Integrated Behavioral and Physiological Assessment of Anxiety-Like Behavior in Rodent Models

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Received July 27, 2025
Accepted November 24, 2025
Electronic access December 15, 2025

Anxiety-like behavior in rodents has been used as a translational model for human anxiety disorders. Unconditioned behavioral tests, which depend on the natural conflict between exploratory drive and aversive behavior to open spaces, are widely employed measurements to assess anxiety-like behavior in rodents. This review focuses on strengths and limitations of different anxiety-like behavioral tests: the open field test (OFT), light-dark box test (LDBT), elevated plus maze test (EPMT), and elevated zero maze test (EZMT). These behavioral assays measure avoidance behaviors of rodents in anxiogenic environments using a simple but ethologically relevant approach. However, they provide challenges in interpretation, given sensitivity to external variables and inconsistencies across pharmacological models. In addition to behavioral paradigms, physiological measures such as corticosterone levels, stress-induced hyperthermia (SIH), heart rate variability (HRV), and infrared thermography provide complementary insight into the biological stress response with increased objectivity. These measurements, however, have similar limitations, including variability in sampling and interpretation. Taken together, this review emphasizes the importance of combining behavioral and physiological assays to enhance construct validity and obtain a more comprehensive assessment of anxiety-like behavior in rodents. A multidimensional approach may improve reproducibility and support more accurate modeling of human anxiety in preclinical research.

Introduction

Anxiety disorders are one of the most common psychiatric diseases in the world characterized by excessive fear and concern, accompanied by behavioral disturbances, even without actual external threats defined by the DSM-V[1]. Since rodent models provide valuable insights into behavioral, cognitive, motor, and psychiatric areas, these models have been widely used in translational research to investigate the underlying mechanisms and potential treatments for these disorders. These models have contributed significantly to the development of anxiolytic treatments[2–4].

In rodents, anxiety-like behavior has been conceptualized as a generalized psychological and physiological response to uncertain or threatening stimuli[5]. Experimental paradigms to study such responses are typically divided into two categories: conditioned and unconditioned tests[6]. Conditioned tests require learning of an association between a neutral stimulus and an aversive event, such as a fear conditioning test, thus depending on intact memory and sensory processing[6]. In contrast, unconditioned tests exploit spontaneous, evolutionarily conserved behaviors, such as the tendency to avoid open, illuminated areas[6]. These tests, including the open field test (OFT), elevated plus maze test (EPMT), and light-dark box test (LDBT), are favored for their simplicity and strong ethological relevance, although they are also criticized regarding interpretability and external validity[6].

In addition to behavioral assays, physiological measures such as corticosterone levels, stress-induced hyperthermia, and autonomic readouts provide complementary perspectives[7,8]. Given these markers reflect internal states associated with stress and arousal, they could provide additional evidence when behavioral data alone are inconclusive or ambiguous[9]. However, physiological measures also have their limitations, such as sampling challenges and variability due to environmental or individual factors[9].

Given the multifaceted nature of anxiety, a growing body of evidence highlights the importance of integrated approaches that combine both behavioral and physiological assessments[9]. This review will examine widely used unconditioned behavioral tests in rodents, discuss their respective advantages and limitations in evaluating anxiety-like behavior, and provide an overview of complementary physiological measures.

Behavioral Assays for Anxiety in Rodents

Open Field Test (OFT)

The open field test (OFT) is one of the most widely used behavioral paradigms for assessing different aspects in rodents, including innate behavior, locomotion, and anxiety-like behavior[10]. It is relatively simple to perform and requires only

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minimal equipment[11]. In this assay, individual experimental animals are placed in the center of an enclosed square-shaped box, typically with high walls to prevent rodents from escaping[11]. OFT is recorded range from 5 minutes to an hour, depending on the experimental design, often with automated tracking systems[11]. Rodents naturally exhibit thigmotactic behavior, a tendency to stay close to the walls of the arena while avoiding the more exposed center area[11]. During OFT, behavioral parameters recorded include total distance traveled, number and duration of rearing and grooming bouts, freezing behavior, fecal boli count, and particularly, time spent in the center area[12]. Among these, reduced time spent in the center area is most commonly interpreted as an indicator of heightened anxiety-like behavior[11].

In the past, reduced locomotion in the OFT was also interpreted as heightened "emotionality" or anxiety[10]. However, subsequent pharmacological studies have demonstrated that overall locomotion is not a reliable index of anxiety since non-anxiolytic drugs can also influence movement patterns[10]. As a result, general ambulation is now considered a poor standalone marker of anxiety-like behavior[10]. In addition, distinguishing between anxiety-driven avoidance and reduced locomotion could be difficult due to other factors, such as sedation, fatigue, or motor deficits[10]. Furthermore, anxiolytic drugs do not always lead to increased center area exploration, which makes the interpretation of pharmacological effects complicated[10]. Due to these issues, results obtained from the OFT are often considered preliminary, and it is recommended that researchers corroborate findings with more specific complementary tests such as the light-dark box test or elevated plus maze test to obtain a more comprehensive evaluation of anxiety-related behavior[12].

Quantification of thigmotaxis, the tendency of rodents to remain close to the walls of the arena, can be performed using automated video tracking software or manual scoring based on time or distance spent within a defined peripheral zone. The definition of the central zone is not standardized and can substantially influence test outcomes: for instance, a larger center area tends to increase apparent anxiety-like behavior by reducing time spent in the center, whereas smaller central zones may mask subtle differences between treatment groups. Therefore, consistent zone definitions and transparent reporting of arena dimensions are critical for cross-study reproducibility[10,11].

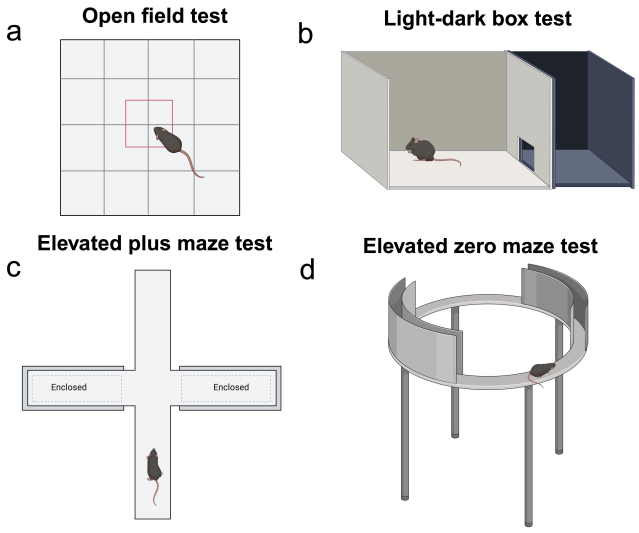

Fig. 1 Schematic representation of commonly used unconditioned anxiety tests in rodents. (a) the open field test (OFT); (b) light–dark box test (LDBT); (c) elevated plus maze test (EPMT); (d) elevated zero maze test (EZMT). The illustration was created using BioRender for conceptual purposes and is not drawn to scale. No empirical behavioral data is shown.

Light-Dark Box Test (LDBT)

The light-dark box test (LDBT) is a modified version of OFT, which offers a more ethologically relevant environment to evaluate anxiety-like behaviors in rodents. The apparatus is typically divided into two equal compartments: a brightly illuminated chamber (∼400 Lux) that is uncovered and a dark chamber that is enclosed with opaque walls and covered. However, some laboratories employ a modified configuration in which approximately two-thirds of the space is illuminated and one-third is enclosed, depending on experimental goals or apparatus design[13,14]. Rodents typically stay in small and enclosed spaces as a natural tendency, thereby preferring the dark compartment[13,15]. However, they are also driven to investigate the bright lit area due to their innate curiosity and exploratory drive[13,15]. During the test, the animal is placed in the illuminated compartment, and its behavior is monitored for 10 minutes[13,15]. The time spent in each compartment and the number of transitions between the light and dark chambers are key measurements for this test[13,15]. An enhancement in the time spent in the illuminated compartment is generally interpreted as an anxiolytic behavior[13,15]. Additional parameters such as latency to enter the dark compartment, rearing behavior, and risk assessment behaviors (e.g., peeking from the dark side before retreating) have also been used to assess anxiety-like behavior, although these are less frequently reported in standard protocols[13,15]. The most reliable and consistent measurement is the time spent in the bright compartment for assessing anxiety-like behavior, particularly in response to anxiolytic compounds[15]. This parameter offers a relatively stable baseline and has demonstrated dose-dependent sensitivity to pharmacological interventions, making the LDBT a reliable tool for screening anxiety-modulating agents in preclinical models[15].

Elevated Plus Maze Test (EPMT)

The elevated plus maze test (EPMT) is the most extensively used behavioral paradigm for assessing anxiety-like behavior in rodents and evaluating the effects of anxiolytic agents[16]. This test was originally developed for rats[17] and later adapted for mice[18]. The EPM apparatus consists of a plus-shaped platform with two closed arms and two open arms, all elevated 50-100 cm above the ground[19]. Unlike the open field test (OFT) and light-dark box test (LDBT), where the anxiety-provoking factor is exposure to an open or brightly lit area, the primary anxiogenic stimulus in the EPMT is the lack of walls or protective cues in the open arms rather than height, which creates a conflict between exploratory drive and fear of open spaces[19]. During the test, the animal is placed in the center of the maze, facing the same direction as the apparatus, and its behavior is monitored for 5-10 minutes[9,10]. The time spent in the open arms and the number of entries into open arms are the two primary analyses used to assess anxiety levels[20]. Rodents with higher anxiety-like states tend to spend more time in the closed arms and avoid the open arms, reflecting their preference for protected environments[20]. Conversely, increased time spent in the open arms or more frequent entries to open arms are interpreted as reductions in anxiety, particularly in response to anxiolytic drug administration[20]. While the EPMT is valued for high sensitivity to pharmacological manipulation and strong ethological relevance, interpretation of staying in the center area, where animals often hesitate, can be ambiguous. Also, variability in baseline anxiety across strains or test conditions should be carefully considered[3].

Elevated Zero Maze Test (EZMT)

The elevated zero maze (EZM) is a modified version of the elevated plus maze (EPM), designed to reduce interpretational ambiguity while preserving the core principle of approach-avoidance conflict[21]. The EZM consists of a circular, annular platform divided into alternating "open" and "closed" quadrants, elevated above the floor[21]. A key advantage of this design is the elimination of the center found in the EPM, which often creates ambiguity in interpreting the meaning of staying in the center area[21]. The circular design also encourages mice to explore more continuously[10]. During a 5-10 minute session, the rodent is placed at the boundary between an open and a closed quadrant, typically facing inward toward the closed area[10,22]. The time spent in the open areas, latency to enter an open area, and number of open quadrant entries are the primary measurements for evaluating anxiety-like behavior[10]. These variables have been shown to be sensitive to anxiolytic drugs such as benzodiazepines, zolpidem, and phenobarbitone, supporting the EZMT's validity as a tool for assessing anxiety-like behavior and pharmacological effects in rodent models[10].

OFT, LDBT, EPMT, and EZMT are widely used to evaluate anxiety-like behavior in rodents due to their simplicity, minimal equipment requirements, and ethological relevance[23]. These tests depend on natural characteristics of rodents, the conflicts between aversion to open or bright spaces and the drive to explore novel environments[23]. They have been used to assess anxiety-like behavior and pharmacological responses as non-invasive and cost-effective methods[23]. Despite these advantages, several drawbacks should be considered[23]. First, these tests primarily rely on the rodents' natural instinct to avoid open/exposed areas, which may not fully capture the multifaceted aspect of anxiety-like behavior[23]. Due to inconsistent data from different anxiolytic drugs, external validity is often questioned[23].

Repeated exposures may also lead to habituation, reducing the sensitivity to detect anxiogenic or anxiolytic effects23. Additionally, other aspects, including strain, sex, age, and environmental variables (e.g., lighting, noise, temperature), can significantly affect outcomes, which may cause unexpected results[23].

Despite these concerns, these assays remain valuable components of anxiety research. When combined with physiological markers, such as corticosterone levels, stress-induced hyperthermia, or heart rate variability, behavioral tests can contribute to a more robust and comprehensive understanding of anxiety-related phenomena in rodents[7,8,24]. To increase reproducibility and translational value, using multiple complementary tests and explaining clearly regarding experimental context should be carefully considered[8]. To ensure ethical standards, researchers need to minimize stress in animals and ensure they are properly habituated before testing.

Physiological Measures of Anxiety in Rodents

Physiological measures could provide a complementary approach for assessing anxiety-like states in rodents. These measures are particularly useful when behavioral outcomes are ambiguous or when researchers aim to investigate the biological mechanisms underlying anxiety[7,8,24].

Corticosterone, the primary glucocorticoid hormone in rodents, is the most commonly used biochemical marker for stress and anxiety[24]. Corticosterone levels could be measured in plasma, serum, saliva, or fecal samples, typically via enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay techniques[24]. Since corticosterone levels increase in response to hypothalamic-pituitary-adrenal (HPA) axis activation, they are considered as a physiological indicator of emotional arousal[24]. However, there are several limitations existing: sample collection itself could be stressful, introducing confounding elevation of hormone levels; and temporal dynamics of hormone secretion may not always align with the acute behavioral observations[8]. Furthermore, studies have

shown that corticosterone levels do not always correlate with anxiety-like behavior results, highlighting the importance of context-dependent interpretation [8,25].

Stress-induced hyperthermia (SIH) is also well-established indicator of emotional arousal [7]. This method is monitoring changes in core or peripheral temperature following mild handling or environmental challenge [7]. An increase of approximately $1.2 - 1.9°C$ of body temperature is typically observed, which is sensitive to anxiolytic drugs and experimental manipulations [7]. Despite SIH is a rapid, non-invasive, and repeatable method, it can vary depending on strain, sex, and environmental conditions [7].

Heart rate variability (HRV) reflects the balance between sympathetic and parasympathetic nervous system activity, and reductions in HRV are often associated with heightened anxiety states [26]. While HRV provides insight into autonomic regulation, its measurement in rodents requires surgical implantation of telemetry devices or non-invasive ECG setups [26], both of which are technically demanding.

Lastly, infrared thermography has emerged as a promising technique for assessing surface temperature changes, particularly in the tail, ears, or periorbital regions [27]. These shifts are correlated with stress-induced vasoconstriction or dilation and are used to infer emotional states [27]. This approach is entirely non-invasive and can be applied longitudinally. However, it remains vulnerable to environmental noise such as lighting, humidity, and cage temperature [27].

Each of these physiological measures offers unique insights, but none are without limitations. Therefore, the current consensus in the field suggests that physiological markers should be interpreted in conjunction with behavioral data, rather than in isolation [7,8,24]. Combining multiple approaches enhances construct validity and provides a more comprehensive understanding of anxiety-related phenomena in preclinical models [7,8,24].

Neural Circuits Underlying Anxiety-Like Behaviors

Anxiety-like behavior in rodents emerges from a complex interaction among neural circuits that process threat, uncertainty, and stress. Central among these are the amygdala, bed nucleus of the stria terminalis (BNST), hippocampus, and prefrontal cortex (PFC), regions collectively referred to as the extended amygdala network.

The central amygdala (CeA) and BNST play critical and partially complementary roles. The CeA is primarily involved in rapid, phasic fear responses to immediate threats, while the BNST mediates sustained anxiety during prolonged or ambiguous stressors. Hyperexcitability of CeA somatostatin-positive (SOM^+) neurons has been shown to suppress inhibitory signaling toward the BNST, increasing its activity and promoting anxiety-like behavior [28]. This CeA–BNST pathway is a major driver of chronic anxiety and stress-related behavioral alterations.

The hippocampus and PFC further regulate anxiety expression through top-down control and contextual processing. The ventral hippocampus (vHPC) encodes environmental context and safety information, while projections from the medial PFC modulate amygdala and BNST activity to regulate adaptive versus maladaptive anxiety responses [29]. Dysfunction within this regulatory circuit may lead to excessive avoidance behavior and impaired fear extinction, as observed in generalized anxiety models.

In behavioral paradigms such as the elevated plus maze or open field test, manipulations that reduce BNST excitability consistently decrease avoidance of open or illuminated areas, indicating a causal link between circuit dynamics and measurable anxiety-like outcomes. Importantly, many of these neural signatures are conserved across species, supporting the translational relevance of circuit-level findings in rodents to human anxiety disorders [30].

Together, these data emphasize that behavioral readouts of anxiety are not merely descriptive but can be mechanistically interpreted through well-characterized neural circuits. Incorporating circuit-level perspectives strengthens the construct validity of rodent anxiety models and enables alignment with human neuroimaging findings

Discussion

Assessing anxiety-like behavior in rodents requires a multidimensional approach that accounts for both observable behavior and underlying physiological changes. While behavioral tests such as the open field test (OFT), light-dark box test (LDBT), elevated plus maze test (EPMT), and elevated zero maze test (EZMT) offer relatively straightforward and ethologically relevant results to examine anxiety-like responses, they have limitations need to consider. Many of these paradigms rely on approach-avoidance conflict and spontaneous exploratory drive, which can be influenced by various factors such as locomotor activity, novelty preference, strain differences, and even ambient noise or lighting. For example, historically reduced locomotion in the OFT was considered as an indicator of increased anxiety-like behavior. After thorough consideration from different studies with inconsistent results using various anxiolytic medications, this assumption is not generally applicable anymore.

Physiological assays could be complementary methods to evaluate anxiety-like behavior since they provide more direct information of internal states accompany anxiety state. Corticosterone quantification, stress-induced hyperthermia (SIH), heart rate variability (HRV), and infrared thermography are commonly employed physiological assessments. To be specific, corticosterone is widely considered as a biomarker of the

hypothalamic-pituitary-adrenal (HPA) axis activation in stress models[24]. However, physiological marker itself is not an impeccable measurement[24]. It is also subject to fluctuation due to handling, sampling methods, and temporal dynamics of secretion[24]. Similarly, while SIH offers a reliable and rapid index of emotional arousal, it is also sensitive to strain, sex, and environmental variability[7]. HRV and thermal imaging are promising as non-invasive techniques, yet they are not always feasible in standard lab settings due to technical complexity or susceptibility to environmental noise[26,27].

Both behavioral and physiological measures provide complementary information to assess anxiety-like behavior, and neither should be interpreted in isolation. Behavioral outcomes will give the information regarding not only anxiety state but also general arousal, attention, or motor function. Meanwhile, physiological markers may or may not align with behavioral changes depending on timing, stressor type, or measurement method. Importantly, studies have shown that behavioral and physiological measures do not always correlate, underscoring the need for integrative, multi-dimensional approaches to assessing anxiety-like states in rodents[7,8,24]. Therefore, future studies should aim to develop standardized testing protocols combining multiple behavioral and physiological endpoints to increase validity. Such integrative approaches may strengthen experimental reliability as well as enhance translational relevance when studying human anxiety disorders. Lastly, careful attention to strain selection, experimental conditions, and ethical considerations will be essential to improve reproducibility and refine our understanding of anxiety-related mechanisms.

Translational Validity of Rodent Anxiety Tests

The utility of rodent models in anxiety research largely depends on their translational validity. That is, how well behavioral outcomes predict or reflect clinical phenomena in humans. Three classical dimensions of validity are typically used to evaluate these models: face, construct, and predictive validity[31,32].

Face validity refers to the extent to which rodent behavior resembles human anxiety symptoms. For example, reduced exploration in the open field or open arms of the elevated plus maze superficially parallels avoidance behaviors in anxious patients. However, not all anxiety manifestations (e.g., cognitive worry or anticipatory tension) have direct behavioral analogs in rodents[31,32].

Construct validity concerns whether the underlying neurobiological mechanisms align with human anxiety pathology. Models incorporating dysregulation of the amygdala-BNST-PFC network, hypothalamic–pituitary–adrenal (HPA) axis alterations, or serotonergic imbalance show stronger construct validity than those based solely on behavioral observation[3].

Predictive validity evaluates whether pharmacological responses in rodents forecast clinical efficacy. Classical anxiolytics such as benzodiazepines consistently reduce avoidance behavior across tests, supporting strong predictive validity. In contrast, serotonergic agents like selective serotonin reuptake inhibitors (SSRIs) exhibit acute anxiogenic effects in rodents despite long-term anxiolysis in humans, highlighting translational limitations[33].

Improving translational validity requires integrating behavioral, physiological, and neural measures within the same experimental framework. Incorporating automated video analysis, physiological biomarkers (e.g., corticosterone, heart rate variability), and circuit-based manipulations can bridge the gap between preclinical and clinical research.

In addition to classical unconditioned paradigms, several cognitive and conflict-based tasks have been developed to assess higher-order dimensions of anxiety. These include the novelty-suppressed feeding test, stress-induced potentiation of the startle response, and operant risk-assessment paradigms, which probe decision-making under uncertainty, attentional bias, and risk evaluation. Integrating such tasks alongside traditional assays broadens the behavioral repertoire captured in rodents and provides a more comprehensive representation of the cognitive and affective components of human anxiety[3,32].

Future studies should explicitly state which dimensions of validity each assay addresses and interpret findings accordingly, rather than assuming direct equivalence with human anxiety disorders[34].

Pharmacological Paradoxes and Predictive Limitations

Pharmacological Paradoxes and Predictive Limitations While rodent anxiety paradigms have been instrumental in identifying compounds with anxiolytic potential, several well-documented pharmacological paradoxes limit their predictive validity. Classical benzodiazepines such as diazepam and lorazepam reliably increase open-arm exploration in the elevated plus maze and light exposure in the light-dark box, confirming strong predictive validity for this drug class. However, newer or non-benzodiazepine agents often display inconsistent or even paradoxical outcomes.

One notable example involves selective serotonin reuptake inhibitors (SSRIs). Acute SSRI administration in rodents typically produces anxiogenic effects, manifested as decreased open-arm entries or increased thigmotaxis, despite their well-established anxiolytic efficacy after chronic treatment in humans[35]. This discrepancy reflects both pharmacodynamic differences across time scales and the complex role of serotonin in modulating defensive behaviors.

Similarly, buspirone, a partial $5-HT_1A$ receptor agonist with proven clinical anxiolytic action, often produces subtle or null behavioral changes in rodent tests[16]. The limited respon-

siveness of such non-benzodiazepine drugs suggests that many current paradigms preferentially capture behaviors linked to acute sedative or motor-relaxant effects rather than sustained anxiety reduction.

To contextualize these discrepancies, Table 1 summarizes the comparative predictive performance of representative anxiolytic classes in rodent assays, and Table 2 compiles major sources of false positives and negatives that contribute to translational failure.

Table 1 Predictive performance of representative anxiolytic drug classes in common rodent tests[3,32,34,35]

Drug class	Example compounds	Typical rodent response	Clinical correlation	Predictive validity
Benzodiazepines	Diazepam, Lorazepam	Robust increase in open-arm/light exploration	Strong	High
SSRIs (acute)	Fluoxetine, Sertraline	Decreased exploration (anxiogenic)	Opposite to chronic human outcome	Low
SSRIs (chronic)	Fluoxetine ($\geq$14 days)	Normalization or mild increase in exploration	Moderate	Moderate
5-HT_{1A} agonists	Buspirone, Tandospirone	Weak or inconsistent	Positive clinical effect	Low-moderate
GABA-B modulators	Baclofen	Sedation confounds interpretation	Limited data	Uncertain

Table 2 Major sources of false positives and false negatives in rodent anxiety tests[3,32,35,36]

Source of bias	Description	Representative outcome
Sedative or locomotor effects	Reduced exploration misinterpreted as "anxiolytic"	False positive
Strain or sex variability	Divergent baseline anxiety profiles	False positive/negative
Habituation and retesting	Diminished avoidance after repeated exposure	False negative
Environmental factors (light, noise)	Altered thigmotaxis and locomotion	False positive/negative
Non-specific pharmacological effects	Motor impairment or altered arousal	False positive

These paradoxes highlight that behavioral readouts alone are insufficient for evaluating anxiolytic efficacy. Integrating physiological and circuit-level biomarkers (e.g., heart-rate variability, corticosterone dynamics, and neural activity mapping) can help discriminate true anxiolytic effects from confounding sedative or motor influences. Establishing standardized multimodal endpoints across laboratories will be critical to enhance the predictive validity of preclinical anxiety assays.

Biological and Environmental Modulators of Anxiety-Like Behavior

Behavioral and physiological measures of anxiety in rodents are highly sensitive to biological and environmental variables. Ignoring these factors can lead to misinterpretation of test outcomes and contribute to the low reproducibility of preclinical findings.

Sex differences represent one of the most consistent modulators. Female rodents often display higher baseline anxiety-like behavior in the elevated plus maze and light–dark box, potentially linked to estrogen-dependent modulation of amygdala and HPA-axis reactivity[37]. Hormonal cycling further influences corticosterone secretion and exploratory drive, suggesting that estrous phase should be monitored or experimentally controlled.

Age also affects test sensitivity. Juvenile and aged animals frequently exhibit exaggerated or blunted avoidance responses relative to adults, respectively, reflecting developmental and neuroendocrine differences[34]. Incorporating age-matched cohorts is therefore essential for consistent interpretation.

Strain and genetic background substantially impact baseline anxiety and drug responsiveness. For example, C57BL/6 mice generally show lower anxiety scores compared to BALB/c mice, and strain-specific differences in locomotion can confound measures such as open-arm entries[7].

Circadian variation further modulates both behavioral and endocrine endpoints. Corticosterone levels can fluctuate more than ten-fold across the light-dark cycle in rats, producing marked differences in anxiety test outcomes depending on the time of day[38]. Standardizing testing periods within the active phase of each species is thus recommended to improve reproducibility.

Environmental factors such as ambient noise, light intensity, and prior handling also exert measurable effects on anxiety-like behavior[39]. Consistent laboratory conditions and adherence to ARRIVE 2.0 Essential 10 guidelines will facilitate transparent reporting and enhance cross-study comparability[40]

Integrative Framework: Combining Behavioral and Physiological Measures

An integrated assessment of anxiety-like behavior in rodents benefits from concurrent measurement of behavioral, physiological, and neurobiological indices within the same experimental framework[41]. This multimodal approach enhances construct validity and provides a more holistic interpretation of anxiety-related phenomena. In a typical experimental timeline (illustrated schematically in Figure 2), baseline physiological parameters such as heart rate, core temperature, or corticosterone levels can be recorded before exposure to the behavioral assay. During the testing phase, for example, the

elevated plus maze or open field, video tracking software captures locomotor trajectories and postural dynamics, while telemetry or infrared thermography continuously monitors autonomic and thermal responses[7,27]. Post-test sampling (e.g., plasma or fecal corticosterone assays) provides delayed physiological readouts of stress reactivity.

Integrating these temporal layers: pre-test baseline, in-test dynamics, and post-test recovery, allows for cross-validation between behavioral and biological responses. For instance, animals exhibiting high thigmotaxis or avoidance behavior typically display concurrent elevations in heart-rate variability or corticosterone output, supporting convergent validity. Conversely, dissociation between behavioral and physiological indices may indicate confounding variables such as sedation or motor impairment rather than true anxiolysis[7,26].

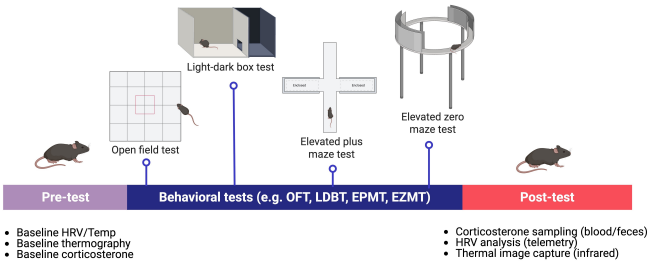

Fig. 2 Schematic timeline illustrating the integration of behavioral and physiological assessments in rodent anxiety testing. Baseline physiological parameters such as heart rate variability (HRV), body temperature (thermography), and corticosterone levels are recorded before testing. During behavioral assays (e.g., open field, light–dark box, elevated plus maze, elevated zero maze), real-time telemetry and thermal monitoring can be performed concurrently with behavioral tracking. After testing, blood or fecal samples are collected for corticosterone analysis, and post-test physiological measures are obtained to assess recovery. This multimodal framework provides temporal alignment between behavioral and physiological endpoints. The illustration was created using BioRender.

This schematic integration also facilitates correlation with neural circuit activity assessed via c-Fos mapping, fiber photometry, or in vivo calcium imaging, thereby linking observed behavior to underlying neural substrates. Collectively, multimodal designs as depicted in Figure 2 can substantially improve reproducibility, mechanistic interpretation, and translational relevance of rodent anxiety research[41].

In recent years, the integration of machine-learning–based behavioral quantification tools has further advanced the precision and reproducibility of anxiety testing in rodents. Systems such as DeepLabCut, B-SOiD, and SimBA employ markerless pose estimation and unsupervised clustering to extract high-dimensional behavioral features from raw video data[42–44]. These approaches enable automated scoring of subtle actions such as head dips, rearing, or stretch-attend postures, behaviors that are often overlooked or subject to observer bias in manual analyses. When combined with physiological recordings (e.g., heart-rate telemetry, infrared thermography), such computational pipelines can yield multidimensional behavioral phenotypes that align more closely with underlying neural dynamics and improve cross-laboratory reproducibility[7,27]

Methods

A focused literature review was conducted to examine how anxiety-like behavior is assessed in rodent models using both behavioral and physiological methods. Searches were performed using PubMed and Google Scholar between January 2000 and June 2025, with the most recent search completed on June 15, 2025. The primary search terms included "anxiety-like behavior" AND "rodent" OR "mice" OR "rat" combined with "open field test," "light-dark box," "elevated plus maze," or "elevated zero maze." Additional terms such as "corticosterone," "stress-induced hyperthermia," "heart rate variability," and "infrared thermography" were used to identify studies incorporating physiological endpoints. Secondary searches targeted neural circuits, machine-learning behavioral analysis, and translational validity. Peer-reviewed, English-language articles were included; however, earlier studies were considered if they described essential or foundational assay protocols. Studies were included if they (1) used rodent models (mice or rats), (2) clearly described the test apparatus and procedures, and (3) reported key outcome metrics such as time spent in anxiogenic zones, entries, and latency. For physiological measures, studies needed to describe methods for assessing corticosterone levels, core or surface temperature changes, or autonomic regulation via ECG or thermography. Data extracted from each study included the test used (e.g., OFT, LDBT, EPMT, EZMT), session duration, initial animal placement, behavioral outcomes (e.g., time in open arms, number of entries), and physiological parameters (e.g., method of corticosterone sampling, thermal camera settings, ECG setup). This information was synthesized to compare the strengths and limitations of each approach and highlight the benefits of integrated assessments. The search strategy and inclusion criteria were reported transparently to enhance methodological rigor and reproducibility.

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