

Effect of Estrous Cycle on Female Behavior in Anxiety

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Abstract: Women have anxiety problems at a higher rate than males do. The menstrual cycle adds another variation to women's lives; in fact, several illnesses, such as premenstrual syndrome, are cycle specific. Almost all animal models of anxiety and terror that are used to study pharmacological therapies have been created on males. There is still a dearth of work including women, and the material that is currently accessible paints an unclear image. One source of confusion is the female ovulation period, which is taken into account by some writers but not by many. Notably, the fast shifting hormonal profile during a rodent's 4-day cycle, there are no recognized, defined criteria for identifying cycle phase. Furthermore, because many behavioral tests with a learning component or that take into account the extinction of a previously learned association take many days to complete, the results may vary depending on the stage of the cycle on both test and training days. In this article, we examine how responsive females are in comparison to males in many widely used behavioral tests of fear and anxiety that were created in male mice. We find that although there may be strain and sex-specific differences in sensitivity, overall, female performance in most tests is qualitatively equivalent to that of male performance. Estrous cycle phase has a considerable impact on tests based on unconditioned threatening stimuli; animals exhibit enhanced response during the late diestrus phase of the cycle, which is comparable to the premenstrual phase in women. The estrous cycle appears to have less of an effect on tests that use conditioned fear paradigms, which have a learning component, however sex- and cycle-related differences in response are still discernible. In females, tests that are important to ethology seem to have greater translational value. Nevertheless, distinct brain pathways may mediate the same outer behavioral response even in cases where sex differences in behavior are not observed. Validating and standardizing experimental techniques for using female animal models of anxiety-related states is crucial to advancing basic research in the fields of female psychiatry and psychopharmacology.

Keywords: anxiety, female behavior, estrous cycle

1. INTRODUCTION

It is often known that women are far more likely than men to experience psychiatric diseases, including pathologies connected to anxiety. Additionally, women are more prone than men to have negative side effects from several psychoactive drugs [1-4]. Another important factor influencing psychiatric disease is the menstrual cycle. For instance, women with psychotic disorders have been found to experience perimenstrual exacerbations of their symptoms; admissions to psychiatric hospitals are more common during the perimenstrual portion of the cycle than during the non-perimenstrual phase [5]. Premenstrual
syndrome and premenstrual dysphoric disorder are two examples of anxiety-related illness states that are particular to the menstrual cycle, while panic disorder is an example of a condition where symptoms worsen during the premenstrual period [6]. It may come as a surprise, given the clinical result, that males have been used virtually exclusively in the development of animal models of fear and anxiety, which serve as the foundation for studies into pharmacological therapies for people. It’s shocking how much sex bias exists in neuroscience and scientific studies. More than 80% of behavioral pharmacology studies involving rats and mice that were published in five respected journals were found to have only employed male models in a 2007 assessment comparing these research [7]. After ten years, not much had changed (7), even though the NIH and more grant-awarding organizations across the globe were requiring research submissions to take gender disparities into account [8]. The cyclical change of female sex hormones during the estrous cycle is thought to present challenges and variability, which is a major reason why working with female animal models can be challenging. Because most steroid hormone molecules are lipophilic, they easily cross the blood-brain barrier, allowing the female brain to operate in a chemical environment that is continuously changing. It should be noted that sex hormones may also have an effect on male behavior, yet research that use male subjects to screen for drugs or identify the neurological or chemical causes of psychopathologies usually ignore this. For instance, testosterone, which affects male dominance, also positively affects the emission of 22-kHz sounds that are triggered by stress [9]. One tactic to address the cause of female hormone fluctuation has been to ovariectomise animals, which stabilizes hormone levels. Exogenous hormones can be carefully added back against a steady baseline to examine their impact on behavior and brain circuitry. This strategy has advantages since it has provided valuable insights into the effects of artificial hormone modification on behavior in addition to the genomic (nuclear) and non-genomic (membrane) actions of several neuroactive steroid hormones on cells [10]. However, neutering eliminates the core of what it means to be a woman by definition. After the procedure, the hormone levels drop dramatically, which could lead to negative behavioral changes. In female rats, ovariectomies can cause anxiety and depression-like behaviors [11, 12]. Hormone replacement treatment is really recommended for young, premenopausal women after surgical hysterectomy and/or oophorectomy, specifically to stop the onset of negative emotional states and cognitive deterioration [13]. There is growing evidence that response to anxiety-inducing substances, such as alcohol, might change over the estrous cycle [14–20]. Hence, a major prerequisite for the creation of focused pharmaceutical treatments for women is a knowledge of the alterations in brain neurochemistry that occur during the estrous cycle. When designing female animal models of psychiatric illnesses, it is important to take into account the effects of the estrous cycle on behavior. Nevertheless, the development of models sensitive to the estrous cycle stage is a prerequisite for progress toward this aim. The literature now paints an unclear picture. The choice of behavioral test and strain of rat or mouse, variations in living conditions, and experimental procedures are probably just a few of the factors contributing to variation throughout laboratories. Clearly, another major cause of diversity is the absence of widely recognized standards for staging the estrous cycle. The application of robust and adequately relevant animal models is a foundational aspect of clinical practice in psychiatry and psychobiology [20–22], particularly when it comes to the creation of sex-specific pharmacotherapy for the treatment of affective disorders in women. In this brief overview, we highlight some widely used tests of fear- and anxiety-related behaviors that were created and verified in male subjects. We take into account the scant data on female behavior in these tests, any gender disparities in response, and specifically if female estrous cycles affect female behavior.

2. LITERATURE REVIEW
Unquestionably, one of the main causes of the variation and unreliability of the results produced by various labs employing mice to simulate anxiety-related problems in women is the classification of estrous cycle stage. It is not practical to sample plasma hormone levels as a standard approach to determine gonadal state in behavioral investigations. On the other hand, as the reproductive system primes for pregnancy, variations in vaginal cytology correspond to increases in peripheral hormone levels. Vaginal cytology allows for the division of the cycle into several stages in females who are single and have not mated. This offers a useful, although not entirely accurate, proxy for the brain’s fluctuating hormonal composition. Samples for cytological analysis are taken from the vaginal wall using one of two methods: lavage (flushing the vagina with buffer or water) or cell sampling with the insertion of a probe. It is easy to distinguish between three types of cells: leucocytes, keratinized squamous cells, and lymphocytes, the proportions of which change during the cycle. The reader is directed to several very good descriptions and examples of different cell types [23, 24]. Proestrus, estrus, and diestrus—which is often separated into two phases—are the four unequally long stages of the cycle that may be distinguished based on the presence and proportion of distinct cell types. The transitions between these stages are gradual rather than step-like. Most laboratories collect a vaginal smear early in the morning and, for convenience, conduct behavioral experiments during the animal’s light period. Proestrus usually lasts for 14 hours, but within that time, the hormone profile rapidly shifts. In numerous labs, progesterone secretion in rats kept on a 12-hour on, 12-hour off light-dark cycle (lights on at 06.00 h) is relatively low from midnight (00.00 h on the day of proestrus) until approximately 15.00 h in the afternoon, when a rapid jump in secretion begins. Early in the dark phase, in the evening, progesterone concentration peaks before dropping quickly to basal levels by midnight (00.00 h) [25]. After progressively increasing over the preceding three days, estradiol reaches its highest concentration at noon (12.00 h), decreases, and approaches its basal level by late afternoon (24, 32). As a result, mornings are marked by high estradiol and low progesterone, while afternoons see a spike in progesterone secretion that results in the cycle’s greatest concentration while estradiol concentration rapidly drops [26]. The timing of behavioral trials on the day of proestrus warrants consideration as a potential source of variability, given the rapidly shifting hormone profile during proestrus. It has been demonstrated that abruptly stopping progesterone can cause GABAergic receptor subtype expression to become plastic, which can alter the excitability of brain circuits linked to anxiety [27]. To take advantage of the growing number of genetically engineered strains that can aid in defining the neurochemistry underlying emotional behavior, mice are being employed more and more for behavioral study. Nevertheless, compared to rats, it is far more difficult to infer a causal relationship between alterations in brain neuroactive steroid hormone levels and behavior in mice. Progesterone and its metabolites are secreted by the adrenal glands on a daily basis; these secretions are significantly greater than those of the ovaries and peak during the dark period. Furthermore, in contrast to rats, the amount of progesterone secreted by the adrenal glands also seems to be dependent on the stage of the estrous cycle [28]. Progesterone metabolism in the mouse brain is different from that in the rat. However, perhaps most significantly, variations in the endogenous brain progesterone supply, as opposed to external sources, are the primary cause of progesterone and its metabolite tetrahydroprogesterone (TH PROG) concentrations in the female mouse brain [29]. Standardizing the classification of estrous cycle stages is crucial in order to enable comparisons of cycle-related behavioral alterations reported by various laboratories. Numerous attempts have been made to create quick, impartial techniques, especially for usage with mice. These include the use of non-stained smear material, adjustments to staining techniques, and the categorization of smears using deep learning technology. On the basis of differences in vaginal wall impedance, changes in skin temperature brought on by the activation of brown adipose
tissue, and physical examination of the vaginal aperture, alternatives to vaginal cytology have been suggested [30]. The gold standard for determining the estrous cycle stage is still vaginal cytology, as none of the other approaches have been widely used to date. Avoiding managing stress is another significant source of unpredictability that must be taken into account. Elevated plasma corticosterone during vaginal lavage may result in spatial memory impairments. Even the acute stress of handling an animal that is not acclimated to the process causes an increase in progesterone levels in the brain in male rats [31]. In females, a comparable outcome might be observed. Smear collection, however, is not very stressful when done correctly, and the stained smears offer a permanent record that blind personnel may examine objectively. Nonetheless, there is still disagreement within research over how to classify the diestrus stage. For the sake of simplicity, we will refer to diestrus I in this article to encompass the phases known by various authors as metestrus, early diestrus, and diestrus I, with the disclaimer that standards for designating these stages may vary throughout laboratories. Stages referred to as late diestrus or diestrus 2 are included in Diestrus II. Diestrus describes research when no differentiation has been made between phases. A widely accepted consensus on cytological criteria for estrous cycle staging is obviously urgently needed in order to make it easier to compare data from various laboratories. There is an adaptive advantage to the emotional states of anxiety and terror. Novel stimuli and events can pose a hazard to an individual’s survival. The most practical course of action for small prey species is typically to flee or, in the event of detection, to become motionless. If this tactic is used arbitrarily in response to every new stimulus, it becomes maladaptive because it interferes with other survival-related tasks like foraging and mate-finding. Rather, the animal must exhibit a certain level of alertness to identify changes in its surroundings, evaluate the danger and threat, and then determine whether to alter its habitual behavior [32]. Animal behavioral tests designed to induce fear and anxiety are often predicated on two principles: (i) The animal flees from danger because of fear. It involves the flight/flight/freeze response, which is elicited more frequently by clear-cut, immediate/proximal threats, like a predator encounter; (ii) Anxiety is a preemptive reaction to potential future threatening events, particularly when there is a conflict between competing goals, like avoiding a potential threat and being drawn to food. It might manifest as actions like interrupting regular routines and engaging in risk-assessment behaviors, which include monitoring the area and being extremely alert to any threats [33]. In general, there are two main subclasses of animal models of anxiety and fear. The first is composed of ethologically grounded paradigms that rely on an animal’s spontaneous or natural (unconditioned) reactions (such as flight, avoidance, freezing, and risk assessment) to stress stimuli that do not directly cause pain or discomfort but instead pose a threat to survival (such as exposure to a new, brightly lit test chamber or a predator). The second consists of the conditioned reactions that animals develop after being subjected to traumatic and frequently unpleasant situations (such as electric footshock). The EPM is the most used animal model for examining the pathophysiological causes of anxiety, as well as for testing mouse genotypes and anxiety-modulating medications. With about 8700 papers published as of May 2021 on PubMed, the NIH National Library of Medicine, and nearly 40 years of use, the EPM is still the gold standard by which other behavioral tests for anxiety are measured, at least when it comes to males [33]. Nevertheless, several people have voiced concerns over the EPM’s use [34]. Even though it is widely used, not many research have looked at how different sexes behave differently or how the female estrous cycle affects things. Based on the scant data that is currently available, it is generally agreed upon that adult female rats exhibit lower overall anxiety levels while behaving in an EPM qualitatively similar to males. Interestingly, older females were shown to be more worried than males, whereas sex differences were not evident in young adult mice (C57BL/6NIA strain) [34]. The picture becomes less obvious when the estrous cycle is
factored in. Some researchers find that rats in proestrus/estrus have lower anxiety levels (i.e., spend more time in the open arms of the labyrinth) than in diestrus, while others find no evidence of any estrous cycle-related effects. Direct comparison between studies is challenging, though, because not all researchers compare every stage of the cycle, and in certain situations, data from two stages of the cycle have been combined. A similarly muddled picture is painted by the findings of research conducted on mice. Some studies found that mice in estrus spent longer time on the open arm than mice in diestrus, while others found that proestrus mice showed more open arm entries and spent more time on the open arm of the EPM than diestrus females or males. Still other studies were unable to find any differences in performance between diestrus II and proestrus (defined as non-receptive stages) and diestrus I and estrus (defined as receptive stages) [35]. Numerous factors that may affect responding in males have been identified, and methodological variations may result in inconsistent results between laboratories. It has been emphasized how crucial it is to identify and precisely manage testing circumstances, especially light level. It has been documented that the behavior of male rats is influenced by the age of the rat, the circadian phase, and the amount of light present during testing [36]. Women’s responsiveness has not been examined to the same extent. Nonetheless, there is some data that suggests circadian phase, light level, and strain—especially in mice—may have an impact on response. The challenge of evaluating the impact of the estrous cycle is exacerbated by the fact that, in many instances, only two phases of the cycle—proestrus and diestrus—have been compared, with no consideration for substages. In other studies, however, data from two phases—proestrus and estrus, for example—are combined, making it impossible to evaluate the impact of a single cycle stage. There’s no agreement as of yet. Because it incorporates two defensive strategies—(1) inhibitory avoidance, which occurs when an animal is in the enclosed arms and does not enter an open arm, and (2) one-way escape, which occurs when an animal retreats from an open arm in search of the enclosed arm—the EPM, which was previously discussed, can be seen as a mixed model of anxiety/panic. The test allows the measurement in the same rat of an approach-avoidance conflict-type response: inhibitory avoidance, which is related to anxiety, and an escape response, which is related to fear/panic. The unstable balance between the expressions of these two types of responses could explain the inconsistent drug effects in males, mainly 5-HT-modulating compounds, frequently reported for the EPM [37]. The rat cannot see the open arms when it is positioned at the end of the enclosed arm until its head protrudes past the arm's walls. When the animal is regularly placed at the end of an enclosed arm and given the freedom to explore the maze, it will develop inhibitory avoidance due to the aversive nature of the open arms. However, if the rat is positioned at the end of an open arm, it may move in the direction of the safer enclosed arm. This is known as a one-way escape, and it is connected to panic and fear attacks. Typically, the delay to exit the open arm remains constant throughout subsequent trials, in contrast to what occurs in the enclosed arm. Anxiolytic medications (such as buspirone and diazepam) affect escape expression but not the acquisition of inhibitory avoidance. Escape expression is inhibited by antipanic medications such as imipramine or fluoxetine, as well as strong benzodiazepines like clonazepam and alprazolam [for a detailed description of the test see [37]. There are no sex-related variations in Wistar rats' acquisition of inhibitory avoidance or expression of escape, according to the few comparisons between male and female rats in this test. On the other hand, Sprague-Dawley or Long-Evans female rats exhibit a lower anxiety phenotype due to a deficit in avoidance learning when compared to males. Thus, it appears that rat strains may differ in how well they do in the test. One study found that female Wistar rats in diestrus II are marginally more reactive to the open-arms than males (i.e., they take longer to exit the enclosed arm, but only in the first trial), suggesting a higher anxiety level in relation to the influence of estrous cycle phases. A recent
investigation using the same breed of rats did not, however, reproduce this slight impact [38]. Since laboratory-based anxiety tests are artificial by design, they are unlikely to capture all aspects of emotionality and, when taken in isolation, cannot give a whole picture of an animal's emotional profile. In order to get over this restriction, it has been suggested that, at least for men, employing a battery of tests could yield a more accurate measurement [39]. In general, female rats have been observed to exhibit higher levels of activity in the open field when compared to their male counterparts. This includes more ambulatory and rearing activity, reduced defecation, and a perceived decreased anxiety level while approaching the center zone. There are numerous instances in the literature where there is no behavioral difference between the sexes, hence the data is far from consistent. A retrospective study of mice's performance in the open field also found that male and female mice performed similarly. Strain differences in male mice have been shown for both anxiety measured by the EPM and locomotor activity in the open field; sex variations in response are noticeable in some strains but not in others [40]. Including data from all females in one pool runs the danger of hiding potential responses related to estrous cycle stage. Still, the results are inconclusive when the estrous cycle is considered. Rats in proestrus, for instance, exhibited more anxiolytic behavior than rats in later stages. On the other hand, no variations were found in fear or anxiety-like behaviors in proestrus versus diestrus rats. Comparably, proestrus wildtype mice BALB/cBy have been reported by some researchers to make more entries and spend more time in the central zone of the open field than their diestrus (stage not subdivided) counterparts (26, 68). However, other researchers who used C57BL/6J mice discovered that the mice's behavior in the open field remained consistent throughout all four phases of the cycle [41]. A new enlightening study evaluating rat behavior in an open field has shown how several variables can acutely regulate each other in various circumstances, underscoring the significance of taking each of these elements into account. Estrous cycle effects were found to be obscured by novelty. Rats perceive white light to be unpleasant, thus it is not surprising that estrous cycle-related effects were not visible in tests done in this environment, but they were visible in tests done under dim red illumination (108). The size of the field appears to be another element that affects response. In a huge open field measuring 129 × 120 × 60 cm with dim red light at 18 lux, female rats entered and stayed in the centre zone more frequently than male rats (60). Smaller venues (70 × 70 × 70 cm, light intensity not specified, and 54.5 × 80 × 33 cm, dim red light 18 lux) were used, yet no sex difference was found in the entry into the middle zone or the distance walked. These seemingly contradictory results raise serious concerns. Large arenas, however, can be seen as a more dangerous challenge than tiny ones due to laboratory rats' poor vision. Therefore, a true sex difference in inherent anxiety level may be the reason for females' greater level of exploratory behavior in big arenas compared to males. Contradictory results have been reported about sex differences and estrous cycle-linked effects on anxiety-like behavior in the same animals exposed to the EPM and the big open field when the open field test is integrated into a battery of tests intended to assess fear and anxiety. Crucially, an influence from the estrous cycle on behavior in the open field does not always translate into an animal's behavior in the EPM [42]. The findings cited above paint an unclear picture. The lack of agreement about sex differences or the impact of the estrous cycle is probably mostly due to methodological discrepancies between laboratories, given the multitude of factors that can affect responding in the open field and the EPM. In both the open field and elevated plus maze tests, it is clear that female behavior is qualitatively similar to that of male behavior, although it is unclear whether there are any differences in response between the sexes. According to the research that have identified sex differences, anxiety levels have generally been lower in women than in men. This finding is entirely at odds with human data, where women are more likely than males to develop anxiety-related pathological
states, and their symptoms are frequently associated with their menstrual cycles. Few studies have examined the effect of cycle phases on female reaction, despite the fact that a fair number of studies in the literature have compared the behavior of males and females in these tests. Most research using either rats or mice has not been able to demonstrate variations in sex in this test. Direct comparisons between strains and/or animal age, which are crucially important variables, were carried out in some of these investigations, but no sex-related effect was discovered. However, a few data indicate that women experience anxiety to varying degrees compared to men. In terms of cycle stages, female rats exhibit reduced anxiety during proestrus or estrus+proestrus in comparison to other phases or while interacting with males. In the single study that is currently available, proestrus and estrous stages in mice were found to have lower levels of anxiety than diestrous or males. It is evident that the influence of a fluctuating hormone profile during the estrous cycle might obscure significant sex differences when pooling data from females is used. In order to organize social interactions and to indicate whether they are in a happy or negative emotional state, rodents make a variety of ultrasonic cries [43]. Male rats in a semi-naturalistic setting (visible burrow system) are stimulated to produce high frequency ultrasonic vocalizations (USV) at around 22 kHz when they are in the company of a domestic cat, their predator. Given that far fewer sounds are made if the encounter with the predator takes place while the rats are separated from their social group, it is believed that the 22-kHz USVs serve as a warning to conspecifics. While female rats also produce 22 kHz USVs, they do so more frequently and for longer periods of time than male rats. It’s interesting to note that in comparable dangerous circumstances, mice—who are also eaten by cats—don’t cry out like this. Adult mice use USVs for communication, although they do so at different frequencies and mostly during social interactions. Emission of 22 kHz USVs is one of the stimuli that have been found to elicit innate protective escape responses in laboratory-bred rats. These consist of air puff, forced swimming, minor constraint stress, overhead looming stimuli that mimic an aerial attack, and inevitable acute or recurrent footshocks [43]. It is often accepted that the 22 kHz USVs released in these environments represent a negative emotional state similar to fear and anxiety [43]. Female rat USVs are still relatively unrecognized in comparison to the enormous body of literature on 22 kHz USVs produced by male rats in lab-based experiments. According to the few research that is currently available, women might not be as receptive as men. Surprisingly, male and female rats responded differently to air puff stress; yet, the amount of freezing induced by the identical stimulus was the same for both sexes. In comparison to male Wistar rats, female rats underwent a brief period of non-noxious confinement stress and produced significantly less 22 kHz sounds. However, there was a noticeable impact of estrous cycle stage within the study’s female population. Very few calls were made by females during their proestrus, estrus, and early diestrus (diestrus I). However, during the late diestrus stage (diestrus II), noises increased five times and reached a level similar to that of males. Inagaki and Mori were similarly unable to distinguish between the 22 kHz USVs that rats emit during proestrus and diestrus I in the air puff test. Nevertheless, it is impossible to determine if the estrous cycle affected this test because responsiveness during other phases of the cycle was not examined. These results, however, are at odds with observations that female Long Evans rats in a semi-naturalistic setting (the visible burrow system) were more likely than males to cry out when a predator was around. It’s unclear if this is due to a variation in strain or the impact of the living environment [44]. The picture becomes less obvious when the estrous cycle is factored in. Some researchers find that rats in proestrus/estrus have lower anxiety levels (i.e., spend more time in the open arms of the labyrinth) than in diestrous, while others find no evidence of any estrous cycle-related effects. Direct comparison between studies is challenging, though, because not all researchers compare every stage of the cycle, and in certain situations, data from two stages of the cycle
have been combined. A similarly muddled picture is painted by the findings of research conducted on mice. Some studies found that mice in estrus spent longer time on the open arm than mice in diestrus, while others found that proestrus mice showed more open arm entries and spent more time on the open arm of the EPM than diestrus females or males. Still other studies were unable to find any differences in performance between diestrus II and proestrus (defined as non-receptive stages) and diestrus I and estrus (defined as receptive stages) [35]. Numerous factors that may affect responding in males have been identified, and methodological variations may result in inconsistent results between laboratories. It has been emphasized how crucial it is to identify and precisely manage testing circumstances, especially light level. It has been documented that the behavior of male rats is influenced by the age of the rat, the circadian phase, and the amount of light present during testing [36]. Many studies have demonstrated that respiratory stressors, such as being in an environment with high or low CO2 concentrations, cause panic episodes in people; these stimuli are widely employed as test subjects in the investigation of panic disorder. There is strong evidence that connects panic disorder to breathing problems, even if the pathophysiological mechanisms behind this mental health issue are yet unknown. It has been more difficult to simulate panic episodes in experimental animals using respiratory challenges, and the outcomes cast doubt on whether a panic-like condition was actually induced in these non-human individuals. Generally speaking, many indicators, mainly autonomic indexes (i.e., arterial blood pressure, heart, and respiration rates) have been employed in these analyses, which were usually carried out in male rats and mice, to infer that a severe fear response, and consequently a panic-like condition, was triggered. Some research has also been done on the behavioral effects of hypoxia or CO2 inhalation, but oddly, this research has typically been conducted after, not during, exposure to the respiratory difficulties. Although studies have been conducted to determine if variations in these cardio-respiratory indices are impacted by the estrous cycle or sex, their findings have not been definitively examined [45]. According to research conducted in our lab, Wistar male rats exposed to acute hypoxia (7% O2) exhibit an escape response akin to panic (i.e., jumping upward to the experimental cage's border), which is lessened when the rats are given common panicolytic medications like fluoxetine and alprazolam. Additionally, we found that these medications have an equivalent impact on the quantity of escape attempts performed by mice when exposed to a high CO2 concentration (20%), confirming the validity of these two behaviorally-based tests for the investigation of panic episodes in male rodents. We have validated the hypoxia model for usage in females recently as well. We found that 7% O2 exposure causes male and female Sprague Dawley rats to exhibit escape behavior akin to panic. Reactivity to this respiratory challenge, however, varied markedly depending on the stage of the estrous cycle in females, with diestrus II showing a much larger response than other cycle stages or men. Since women with panic disorder suffer an increase in anxiety and panic symptoms during the premenstrual period of the menstrual cycle, which corresponds to diestrus II in mice, this study has significant translational value. It is common practice to evaluate protective behavior in rats by exposing them to predators or stimuli associated with them (such as predator odor) [44]. Research in this area has long been influenced by the significant ethoexperimental experiments carried out by Caroline and Robert Blanchard. These researchers examined the pattern of defensive behavior displayed by male and female rodents exposed to these realistic dangers using two clever test batteries: the Fear/Defense Test Battery (F/DTB) and the Anxiety/Defense Test Battery (A/DTB). The later battery looks into responses to prospective threats (like cat odor), like risk-assessment behaviors. The previous battery provided information on the defensive behaviors that rats have shown up to now, approaching predators (a live cat), such as flight/escape, freeze, and defensive assault. Overall, their findings indicate that when faced with these cues, females are
more protective than males. This tendency is especially prevalent when the threat is hypothetical rather than real or immediate. In reaction to cat scents, for example, females exhibit greater risk-assessment and avoidance responses than do males. It is interesting, nevertheless, that other groups have also reported contradictory findings. According to Perrot-Sinal et al. [44], male and female rats exposed to cat odor displayed higher expressions of risk-assessment behaviors; however, the frequency of these actions was significantly lower in the female rats. Nevertheless, females showed a higher frequency of these behaviors than males when animals were subjected to prolonged restraint stress before testing. This suggests that the animals' baseline levels of stress and anxiety prior to the test may have an impact on how they react to stimuli associated with predators. Conversely, rats exposed to a different predator's odor stressor (trimethyl thiazoline, the primary constituent of fox feces) expressed more defensive behaviors, such as risk-assessment and defensive burying, in a sex-independent way [45]. Remarkably, sexually dimorphic alterations in cell growth and death in the hippocampus dentate gyrus were concealed by identical behavioral responsiveness [46]. More recently, [47] examined how the stages of the estrous cycle affected the protective reactions, both conditioned and unconditioned, of female rats to the smell of cats. When exposed to a cloth infused with cat odor, rats in diestrus 2 showed noticeably higher levels of risk assessment reactions than those in estrus or proestrus. Aversive learning was demonstrated when the animals were reintroduced to the odor-presenting cage 24 hours later, with a control cloth in place of the cat odor, which acted as a stimulus-paired cue. This was done to examine the conditioned responses to the experimental context/cue, and it was found that the animals previously exposed to the cat odor were significantly more defensive, spending more time avoiding the cue and engaging in risk-assessment activities. The cycle phases had no effect on the learnt response, in contrast to the initial exposure (unconditioned response). Last but not least, it is noteworthy that research has demonstrated that, in addition to the effects of predator odors on defensive behaviors, exposure of weaning female rats to cat odor for ten consecutive days interferes with the hypothalamic-pituitary gonadal axis' maturation, delaying first estrus and vaginal opening in addition to upsetting the estrous cycle [47]. The Vogel test is predicated on the approach-avoidance conflict that rodents experience between an appetitive impulse to drink water during a period of dehydration and a fear of doing so because the animal will be shocked with electricity if it consumes any more water. This test was first developed in 1971 and has been extensively used to identify the pathophysiological causes of anxiety as well as to screen for anxiolytic medications. Anxiolytic medications, including the benzodiazepines diazepam and chlordiazepoxide, regularly increase the frequency of penalised reactions, as is mostly suggested by research conducted on male subjects. Few research have explicitly compared the conduct of males and girls in this test, as is the case with other models. Overall, compared to male rats and mice, females show less penalized responses, which may indicate a higher level of fear. Evidence suggesting that female rats may be more sensitive to pain than male rats, perceive a lower shock threshold, and have fewer punished drinking responses has called into doubt this result. The latter result, which denotes a distinct baseline water intake, was noted following adjustment for both sexes' body weight—a significant and sometimes disregarded confounding factor [48]. Based on the correlation between particular stimuli (cued or contextual) and stressful and frequently painful occurrences (e.g., electric footshock), there are two widely used indices of fear responses in male animals. Rats are trained to link an unpleasant unconditioned stimulus (US; footshock) with a conditioned stimulus (CS), usually light or music, in conditioned fear responses (CF). After then, the animals are exposed to the CS alone once more in a different setting. It is consequently assumed that freezing in reaction to the CS is a sign of conditioned fear. A related test called fear potentiated startle (FPS) gauges the amplified impact of a CS presentation that has previously been combined with an
unpleasant US (footshock) on the startle response to a loud sound. Overall, female rats do qualitatively similarly to male rats in both tasks, and while some researchers report that females are less receptive than men (200–203), others have not been able to identify sex differences [49]. In mice, also, conflicting results have been seen. For instance, depending on the strain and exact experimental protocol, there may not be a gender difference in contextual fear conditioning; females have been reported to exhibit stronger context fear conditioning and greater generalization to a similar context than males, while males exhibited a faster rate of extinction for conditioned freezing to a tone. Females showed higher freezing behavior than males when using a serial compound conditioned stimulus (tone and white noise that evokes distinct transitions between freezing and flight behaviors within individual individuals); nevertheless, there was no difference in flight behavior between the sexes [49]. The consensus from the few research that are currently available when estrous cycle phase is taken into account is that it has no effect on conditioned fear to context or the expression of fear-potentiated startle, while a more recent study offers a different perspective. Conversely, Milad et al. [50] discovered that extinction training during the proestrus phase (high estrogen/progesterone) was more fully consolidated, as seen by low freezing during a recall test, in tests of cued fear (conditioned freezing). Some researchers found that rats in diestrus II or in the diestrus phase had less extinction during training than rats in proestrus. This means that during the test session, rats in diestrus II remained responsive to presentations of the unreinforced CS, but in proestrus, the response quickly vanished. When administering exams with a learning component, keep in mind that they usually require two or more sessions and take many days to finish. This implies that females may undergo conditioning during one phase of their estrous cycle and then be tested during a different phase on a different day. It is possible that responsiveness during the test session will be impacted by cycle stage during conditioning, and vice versa. The training or testing components of the conditioned fear paradigm do not appear to be greatly impacted by the estrous cycle phase, according to the few research that have addressed this issue [51]. However, gonadal state does have an impact on fear-induced startle. Although performance improved as the test session went on, females who had been trained in proestrus or diestrus II of the previous cycle and were tested in proestrus initially didn't seem to be able to discern between a positive and neutral conditioned stimulus. High amounts of circulating estrogen are associated with proestrus, and it has been demonstrated that estradiol enhances fear generalization to context. Rats examined during proestrus may appear to have failed in discriminate learning, although this may really be the result of generalization to positive and negative conditioned stimuli rather than a learning deficit [51]. In a recent study, female rats in diestrus I or II exhibited significantly lower safety memory compared to females in the proestrus or estrus phase, using startle training within a fear safety conditioning paradigm [51]. It's intriguing to wonder why the estrous cycle stage influences some conditioned fear tests but not others. Unlike other widely used tests of fear, fear potentiated startle measures amplification rather than suppression of ongoing activity. It's possible that this element makes the test more susceptible to the impacts of female hormone variations. Nonetheless, the aforementioned instances highlight the need of drug testing for both genders as well as the selection of behavioral tests. Furthermore, they emphasize that failing to include the impact of the female estrous cycle could result in incorrect interpretation of results from various behavioral tests. The eradication of conditioned fear reactions has received a lot of attention in recent years. Deficits in the ability to eradicate conditioned fear in people after repeatedly seeing an unreinforced stimulus are thought to be a contributing factor in the development of anxiety disorders. Compared to healthy controls, anxious people exhibit greater increased dread in response to a CS during extinction. Even after receiving extinction training, patients with posttraumatic stress disorder (PTSD) also maintain a strong
conditioned fear response. Fear extinction in animals, which is defined as the reduction in conditioned fear responses following repeated exposure to an unreinforced conditioned fear stimulus, may thus offer a helpful model for comprehending the psychopathology behind anxious states. PTSD may be primarily caused by deficiencies in the extinction of fear memories and how these affect how one interprets and reacts to sensory events in the future. However, it is important to remember that extinction, as defined by conventional Pavlovian theory, refers to the progressive loss of the conditioned response brought on by the conditioned stimulus (CS) not being reinforced. The person with PTSD typically does not relive the same CS. Instead, they seem to extend to the initial traumatic experience, causing other stimuli to serve as a CS and set off an unpleasant response. However, extinction recall/retention after fear conditioning has become more popular in PTSD models based on conditioned fear. Women experience PTSD twice as frequently as men do, and research suggests that the menstrual cycle phase affects extinction retention in women. It is crucial—and impossible to emphasize—to include females in animal experiments on fear of extinction. Extinction recall in rats can be influenced by the phase of the estrous cycle that occurs before extinction training (i.e., measuring the reaction to the unreinforced CS 24 hours after training). When extinction learning occurred in diestrus I, rats showed a lower retention of extinction than when extinction learning occurred in proestrus. It is becoming evident that different processes may mediate the same external behavioral response even in cases when sex differences in behavior are not observed, as more and more females are being increasingly included into experimental methods. Male and female mice used in a relevant study that used conditioned fear reported comparable degrees of fear extinction. But behavioral similarities across the sexes concealed variations in the underlying pharmacological. Administering a presynaptic GABA B receptor antagonist improved fear extinction and renewal in males, but had no effect on females. In a similar line, considerable overexpression of the early gene ARC was found in the bed nucleus of stria terminalis in male rats but not in female rats, even though no sex differences could be established in freezing recall in rats evaluated in a contextual fear paradigm. Rats have also been shown to exhibit sexual dimorphism in relation to the role of endocannabinoid pathways in the extinction of conditioned fear [51]. Traditional rodent tests of fear and anxiety behavior only provide a "snapshot" of the behaviors associated with these emotions; they evaluate certain behaviors (like freezing) across short sampling times and in a synthetic laboratory environment. The quest for more ethologically appropriate environments to research fear and anxiety-like behaviors has been sparked by this constraint. Animals in the Risky Closed Economy (RCE) are kept in solitary housing but live in a semi-naturalistic setting free to forage for food and drink by pressing a lever in a designated area. To simulate the risk of predation, an unidentified, unpredictable hazard (footshock) is placed into the feeding zone. This test, it may be argued, could provide a more comprehensive understanding of the everyday effects of anxiety and terror because a wide range of variables' data can be automatically and constantly collected over the course of several weeks to months. The ability to track the same animal at various phases of its estrous cycle is another benefit of using the RCE on females. Regarding their behavior when foraging in the RCE, female rats exhibited greater dread than male rats. Furthermore, the proestrus period was linked to higher risk-taking, suggesting that the estrous phase influenced riskier foraging decisions. This discovery is noteworthy because, in contrast to the majority of behavioral tests that use scenarios that are less relevant to ethology, the elevated level of anxiety or terror shown in female rats in the RCE during the diestrus phase is consistent with human experience. It is becoming more and more obvious that the brains of men and women do not always use the same neurological mechanisms to produce the same behavioral outcomes. Furthermore, evidence demonstrating sex variations in drug responsiveness as well as a variable reactivity within females based on the stage of the estrous cycle is
accumulating as females are progressively incorporated into drug testing regimens. It has long been known that there are gender variations in how the traditional anxiolytic benzodiazepines affect the body. It’s commonly believed that men are more responsive than women. The outcomes, though, need to be interpreted cautiously. For instance, rather than a distinct reaction to the medication, it was discovered that the high baseline activity levels observed in females were the cause of the lack of female responses to the effects of diazepam in the EPM. These results show that in order to enable clear-cut result extrapolation, baseline behavior variations between the sexes must be taken into account. Although no sex difference in brain concentration was identified, at least not in Long Evans rats, sex and strain differences in benzodiazepine metabolism have been documented, which may possibly bias results [54].

The estrous cycle has an effect on benzodiazepine reactivity as well, albeit the results are unclear once more. The majority of workers don’t look into every phase of the cycle, which results in incomplete data sets. Certain rat investigations failed to find any variations in diazepam response related to the estrous cycle. Nonetheless, the general agreement in the EPM is that, in contrast to diestrus, particularly diestrus II, rats and mice are more responsive to diazepam during the proestrus/estrus stages. Similarly, Rodriguez-Landa and colleagues [18] discovered that diazepam had anxiolytic effects in female Wistar rats during their proestrus or estrus phases, but not during their diestrus phase, in the light-dark transition test. This could be the result of proestrus having a higher level of diazepam binding to brain membranes than the other cycle stages. Various behavioral experiments have also found variations in response to various anxiolytic medications that are related to sex and the estrous cycle. While there was no gender difference in the effects of the serotonin and noradrenaline reuptake inhibitor sibutramine in rats tested in the EPM, sibutramine inhibited escape expression (latency to leave the open arm) in both sexes but impaired inhibitory avoidance (withdrawal from the enclosed arm) in the ETM in males but not females. The antipanic-like effect of the medication on escape performance was shown to be absent in females in diestrus II but preserved in the other cycle phases when the estrous cycle was taken into account [54]. In the Vogel conflict test, the sensitivity of male and female rats to the effects of anxiolytic medications varies as well. While an increase in punished response was seen in both sexes following acute administration of diazepam and chlordiazepoxide, only males showed signs of anxiolysis from buspirone, fluoxetine, paroxetine, or propranolol. Furthermore, compared to male rats, female rats appear to be more susceptible to the sedative effects of buspirone and chlordiazepoxide [54]. In a different recent study, female rats in diestrus I and II exhibited considerably less safety memory than females in the proestrus or estrus stages when startle was used in a fear safety conditioning paradigm. Applying oxytocin intranasally could correct this difference, however surprisingly, it had no effect on males. The commonly used serotonin reuptake medication fluoxetine is susceptible to changes in estrous cycle stage and sex. In male mice, but not in female mice, chronic fluoxetine reduced inhibitory avoidance in a one-trial step-through test. Chronic (14-day) dosing of rats decreased fear responses in female rats during diestrus I and II during extinction learning and extinction recall, but not in male or proestrus/estrus female rats. It usually takes a long time for fluoxetine to start having anxiolytic effects. On the other hand, regardless of sex, acute administration has anxiogenic effects. Fluoxetine can, however, be anxiolytic in females at low doses that are subthreshold for its effects on 5-HT systems; this effect is contingent on the stage of the estrous cycle. The rise in unconditioned dread that is typical of this stage of the cycle was fully reversed by administering a low dosage of fluoxetine during diestrus 2. Therefore, fluoxetine in diestrus 2 reversed the rise in ultrasonic vocalizations caused by restraint stress, escape behavior induced by hypoxia, and hyperalgesia induced by vibration stress that are characteristic of this stage of the cycle, but it had no effect when provided at other phases. Additionally, fluoxetine corrected the elevated
excitability of the panic circuitry in the periaqueductal gray matter that arises during diestrus 2 and brought back the EPM’s sensitivity to diazepam. These benefits are believed to be due to fluoxetine’s quick steroid-stimulating qualities, which increase allopregnanolone concentration in the brain and counteract the abrupt natural drop that happens at diestrus 2 [55].

3. CONCLUSION

Based on existing evidence, most behavioral tests used to measure fear and anxiety in male animals show qualitatively similar responses from females as compared to men. Overall, it may be inferred from female conduct in "male" models of dread and anxiety that women experience anxiety at lower levels than men. However, this conclusion runs counter to clinical practice, which shows that women are more likely than men to suffer from anxiety-related problems. However, it’s important to keep in mind that the frequently employed animal experiments replicate the adaptive states of fear and anxiety rather than the psychopathology that underlies human anxiety disorders including panic disorder, generalized anxiety disorder, and post-traumatic stress disorder. When examining the biological underpinnings of anxiety behavior, it’s possible that females' lower intrinsic (baseline) levels of worry relative to males are typical in rodent cultures and shouldn’t be taken into account. Because animal tests primarily use locomotor data, the overall higher activity level in females may skew the results. Nevertheless, a recent meticulous examination of locomotor activity in three anxiety tests—open field, social interaction, and EPM—failed to find a significant impact. Rather than exhibiting reduced anxiety, it’s possible that female rats exhibit distinct anxiety-like behaviors that are not adequately represented by the testing protocols that have been established and refined utilizing male rats. Therefore, in order to assess the same emotional states in females, the readouts of many popular behavioral tests that were designed and validated in male animals may need to be modified. One example is the traditional fear conditioning paradigm, in which animals freeze in reaction to a context that indicates footshock or a conditioned stimulus. Males froze more frequently than females generally, although some females were more prone to “darting” behavior, which was not related to general hyperactivity. One should exercise caution while analyzing the behavior of females in the classic "male" animal studies and take into account how closely the present animal models of fear and anxiety resemble similar human emotions. Indeed, since the unnatural tasks carried out by rodents housed in standard laboratory conditions may not accurately represent the behavior of the animals in the wild, where survival challenges and living conditions differ significantly, it has been questioned whether current rodent fear conditioning studies actually take place in the natural world. Because of the well-established effects of housing settings on the brain and behavior of rats, for instance, experimental housing arrangements for rodents have incorporated varying degrees of enrichment. Experimental research paradigms have successfully integrated an ethological perspective on fear in humans. In this regard, research on animals lags behind; nevertheless, the use of the visible burrow system, which was initially introduced thirty years ago and allows for the study of rats' behavior in mixed-sex colonies, was an early indicator of how the environment affects behaviors related to fear. Since anxiety-related psychopathology is significantly more common in women than in men, the fact that females exhibit lower levels of anxiety in "male" models raises serious concerns about the translational validity of the majority of currently utilized tests. However, females look more nervous and risk averse than males in more ethologically relevant settings, such as the dangerous closed economy, an open field with cover, or when living in a conspicuous burrow system.

During the premenstrual phase, when progesterone is rapidly decreasing and estrogen is low, anxiety, fear, and avoidance feelings tend to rise in women with anxiety disorders, including panic and PTSD.
this regard, it is relevant to note that rat responses to tests of unconditioned fear behavior closely resemble clinical experiences. This is especially true given research showing that women's menstrual cycles primarily affect emotions and have little effect on cognitive function. While the cycle has varying effects in experiments using conditioned threatening stimuli, which have a learning component, in female rats, unconditioned fear is greatly heightened in diestrus 2 (akin to the premenstrual period in women). It's possible to interpret the negative symptoms of the late luteal (premenstrual) phase as an unwarranted overreaction to common psychological stressors, which don't cause an undesirable response in other phases of the cycle. The pathophysiology of premenstrual dysphoric disorder, which manifests as affective symptoms and poor regulation of the physiologic stress response, is thought to be rooted in impaired GABAA-receptor response to dynamic fluctuations in allopregnanolone across the menstrual cycle. This theory is supported by the clinical literature. It is impossible to overestimate the significance of incorporating females into all drug development procedures, from fundamental research with animal models through human clinical trials. To make it easier to find and characterize new anxiolytic drugs for both sexes, there needs to be more standardization of experimental psychopharmacology procedures. Although most behavioral experiments designed to simulate fear or anxiety in male mice seem to produce qualitatively identical responses from females, it is becoming more evident that male and female brains do not always use the same neural processes to produce the same behavioral output. Moreover, the shifting chemical environment of the brain during the estrous cycle may have an impact on a female's behavioral response and pharmacological effect. Development of new drugs must take into account the psychopharmacology of women and take adequate behavioral testing into account.

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