking, aversion, spatial exploration, and social behavior to activity in defined circuits and neuromodulatory systems.

5.3 Space-Based Analysis

Another approach to analyze neural activity recordings is to use a space-based framework, in which spatial heatmaps of neural signals are used to relate activity to the animal's position in the environment (Fig. 4C). In this strategy, spatially defined behavioral states serve as anchors: neural activity is compared across predefined zones, such as open versus closed arms of the elevated plus maze or center versus periphery of the open field. Heatmaps of neural activity overlaid on the arena layout then provide an intuitive visualization of how activity varies across anxiogenic versus protected locations.

For example, Nicolas et al. measured aIC and pIC activity during exploration of anxiogenic environments by sorting calcium signals according to the animal's position in the elevated plus maze and open field [43]. They found that anterior insula activity was significantly increased when mice occupied anxiogenic locations, compared to protected zones. A similar space-based strategy was used by Jimenez et al., who examined ventral CA1 activity in the elevated plus maze and compared neural signals between open and closed arms [109]. This study identified a subset of ventral CA1 neurons that were preferentially active in the open arms, consistent with a specialized representation of anxiogenic spaces in the hippocampal circuit. Collectively, these findings highlight space-based analysis as a powerful complement to event-locked analysis methods, enabling visualization and quantification of how anxiety-related circuits track the animal's position along gradients of threat and safety.

5.4 Correlation of Neural Activity at Behavioral Events

Another important analysis tool for neural activity recordings is to correlate neural signals with event-based behavioral measures and with indices of anxiety, which can provide insight into both state and trait components of anxiety (Fig. 4C). For example, Couderc et al. reported that D1-positive anterior insular cortex activity is negatively correlated with the time animals spend in anxiogenic spaces, suggesting that activity patterns in this population relate not only

to momentary state anxiety during individual entries but also to stable, trait-like differences in avoidance of anxiogenic spaces [98]. These correlation analyses are particularly useful when combined with longitudinal or across-individual comparisons, where consistent relationships between neural activity and anxiety measures can reveal trait-like circuit signatures.

5.5 Unsupervised Analysis Tools

The current state-of-the-art tools, including Neuropixels probes and miniscopes, can measure hundreds to thousands of cells simultaneously, making downstream analysis increasingly challenging. Unsupervised and semi-supervised representation-learning methods, such as CEBRA, address this by learning low-dimensional latent spaces that jointly organize neural activity and behavior [92]. Schneider et al. showed that CEBRA could display low-dimensional embeddings that organized neural activity according to behavioral variables such as position and movement without manual intervention [92]. More recently, Chang et al. applied CEBRA to extended amygdala recordings collected during different anxiety-related assays and showed that the learned latent spaces segregate activity by behavioral context and internal state, revealing latent "anxiety maps" distributed across the neural network [110]. Notably, anxiety-associated states occupied distinct regions of the latent space and could be decoded from population activity, indicating a robust, distributed code for anxiety in these circuits. Beyond unsupervised discovery, CEBRA's latent spaces can be used for decoding and cross-session alignment, enabling prediction of behavioral variables from neural activity and integration of data across days or animals, which is particularly useful for high-dimensional anxiety-circuit recordings. In the context of the analyses described above, such latent-space methods provide a complementary way to summarize how event-locked and space-defined anxiety behaviors are embedded in large-scale population codes.

6 Conclusions and Outlook

Over the years, methods for manipulating and recording the activity of neuronal populations have expanded rapidly, from optogenetics and chemogenetics to large-scale calcium imaging and high-density electrophysiology. Other less invasive approaches, such as magnetogenetics, are being developed and will further broaden the possibilities for dissecting neural circuits involved in anxiety-related behavior. The combination of these manipulations and recording techniques with cutting-edge tools such as single-cell RNA sequencing and whole-brain clearing now enables cell-type-specific circuit mapping across the entire brain, linking molecular identity, connectivity, and function. A major challenge for the coming years will be to develop computational frameworks capable of integrating information across

these levels of analysis, from gene expression and circuit architecture to physiological and pathological behaviors, including coding properties and plasticity of neural circuits.

6.1 The Push for Automated Behavioral Phenotyping Tools Using Artificial Intelligence

In parallel with these advances in circuit-level techniques, there has been a strong push to change how behavior itself is measured and analyzed. In Sect. 1, we described established behavioral tasks that have been and will remain highly valuable for measuring key aspects of anxiety-like behaviors. However, as noted before, they have important limitations, including a restricted number of read-outs and habituation effects during repeated exposure, which have continuously motivated scientists to explore additional assays using more advanced analytical approaches.

Recent developments in machine-learning tools have greatly improved markerless animal pose estimation using open-source software such as DeepLabCut [111] and SLEAP [112]. These tools enable the extraction of detailed body part trajectories without physical markers, providing a much richer behavioral description than was previously possible and allowing more detailed, automated analysis of behavior, including anxiety-related behavior. Since the initial release in 2019, there has been an explosion of advanced machine-learning pipelines for behavioral dissection, using both supervised classification (to detect predefined actions or states) and unsupervised clustering (to uncover latent structure without labels). Using a combination of supervised and unsupervised approaches, Bordes et al. showed that chronic social stress produces a robust increase in anxiety-related behaviors specifically during social interaction, which is not captured to the same extent using traditional read-outs [113].

These computational tools are particularly powerful in longitudinal recordings of behavior. In recent years, there has been increasing interest in semi-naturalistic environments that allow home-cage-like behavior to be tracked over days to weeks, often in group-housed animals [114]. Systems such as the Social Box [115] and Live Mouse Tracker [116] exemplify how continuous multi-animal tracking and automated classification can be used to quantify social behavior and social dominance hierarchies. For example, they have revealed strong links between social structure, stress-related phenotypes, and responses to pharmacological interventions [117, 118]. Although very few studies have explicitly focused on anxiety-related behaviors in such semi-naturalistic environments, relevant information is often implicitly present in these datasets. For example, in metrics such as the latency to leave the nest and approach food zones, the amount and spatial distribution of exploratory behavior across light/dark phases, or the frequency and structure of escape responses following aggressive and non-aggressive chases. As analysis methods mature, these features can be formalized into continuous, ethogram-based anxiety measures that complement canonical tests, such as the elevated plus maze.

Overall, artificial intelligence-based behavioral analysis offers a path to move from low-dimensional, assay-specific read-outs (e.g., time in open arms) to high-dimensional, task-agnostic behavioral signatures of anxiety. By applying advanced pose-estimation as well as supervised and unsupervised behavioral segmentation in both standard and semi-naturalistic settings, future work can derive more sensitive, longitudinal markers of anxiety-like states and better capture individual differences and treatment responses.

6.2 Toward Holistic, Longitudinal Circuit–Behavior Models

A natural next step is to integrate such rich behavioral descriptions with other physiological measures, including heart rate, respiration, and endocrine read-outs, to obtain a more holistic picture of anxiety states in individual animals. In parallel, the continued development of wireless recording and manipulation systems will facilitate longitudinal measurements of neural activity in freely moving animals, enabling stable tracking of circuit dynamics across days, contexts, and interventions. Together with advanced analytic tools, such as CEBRA, these developments promise a future in which anxiety-related circuit function is characterized at multiple scales: molecular, cellular, circuit, behavioral, and physiological in the same subjects over time.

Competing Interests

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Chapter 18

In Vivo Two-Photon Calcium Imaging of Cortical Neurons in Mice with Traumatic Brain Injury

Xingjie Ping, Wenhui Xiong, Jodi L. Smith, Jeffrey M. Witkin and Xiaoming Jin

Abstract

Aberrant neuronal activity is a neurological consequence of traumatic brain injury (TBI) that can lead to cognitive, emotional, and behavioral deficits, including anxiety- and mood-related disturbances. However, the mechanisms by which these activity alterations arise and their stability over time remain poorly understood with conventional electrophysiological recording methods. Two-photon calcium imaging is a powerful technique that allows high spatiotemporal resolution live imaging, enabling long-term monitoring of the activity of individual neurons in brain tissue in vivo. We here describe a protocol for long-term in vivo two-photon imaging of calcium signals in mice expressing a genetically encoded calcium indicator to monitor the dynamic changes in neuronal activity after brain trauma. This approach offers valuable insights into the neuronal mechanisms underlying anxiety and mood disorders following TBI.

Keywords In vivo two-photon calcium imaging, Traumatic brain injury (TBI), Controlled cortical impact (CCI), GCaMP6s transgenic mice, Neuronal activity

1 Introduction

Traumatic brain injury (TBI) is a leading cause of death and disability in both children and adults, and invariantly results in impairments of sensory, motor, and cognitive function [1–3]. TBI leads to structural damage and neuronal loss, accompanied by dynamic changes in the pattern and level of neuronal activity. These alterations trigger complex biological cascades that contribute to secondary consequences such as epilepsy, cognitive decline, chronic pain, and mood-related dysfunctions, including anxiety and depression [4–12]. While electrophysiological recordings have been widely used to study these neural activity changes, tracking long-term dynamics in response to brain injury with in vivo electrophysiology alone remains challenging [13–16].

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In recent decades, two-photon calcium imaging has become a powerful method for studying neuronal ensemble dynamics in the intact brain [17–19]. This technique monitors neuronal activity by detecting increases in intracellular calcium levels, which occur when neurons fire action potentials [20, 21]. Fluorescent molecules that bind to calcium allow for visualization of these changes. Compared to electrophysiological recordings, in vivo two-photon calcium imaging offers several advantages over electrophysical methods. It is less invasive, as it typically does not require disrupting the dura or the underlying brain tissue. Additionally, it enables the recording of neural activity in small structures such as dendrites and axons. When combined with genetic tools, this method facilitates targeted recording in specific neuronal populations. Moreover, two-photon calcium imaging allows for longitudinal tracking of identified cells over weeks or months, providing submicron-level precision in locating recorded neurons [22–27].

Here we describe a method utilizing in vivo two-photon calcium imaging to monitor long-term changes in neuronal activity and neural network following TBI. This approach provides a framework for elucidating the cellular and circuit mechanisms underlying a wide spectrum of post-traumatic neurological outcomes—including anxiety and mood disorders. Moreover, it can be applied to evaluate the long-term effects of specific therapeutic interventions on TBI-induced neurological consequences [28, 29].

2 Materials

2.1 Animals

Either genetic encoded calcium indicators (GECIs) transgenic mice or viral delivery of calcium indicators can be used for in vivo calcium imaging. For longitudinal calcium imaging, transgenic mice are preferred since the expression of calcium indicator is more stable over time than viral induction. Here we use 2- to 3-month-old Thy1-GCaMP6s transgenic mice (JAX stock #024275), which express the green, fluorescent calcium indicator GCaMP6s in subsets of excitatory neurons in the brain [30]. The reagents, resources, software, and equipment used in this protocol are summarized in Table 1.

3 Methods

3.1 Procedures

Controlled Cortical Impact

The controlled cortical impact (CCI) model is a widely used experimental approach for inducing traumatic brain injury in rodents. This technique produces a focal injury by delivering a precisely regulated

Table 1
Key resources table

Reagent or resource	Source	Cat. no.
Chemicals		
Ketamine	Covetrus	080524
Xylazine	Covetrus	061035
Ethiqa XR (buprenorphine)	Covetrus	072117
Carprofen	Sigma-Aldrich	SML1713
Sterile saline	Fisher Scientific	Z1376
Betadine surgical scrub	Fisher Scientific	19-066452
Chlorprothixene hydrochloride	Fisher Scientific	18-620-476
Ethanol	Fisher Scientific	A405P-4
Dental acrylic powder and liquid	Lang Dental	124287/124299
Pro CA-Gel cyanoacrylate glue (thick glue)	Ted Pella	14478
Pro C5 cyanoacrylate glue (thin glue)	Ted Pella	14474
Ophthalmic ointment	Fisher Scientific	NC2671618
Surgical tools		
Dissecting scissors	Fine Science Tools	14060-09
Fine forceps		11923-13
Dumont forceps	Fine Science Tools	11252-00
Scalpel handle	Fine Science Tools	10003-12
Scalpel blades	Fine Science Tools	10010-00
Stereotaxic frame	Narishige	SGM-4
Stainless steel bar	Custom-made	N/A
Dental spatula	Fisher Scientific	HS15907
Dental drill	Handler Redwing International	K. 107018
Devices		
CCI equipment	Leica Biosystems	N/A
Motorized micromanipulator	Sutter Instrument	MP-285
Two-photon imaging microscope	Olympus	N/A
Ti: sapphire excitation laser	Newport	N/A
photomultiplier tube (PMT)	Prairie Technologies	N/A
Imaging frame	Custom-made	N/A
4× / N.A. 0.1 air objective	AmScope	A4X-490

(Continued)

Table 1 (continued)

Reagent or resource	Source	Cat. no.
20× / N.A. 1.00 water immersion objective	Olympus	XLUMPlanFLN20X
Others		
Syringes	Fisher Scientific	14-841-31
Gelfoam	VWR International	10611-586
Sterile cotton swabs	Fisher Scientific	22-363-173
Cover glass	Warner Instruments	D263, 5 mm round
Heating pad	Kent Scientific	TPZ-814EA/HTP-1500
Compressed air	Built-in	N/A
Vacuum	Built-in	N/A
toothpick	Amazon	N/A
Binder clip	Office Depot	N/A
Softwares		
PrairieView	Prairie Technologies	N/A
ImageJ	NIH	N/A
OriginLab2021b	OriginLab	N/A

mechanical impact to the exposed cortex, resulting in well-defined cortical contusion and secondary pathophysiological responses, recapitulating key features of human TBI, including neuronal loss, axonal injury, blood-brain barrier disruption, and robust neuroinflammatory responses [31–36]. Animals subjected to CCI typically develop long-lasting deficits in cognition, motor function, and emotional behaviors, paralleling clinical outcomes [37–40]. The severity of injury can be adjusted through parameters such as impact velocity, depth, and dwell time, and ensuring high reproducibility [41–43].

1. Anesthetize the mouse with an intraperitoneal injection of ketamine/xylazine (87.7/12.3 mg/kg).
2. Once the mouse is fully anesthetized, shave its head and secure it in a stereotaxic frame.
3. Apply ophthalmic ointment to prevent dehydration and irritation. Position the mouse on a heating pad ($T = 33°C$) to maintain body temperature during the procedure. Administer a single subcutaneous injection of SR buprenorphine (3.25 mg/kg) for analgesia.
4. Clean the scalp with alternating swabs of betadine and 70% ethanol. Carefully remove a small section of scalp skin using scissors. Gently scrape the skull with a sterile scalpel blade to

remove the periosteum, which ensures better adhesion of the glue to the bone when performing window implantation.

5. Use a dental drill to gently outline a 3–4 mm circle over the area of interest, then create a craniotomy by carefully drilling along the border under the microscope, irrigating with sterile saline regularly to prevent overheating, which could damage the dura. Remove bone dust with compressed air.
6. Stop drilling when small cracks begin to form around the engraving, or when the bone within the engraving can be easily moved with gentle pressure from forceps.
7. Place a drop of sterile saline on the circular groove, and carefully insert the tips of Dumont forceps into the trabecular bone along the edge of the groove. Keep the forceps horizontal to avoid puncturing the thinned bone, which could injure the dura. Slowly lift and remove the island of cranial bone (Fig. 1A). If minor bleeding occurs from torn superficial capillaries, apply saline-soaked Gelfoam to the dura to stop bleeding.
8. Attach the CCI control box to the stereotaxic frame by securing the cable of the control box to the ear bar. Set the velocity to 3 m/s and dwell time to 100 ms. The exposed cortex should be covered with saline-soaked Gelfoam to keep

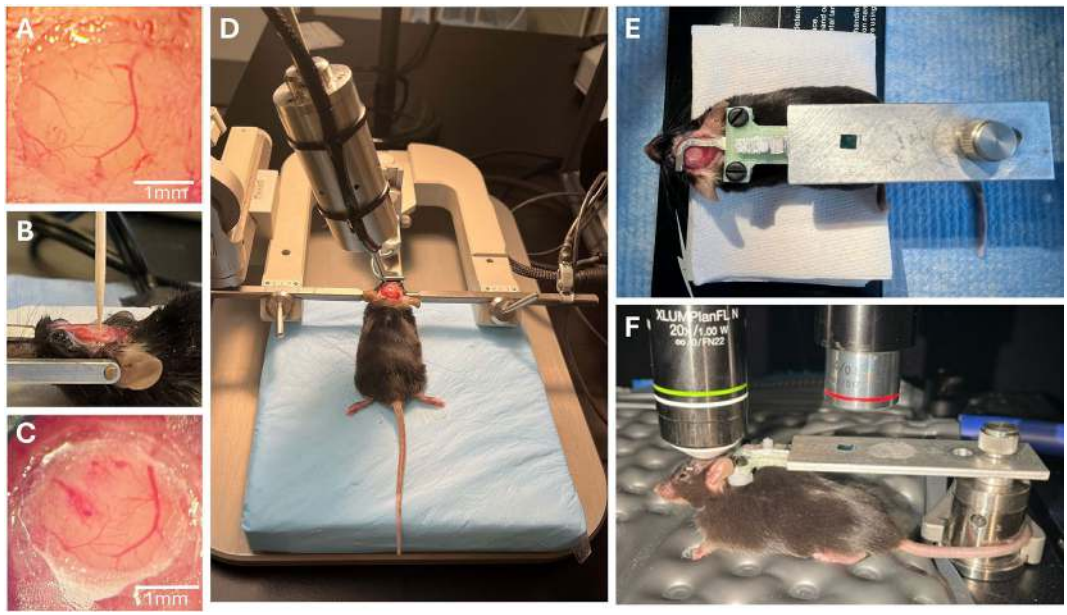


Fig. 1 Scheme of cranial window preparation, CCI, and imaging setup. (**A**) Mouse cranial window before CCI. (**B**) The coverslip was held in place with a toothpick, which was secured in a manipulator. (**C**) Cranial window after CCI and covered with a coverslip. (**D**) CCI setup. (**E**) Mouse fixed on the imaging frame. (**F**) In vivo two-photon calcium imaging setup

it wet when preparing the setup for CCI surgery. Remove the Gelfoam from the cortex, ensuring that any bleeding has stopped. Adjust the angle of the actuator so that the impactor tip is parallel to the exposed cortex (Fig. 1D).

9. Set the zero point by lowering the actuator until the impactor tip touches the dura, then calibrate the Z channel on the stereotactic control panel to zero.
10. Retract the impactor tip and lower the actuator by 0.5 mm, depending on the desired injury severity. Press the impact button to strike the injury site, aiming for a deformation depth of 0.5 mm, which will induce a mild TBI. Moderate to severe TBI can be induced by increasing velocity, depth and/or dwell time [44, 45].
11. Gently apply saline-soaked Gelfoam to the injury site until bleeding ceases.
12. Detach the CCI box by releasing the cable from the ear bar of the stereotaxic frame.

Window Implantation

1. When the bleeding stops, place a circular cover glass (5 mm in diameter) over the lesion area, ensuring the injury site remains wet. To avoid air bubbles under the window, apply a drop of sterile saline onto the exposed cortex, and the cover glass can be held in place by a toothpick, which is secured in a micromanipulator [46] (Fig. 1B). Then, gently dry the surrounding skull area with a vacuum (cover glass should be sterilized by soaking in 70% ethanol prior to the experiment and rinsed with sterile saline before being placed over the cranial window).
2. Seal the cranial window to the skull by applying thick glue to the edges of the cover glass. Ensure there are no air bubbles under the window (Fig. 1C). Next, apply a layer of thin glue to the surrounding skull to create a better base for the dental acrylic to adhere to. Then remove the toothpick.
3. Secure a stainless-steel bar to the skull using thick glue. Then, cover the bar and the surrounding intact hemispheres with dental cement to stabilize the animal for imaging sessions. Under the microscope, visually adjust the bar to ensure that it is parallel to the glass window surface, and adjust further until the window surface shows an even reflection, indicating that it is horizontal and perpendicular to the optical axis (Fig. 1E).
4. Place the mouse in a cage and allow it to recover on a heating pad until fully awake. Add a recovery food supplement to the cage. Carprofen (7.5 mg/kg, i.p.) can be injected daily if there is fog or blood under the window during the 2 weeks following the surgery to reduce inflammation.

3.2 In Vivo Two-Photon Imaging of Spontaneous Neuronal Activity

1. Prepare all required equipment and materials, power on the laser, and any microscope components needing warm-up time.
2. Sedate the mouse with chlorprothixene HCl (1.0 mg/kg, i.p.). Calcium responses of neuronal somata in cortex are notably suppressed by anesthesia (e.g., isoflurane, ketamine, or barbiturate), but minimally affected by sedation, in terms of the response amplitude and spatial extent of neuronal activation [47–49]. Therefore, we recommend sedation instead of anesthetics to preserve the integrity of cortical activity during imaging. Alternatively, recordings can also be performed in fully awake animals, with the head fixed while the mouse runs freely on a running disk or treadmill. In this case, the animal should be habituated to the head-fixed running disk or treadmill prior to imaging to reduce stress and allow for natural cortical activity during recording [50–52].
3. Gently wipe the window with 70% ethanol-soaked cotton applicator to remove dust.
4. Secure the mouse under a two-photon microscope with a head fixation device and maintain its body temperature on a heat pad during imaging (Fig. 1F).
5. Adjust the angles of the animal stage and the objective lens to ensure the glass window is perpendicular to the light path of the objective lens.
6. Use the microscope in bright-field mode with a low magnification objective lens (4×) to locate the area for imaging. Apply distilled water onto the cranial window and switch to a high magnification (20×) water-immersion objective lens. Adjust the focus and verify the presence of blood flow.
7. Switch to two-photon imaging and turn on the laser for two-photon excitation at 900 nm. Tune the detector gain and laser power to achieve optimal imaging quality, and slowly lower the object lens to reach the desired imaging depth.
8. Select and crop a specific region of interest (ROI) and start calcium imaging. Record spontaneous activity in a single trial lasting 120–300 s, using a scan rate of approximately 2-4 Hz at 512 × 512 pixels.
9. Switch to bright-field mode to capture the vasculature pattern under 20× objective lens as well as 4× objective lens as a reference image for future imaging sessions.
10. Repeat Steps 9 and 10 for all required fields of view (FOVs). Check regularly to ensure sufficient water under the objective, as it may evaporate over time.
11. When the scan is complete, carefully remove the mouse from the head fixation device and place it back in its home cage. Keep the cage on a heating pad until the mouse is fully awake.

12. Use lens paper to clean the objectives, close the software, turn off all microscope components that require daily shutdown, and put the laser into standby mode.
13. For repeated imaging, locate the target FOVs by matching the vasculature patterns with the reference images, then adjust the focus to reach the same depth used previously.

3.3 Histological Characterization of CCI Severity

After imaging, mouse brains can be fixed in a suitable fixative (e.g., 4% paraformaldehyde) for subsequent histological analysis, which provides a direct and reliable approach to assess the extent of structural brain damage following TBI. Brains are typically sectioned coronally at a thickness of 30–50 μm and processed for Nissl staining (Cresyl violet). Nissl staining is widely used to visualize neuronal cell bodies and cortical architecture. Quantification of lesion volume or cortical cavity size by serial section reconstruction serves as a direct measure of injury severity [53, 54]. Several studies have used the loss of cortical and hippocampal tissue as criteria for defining TBI severity. Although definitions vary across studies, mild TBI is generally characterized by absent or confined tissue loss to the cortical layer; moderate TBI is associated with substantial cortical loss with little to no overt hippocampal involvement; and severe TBI is typically defined by extensive hippocampal lesions in addition to cortical tissue loss [42, 44, 55].

3.4 Neuronal Activity Analysis

1. Import image stacks with appropriate software such as ImageJ.
2. Inspect the entire video. If necessary, correct and align lateral movement (X–Y direction) using the StackReg plugin in ImageJ and discard any images where the drift cannot be corrected by this method [56].
3. For single-cell fluorescence analysis, outline individual GCaMP-expressing cells as multiple regions of interest (ROIs).
4. Obtain $\Delta F/F$ wave, expressed as $\Delta F/F = (F–F_0)/F_0$, where F_0 is the background fluorescence, measured as the means of two sites that are just around the cell and contain non-neuronal structures [29, 57].
5. Neuronal activity can be measured as both the mean integrated fluorescence and the ratio of active neurons [58–60]. To measure the integrated fluorescence, apply a threshold of approximately 20% $\Delta F/F$ to minimize noise, and calculate the area under the curve (AUC) of the peaks in each trace using commercial software such as OriginLab. Automated analysis scripts are also available for this purpose [61, 62]. Active neurons are defined as those that have fluorescence intensity above the threshold at least once. Calculate the ratio of active neurons by dividing the number of active neurons by the total number of neurons in the specific imaging field.

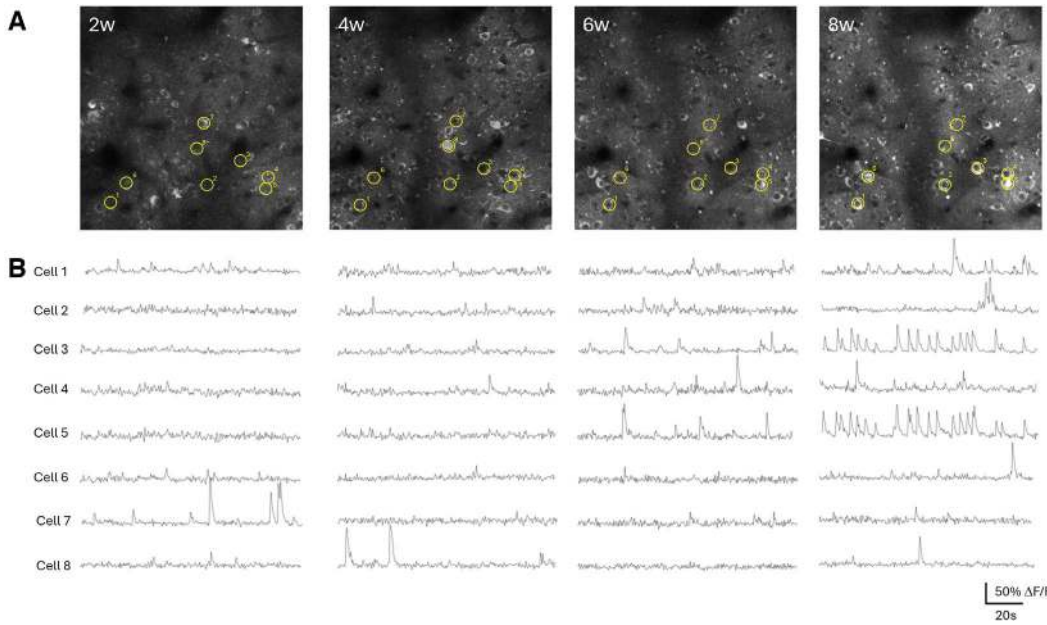


Fig. 2 Two-photon images of the same cortical regions received CCI at 2, 4, 6, and 8 weeks after injury. (**A**) Maximal projections of the same regions of cortical layer II/III GCaMP6s-expressing neurons at different time points after injury. (**B**) Traces of calcium transients of the same population of neurons at different time points after injury

4 Representative Results

This protocol enables monitoring the longitudinal changes in neuronal activity in the same cortical regions after TBI (*see* **Note 1**). Example images from the same FOV in the somatosensory cortex at 2, 4, 6, and 8 weeks after CCI are shown in Fig. 2A. The calcium transients of the same population of neurons were extracted for each imaging session (Fig. 2B).

The alterations in neuronal activity after TBI depend on the location and type of injury. In a mild TBI model, both integrated fluorescence and ratio of active neurons in layer II/III somatosensory cortical neurons were significantly higher than those of sham mice two months post-injury, indicating an increase in spontaneous neuronal activity (Fig. 3A, B). Following repeated closed head injuries, enhanced calcium transients were observed in motor cortical neurons two days after injury [63]. Another study noted a reduction in the number of active neurons in the motor cortex up to 2 weeks after moderate CCI, although the firing frequency of those active neurons was increased [64].

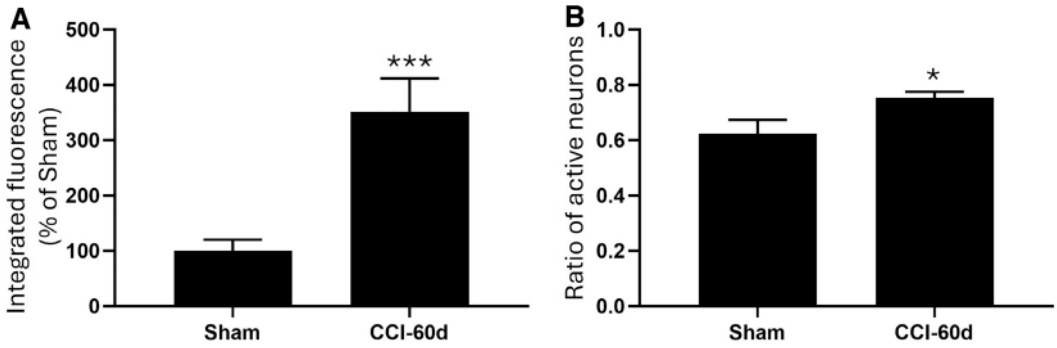


Fig. 3 Changes in spontaneous neuronal activity in layer II/III cortical neurons at 2 months after mild TBI. (**A**) Elevated mean integrated fluorescence of cortical neurons at 2 months after mild TBI compared with sham mice. (**B**) Increase in the ratio of active neurons in injured cortex at 2 months after mild TBI

5 Limitations and Applications of this Protocol

A major limitation of this protocol is the difficulty of obtaining baseline recordings prior to injury, as disrupting the glass window to induce TBI inevitably causes cortical damage. To overcome this issue, recent approaches have replaced glass with flexible polymer-based cranial windows. These polymer materials provide optical access comparable to glass while allowing easier penetration to induce injury after baseline imaging [65]. The window can then be resealed, enabling longitudinal monitoring of neuronal activity before and after injury within the same animal [66].

In addition to in vivo two-photon calcium imaging, the described cranial window preparation allows for visualization of morphological changes in somas, dendrites, spines, glial cells, and vasculature after TBI. By utilizing transgenic mice that express fluorescent protein, researchers can perform longitudinal studies to monitor structural alterations in neuronal architecture over time (41-43). Additionally, vascular changes following TBI can be observed with intravenously injected fluorescent dyes such as Texas red-labeled Dextran (44, 45). This approach provides valuable insights into the progression of neurodegenerative processes and the efficacy of potential therapeutic interventions post-TBI.

6 Troubleshooting

1. Damage to the dura when making a cranial window. When preparing the cranial window, avoid pressing vertically with the dental drill. Instead, try to drill horizontally along the skull surface to prevent undue pressure on the dura.

2. The Cranial window may become cloudy due to inflammation. Administer Carprofen (7.5 mg/kg, i.p.) daily as needed. Also, ensure there are no air bubbles under the coverslip when sealing it on the skull with glue, as this can help maintain window clarity.
3. The stainless-steel bar and/or dental cement may come off several days after surgery. To ensure stability of the cranial window, start by removing the periosteum and connective tissue on the skull with a sterile scalpel, and make sure the skull is dry. Apply a layer of cyanoacrylate glue onto the skull to provide a solid base for the dental cement.

7 Mood/Anxiety-Related Behavioral Test in TBI Mice

Rodent models of TBI often exhibit emotional disturbances resembling anxiety and depression in humans, which are commonly evaluated using standard behavioral paradigms [67, 68] (Fig. 4). For anxiety-like behaviors, TBI rodents frequently display reduced exploration of the central zone in the open field test [69, 70], spend less time in the open arms of the elevated plus maze [71, 72], and avoid the light compartment in the light-dark box [73], all indicative of heightened anxiety. For depression-like phenotypes, animals often show increased immobility in the forced swim and tail suspension tests [72, 74], reflecting behavioral despair, and reduced sucrose

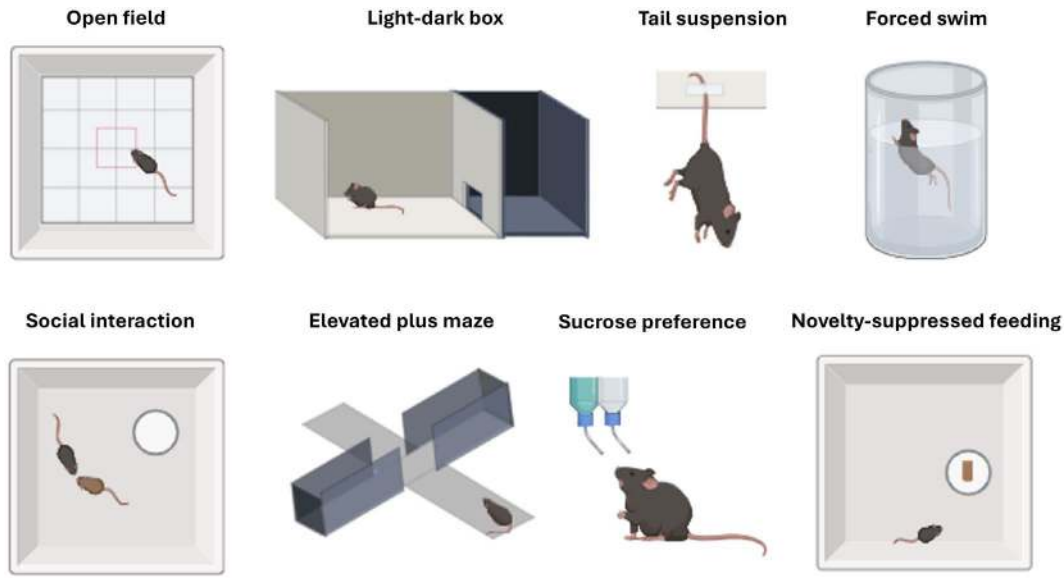


Fig. 4 Suggested behavioral test battery for mood/anxiety-like behaviors. *This schematic figure was created using BioRender.com*

consumption or preference [72], an indicator of anhedonia. Additional measures, such as decreased social interaction [75, 76] and increased latency to feed in the novelty-suppressed feeding test [77, 78], further suggest mood-related dysfunction after injury.

However, not all findings in this area have been consistent. Some studies report reduced anxiety-like behavior in the elevated plus maze or no significant differences in sucrose preference or immobility compared to non-injured animals [55, 79, 80]. These discrepancies are likely due to variations in injury severity, affected brain regions, and the timing of behavioral assessments [81]. Consequently, interpretation of mood- and anxiety-related outcomes after TBI requires careful consideration of these variables.

Importantly, combining behavioral data with in vivo two-photon calcium imaging offers a powerful framework to link observed emotional phenotypes with underlying neuronal activity and circuit-level changes, offering deeper insights into the mechanisms of post-TBI mood disturbances. Such data can then be leveraged to evaluate potential treatment methods.

Notes

1. Although this chapter has focused on cortical neuronal activity following traumatic brain injury using in vivo two-photon calcium imaging, a broad literature on other post-traumatic neurological outcomes is not covered here. Complementary imaging approaches, such as in vivo voltage imaging or miniature endoscopic calcium imaging, can further illuminate neural network dynamics [82–84]. Notably, the use of gradient-index (GRIN) lenses enables optical access to deeper brain regions, such as the hippocampus, amygdala, and nucleus accumbens, which are implicated in anxiety- and mood-related behaviors [85–87]. These structures are otherwise inaccessible through conventional craniotomy windows, but recent technical advancements have improved imaging quality and signal stability in these regions [88]. These studies provide valuable insights for developing therapeutic interventions to mitigate the neurological consequences of traumatic brain injury.

Conflict of Interest

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responsibility of the authors and do not necessarily represent the official views of the Indiana Department of Health. The authors declare that they have no competing interests or conflicts relevant to the content of this chapter.

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Chapter 19

Decoding Depression: Advances in Selectively Bred Rodent Models

Lia Parada Iglesias, Gregers Wegener and Livea Dornela Godoy

Abstract

Depression significantly impacts global health and economics, necessitating good preclinical models to understand its underlying neurobiology and develop effective treatments. This review explores the use of selectively bred rodent models in depression research, focusing on their historical development, behavioral phenotypes, translational relevance, and important methodological details. Key models discussed include Flinders Sensitive Line (FSL) rats, Wistar Kyoto (WKY) rats, Fawn-Hooded (FH) rats, congenitally learned helpless (cLH) rats, and high/low stress-reactivity mice. These models exhibit distinctive traits such as anhedonia, cognitive deficits, and stress-induced behaviors, offering insights into the pathophysiology of depression and related comorbidities.

Keywords Selectively bred animal models, Depression, Neurobiology

1 Introduction

Depression is recognized as a major contributor to the global burden of disease, exerting significant influence on both public health and economic systems. The World Health Organization (WHO) estimates that over 300 million individuals are affected by depression worldwide [1, 2], with particularly high prevalence rates observed among women, adolescents, and populations residing in low- and middle-income countries [3]. The disorder is characterized by substantial direct and indirect costs, including healthcare expenses, loss of productivity, and the economic impact of absenteeism. Depression's chronic and recurrent nature often leads to significant impairments in social and occupational functioning, thereby exacerbating its societal impact [4].

The interplay between depression and comorbid chronic medical conditions, such as cardiovascular disease and diabetes, further complicates the clinical management of these patients and increases the overall risk of mortality [5]. Depression is also a significant

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contributor to disability-adjusted life years (DALYs) worldwide [1], particularly in regions like South Asia and Brazil, where the burden has been steadily increasing [6, 7]. Despite its high prevalence and profound impact, depression remains underdiagnosed and undertreated, particularly in resource-constrained settings where mental health services are often insufficient [1].

Given the complexity of depression's etiology and its impact on various physiological systems, the study of this disorder in preclinical models is of paramount importance. Preclinical models, particularly those using animals, offer critical insights into the underlying neurobiological mechanisms of depression, which are difficult to study directly in humans due to ethical and practical constraints. In the evaluation of animal models of depression, the concepts of face, construct, and predictive validity are central to determining their relevance and applicability to human depression. A comprehensive coverage of the area can be found elsewhere [8–10].

Face validity refers to the extent to which an animal model exhibits behaviors that are phenomenologically similar to the symptoms of human depression. For example, the Chronic Mild Stress (CMS) model demonstrates high face validity as it induces anhedonia, a core symptom of depression, by decreasing the animal's consumption of a sweet solution, which parallels the loss of interest or pleasure seen in human depression [11]. Construct validity assesses whether the model accurately reflects the theoretical mechanisms underlying depression. This validity is crucial for understanding the pathophysiology of the disorder. Models that manipulate genetic, neurochemical, or environmental factors implicated in human depression, such as alterations in the serotonergic system or chronic stress exposure, are considered to have high construct validity. For instance, the Flinders Sensitive Line (FSL) rats, which display increased sensitivity to cholinergic agonists, are considered to have good construct validity because they mimic the cholinergic model of depression, where dysregulation of the cholinergic system is hypothesized to contribute to the disorder [12, 13]. Predictive validity is concerned with how well the model responds to treatments that are effective in humans. A model with high predictive validity would reliably show improvement in depressive-like behaviors following the administration of standard antidepressant drugs. The CMS model, for instance, exhibits good predictive validity as chronic treatment with a variety of antidepressants reverses the behavioral changes induced by mild stress, mirroring the therapeutic effects observed in human patients [11].

Importantly, no single model can perfectly replicate human depression. The combination of these validity criteria helps in selecting and refining animal models that can contribute to the understanding of depression and aid in the development of effective treatments. An updated overview of rodent tests of depression and anxiety, focusing on construct validity and translational relevance, can be found elsewhere [14].

Individuals exhibiting responses at the extreme of the response curve can be selectively bred for bidirectional trait phenotypes over multiple generations. Thus, after later generations of these inbred lines, the heritability characteristics of a specific trait can be evaluated, so underlying genetic variations, endophenotypes [15], such as behavioral phenotypes and neurobiological features associated with depressive disorder, can be examined. In this chapter, we will explore

Table 1
Overview of the models

Strain	Controls	Strengths	Limitations	Main comorbidities
Flinders Sensitive Line (FSL)	Sprague-Dawley, Flinders Resistant Line (FRL)	Genetic model with robust depressive-like phenotypes, responsive to a range of treatments	Inconsistent anhedonia evidence	Cognitive deficits, stress sensitivity
Wistar Kyoto Rats (WKY)	Wistar rats, spontaneously hypertensive rats (SHR)	Resistant to traditional antidepressants, valid for studying anhedonia and chronic treatment effects	Phenotypic and genetic variability across substrains	Anxiety, cardiovascular function
Fawn-Hooded Rats (FH)	Fisher 344, Wistar, Sprague-Dawley, Long-Evans	Monoaminergic dysfunction, alcoholism model, high corticosterone release	Variability between substrains, challenges with control selection	Alcoholism, binge drinking
Congenital learned helplessness (cLH)	Congenitally not learned helpless (cNLH), Sprague-Dawley	Models treatment-resistant depression and reward-processing deficits	Stress effects not always coupled with neural plasticity changes	Anxiety, eating behavior alterations
Swim low rats (SwLo)	Swim high rats (SwHi), Sprague-Dawley	Useful for studying pharmacological responses and immobility-related phenotypes	No basal hedonic changes; limited application outside stress contexts	Epilepsy, drug abuse, ethanol abuse
High-stress reactivity mice (HR)	Low-stress reactivity mice (LR), CD1	Hyper-reactive HPA axis, valuable for stress-related depression research	Investigation primarily limited to stress-related contexts	Cognitive deficits, psychotic behavior

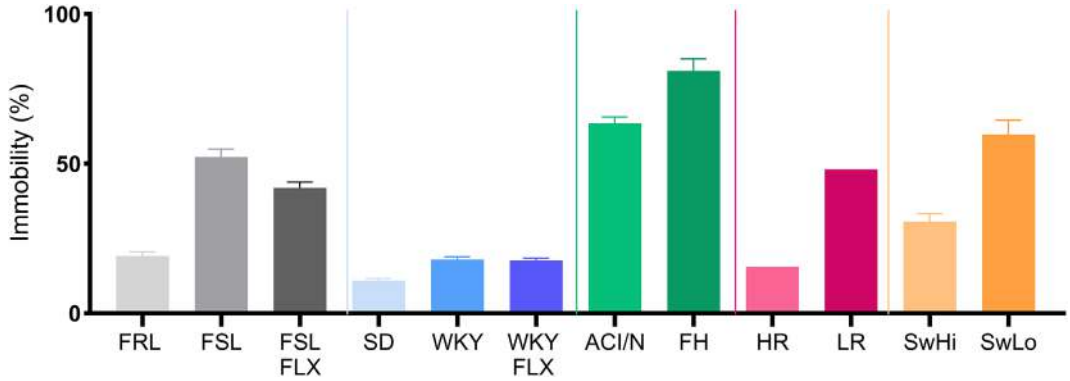


Fig. 1 Animal strain behavior in the Forced Swim Test (FST). Mean and SEM were reversely extracted from [16–20] using Automeris except for HR/LR (median provided in the article) and SwHi/SwLo (SD instead of SEM). Data was transformed into percentage for comparative purposes. Flinders Resistant Line (FRL), Flinders Sensitive Line (FSL), Sprague-Dawley (SD), Wistar Kyoto (WKY), Fawn-Hooded FH/Wjd (FH), high reactivity (HR), Low-reactivity (LR). FSL FLX were animals treated once with Fluoxetine (10 mg/kg), WKY FLX were animals treated three times in the 24 h preceding the FST with Fluoxetine (10 mg/kg)

rodent strains developed to study depression, their developmental history, hallmarks, strengths, and translational relevance. While we describe how the models have historically been developed, it is highly recommended to acquire the models by contacting the contacts mentioned in the text. A summary of the model characteristics can be found in Table 1 together with typical model characteristics in the Porsolt test in Fig. 1.

2 Materials

- **Animal models:** selectively bred strains, including Flinders Sensitive Line (FSL) rats and their control Flinders Resistant Line (FRL); Wistar Kyoto (WKY) rats; Fawn-Hooded (FH) rats; congenitally learned helpless (cLH) rats and their non-helpless control (cNLH); High stress-reactivity (HR); and Low stress-reactivity (LR) mice.
- **Reagents:** diisopropyl fluorophosphate (DFP) for cholinergic sensitivity screening, and other standard reagents (e.g., sucrose solution for anhedonia tests) as required.
- **Equipment:** apparatus for behavioral testing (e.g., cylinders for forced swim test, shuttle boxes for learned helplessness tests, two-bottle preference test setup for anhedonia, maze and open-field equipment for cognitive and anxiety assessments).
- **Laboratory facilities:** standard rodent housing, breeding facilities, and biosafety equipment for handling animals and chemicals.

3 Methods

3.1 Flinders Sensitive Line (FSL) Rats

Brief historical background. The development of the Flinders strain was initially driven by the objective of creating an animal model resistant to irreversible anticholinesterase agents, specifically diisopropyl fluorophosphate (DFP) [21, 22]. However, the research unexpectedly yielded a strain progressively more sensitive to this agent—the Flinders Sensitive Line (FSL). Paradoxically, the line that failed to exhibit the desired resistance, and seemed to resemble the original outbred strain, ended up being called the Flinders Resistant Line (FRL). Together with the high sensitivity to DFP, FSL rats presented enhanced central expression of muscarinic receptors [23] and were more susceptible to the effects of other cholinergic drugs [24]. Some years before the establishment of the FSL, Janowsky had described a high vulnerability to cholinergic stimulation in depressive patients [25]. Therefore, in 1984, the similarities between FSL rats and depressive patients were recognized [26]. In the subsequent years, Overstreet and colleagues further characterized FSL animals and described a variety of phenotypic peculiarities that fit with an animal model of depression: high immobility in the forced swim test (FST) [27], alterations in REM sleep [28], and stress-induced anhedonia [29]. Colonies of FSL rats have since been established in multiple countries (Australia, Canada, the United States, Denmark, Israel, and South Africa). Despite all of these colonies presenting the core FSL characteristics, other specific phenotypic peculiarities may have appeared over the years in specific colonies, which may account for controversies in the literature (e.g., some inconsistent findings across labs).

Development of the model

The outbred strain used for the development of FSL was Sprague-Dawley (Hawthorn Park Research Australia). For each generation, animals were sorted as sensitive or resistant to DFP (1 mg/kg, i.m.) considering three different parameters: core body temperature (central cholinergic action), drinking behavior (central and peripheral action), and body weight (non-specific effect) [21]. Breeding within the sensitive and resistant subpopulations provided a significantly DFP-sensitive strain after the 6th generation [21]. The strain obtained from the resistant subpopulation did not differ from a random-bred strain [21].

Procedure

1. Acquire the FSL/FRL from existing colonies (Table 2).
2. Develop new "FSL/FRL"-like pairs
   a. Begin with an outbred Sprague-Dawley rat colony. Administer diisopropyl fluorophosphate (DFP) at 1 mg/kg

Table 2
Model sourcing

Model/strain	Abbreviation(s)	Designated control strain(s)	Sourcing type	Commercial vendor(s) & strain code(s)	Primary academic/ repository contact(s)	Repository status	Critical sourcing & experimental considerations
Flinders Sensitive Line rat	FSL	Flinders Resistant Line (FRL); Sprague-Dawley	Academic only	N/A	**Denmark:** Gregers Wegener, Aarhus University. **South Africa:** Brian Harvey, North-West University. **Israel:** Gal Yadid, Bar-Ilan University	Not listed in RRRC	**Phenotypic drift is a major factor:** due to decades of maintenance in isolated academic colonies, minor but significant phenotypic differences (e.g., in anxiety) have emerged. **In-house validation of core phenotypes (high FST immobility, DFP sensitivity) is mandatory** upon acquiring animals from any source. Anhedonic phenotype is inconsistent and often requires a stressor to be unmasked

Wistar Kyoto rat	WKY	Wistar; spontaneously hypertensive rat (SHR)	Commercial	**Charles River:** WKY/NCrl (Code: 008) **Inotiv:** WKY/NHsd (Code: 023) **Janvier Labs:** WKY/KyoRj **Taconic:** NTac:WKY (live production has ceased)	N/A	Not listed in RRRC	**Profound substrain divergence:** WKY substrains from different vendors are genetically and behaviorally distinct and must be treated as separate models. **WKY/NCrl (Charles River)** exhibits an ADHD-like inattentive phenotype, a potential confound for cognitive studies. **WKY/NHsd (Inotiv)** does not show this deficit and is considered a better behavioral control. Sourcing must be consistent from a single vendor and substrain, and full nomenclature must be reported in publications

(continued)

Table 2
(continued)

Model/strain	Abbreviation(s)	Designated control strain(s)	Sourcing type	Commercial vendor(s) & strain code(s)	Primary academic/ repository contact(s)	Repository status	Critical sourcing & experimental considerations
Fawn-Hooded rat	FH	Wistar; Sprague-Dawley; FHL	Limited commercial; academic	**Janvier Labs:** RjIbm(m):FH (descended from the FH/Wjd line)	**USA**: FH/Har: University of Iowa, Animal Shipping Coordinator **Netherlands**: FHH/FHL: Erasmus University Research Office **Japan**: FH/HamSlc: Tezukayama University	Not listed in RRRC	**Substrains differ significantly:** the commercially available **FH/Wjd** line is known for high alcohol preference.Other academic substrains (e.g., FH/Har) have lower alcohol intake. **Inherent bleeding disorder:** all FH lines carry a platelet storage pool deficiency (due to the r/r genotype) that causes impaired coagulation.21 This is a critical comorbidity that requires refined surgical and handling protocols to prevent animal loss

Congenitally learned helpless rat	cLH	Congenitally not learned helpless (cNLH); Sprague-Dawley	Academic only	N/A	**Germany**: Barbara Vollmayr, Central Institute of Mental Health (CIMH), Mannheim, Germany	Not listed in RRRC	**Exclusive academic source:** this model is only available via collaboration with the maintaining laboratory. **Profound treatment-resistant phenotype:** represents an extreme endophenotype of depression. Good for studying refractory depression, but less representative of the general condition. Cognitive deficits often emerge only after stress exposure.

(continued)

Table 2
(continued)

Model/strain	Abbreviation(s)	Designated control strain(s)	Sourcing type	Commercial vendor(s) & strain code(s)	Primary academic/ repository contact(s)	Repository status	Critical sourcing & experimental considerations
Swim low-active/ high-active rat	SwLo / SwHi; SUS (for SwLo)	Sprague-Dawley (random-bred)	N/A	N/A	N/A	Not listed in RRRC	**Model of Coping Style, Not General Depression:** these lines were selected specifically for passive (SwLo) vs. active (SwHi) coping in the FST. Good for studying the neurobiology of stress resilience and vulnerability. No basal differences in anhedonia are observed.
High/low stress-reactivity mouse	HR / LR	CD-1 (outbred stock); intermediate-reactivity (IR) mice	Academic only	N/A	**Germany:** Prof. Chadi Touma, Osnabrück University, Germany	Not listed in IMSR	**MOUSE model**. Bred from a genetically heterogeneous outbred stock (CD-1) to model human diversity.

intramuscularly to all rats in each generation and measure **(a)** core body temperature, **(b)** drinking behavior, and **(c)** body weight changes.

b. Classify each rat as **DFP-sensitive** or **DFP-resistant** based on significant hypothermia, reduced drinking, and weight loss (sensitive rats show greater changes).

c. Selectively breed the DFP-sensitive rats with each other to propagate the **Flinders Sensitive Line (FSL)**. Similarly, breed DFP-non-responsive (resistant) rats to maintain a comparison line (**FRL**).

d. Continue this breeding and testing process for multiple generations (a robust FSL phenotype typically emerged by ~6 generations).

3. Confirm the FSL phenotype in new generations by retesting DFP sensitivity (exaggerated hypothermic response) and performing a **forced swim test (FST)** to verify high immobility.
4. Optionally assess anhedonia in FSL rats (e.g., sucrose preference test), especially after exposure to mild stressors (FSL rats often require stress to reveal reduced reward sensitivity; *see* **Key Consideration Note 5**).
5. Maintain a colony of FRL rats under similar conditions to serve as a control group when evaluating behavioral and physiological differences (FSL vs. FRL). *Note:* minor phenotypic differences can occur between different FSL colonies (*see* **Key Consideration Note 1**).

Behavior and neurobiological correlates

In FSL rats, the hallmark depressive-like behavior is a remarkably high immobility time in the FST [27]. This phenotype has been consistently reported in the literature across different colonies and generations of FSL rats. Furthermore, several environmental and pharmacological interventions are able to decrease the immobility time of FSL rats, such as physical exercise [30], fast-acting antidepressants like ketamine [16, 31, 32], SSRIs [33, 34], multimodal antidepressants [35], tricyclic antidepressants [36, 37], acetyl-L-carnitine supplementation [38], or electroconvulsive therapy [39, 40].

In contrast to the clear immobility phenotype, evidence for anhedonia in FSL rats is less cohesive. FSL rats do not appear to exhibit a baseline anhedonic phenotype; [29, 41] however, under certain conditions (e.g., exposure to specific types of stress [29, 42]), FSL rats show stronger anhedonic responses compared to their control counterparts. Overall, FSL rats present an enhanced susceptibility to adverse environmental interventions such as early-life stress [43], chronic mild stress [42], high-fat diet [44, 45], or maternal separation [46, 47]. In addition, FSL rats exhibit impairments in cognitive tasks, including operant delayed matching-to-position/visual

discrimination [48], radial arm maze performance [48], novel object recognition [37, 49], and olfactory discrimination [50], indicating a robust cognitive deficit associated with the line.

Although FSL is considered an animal model of depression, these rats also display abnormalities in other behavioral domains, making them useful for studying certain comorbidities. For instance, FSL rats showed subsensitivity to cocaine-seeking behavior [51], 3,4-methylenedioxymethamphetamine (MDMA, "ecstasy") [52], and nicotine [53]. However, other studies reported no difference in rewarding electrical brain-stimulation thresholds [54] or even blunted responses to cocaine with reduced drug intake [55]. Anxiety is another important comorbidity of depression; however, most studies suggest that FSL rats do not display alterations in the elevated plus maze [56–59] or even show lower anxiety-like levels than controls [41, 45]. Similarly, FSL rats showed impairments in processing fear memory in both active [60] and passive avoidance tasks [61]. Other findings suggest that FSL rats have intact fear memory acquisition but profound deficits in the extinction of fear memory [62], a key characteristic relevant to PTSD. In turn, FSL rats appear to display an exacerbated susceptibility to certain environmental models of PTSD [63, 64].

Regarding the neurobiology of depression, FSL rats display many remarkable features that make them suitable for studying specific aspects of this disorder. In addition to the aforementioned cholinergic hypersensitivity, FSL rats have alterations in REM sleep [28, 65, 66] that resemble those observed in patients: more REM sleep, shorter REM latency, and a faster REM–REM cycle length. Neuroendocrine abnormalities are also well described in both depression and in FSL rats: for example, FSL rats exhibit higher corticosterone release upon stress [62], decreased ACTH [67, 68], decreased CRF [68], and increased GR and FKBP51 expression in the hippocampus [62]. Furthermore, major serotonergic alterations have been described in FSL rats, including decreased 5-HT_1A [61] and 5-HT_2A receptors [69], increased 5-HT_1B receptors [70, 71], elevated 5-HT transporter expression [72] and 5-HT levels [56, 73, 74], and reduced serotonin turnover [56]. In addition to the serotonergic system, differences have also been observed in glutamatergic neurotransmission [32, 75, 76], dopaminergic neurotransmission [70, 77], and neuropeptide Y levels [78, 79]. In the hippocampus, FSL rats present clear atrophy [16], impaired LTP [43, 80], and disrupted excitatory neurotransmission [81] with no reduction in neurogenesis [82, 83]. These hippocampal alterations may account for the depressive-like behaviors and cognitive and fear-extinction deficits observed in FSL rats. Moreover, alterations in the mesocorticolimbic pathway [70,74] and aberrant connectivity among its subregions [84] have been related to the subsensitivity to drugs seen in FSL rats. Finally, FSL rats have hyperactivation of the amygdala and other limbic regions [85, 86], paralleling findings in depressed patients, and display

changes in growth factor signaling [87] and immune/inflammatory pathways [88, 89] that are relevant to depression pathophysiology.

3.2 Wistar Kyoto (WKY) Rats

Brief historical background. The history of the Wistar Kyoto rat (WKY) is fundamentally connected to the development of the Spontaneously Hypertensive Rat (SHR). The SHR was derived from a colony of Wistar rats at Kyoto University's School of Medicine [86] and later distributed to other centers such as the NIH. However, the use of SHR for hypertension research was initially limited by the lack of an appropriate normotensive control strain with the same genetic background [87, 89]. At that time, it was thought that an ideal control strain could be obtained by inbreeding within the same colony of Wistar rats used to generate SHR (i.e., the Kyoto colony) [87, 88]. Thus, instead of a congenic development of strains, the SHR and its "normotensive control" (the WKY) were produced more than ten years apart [87, 88]. In addition, distribution of WKY rats to research laboratories began before the strain was fully inbred [87, 88, 90]. For instance, the original NIH WKY colony was obtained from Kyoto in 1971 (WKY/N) and then distributed to different vendors: Charles River Laboratories in 1971 (WKY/NCrl), Taconic Farms in 1974 (WKY outbred), and Harlan Laboratories in 1982 (WKY/NHsd) [90]. Considering the atypical course of development and distribution of WKY rats, it is not surprising that early reports pointed to high variability in growth rate and blood pressure among different WKY colonies [88].

Moreover, the notion of WKY as a simple control strain was quickly challenged by W. P. Paré, who observed that WKY rats were highly vulnerable to stress-induced gastric ulceration [91]. Later, in a seminal work [92], Paré summarized evidence of aberrant emotional processing in WKY rats, making the case for repurposing this strain as a model of depression. Key observations included hypoactivity in the open field, high vulnerability to learned helplessness and passive avoidance deficits, high immobility in the FST, and elevated ACTH levels after stress [92]. Paré also demonstrated differences in stress responses among WKY rats from different suppliers [86]. After decades of breeding, there are now at least ten substrains of WKY rats, many of which show differences in stress-related behaviors and genetics [89]. While this substrain divergence poses a challenge, it has also been exploited: researchers selectively bred WKY rats for extremes of FST immobility, yielding new lines with **high immobility (WMI)** and **low immobility (WLI)** [93].

Procedure

1. Obtain **Wistar Kyoto (WKY)** rats from a consistent source or substrain (Table 2, significant behavioral and physiological variability exists between WKY substrains—*see* **Key Consideration Note 2**).

2. Develop new **"WKY"**-like pairs

    a. Evaluate baseline depressive-like behavior; for example, conduct an FST. WKY rats typically exhibit very high immobility (indicative of passive coping).

    b. Test antidepressant responses: acute administration of a standard antidepressant (e.g., imipramine or fluoxetine) usually does **not** reduce WKY immobility. However, chronic antidepressant treatment or a fast-acting intervention (e.g., ketamine or electroconvulsive therapy) can significantly decrease immobility.

    c. Assess additional behaviors to characterize the depressive phenotype: WKY rats often show reduced motivation (e.g., low sucrose intake, suggesting anhedonia) and cognitive deficits (e.g., poor performance in the Morris water maze).

    d. Include both male and female WKY rats in studies when possible (female WKY typically display even more pronounced depressive-like behaviors than males—*see* **Key Consideration Note 4**).

Behavior and neurobiological correlates

WKY rats can present relatively high immobility in the FST [94], which is not reduced by acute administration of tricyclic antidepressants [95] or SSRIs [96, 97]. However, chronic treatment with these classic antidepressants [17, 95], as well as acute administration of a fast-acting antidepressant (ketamine) [40, 98] or electroconvulsive therapy [99], significantly reduces immobility in WKY rats. In addition to their performance in the FST, WKY rats display a general deficit in motivated behaviors that suggests an anhedonic trait [57, 100]. Consistent with a depressive phenotype, WKY rats have an enhanced susceptibility to the learned helplessness model [92] and show heightened responses to other stressors such as restraint stress [101] and chronic mild stress [101, 102]. Moreover, WKY rats perform poorly in the Morris water maze and novel object recognition tests [103, 104], indicating a cognitive deficit.

At the same time, WKY rats exhibit enhanced avoidance and fear learning [92, 105, 106] and deficits in fear extinction [105]. Additionally, WKY rats consistently show elevated anxiety-like behaviors in the elevated plus maze [107, 108], open field [109], and novelty-induced hypophagia tests [109].

Similar to what is observed in many depressed patients, WKY rats have increased REM sleep [110, 111] and hypothalamic–pituitary–adrenal (HPA) axis disturbances. Specifically, WKY rats exhibit an enhanced release of ACTH and corticosterone, both under basal conditions and in response to stress [92, 112, 113], along with a deficient glucocorticoid negative feedback [112]. Moreover, WKY

rats have lower levels of serotonin in mesocorticolimbic structures [114, 115] and decreased 5-HT_1A receptor sensitivity [116]. They also present alterations in other neurotransmitter systems, including noradrenaline, dopamine, GABA, and glutamate [for a review, see [117]]. Major structural and neuroplastic changes have been found in the hippocampus of WKY rats, such as hippocampal atrophy [118], impaired neurogenesis [119], and impaired LTP [117].

3.3 Fawn-Hooded (FH) Rats

Brief historical background. The exact origin of Fawn-Hooded rats (FH) is not extensively documented, but some authors have noted that FH rats were originally developed from cross-breeding German Brown, Lister (perhaps "Lashley") Albino, and Long-Evans rats at the University of Michigan [120, 121]. FH rats were initially characterized as animals with a mild bleeding disorder and reduced platelet serotonin content [121]. Over time, various peculiarities of FH rats were reported: hemostatic alterations [122], attenuated hypothermic responses to serotonergic agents [123], high blood pressure and proteinuria [124], increased immobility in the FST [125], high voluntary ethanol consumption [125], hypercortisolemia [126], and elevated saccharin consumption [127]. However, some of these observations were not replicable across different FH substrains [128]. From the original colony, different groups established their own inbred substrains [120, 129]. These include FH/Wjd, developed by W. Jean Dodds at the New York Department of Health; FH/Har (also known as **IR**—Iowa Reactive), developed by Gordon Harrington at the University of Iowa (later transferred to the National Cancer Institute); and FHH/FHL, developed by Professor Provoost at Erasmus University, who selectively bred FH rats for divergent blood pressures, yielding **FHH/Eur** (high blood pressure) and **FHL/Eur** (low blood pressure) lines. When FH/Wjd rats were compared to FH/Har rats, the former showed higher alcohol/saccharin intake and higher FST immobility than the latter [120]. Similarly, a study examining all four strains found that none of FHL, FHH, or FH/Har lines had ethanol consumption levels comparable to FH/Wjd rats [130]. However, FHL, FHH, and FH/Wjd rats exhibited similar performance in the FST [130].

Development of the model. (Not formally a selective breeding for depression—multiple substrains arose via different selection criteria, as described above.)

Procedure

1. Acquire the **Fawn-Hooded (FH)** from existing colonies (Table 2). **Fawn-Hooded (FH)** rat substrain appropriate for the study objectives. (Different FH substrains, such as FH/Wjd vs. FH/Har, vary in traits like alcohol intake and FST immobility—*see* **Key Consideration Note Note 3**.)

2. Develop new **"Fawn-Hooded (FH)"** -like pairs

    a. Measure baseline saccharin or sucrose intake to gauge hedonic state. FH rats characteristically consume high amounts of sweet solutions (this was initially interpreted as an anhedonic trait of the model).

    b. Perform an FST to assess depressive-like behavior. FH rats generally exhibit elevated immobility, although their vigorous struggling can sometimes complicate interpretation.

    c. If studying comorbid addiction or reward, utilize the FH strain's propensity for high voluntary alcohol consumption. For example, offer a two-bottle choice to quantify ethanol preference, and test pharmacological interventions (e.g., naltrexone) to reduce drinking.

    d. Monitor for any physiological anomalies. (FH rats have known health peculiarities, such as a bleeding tendency and high platelet serotonin uptake, which may affect experimental outcomes—*see* **Key Consideration Note 7**.)

Behavior and neurobiological correlates

Two main features led to FH rats being considered an animal model of depression: high intake of sweet solutions (interpreted as an anhedonia-relevant trait, given the altered hedonic processing) [127] and high immobility in the FST [125, 131, 132]. However, some have argued that this strain displays unusually high levels of active struggling in the FST [131], which may confound conclusions drawn from this test. Nevertheless, depressive-like behaviors in FH rats can be reduced by antidepressant treatment such as fluoxetine [133].

In addition to the depression-like phenotype, the defining characteristic of FH rats is their high voluntary consumption of ethanol [125]. This excessive alcohol intake can be attenuated by interventions like ibogaine [134], naltrexone [135], MDMA [136], or a CB1 receptor antagonist [137]. Intriguingly, despite their high ethanol consumption, FH rats have poor ethanol discrimination in operant tasks [138]. They do, however, display normal conditioned place preference responses to certain drugs like LSD [139].

Other behavioral characteristics of FH rats include higher anxiety-like behavior in the elevated plus maze [140], low social interaction and aggressiveness [141], a deficient response in contextual fear conditioning [106, 142], and higher acoustic startle with lower prepulse inhibition [143].

Regarding depression-relevant neurobiology, FH rats present basal hypercortisolemia [126] and higher corticosterone release after FST exposure [132]. Treatment with tricyclic antidepressants (imipramine, clomipramine) or monoamine oxidase type A inhibitors can prevent some depressive behaviors in FH rats, indicating pharmacological validity. Neurochemically, FH rats were noted to

have differences in brain monoamine levels; for instance, early reports highlighted their reduced platelet and brain serotonin storage [121], which aligns with certain endophenotypes observed in depression.

3.4 Congenitally Learned Helpless (cLH) Rats

Brief historical background. The cLH strain (also known as "therapy-refractory" or **congenitally helpless** rats) was initially generated in the United States and later re-derived in Germany. In 1990, researchers began a breeding program to create helpless lines of rats from a Harlan Sprague-Dawley outbred stock at the State University of New York (SUNY). The goal was to achieve a higher yield of animals exhibiting learned helplessness after exposure to inescapable shocks. Helplessness-prone rats were selectively bred (and, as a control, rats that did not develop helplessness were bred separately). After ~20 generations, this effort produced a **congenitally learned helpless (cLH)** line—rats that exhibit a helpless phenotype **without any exposure to uncontrollable shock**—and a **congenitally non-learned helpless (cNLH)** line that is resistant to the effects of inescapable shock [144].

Development of the model

Once the lines were established, researchers characterized their behavioral and neurobiological features. For example, cLH rats show reduced lever-pressing for sucrose, which could indicate anhedonia and/or reduced behavioral persistence [145]. Following an acute inescapable footshock stress challenge, cLH rats demonstrate a marked decrease in sweetened condensed milk intake and a diminished pleasure-attenuated startle response compared to cNLH controls [146]. Providing environmental enrichment to cLH rats in youth improved social behavior and modestly reduced helpless behavior, but it did not prevent the stress-induced reduction in sweetened milk consumption—suggesting a profound, hard-wired change in the reward system [147]. A high dose of a monoamine oxidase inhibitor was able to attenuate helpless behavior in cLH rats; however, full reversal of helplessness required both the antidepressant treatment and repeated testing (i.e., a learning effect was needed) [148]. Ventromedial prefrontal cortex deep brain stimulation (DBS) failed to restore sucrose consumption or reduce helpless behavior or FST immobility in cLH rats (even though the same DBS protocol was effective in FSL rats), underscoring the pronounced treatment resistance of the cLH strain [149].

Procedure

1. Acquire the cLH from existing colonies (Table 1).
2. Develop new "cLH"-like animals
    a. Begin with an outbred Sprague-Dawley rat population. Expose rats to **inescapable footshocks** (e.g., shuttle box sessions) to induce learned helplessness.

b. Identify and separate the rats that **fail to escape** (helpless) from those that **successfully escape** or avoid shocks (non-helpless).

c. Establish a **congenitally learned helpless (cLH)** line by breeding the helpless rats together. In parallel, breed the non-helpless rats to form a resilient control line (**cNLH**).

d. Repeat the selection process over successive generations. By ~20 generations, the cLH rats will exhibit a stable helpless phenotype *even without any shock exposure* (*see* **Key Consideration Notes 6**).

e. Verify the cLH phenotype in naïve offspring by conducting a standard learned helplessness test (e.g., exposure to escapable shocks). cLH rats will show significantly impaired escape performance compared to controls.

f. Assess reward-related behavior to confirm anhedonia and motivational deficits. For instance, cLH rats typically consume less of a sweet solution following stress and have blunted pleasure-attenuated startle responses.

g. Recognize that cLH rats tend to be **highly treatment-resistant**. Even intensive interventions (such as high-dose antidepressants or deep-brain stimulation) produce limited reversal of their helpless behavior.

Behavior and neurobiological correlates

In cLH rats, the major behavioral abnormality is an impaired perception of reward [146]. When evaluating reward processing, cLH rats are more sensitive to negative feedback: for example, they display a greater depressive-like response to a downshift in reward size compared to Sprague-Dawley controls [150]. This behavior correlates with risk-averse decision-making and suggests abnormal habenula function in processing rewarding vs. aversive events. Indices of anxiety-like behavior are also elevated in the cLH strain and correlate with helpless behavior [148]. Interestingly, there are no differences in general locomotor activity between cLH and control rats in an open field, although cNLH rats show slight hyperactivity in the initial phase of open field testing [145]. In cognitive testing, cLH rats exhibit no baseline memory deficits in the Morris water maze [145], but spatial memory becomes compromised after footshock stress [151]. Working memory appears intact; however, cLH rats show a short retention interval impairment, which could reflect a stress-related disruption of attention [152].

More recently, resting-state high-field magnetic resonance spectroscopy was used to assess metabolic profiles in the hippocampus and frontal cortex of cLH vs. Sprague-Dawley rats. Subsequent behavioral testing one week later allowed correlations between brain metabolic changes and stress responses. cLH rats showed reduced glutamate

metabolism in the frontal cortex and reduced glutamate and choline levels in the hippocampus [153]. Electrophysiological manipulation of mGlu_5 receptors in hippocampal slices suggests that altered mGlu_5 receptor expression and function in the cLH hippocampus may contribute to the synaptic long-term plasticity changes linked to their depressive-like behavior [154]. In the prelimbic cortex, cLH rats have substantially lower levels of the synaptic marker PSD-95 (both in volume and intensity), whereas cNLH rats had ~25% more PSDs than both cLH and control rats. This reduction in prelimbic synaptic markers in cLH rats is accompanied by reduced numerical density of the AMPA receptor subunit GluA1; both cLH and cNLH strains also show a reduction in GABAergic interneuron density [155]. Additionally, there is a brain-wide downregulation of the GABA transporter GAT-3 in cLH rats compared to cNLH [156].

On postnatal day 14, cLH rats display a dramatic increase in ACTH (adrenocorticotropic hormone) and a blunted corticosterone response to a cold stress, suggesting an altered stress reactivity and HPA axis dysfunction in the cLH strain [157]. Although BDNF levels are only modestly reduced in cLH vs. cNLH [158], cLH rats show no BDNF reduction after stress (unlike other strains). Thus, even when corticosterone levels are elevated in stressed cLH rats, BDNF does not decrease as it normally would—indicating an uncoupling of the corticosterone–BDNF response. This could reflect defective glucocorticoid receptor (GR) function, which has been implicated in the pathogenesis of depression [159]. Collectively, it seems that the cLH strain lacks certain adaptive responses mediated by the HPA axis under stress, responses that are uncoupled from the neuroplastic changes typically observed in depression models.

3.5 Swim High-Active/Low-Active (SwHi/SwLo) Rats

Brief historical background. In 1987, at Emory University School of Medicine, researchers obtained a cohort of Sprague-Dawley rats (84 males and 42 females) and assessed their behavior in the FST. They then initiated selective breeding by mating rats that showed either very low or very high levels of motor activity in the test (approximately 6–9 breeder pairs per extreme group were used). After 18 generations, two rat lines were established: the **Swim Low-Active (SwLo)** line and the **Swim High-Active (SwHi)** line [20]. The SwLo line is sometimes also called the **Swim Test Susceptible (SUS)** line (with SwHi being implicitly the less susceptible). After the year 2000, the SwLo/SwHi model was further explored for comorbidities with epilepsy [160–164], vulnerability to drugs of abuse [160, 165], and alcohol preference—SwHi rats in particular consume large amounts of ethanol voluntarily [166–168].

Development of the model

In each successive generation, rats were tested in the FST and then selectively bred. Inbreeding was minimized by avoiding close relatives as breeding pairs (breeders rarely shared even the same

grandparents). A control line of random-bred rats was maintained in parallel; these control rats underwent periodic FST assessments but were bred without regard to their swim-test scores. A difference in FST activity (immobility vs. struggling) appeared in the first generation of selection and grew larger over subsequent generations. The divergence was considered stable (significantly different from controls) by the 13th generation [20].

Procedure

1. Acquire the SwLo/SwHi from existing colonies (no current contact available).
2. Develop new "SwLo/SwHi"-like pairs
    a. Use a large foundation colony of Sprague-Dawley rats for selective breeding. In each generation, perform a **forced swim test (FST)** on all rats.
    b. Rank rats by FST activity (struggling versus immobility). **Select ~10% with the least activity** (high immobility) to breed the **Swim Low-Active (SwLo)** line, and **~10% with the most vigorous activity** (low immobility/high struggling) to breed the **Swim High-Active (SwHi)** line.
    c. To preserve genetic diversity, avoid sibling matings when establishing each line (prefer breeding pairs that are minimally related).
    d. Continue FST screening and selective breeding each generation. Divergence in swim-test behavior typically becomes significant within a few generations and stabilizes by around the 13th generation (*see* **Key Consideration Note 6**).
    e. Maintain a **random-bred control** group in parallel by breeding unselected rats from the same base population without regard to FST performance.
    f. After the lines are established, characterize their phenotypes: SwLo rats will consistently show greater immobility (depressive-like behavior) than SwHi rats. Check for other differences, such as coping behaviors (for example, SwHi rats often exhibit active behaviors like diving during FST) and responses to chronic stress (SwHi may show stress-induced anhedonia that SwLo do not).

Behavioral phenotype and neurobiological correlates

Besides the expected differences in struggling and immobility during the FST, SwHi rats showed more *diving* behavior than SwLo rats [20]. Under baseline conditions, neither line exhibits differences in sucrose intake, but under chronic unpredictable stress, SwHi rats display an initial increase in sucrose consumption followed by an anhedonic-like drop, whereas SwLo (and non-selected control) rats

show no significant change in sucrose intake. SwHi rats also tend to show increased general food intake relative to SwLo rats.

Beyond depressive-like behaviors, the SwHi/SwLo model has been used to study comorbid conditions. For example, SwLo rats have been found to display greater susceptibility to kindling in an epilepsy model, whereas exercise has antidepressant and anticonvulsant effects preferentially in SwLo (but not SwHi) rats [161]. In addiction research, differential responses to stimulants have been noted: in one study, SwLo rats exhibited more locomotor activation than SwHi rats in response to amphetamine infusions into the nucleus accumbens, suggesting differing sensitivity to dopaminergic drugs [164].

Neurobiologically, early comparisons indicated that SwLo and SwHi rats differ in certain stress-regulatory mechanisms (e.g., noradrenergic and serotonergic receptor function in the brainstem) [169]. However, more extensive profiling of these lines—including genetic, molecular, and electrophysiological analyses—has not been as widely reported as for some other models. The differences observed in FST behavior and stress responses imply underlying divergences in stress reactivity pathways and coping styles between the SwHi and SwLo lines, which could be valuable for studying the biology of resilience versus susceptibility to depression.

3.6 High/Low Stress-Reactivity Mice (HR/LR)

Brief historical background. In a series of studies by Landgraf, Holsboer, and colleagues, outbred CD-1 mice were selectively bred for extremes in stress reactivity as a model for affective disorders. Starting from a population of 200 young adult CD-1 mice (100 males and 100 females, age ~7–8 weeks), three breeding lines were established by bidirectional selection based on the outcome of a "stress reactivity test" involving a brief period (15 min) of confinement as a standardized psychological stressor. The initial experiments and characterization of these lines were led by Dr. Rainer Landgraf's group and later by Dr. Chadi Touma and others. A comprehensive battery of behavioral tests, along with neuroendocrine profiling, was used to validate the selected lines [170].

Development of the model. To ensure sufficient genetic heterogeneity in the founder population, CD-1 outbred mice were chosen as the background. Mice underwent the confinement stress test, after which their stress responses were quantified (likely through measures such as corticosterone levels and behavioral scores). Starting from the results of this test, the researchers established three lines: **High-reactivity (HR), Intermediate-reactivity (IR)**, and **Low-reactivity (LR)**. Only the HR and LR lines were further propagated for the extremes, while IR mice served as an unselected intermediate group.

Assuring robust separation, each generation involved selecting breeders from the highest- and lowest-responding offspring (with the intermediate animals generally excluded from breeding). This process was repeated for multiple generations until clear, heritable differences in stress reactivity were achieved in the HR vs. LR lines

(reports suggest on the order of >10 generations). The mice selected for high vs. low stress reactivity revealed key endophenotypes relevant to major depression and anxiety disorders [171].

Procedure:

1. Acquire the HR/LR from existing colonies (Table 2).
2. Develop new "HR/LR"-like pairs

    a. Start with an outbred strain of mice (e.g., CD-1). Expose all mice to a **15-min confinement stress** as a standardized stress reactivity test.

    b. Measure stress responses immediately after confinement (for example, assay plasma corticosterone levels or observe anxiety-related behaviors).

    c. Rank the mice by stress reactivity. Designate the top responders as the **High-reactivity (HR)** group and the lowest responders as the **Low-reactivity (LR)** group.

    d. Begin selective breeding by mating HR mice with each other and LR mice with each other (exclude intermediate responders from breeding). Ensure a large breeding population to maintain genetic variation.

    e. Continue the selection across generations until the HR and LR lines show clear, consistent differences in stress response (usually within 10–15 generations).

    f. Validate the phenotypes of the established lines: HR mice typically exhibit heightened anxiety-like behavior and cognitive impairments under stress, whereas LR mice tend to be more stress-resilient or even hypo-responsive.

    g. Monitor metabolic and neuroendocrine parameters in each line. (Prior studies found differences in hippocampal energy metabolism gene expression between HR and LR mice.) These physiological endophenotypes underscore the relevance of these lines to depression and anxiety research.

Behavior and neurobiological correlates

HR mice, by selection, exhibit an exaggerated behavioral and endocrine response to stress. For example, HR animals explored more in a novel environment, while LR mice explored less, compared to intermediate responders [19]. Additionally, in behavioral despair tests like the tail suspension test (TST) and FST, LR mice (both males and females) spent more time immobile than HR (and intermediate) mice [19]. Surprisingly, HR mice did not show the expected *reduction* in mobility (they were not simply the inverse of LR in every measure), indicating complex interactions between stress reactivity and behavioral outcomes.

On the neurobiological side, one study reported significant differences in the hippocampus of HR mice related to glucose metabolism and transport, lipid metabolism, and mitochondrial protein synthesis, with no differences in measures of brain insulin signaling or oxidative damage [170]. These metabolic changes suggest mitochondrial dysfunction may play a role in the high-stress reactivity phenotype, linking it to pathophysiological mechanisms relevant to affective disorders.

HR mice also exhibit hippocampus-dependent memory deficits (e.g., in object recognition and spatial memory tasks) along with decreased hippocampal BDNF levels [171]. These cognitive deficits emerge in early adulthood. Moreover, early-life stress can exacerbate memory impairments in HR mice, whereas LR mice show relatively good memory performance in comparison. HR mice also display perseverative behavior in reversal learning tasks (i.e., difficulty with cognitive flexibility, which depends on prefrontal cortex function) and have disrupted latent inhibition (a form of selective attention often used to model certain cognitive aspects of psychiatric conditions) [172]. These findings highlight that the HR line captures not only heightened anxiety-like behavior but also cognitive and neurotrophic alterations reminiscent of those seen in stress-related psychiatric disorders.

Finally, consistent with their selection criteria, HR and LR mice have been used to examine sex differences in stress responses. While both sexes were included in the selection procedure, subsequent analyses revealed nuanced differences [173]. Additionally, the HR vs. LR lines have enabled investigations into gene–environment interactions—for instance, exposing these lines to chronic social stress or developmental stress to see how innate reactivity influences outcomes [174]. The high vs. low stress-reactivity mice thus serve as a valuable complementary model to the rat lines, emphasizing the role of inherent stress response traits in the etiology of depression and anxiety.

4 Gender Aspects

Given the well-documented higher prevalence of depression in women, sex as a biological variable has increasingly been explored in animal models. The complex interaction between sex and depression-related behaviors underscores the necessity of investigating both structural differences and hormonal influences that contribute to these behavioral phenotypes. This approach enabled a deeper understanding of the sex-specific mechanisms underlying depression, which can further expand into the development of more effective therapeutic strategies.

In studies using the Wistar Kyoto (WKY) rat model, sex differences in depressive-like behaviors have been consistently observed, with females displaying exacerbated symptoms compared to males [109, 174]. Female Flinders Sensitive Line (FSL) rats have also displayed increased immobility in the FST compared to Flinders Resistant Line (FRL) rats, an interesting behavioral phenotype that remains consistent throughout the estrous cycle [175]. Moreover, the estrous cycle does not appear to significantly affect the response to ketamine treatment in this strain [176].

Similarly, in studies involving selective breeding of the SwLo and SwHi rat strains, female SwLo rats demonstrated reduced anxiety-like behaviors and increased exploratory behavior in novel environments compared to SwHi females, a difference that was not observed in males under similar conditions [20]. Conversely, HR females exhibited more anxiogenic behaviors than their male counterparts, although both sexes of HR rats showed equally higher levels of immobility in the tail suspension test (TST) and forced swim test (FST), when compared to LR controls [19]. For cLH rats, most papers have pooled together both sexes. Although, in this strain selection, the proportion for learned helplessness congeniality is higher for females (94% compared to 88% for males) [177], a more recent paper has stated there were no significant anatomical differences between females and males [178].

5 Key Consideration Notes

1. **Colony variability (FSL rats):** FSL colonies maintained in different laboratories have developed minor phenotypic differences over time. For example, some colonies report subtle variations in anxiety-like behavior or stress responsivity. Such divergence is likely due to genetic drift or environmental/husbandry differences between colonies. Researchers should verify key traits (like FST immobility and DFP sensitivity) when obtaining FSL rats from a new source.
2. **WKY substrain differences:** there are numerous WKY substrains (e.g., WKY/NCrl, WKY/NHsd, etc.), and they can differ markedly in behavior and physiology. For instance, some substrains have higher baseline immobility or different stress hormone levels than others. It's advisable to use WKY rats from the same source throughout a study and to report the substrain or vendor to enhance reproducibility.
3. **FH substrains and controls:** when working with Fawn-Hooded rats, the choice of substrain and control strain is crucial. FH/Wjd rats, for example, have much higher alcohol intake and saccharin preference than FH/Har rats. Also, some

studies compare FH rats to Wistar controls, while others use Sprague-Dawley or even FHL (low blood pressure line) as controls. These choices can affect outcomes and interpretations.

4. **Sex differences:** many selectively bred models show sex-specific effects. Female WKY rats tend to exhibit more severe depressive-like behavior (e.g., greater immobility) than males, and female FSL rats have been reported to show higher FST immobility and different neurochemical profiles than males in some studies. Conversely, certain outcomes (like stress-induced ulceration in WKY or corticosterone responses) may be more pronounced in males. It's important to include both sexes when possible or, at a minimum, acknowledge which sex is being studied, as this can influence the model's phenotype and its responsiveness to interventions.
5. **Need for stress to reveal phenotypes:** in some models, certain depressive-like phenotypes only emerge under challenge conditions. For instance, FSL rats do not consistently show reduced sucrose intake (anhedonia) unless they are subjected to chronic mild stress or other precipitating factors. Similarly, cLH rats display cognitive deficits primarily after a stress exposure (e.g., memory impairments post-footshock). When using these models, researchers should consider incorporating mild stress paradigms if the experimental readout is anhedonia or cognitive dysfunction, to ensure the phenotype is unmasked.
6. **Generational timeline for selective breeding:** selective breeding for complex traits often requires many generations to achieve a stable phenotype. The examples in this chapter illustrate this: FSL rats needed ~6 generations, SwLo/SwHi rats about 13 generations, and cLH rats around 20 generations. Investigators attempting to create new selectively bred lines (for instance, selecting for another behavior) should plan for a multi-year project and regularly quantify the trait of interest to track the divergence from control populations.
7. **Additional physiological traits in certain lines:** some selectively bred models carry traits that can impact experiments. FH rats, for example, have a bleeding disorder due to platelet serotonin storage pool deficiency [121] and a genetic predisposition to high blood pressure, which may confound studies on stress reactivity or drug responses. WKY rats were originally derived as a control for hypertension; indeed, some WKY substrains exhibit elevated basal blood pressure or exaggerated stress tachycardia. Such comorbid traits should be considered when designing experiments—they might necessitate additional control measurements (like blood pressure or coagulation tests) or influence the interpretation of results (e.g., distinguishing depressive behavior from illness-related lethargy).

6 Conclusion

Selectively bred rodent models provide invaluable insights into the complexities of depression, bridging the gap between preclinical research and clinical understanding. Although no single model can encompass the full spectrum of human depression, each selectively bred line offers unique strengths in simulating specific aspects of depressive disorders—including distinct behavioral phenotypes, neurochemical dysfunctions, and stress-induced traits. The use of these models has greatly advanced our understanding of the etiology and pathophysiology of depression, particularly in exploring genetic vulnerabilities, neurochemical pathways, and the efficacy of antidepressant treatments.

Each model also comes with limitations that must be acknowledged. For instance, FSL rats capture a cholinergic-linked subtype of depression with cognitive impairment, but their baseline anhedonia is not robust without stress. WKY rats model a treatment-resistant depression endophenotype, yet their origin as a hypertensive control means they carry other physiological baggage. FH rats present an intriguing coupling of depressive-like behavior with high alcohol preference, but interpretations of their hedonic state are complex. cLH rats stand out for their profound treatment resistance and are valuable for studying refractory depression, though their extreme phenotype may oversimplify the polygenic nature of human depression. SwLo/SwHi rat lines and HR/LR mice explicitly demonstrate how selective pressure on stress-coping behaviors can yield divergent phenotypes, underscoring the interplay between innate temperament and depression risk. However, these latter models often represent extremes that, while informative, may be relatively rare in human populations.

In applying these models, researchers can leverage their specific strengths: for example, using FSL or cLH rats to probe mechanisms of antidepressant resistance, or WKY rats to study HPA axis dysregulation and chronic treatment effects. Meanwhile, the SwLo/SwHi and HR/LR models emphasize the value of studying inherent resilience vs. susceptibility to stress. Importantly, careful experimental design is required to account for each model's idiosyncrasies—such as substrain differences, sex-specific effects, and comorbid behavioral traits. By integrating findings across these diverse models, we can achieve a more comprehensive understanding of depression's multifaceted nature and improve the translational relevance of preclinical research in developing novel therapeutic strategies.

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