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UPDATE ARTICLE

Animal models of anxiety disorders and stress

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Anxiety and stress-related disorders are severe psychiatric conditions that affect performance in daily tasks and represent a high cost to public health. The initial observation of Charles Darwin that animals and human beings share similar characteristics in the expression of emotion raise the possibility of studying the mechanisms of psychiatric disorders in other mammals (mainly rodents). The development of animal models of anxiety and stress has helped to identify the pharmacological mechanisms and potential clinical effects of several drugs. Animal models of anxiety are based on conflict situations that can generate opposite motivational states induced by approach-avoidance situations. The present review revisited the main rodent models of anxiety and stress responses used worldwide. Here we defined as ''ethological'' the tests that assess unlearned/unpunished responses (such as the elevated plus maze, light-dark box, and open field), whereas models that involve learned/punished responses are referred to as ''conditioned operant conflict tests'' (such as the Vogel conflict test). We also discussed models that involve mainly classical conditioning tests (fear conditioning). Finally, we addressed the main protocols used to induce stress responses in rodents, including psychosocial (social defeat and neonatal isolation stress), physical (restraint stress), and chronic unpredictable stress.

Keywords: Anxiety disorders; stress; animal models

Introduction

Anxiety and fear are normal emotions with great adaptive value that have been selected along the evolutionary process. While fear occurs in response to specific threats, the source of anxious behavior is usually undefined or unknown.[1] In contrast to normal/adaptive anxiety, anxiety disorders affect the individual performance of daily life tasks,[2] representing a high cost for public health care all over the world.[3-6]

Experimental anxiety: animal models

The initial observation of Charles Darwin that the expression of emotion in humans and other mammals was phylogenetically preserved (''... the young and the old of widely different races, both with man and animals, express the same state of mind by the same movements...,'' Charles Darwin, 1872)[7] brought the evolution theory close to behavioral neuroscience. Based on this assumption, animal models of emotional disorders attempt to reproduce features of human psychiatric disorders in laboratory animals, correlating the physiological and behavioral changes associated with specific emotional states (face validity), the etiology of diseases (construct validity), and responses to pharmacological treatments (predictive validity). Even if one considers that current animal models aim to reflect several factors (such as low cost, speed, and reproducibility) in addition to the dominant theoretical views related to the pathogenesis of specific disorders and the accepted action mechanism of psychotropic drugs, they have produced a significant contribution to the discovery of new drugs and the understanding of the neurobiology of psychiatric diseases.[8]

Despite the theoretical idea that a model should reproduce all features of the phenomenon under investigation, this is rarely (if ever) achieved, reflecting the complex manifestations of psychiatric disorders and the huge cognitive differences between humans and laboratory animals (mainly rodents, e.g., rats, mice, hamsters, gerbils, guinea pigs). Animal models of anxiety, therefore, do not intend to replicate all features and symptoms of a specific anxiety disorder but rather generate a state of anxiety that could be related to these disorders.[9]

In rodents, conflict situations can be generated by opposite motivational states induced by approach-avoidance situations. For example, approach behaviors can be observed in new environments, reflecting an unconditioned general exploratory drive, or in seeking responses that have been previously conditioned. On the other hand, avoidance drives can also be unlearned, such as

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aversion to new, brightly lit, open and elevated places, or learned, including by punished responses such as electric shocks. In this review, animal models that measure unconditioned conflicts are defined as ethological (such as the elevated plus maze, light-dark box, open field, novelty suppressed feeding, and predator exposure tests), since they are all based on unlearned fear/avoidance behavior, whereas models that involve learned/punished responses are referred to as conditioned operant conflict tests (such as the Vogel conflict test, VCT). Finally, models that involve mainly classical conditioning are discussed as classic conditioning tests. Table 1 summarizes the main animal models used to evaluate anxiety-like responses in rodents.

Ethological (unconditioned) behavioral-based models

The study of unconditioned/ethological responses to different forms of external threats is a logical extension and simulation, in laboratory conditions, of what occurs in nature (innate fear/avoidance). These models are proposed to have a high ethological validity, permitting a more detailed characterization of the behavioral changes induced by the tests.[10] The basic premise of most of these models is the set of behavioral responses induced by exposure to a new environment, which simultaneously evokes fear and curiosity, creating a typical approach-avoidance conflict. The first study that investigated this phenomenon was done by Montgomery[11] using a Y-shaped maze with one enclosed arm and two open arms. He observed that rats consistently show high levels of exploration and preference for the enclosed paths and concluded that, since the open and enclosed arms should evoke the same exploratory drive, the greater avoidance of the open arms was due to higher levels of fear/avoidance evoked by open places.[11] However, as we will discuss latter, other factors can also interfere in exploratory behavior of novel environments, such as the complexity of the situation, the degree of novelty (which changes from the beginning to the end of the experimental session), and the basal state of the animal.[9,12-15]

Table 1 Animal models of anxiety

Unconditioned tests	Conditioned tests
1. Exploration-based models a. Elevated plus maze b. Elevated zero maze c. Elevated T maze d. Light-dark box e. Hole-board test f. Novelty-suppressed feeding g. Social interaction test	1. Conditioned operant conflict tests a. Geller–Seifter test b. Vogel conflict test 2. Classic conditioning tests a. Emotional conditioning responses b. Ultrasonic conditioning vocalization c. Fear-potentiated startle d. Place aversion test
2. Predator-based models a. Cat exposure test b. Rat exposure test	

The elevated plus maze

The elevated plus maze (EPM), perhaps the most employed animal model of anxiety in current practice, was first proposed by Handley & Mithani[16] and further validated by File et al.[17] The apparatus is raised above floor level, and is composed of two enclosed arms opposed perpendicularly by two open arms. The test is based on the natural tendency of rodents to explore novel environments and their innate avoidance of unprotected, bright, and elevated places (represented by the open arms). Confinement to the open arms induces physiological signs of stress (increased defecation and corticosterone levels),[17] whereas exposure to classical anxiolytic drugs, such as benzodiazepines, increases exploration of these arms.[17]

The basal activity of the animals in the EPM is affected by several factors, such as housing conditions, lighting levels, circadian cycle variations, prior handling or stress exposure, and familiarity with the maze. For instance, individual housing increases anxiety in rats but decreases it in mice, probably due to distinct social organization patterns between the species, while prior stress exposure (foot shock, social defeat, predator exposure) markedly increases anxiety. Moreover, re-exposure to the EPM results in marked reductions in open arm exploratory behavior and can totally abolish the anxiolytic effect of benzodiazepines.[18-20] In addition, the presence of the experimenter in the same room can also interfere with the results. This caveat, however, has been overcome by videotaping the experimental session for later behavioral analysis (with or without the help of specialized software).

Regarding the different variables that can be recorded in a 5-min EPM session, studies employing factor analysis suggested that the enclosed arm entries can be used as an uncontaminated measure of locomotor activity, while percentage of entries and time spent in the open arms constitute the primary anxiety index.[21] Administration of anxiolytic drugs such as diazepam, at non-sedative doses, promotes an increase in the percentage/ratio of open arm exploration without affecting enclosed arm exploration.[17,18] In addition to these classical measures, different groups have proposed the evaluation of other ethological variables, such as risk assessment of the open arms and head dipping in these arms, to increase the sensitivity of this model.[22]

The elevated zero maze

The elevated zero maze (EZM) is a modification of the EPM that incorporates both traditional and novel ethological measures for the analysis of drug effects while eliminating the ambiguous interpretation of animal location in the center area of the EPM.[23]

The EZM is a circular runway elevated from the floor that alternates open, brightly lit areas with enclosed, dark paths. It is proposed that the uninterrupted nature of the open versus enclosed segments of the circular arena alleviates the problems concerning the center zone of the EPM. Similar to the behavioral measures scored in the EPM, the percent of time spent and the percentage of

entries in the open areas of the EZM during the 5-min session are related to anxiety index. In this model, diazepam and chlordiazepoxide significantly increase the percentage of time spent in the open quadrants, as well as other ethological measures, such as frequency of head dips and reduced frequency of stretched attend postures in the enclosed towards the open quadrants.[23] To minimize environmental variables introduced by the presence of the investigator that may impact anxiety-like behaviors, videotaping of the session is also recommended.

Elevated T maze

The elevated T maze (ETM) was originally proposed by Graeff et al.[24] It is based on the EPM and consists of three arms: one enclosed by a lateral wall standing perpendicular to two opposite open arms of equal dimension. The whole apparatus is elevated from the floor. This model allows measurement of two different behaviors in the same animal: the conditioned response represented by inhibitory avoidance of the open arms and the unconditioned response represented by escape behavior when the animal is placed in the extremity of these arms. These responses have been related to generalized anxiety and panic disorders, respectively. The ETM was developed in response to the inconsistencies found in other animal models of anxiety, particularly the EPM, regarding drugs that interfere directly with serotonergic neurotransmission.

On the day before the test, animals are exposed to one of the open arms of the T-maze for 30 min. This prior forced exposure to one of the open arms of the maze decreases the latency to leave this arm on a later trial. This result has been attributed to the habituation of behavioral reactions to novelty, which may interfere with one-way escape.[25] Twenty-four hours after pre-exposure to the open arm, the animals are tested in the ETM to measure inhibitory avoidance acquisition. To this end, each animal is placed at the distal end of the enclosed arm of the ETM facing the intersection of the arms. The time taken by the rat to leave this arm with all four paws is recorded (baseline latency). The same measurement is repeated in two subsequent trials (avoidance 1 and 2) at 30-s intervals. Following avoidance training (30 s), each rat is placed at the end of the same previously experienced open arm and the latency to leave this arm with all four paws is recorded for three consecutive trials (escape 1, 2 and 3) with 30-s intertrial intervals. A cut-off time of 300 s is usually established for the avoidance and escape latencies.

The light-dark box

The light-dark exploration test was developed before the EPM test by Crawley & Goodwin in the early 1980s.[26] Similar to the EPM, this animal model is based on the innate aversion of rodents to places with bright light. During a 5-min session, animals are allowed to freely explore a novel environment composed of two different compartments: protected (dark) and unprotected (lit). In rodents, this model generates an inherent conflict between their exploratory drive and their avoidance of the lit compartment.[26,27] Treatment with anxiolytic drugs such as benzodiazepines increases the time spent in the lit compartment as well as the number of transitions between the two areas.[26,27] In this test, as in others that measure exploratory activity, particular attention should be given to drug- or genetic-induced changes in basal locomotor activity or novelty-seeking behavior (e.g., amphetamine treatment), since they could produce false positive results.

The hole-board test

The hole board consists of a square arena with a number of holes in the floor that the rodents can explore by poking their heads. The test is based on this latter behavior, named head dipping,[28,29] which has been validated as a measure of exploratory activity and anxiety.[29] The number of head-dips is assumed to be inversely proportional to the anxiety state.[30] Drug effects on this test, however, can be influenced by the familiarity of the animal with the test environment.[31,32] In mice naive to the testing apparatus, benzodiazepines exert a biphasic effect on exploratory head dipping, with lower doses increasing and higher doses decreasing this behavior.[32] However, the doses of benzodiazepines that increase exploration in naive mice fail to do so in mice that have been previously exposed to the apparatus.[32]

The social interaction test

The social interaction test, developed by File & Hyde,[33] was the first model of anxiety-like behavior based on ethologically relevant concepts. This test differs from the others because it involves the important component of eliminating the need to introduce aversive or appetitive conditions. In addition, it does not require previous animal training. Pairs of rodents (rats or mice) are allowed to freely interact in an arena while the time spent on social interaction is recorded. This interaction time for each of the rodents in the pair is directly impacted by the behavior of the partner animal. Therefore, the pair counts as one unit for data collection purposes. If the experimental design involves one rat receiving treatment while the other serves as a control, interaction time initiated by the former is used as the dependent measure. Anxiolytic-like behavior is inferred by an increase in social interaction time while general motor activity remains unaffected. Conversely, decreased time spent engaging in social behavior would indicate anxiogenic-like behavior.

Hyponeophagia-based model: novelty suppressed feeding test

The first report of hyponeophagia in rodents, i.e., the suppression of feeding generated by the increase in anxiety-like states of animals exposed to a novel environment, was made in 1934 by Hall.[34] In 1988,

Bodnoff et al.[35] validated the novelty suppressed feeding (NSF) test. In this model, animals deprived of food for 24 hours are exposed to a transparent box consisting of a sawdust-covered floor, a central platform holding a single pellet of chow, and focused lighting. The latency for the animal to reach the center of the box and initiate food intake is measured, being directly correlated with anxiety levels. Thus, this model creates a conflict between the natural tendency to feed after food-deprivation and the ethologic aversion of novel, brightly lit, and central places. In this test it is also important to control for any drug-induced changes in food intake, which is usually done by measuring this variable in the animals' home cages.[36]

In the NSF, acute and chronic administration of diazepam induces an anxiolytic-like effect, represented by a decrease in the latency to onset of eating.[37] Furthermore, in the case of antidepressants, the model exhibits good predictability, since it responds only to chronic treatment (minimum of 2 weeks), mimicking the time course required for the therapeutic effects of these drugs in humans. Due to this characteristic, the NSF is often employed after the chronic unpredictable stress procedure (where animals are exposed to daily different stressors for, at least, 14 days), which increases anxiety-like behaviors, to evaluate the anxiolytic and antidepressant properties of chronic treatments.[35,37] Although it involves hunger, this test is well accepted since it does not require painful procedures or previous training.

Conditioned operant conflict tests

Operant behaviors relate to spontaneous responses emitted by the animal to an environmental change, known as reinforcement, which can be positive or negative. Positive reinforcement, also known as reward, occurs when the stimulus exposure increases the possibility of a future response in relation to this stimulus, such as progressive lever-pressing to obtain a pleasurable food. On the other hand, negative reinforcement is seen when a trained animal executes a response to avoid an unpleasant stimulus, usually observed in punished paradigms, such as electric shocks. This latter procedure is used in the so-called conflict tests described below.

The Geller–Seifter and Vogel conflict tests

The operant conflict test was firstly developed by Geller & Seifter[38] and later modified by Vogel,[39] and shows a high predictive value for classical anxiolytic drugs. In the Geller–Seifter test, rats deprived of food for 24 hours are trained to press a lever and obtain a sugar-sweetened drink at variable intervals (the non-punished component). In the test session, a signaling stimulus (such as a tone or a light) is introduced, indicating now that the lever-press behavior will always yield a reward but, at the same time, will be punished by an electric shock, producing a conflict between drinking the palatable water and receiving the shocks. In the control condition, the animal's tendency to press the lever decreases, whereas anxiolytic drugs show an anti-conflict effect, increasing the probability of punished responses. This effect is not due to antinociception, since is not observed after treatment with opioid agonists such as morphine. Psychostimulant drugs such as amphetamine also fail to produce this effect.[40]

Some years after the introduction of this model, Leaf & Muller[41] reported that shocks suppress the licking behavior of water-deprived rats. However, these researchers did not test usual anxiolytic drugs, an experiment that was later performed by U.S. researcher John Vogel in 1971.[39] Vogel introduced a more simplified test, in which animals were deprived of water for 24 hours and briefly trained to find a bottle of water in an experimental box. On the next day (after another 24-hour period of water deprivation), the animals are re-exposed to the same box, which contains a stainless steel grid floor. The contact of the animal with the bottle spout and the grid floor closes an electrical circuit controlled by a sensor. After each 20 licks at the bottle of water, the animal receive a mild shock (0.5 mA).[39] In this model, anxiolytic drugs also show anti-conflict properties, inducing an increase in the number of punished licks. Similar to the Geller–Seifter procedure, control experiments to avoid any drug effect in nociception and thirst should be performed.

Even though both models described above have a good predictive value for benzodiazepines and barbiturates, the VCT also responds to some non-anxiolytic drugs, producing false-negative results.[40] Moreover, antidepressants produce inconsistent results in these models. Chronic treatment with tricyclic antidepressants and monoamine oxidase inhibitors, such as imipramine and phenelzine respectively, increases punished responses, but the serotonin reuptake inhibitor (SSRI) fluoxetine does not.[42,43] Chronic administration of the partial $5HT_{1A}$ agonist buspirone also produces anti-conflict effects in rats[44,45] but not in mice.[46]

In comparison to the Geller–Seifter test, the VCT has the advantage of avoiding a prolonged training period. However, despite good predictive value regarding classical anxiolytics, these tests are susceptible to interference from several variables, such as hunger, thirst, pain, learning and memory, which can sometimes hinder interpretation of the results.

Classic conditioning tests

Pavlovian or classical conditioning experiments involve an associative learning process in which a neutral conditional stimulus (CS) is repeatedly paired with an unconditional stimulus (US). After the repeated pairings, the CS presentation alone will induce affective responses manifested as a conditional emotional response.[47]

Fear conditioning

Fear conditioning is a form of Pavlovian conditioning that involves learning the association of a neutral CS, such as a light, tone, or setting, with an aversive stimulus (US),

such as an electric shock. Re-exposure to the CS will activate a conditioned fear response which resembles the responses that occur in the presence of danger.[48,49] Conditioning learning can be elicited in several species, including humans.[50] The defensive responses elicited by the CS in animals are characterized by freezing (complete immobility except as required for breathing), reflex expression (characterized by fear-potentiated startle), and autonomic (increase in heart rate and in the mean arterial pressure) and endocrine (stress-related hormone release) responses.[50-53]

Fear conditioning models involve the encoding of traumatic memories, representing a psychological stress without physical stimuli.[51,54] They have been associated with a vulnerability to phobic fears and other anxiety-related disorders, such as panic disorder (PD), agoraphobia, and posttraumatic stress disorder (PTSD).[55,56]

In this model, administration of anxiolytic drugs immediately before the pairing of CS and US (during the memory acquisition process) affects the formation of conditioned learning. If administration occurs before the re-exposure to CS, it will affect fear and anxiety expression acquired during the conditioning. The drug could also affect extinction of the conditioned response, where a new learning process (that the CS no longer predicts the occurrence of the UCS) occurs after repeated exposure to a CS in the absence of the US.[57]

Systemic administration of benzodiazepines or SSRIs reduces the freezing behavior observed during the expression of conditioned fear.[58-60] This is in agreement with clinical findings indicating that they are effective for the treatment of anxiety disorders.[57] However, in contrast to their clinical effects, SSRIs are effective after acute administration in this model.[61,62] Li et al.,[63] however, showed that chronic treatment with SSRIs induces a greater attenuation of conditioned emotional responses after repeated rather than acute administration.[63] In addition, several other factors can influence the effects of SSRIs, including the timing of drug administration, the kind of CS stimulus, and the intervals between acquisition and expression of conditioned fear.[54,64]

Other animal models of anxiety

Predator encounter-based models

Defensive behaviors are observed in all mammalian species and occur in response to threatening cues, such as the presence of live predators and environmental hazards.[22,65] Therefore, exposure to an ethological stimulus evokes defensive responses that resemble emotional states related to fear and anxiety.[66,67] Accordingly, predator exposure constitutes an important animal model for identification of the impact of threatening situations on different brain regions and the relationship between defensive behaviors and fear-related disorders, such as panic attacks and PTSD.[68-70]

In rats, exposure to a live cat or to its odor elicits specific behaviors, such as fight, freezing, risk-assessment, and autonomic activation. These responses are accompanied by a reduction in locomotor activity and in non-defensive behaviors, such as grooming and reproduction.[66,71,72] Although both stimuli elicit defensive responses, exposure to a live cat induces more robust responses than exposure to its odor, accompanied by freezing and ultrasonic vocalizations. Furthermore, live cat exposure is usually resistant to habituation, has a strong contextual conditioning component, and induces anxiogenic-like effects in animals that are subsequently exposed to other anxiety models, such as the EPM (see below).[68,73,74]

This model was pharmacologically validated with the observation that chronic administration of panicolytic drugs decreases the fight reactions induced by the presence of the predator, whereas benzodiazepines preferentially inhibit the avoidance behavior.[75,76] These latter effects were also described in cat odor models, as pretreatment with chlordiazepoxide reduced the subsequent anxiogenic-like behavior observed in the EPM and light-dark box. However, acute treatment with benzodiazepines did not reduce the defensive behaviors elicited by odor itself.[77] On the other hand, other studies showed that this treatment is able to reduce risk assessment behaviors and increase approach to the odor.[70,78]

Escape behavior induced by electrical/chemical stimulation of dorsal portions of the periaqueductal grey matter (dPAG) as a model of panic disorder

PD is a chronic and disabling psychiatric disorder characterized by unexpected and recurrent panic attacks that affects about 5% of people worldwide.[2] PD patients experience psychosocial impairment and a high risk of psychiatric comorbidities and suicide.

The periaqueductal grey matter (PAG) is a midbrain structure that, among other functions, integrates defensive behavior. In humans, electrical stimulation of this structure evokes strong feelings of fear, impending death, non-localized pain, and marked autonomic changes.[79] Given the striking similarities between the autonomic and behavioral effects of dPAG stimulation and symptoms of panic attacks, it has been suggested that this structure is involved in the genesis of PD in humans and that stimulation of this midbrain area in animals can model panic attacks.

Stimulation of the dPAG is usually performed in a circular arena (40 cm in diameter) with 40 cm-high walls made of transparent Plexiglas. For chemical stimulation, direct injection of an N-methyl-D-aspartate (NMDA) agonist or GABAergic antagonist induces defensive behaviors. For electrical stimulation, a brain electrode is connected to the stimulator by means of an electromechanical swivel and a flexible cable, allowing ample movement of the animal inside the experimental cage. The current is generated by a sine-wave stimulator and monitored on the screen of an oscilloscope.[80] After stimulation of the dPAG, a vigorous reaction is observed, with freezing response, piloerection, miosis, vertical jumps, and strong flight reactions represented by an increase in locomotion and average speed.

The influence of stressful situations on anxiety-like behavior: animal models of stress

Several studies conducted on animals and volunteers have suggested that stressful experiences occurring throughout life may contribute crucially to the development and pathogenesis of several psychiatric disorders, including mood disorders, schizophrenia, and anxiety.[81] Moreover, most of the symptoms of anxiety disorders are accompanied by activation of the hypothalamic-pituitary-adrenal (HPA) axis and changes in hormonal mediators and glucocorticoid biomarkers of stress responses.[81-83] For instance, studies conducted by McEwen et al. at Rockefeller University in New York suggest that laboratory animals subjected to chronic stressors exhibit behavioral changes in models related to anxiety disorders.[84]

Several studies have reported the association between exposure to stressful situations and subsequent episodes of major depression.[85-88] The same is true for anxiety disorders. For example, patients who apparently experienced some stressful situation in the course of their lives have more intense episodes of panic attacks[89,90] and are more vulnerable to the development of PTSD, a disorder that involves an individual overreaction to an initial exposure to traumatic event.[91-94]

It was only in the last three decades that the relationship between somatic and psychological consequences promoted by exposure to extreme stressors and the neurobiological substrate involved in these processes started to be better understood. This advance was made possible by the development of models that aim to evaluate behavioral changes induced by acute or chronic exposure to stressors (predators, shocks, movement restriction), which respond to clinically effective drugs. The main differences among these models relate to the duration (chronic vs. acute) and nature of stressor exposure.

Predator exposure-based models: PTSD

PTSD is a debilitating chronic condition that reflects emotional and physiological modifications following an initial reaction to a traumatic experience.[2] Patients with PTSD exhibit persistent re-experience of traumatic memories (nightmares, intrusive thoughts) and increased avoidance of trauma-related stimuli (hypervigilance and hyperarousal) even though the traumatic event is no longer occurring.[2]

PTSD modeling in laboratory animals has been a particular challenge, since some of the symptoms of this disorder (nightmares, invasive thoughts) cannot be evaluated.[95] Among proposed models for PTSD, those based on predator exposure have been widely used because they can mimic several symptoms of the disorder, such as hyperarousal and chronic generalized anxiety.[96-98] The anxiogenic effects of this procedure are long-lasting, persisting for at least 3 weeks, and reflect the non-associative sensitized fearful manifestations that are observed in PTSD patients.[95] For example, in rats, a single cat exposure modifies the function of brain areas (such as the amygdala, prefrontal cortex, and hippocampus) that have been associated with the genesis of PTSD symptoms in humans.[99,100] In this model, the animals are exposed to a live cat or its odor for 5-30 min and, after 7 to 21 days, are exposed to an animal model of anxiety such as the EPM, fear conditioning, or startle-potentiated responses.[74,96,98]

Psychological and physical stress models

Essentially, these models induce stress by exposing the animals to psychological or physical challenges. These procedures may be used in acute or chronic studies depending on the objectives and parameter chosen by the experimenter to evaluate the impact of stress on anxiety. The main protocols used are presented in Table 2 and briefly described below.

Neonatal isolation stress

Early-life stressful experiences, such as maternal separation or neonatal isolation, promote long-lasting neural and behavioral effects and have profound consequences on subsequent quality of life.[101] During the neonatal separation procedure, on the 2nd day after birth, the litter of the inbred strain is removed from the cage and placed in another cage for 1 hour (9 a.m./12 a.m.) in a room located apart from the animal facility. White noise is played in the background to mask the vocalizations of other pups. After the 1-hour period, the litters are placed back with their dams in their home cages.[102,103] The separation procedure is repeated for 8 days. This model has been used extensively to demonstrate the effect of early lifetime stress on vulnerability to addiction and in the generation of anxiety-like behaviors, which are usually observed in the adult rodents subjected to the contextual fear conditioning, EPM, or social interaction tests.[104-107]

Stress induced by circadian rhythm changes

Alterations in circadian rhythm have a profound impact on the physical and psychological homeostasis of an individual.[108] Rodents subjected to unexpected changes in the day-night light cycle exhibit acute stress responses.[104] Circadian rhythms are controlled by the pineal gland via melatonin secretion.[109] The stress procedure consists of lighting the home cage of the rodents during the dark phase of the cycle (e.g., lights on from 7 p.m. to 7 a.m.) and leaving it unlit in the light phase (lights off from 7 a.m. to 7 p.m.). Another possibility is to promote four or five cycles of dark-light phases (60-180 minutes) during the circadian cycle. This is a good

Table 2 Stress protocols

Psychosocial stress	Physical stress
a. Neonatal isolation	a. Restraint stress
b. Noise stress	b. Immobilization stress
c. Circadian rhythm changes	c. Temperature variation stress
d. Predator stress	d. Electric foot shock stress

method for induction of short-term stress responses, but repeated exposure may lead to adaptation. Responses to this stressor can be evaluated by measuring biochemical parameters associated with stress response and using the previously described animal models of anxiety.[110-112]

Stress induced by a noisy stimulus

Humans are constantly exposed to potentially hazardous levels of noise in modern daily life. In model animals, noise stress can be induced by using loudspeakers (15 W) connected to a white noise generator (0-26 kHz) located 30 cm above the cage. The noise can be set at a certain level (e.g., 100 dB or higher) and the animals can be exposed to the noise protocol either acutely or repeatedly (4 hours/day/15 days).[113,114] Like those of other protocols, the behavioral effects of noise stress can be observed in animal models of anxiety and depression.[115,116]

Low temperature-induced stress

Changes in body temperature lead to stressful responses due to activation of the thermoregulatory center and, subsequently, of the HPA axis.[117] Abrupt reductions in temperature by using either cold water or freezer compartments have frequently been used to induce stress in laboratory animals. The most widely used protocols consist in the immersion of the animals in cold water (15-18°C for 15-30 min) or placing the animals (in their home cages) in a cold, isolated environment (4°C for 15-30 min). This procedure can be used in acute or chronic protocols (7-14 days).[118]

Restraint and immobilization stress

Restraint stress and immobilization protocols are one of the most commonly employed procedures to induce stress-related behavioral, biochemical and physiological changes in laboratory animals.[119] Restraint stress is generally induced by keeping the animals in a cylindrical or semi-cylindrical tube with ventilation holes for 120-180 min.[120,121] In an immobilization stress protocol, animals are restrained by gentle wrapping of their upper and lower limbs with adhesive tape for 120 min.[122,123] Head movement is restricted by a metal loop wound around the neck. The procedure can be used to induce either acute or chronic stress (7-21 days). Immobilization models produce an inescapable physical and mental stress with a low rate of adaptation.[124] After restraint or immobilization stress, animals exhibit higher levels of anxiety in the EPM and other tests of anxiety.[120,121]

Electric foot shock-induced stress

This protocol is very similar to the pre-test session described in fear conditioning-based models. Rodents are very susceptible to mild shocks, exhibiting a remarkable stress response after foot shock delivery. The protocol consists of placing rodents in a chamber with a metal grid floor connect to a shock generator. After a habituation period, animals receive mild (05-2 mA), brief (1-2 s duration) foot shocks. Like other stress protocols, electric foot shocks can be combined with anxiety tests.[125,126]

Social defeat stress

The social defeat stress (SDS) model was initially proposed by Klaus Miczec.[127] The SDS protocol consists of the introduction of a single mouse (known as the intruder) in the home cage of a resident male mouse (known as the aggressor).[127-131] During the test, behaviors related to confrontation of the intruder mouse by the resident aggressor is recorded. The time spent by an intruder mouse in social defeat posture induced by the presence of an aggressor is computed throughout five trials by a blind observer. Defeat posture is identified by the followed criteria: immobility (four paws on ground, oriented toward the aggressor), escape (escaping from the aggressor), crouching (four paws on ground, not oriented toward aggressor), or defensive upright stance (standing erect with forepaws extended).[127] The procedure can be used in acute or chronic stress protocols.[130,132]

Chronic unpredictable stress

The chronic unpredictable stress (CUS) model has been widely used to induce persisting stress-related behavioral changes in rodents.[133] It consists of randomly presenting different stressors to the rodents on a daily basis. This scheme prevents the stress adaptation process observed in other models of chronic stress.[134] In this model, animals are exposed for 2 to 5 weeks to a wide range of stressors, including foot shocks, restraint stress, light-dark cycle reversal, unpleasant noises, changes in the home cage (removal of sawdust, replacement of sawdust with water, heating [37°C] or cooling [4°C] of the home cage). After several days of exposure to this regimen, the animals exhibit a gradually increased HPA axis sensitivity and a decrease in responses to pleasant stimuli, without, however, any change in exploratory activity.[135]

This protocol has good face validity and seems to represent the stressors faced by humans in everyday life more realistically. Moreover, it has excellent predictive validity, since repeated treatment with antidepressants (fluoxetine, desipramine, or imipramine) is able to reverse the behavioral effects induced by this model.[36,136]

Conclusions

The number of stress and anxiety animal models currently available is significantly greater then when these models first entered research use 50 years ago. This means the choice of the most appropriate model for a specific experiment is not always a straightforward task. Ideally, this choice should be based on the hypothesis being tested, the design of the experiment, the experience of the investigator, and knowledge of the limitations

of the model. Particular attention should be paid to procedures that can control for false-positive or false-negative results and bias induced by local laboratory conditions. Some of these aspects have been addressed in the current review. Despite their drawbacks, animal models are invaluable tools for investigation of the neurobiology of anxiety- and stress-related disorders.

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Disclosure

The authors report no conflicts of interest.

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