Article

The Difference between Male and Female Rats in Terms of Freezing and Aversive Ultrasonic Vocalization in an Active Avoidance Test

Pavel Alexandrov, Maria Pupikina, Zabava Adaeva and Evgenia Sitnikova *

Institute of the Higher Nervous Activity and Neurophysiology of Russian Academy of Sciences, Butlerova Str., 5A, 117485 Moscow, Russia; mariapupikina@yandex.ru (M.P.)
* Correspondence: eu.sitnikova@ihna.ru

Abstract: We examined sex differences in behavioral responses to fear-related learning through ultrasonic vocalization. Adult male and female rats of two inbred rat strains (WAG/Rij rats with genetic predisposition absence epilepsy, \(n = 25\); and the minor substrain NEW with non-epileptic phenotype, \(n = 32\)) were examined in the two-way active avoidance task (50 trials). Three behavioral responses were defined: successful learning, unsuccessful learning, and freezing (motionless behavior). We found that males were more likely to experience freezing (40%) than females (3.7%). Females performed significantly better than males: 93% of females showed “successful learning”, while only 43.2% of males did. Long-lasting 22 kHz ultrasonic calls, so-called aversive ultrasonic vocalizations aUSVs, were recorded in 26% of females (only successful learners) and in 50% of males. The density of aUSVs in females was lower than in males. WAG/Rij males performed much poorer than the NEW males. In general, males, in contrast to females, showed significantly poorer avoidance learning, more frequently experienced freezing, and produced more aversive USVs. Males thus experienced a negative emotional state, rather than exhibiting poor cognitive abilities. Perhaps, WAG/Rij rats and a minor NEW substrain showed an increased emotionally in fear-related tasks, because they are prone to emotional disorders or emotional exhaustion. Sex differences in freezing and in aUSVs might result from the poorly understood neuromodulatory effects of the cholinergic system.

Keywords: fear conditioning; adult rats; behavioral response; aversive ultrasonic vocalization; 22 kHz calls; Sonotrack

1. Introduction

Active avoidance is a classic behavioral paradigm that is widely used to study conditioned learning in animals and humans [1–4]. In the active avoidance paradigm, a subject learns to associate a negative outcome with an event that typically precedes it. Avoidance has been a longstanding technique since the late 1960s. In 1968, Riccio and colleagues presented their findings on the application of the active avoidance test in rats [5]. This study demonstrated that passive avoidance learning significantly improved with age, whereas active avoidance performance in a simple task was relatively unaffected by age. The test for active avoidance learning in rats is typically performed in the two-alternative choice paradigm, where electric shock is used as an unconditioned stimulus. Rats are trained to shuttle between compartments when a warning signal (tone or flash) is presented. This test does not provide a permanent safe place for the rat, as long as the rat appears in a place where it has already received a shock [6].

Currently, multiple protocols utilizing active avoidance are employed to investigate questions related to emotional reactivity [7,8], fear learning and fear extinction [9,10], stress reactivity [3,6,10,11], and depression-like behavior [12–14]. The amygdala is recognized as a major control center for acquiring and exhibiting avoidance behaviors [15–17]. The medial prefrontal cortex has the capability to override this response through its top-down
The amygdalo-cortical circuitry is known to regulate active avoidance behavior and control responses to conflicting cues [1,2,9,15,17]. The hippocampus seems to have an influence on avoidance behavior, yet its involvement in learned active avoidance is still a mystery [9,19]. Active avoidance behavior has been shown to involve similar neural circuits in both animals and humans [1–4,9], and this test is used to validate animal models of neuropsychiatric disorders [1,6–8].

Active avoidance is regarded as an adaptive avoidance behavior in which individuals avoid a potentially noxious stimulus, in contrast to maladaptive avoidance, which occurs when individuals avoid relatively safe situations (in anxiety and related disorders, such as obsessive–compulsive disorder or post-traumatic stress disorder) [3]. Active avoidance elicits a high level of fear in rats, leading to the production of long-lasting 22kHz calls [20–23], so-called aversive ultrasonic vocalizations (aUSVs). This type of vocalization was thoroughly examined by Stefan M. Brudzynski [20,21,24–26], who wrote the following: “rats emit 22 kHz USVs any time there is a dangerous or potentially dangerous situation, anxiety-inducing circumstances, external threat, or uneasy uncertainty” [27]. Moreover, the “emission of rat 22 kHz vocalizations may be treated as an evolutionary vocal homolog of human crying” [27].

Ultrasonic vocalization in a rat subject is a marker of its emotional state. More than twenty years ago (in 1992), V. Cuomo and co-authors wrote the following [28]: “ultrasonic calling during stressful situations may be sensitive indicator of underlying emotional states that Interfere with the acquisition of a complex task”. It is surprising how few scientists have documented USVs during learning sessions in rats and used aversive ultrasonic calls as a sign of a negative emotional state. Recently, Shuichi Chiba et al. (2022) analyzed 22 kHz ultrasonic calls as a measure of emotional reactivity in contextual fear conditioning in rats [7]. We recently demonstrated that adult males of the NEW substrain more often produced aversive USVs than females [29]. Therefore, males were more likely to express their negative emotional state through vocalizations, unlike females. Similarly, male rats that experienced isolation restraint emitted more aversive 22 kHz USVs than females did [30].

Our study was performed in two inbred strains of rats: Wistar Albino Rats from Rijswijk (WAG/Rij rats)—a genetic rat model of absence epilepsy [31,32] and in non-epileptic substrain. The latter is a minor rat substrain that has been selected since 2016 as a non-epileptic control for WAG/Rij rats (NEW abbreviation for non-epileptic WAG/Rij), in which spontaneous spike-wave seizures were absent during the entire life [29,33].

Our experiments were performed in both sexes. Females were tested strictly during their diestrus period. Rats were trained to avoid footshock by moving to the adjacent “safe” compartment of the shuttle box after presentation of a conditioned stimulus (a 4.5 s tone). The test consisted of 50 trials. There were three main behavioral responses:

1. Successfully learned avoidance: The rat was trained to move to the “safe” chamber during tone stimulus (before electric stimulation) in five out of six trials.
2. Unsuccessful learning: The rat showed less than five avoidances in a sequence of six trials.

2. Results

The study was performed with 57 adult rats of both sexes (30 males and 27 females), at the age 8.53 ±1.15 months. All of the rats were examined in the standard active avoidance learning test. Females were tested strictly during their diestrus period. Rats were trained to avoid footshock by moving to the adjacent “safe” compartment of the shuttle box after presentation of a conditioned stimulus (a 4.5 s tone). The test consisted of 50 trials. There were three main behavioral responses:
3. Freezing reaction: The rat stayed immobile, usually in a corner, and endured electrical stimulation. The test was stopped when the rat stayed immobile during 20 trails.

Table 1 shows the number of rats with different types of test performances in two rat strains and two sexes.

Table 1. The number of rats of two strains (NEW and WAG/Rij) demonstrating different behavioral reactions in the active avoidance test, i.e., different types of test performances.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Test Performance</th>
<th>N</th>
<th>Strain</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>Successful</td>
<td>14</td>
<td>NEW</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>WAG/Rij</td>
<td></td>
<td></td>
<td>1 **</td>
</tr>
<tr>
<td></td>
<td>Unsuccessful</td>
<td>4</td>
<td>NEW</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>WAG/Rij</td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Freezing</td>
<td>12 *</td>
<td>NEW</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>WAG/Rij</td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td>Successful</td>
<td>25</td>
<td>NEW</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>WAG/Rij</td>
<td></td>
<td></td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Unsuccessful</td>
<td>1</td>
<td>NEW</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>WAG/Rij</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Freezing</td>
<td>1</td>
<td>NEW</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>WAG/Rij</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>27</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The number of males demonstrating freezing during active avoidance was higher than the number of females, p < 0.05, chi-square test. ** The number of WAG/Rij males who successfully performed the test was lower than the number of NEW males, p < 0.05, chi-square test.

The WAG/Rij and NEW males showed significantly different learning profiles and demonstrated a proportion of successful learners, unsuccessful learners, and subjects freezing (Table 1; Freeman–Halton extension of Fisher’s exact test, p = 0.047). A higher number of NEW males successfully learned the task in comparison to the number of WAG/Rij males (Table 1; Fisher’s exact test, p = 0.041). The number of NEW and WAG/Rij males showing freezing behavior did not differ significantly (Table 1; p = 0.41). Moreover, there was no difference in the number of NEW and WAG/Rij males who could not learn (Table 1; p = 0.086).

The number of females who successfully performed the test (25 out of 27 subjects) was significantly higher than the number of “good learners” in males (14 out of 30 subjects, Fisher’s exact test, p = 0.014).

In females, the freezing reaction was exceptional, and it was found only in 1 out of 27 females (3.7%). In contrast, almost half of males showed freezing (12 out of 30 males or 40%), and this was significantly more often than in females (Table 1; Fisher’s exact test, p = 0.006). Therefore, males seemed to avoid taking risks and held back in order to protect themselves from potential negative outcomes.

The active avoidance test could be considered an artificial aversive behavioral situation. In this situation, rats often emit ultrasonic calls that can be interpreted in many ways (as a signal of pain, fear, or anxiety; as a signal of unescapable danger; or as a signal of their aversive emotional state and learned helplessness [20,26,27,35]). Here, we recorded ultrasonic vocalization in a rat during the entire test session. Figure 1 shows two examples of ultrasonic calls with different durations and different frequency structures. The first call (Figure 1a) was a typical aversive US call with a frequency around 22–25 kHz, long duration of 0.972 s, and a “flat” spectrum. The other type of short US call is shown in Figure 1b, in which the main frequency is around 50 kHz, and there is a peculiar frequency spectrum. This type of call is more related to an explorative behavior and rarely occurred.
during active avoidance (i.e., in a few rats at the very beginning of the test). The recorded US signals were analyzed automatically with Metris Sonotrack 2.5 software, and US calls were detected based on their time and amplitude parameters. Our analysis included only US calls with the frequency below 30 Hz, i.e., aversive US calls.

**Figure 1.** Sonograms of ultrasonic rats’ vocalization during active avoidance test. Signals were recorded and processed with Sonotrack software. The scale of the spectra is in db. (a) A typical long-lasting 22–26 kHz ultrasonic aversive call emitted by a male subject. This type of vocalization was automatically detected and statistically analyzed. (b) Rarely occurring short ~50 kHz ultrasonic calls produced by a female rat; her behavior in the experimental chamber had an explorative character. This type of vocalization was extremely rare in the current paradigm and was not analyzed.

Half of males produced aversive ultrasonic vocalizations (aUSVs) (50%, 15 out of 30; Figure 2). Males emitted aUSVs during freezing (53.3% from vocalized males; Figure 2), as well as during successful and unsuccessful learning (33.3% and 13.3%, respectively; Figure 2a). For each type of test performance, the number of males that produced aUSVs and those that did not produce aUSVs did not statistically differ (Figures 2a and 3; Freeman–Halton extension of Fisher’s exact test, \( p = 0.20 \)). This means that males vocalized irrespective of how they acted.

**Figure 2.** The distribution of behavioral responses in active avoidance in female and male rats: (a) subjects who emitted aversive US calls and (b) those who did not vocalize.
Almost one-third of females emitted aUSVs during the avoidance learning task (7 out of 27 females, 26%; Figure 2), and all of them vocalized during successful learning (100% from vocalized females; Figure 2a). There was one female that could not learn the task and one that was freezing. Neither of them produced aUSVs (Figures 2b and 3).

Figure 3 shows the density of aversive ultrasonic vocalizations, as measured in a 30min period with 5 min bins. The duration of the task differed between different rats, depending on behavioral reaction and the speed of learning (each rat was given 50 trials of the task). In rats exhibiting freezing behavior, the test was terminated after the 20th trial. Consequently, the entire test in three subjects with freezing reaction lasted only 15 min, and in one subject, around 20 min.

The density of aUSVs was statistically analyzed in subjects who vocalized in the active avoidance task—15 males and 7 females during 30 min of the test (Figure 4). In males, the density of aUSVs demonstrated a U-shaped distribution, whereas in females, it was flat. The density of aUSVs in males was higher than in females during the 10–15 min (Mann–Whitney U test, $p = 0.037$) and 15–20 min (Mann–Whitney U test, $p = 0.040$) intervals.
test performance was either successful (5 avoidances out of 6 successive trials) or unsuccessful (less than 5 avoidances out of 6 trials) and resulted in freezing behavior in males.

Figure 3 shows the density of aversive ultrasonic vocalizations, as measured in a 30min period with 5 min bins. The duration of the task differed between different rats, depending on behavioral reaction and the speed of learning (each rat was given 50 trials of the task). In rats exhibiting freezing behavior, the test was terminated after the 20th trial. Consequently, the entire test in three subjects with freezing reaction lasted only 15 min, and in one subject, around 20 min.

The density of aUSVs was statistically analyzed in subjects who vocalized in the active avoidance task—15 males and 7 females during 30 min of the test (Figure 4). In males, the density of aUSVs demonstrated a U-shaped distribution, whereas in females, it was flat. The density of aUSVs in males was higher than in females during the 10–15 min (Mann–Whitney U test, \( p = 0.037 \)) and 15–20 min (Mann–Whitney U test, \( p = 0.040 \) ) intervals.

Figure 4. The density of aversive ultrasonic calls in rats who vocalized during their performance of the active avoidance task. * Significant differences between males and females (Mann–Whitney U test, \( p < 0.05 \)).

3. Discussion

Here, we categorized the outcomes of the 50-trials avoidance test in three categories, which, together with USVs, could better characterize the behavioral phenotype in males and females.

3.1. Sex Differences in Performance of the Active Avoidance Test

Males and females demonstrated a divergent behavioral performance in the active avoidance test.

- Males more likely experienced freezing (40% from males) than females (3.7% from females).
- Only 43.2% of males successfully learned the task, in contrast to females, who performed significantly better than males (93% of females successfully learned the task).

Particular attention was paid to freezing behavior when a rat remained motionless and tolerated 40s of electrical stimulation in over 20 trials. This freezing behavior is known to be the most common and best-understood fear response in rats [36–39]. Freezing was observed in one female (3.7%) and in twelve males (40%) in our tests. This finding is consistent with the existing literature, which indicates that female rats generally display less freezing behavior than males (see references in Reference [38]).

We found that two-thirds of the males who froze emitted aversive USVs and one-third were salient. Therefore, freezing may be related to learned helpfulness, although it was not necessarily accompanied by aversive ultrasonic vocalizations.

Freezing in rodents is a specific behavioral response to an unpredictable threat (such as a footshock in a laboratory) or in a natural situation with a high risk of predation. Here, in the avoidance task, we found that around 26% of males, but no females, experienced freezing and were not able to learn. This type of reaction might be classified as helplessness-like behavior. The conceptual statement that “Females do not express learned helplessness like males do” was introduced by Christina Dalla et al. [13] (see references in their review [40]). Namely, “when male rats are exposed to uncontrollable stress, they do not learn to escape the shock in the active avoidance task and thus they become helpless. On the contrary, females exposed to the
same regime of uncontrollable stress learn to escape and thus they do not express learned helplessness behavior” [40]. According to our results, freezing is almost entirely the male’s reaction in the active avoidance task.

We demonstrated that females performed significantly better than males. Similar results were obtained in many studies: a reduced contextual fear conditioning was found in females as compared to males (see references in Reference [41]). In Pavlovian fear conditioning and memory retrieval, females were characterized by a decreased freezing during the later retrieval of contextual fear memory (see references in Reference [41]). Sex differences in nociception during conditioning are unlikely to explain this finding [40,41].

### 3.2. Ultrasonic Vocalization

In this study, we approached the problem of individual emotional reactions in two-way active avoidance via ultrasonic vocalization. Aversive USVs were recorded in 25.9% of females (only successful learners) and in 50% of males (successful learners, unsuccessful learners, and freezing response). The density of aversive USVs in females during 10–20 min of the avoidance test was lower than in males.

Ultrasonic vocalization is a common form of vocalization in rats. The emission of rat 22 kHz calls represents the evolutionary vocal homolog of human crying [27]. Multiple footshocks in rats are known to elicit 22 kHz ultrasonic calls [37]. Therefore, aUSVs in our experiment might be elicited by foot-shock stimulation, but they reflect a negative emotional state.

Aversive vocalization was absent in two-thirds of females and approximately in half of males. These subjects did not vocally express their negative emotional state.

In our experiments, rats extremely rarely emitted brief 50 kHz appetitive calls (Figure 1b). Calls of 50 kHz in rats are part of a specific class of USVs that is associated with positive outcomes, reward-seeking behavior, and approach motivation [21,42–44]. These calls are produced in “friendly” and non-aggressive behavioral situations and cannot be acoustically mixed with the aversive 22 kHz calls [21]. At least behaviorally ambiguous situations associated with emission of 22 kHz and 50 kHz calls are extremely rare.

It is known that the 22 kHz calls are controlled by the core limbic mid-brain areas. The ascending cholinergic system is responsible for producing 22 kHz calls, and the ascending dopaminergic system is responsible for producing 50 kHz calls [20,21,26]. The incompatibility between these two ascending systems causes rats to produce only one type of vocalization. The emission of both 22 kHz and 50 kHz calls during behaviorally aversive situations may be indicative of the impairment of ascending neuromodulatory systems (cholinergic and dopaminergic). The study of ultrasonic vocalizations in rat models of neuropsychiatric diseases may provide insight into the disturbances of cholinergic and dopaminergic control of behavior.

It is widely accepted that the initiation of 22 kHz calls is regulated by the ascending cholinergic system, which has a center in the brainstem tegmentum (laterodorsal tegmental nucleus) and targets in the basal forebrain and limbic regions (anterior hypothalamus, preoptic area, bed nucleus of stria terminalis, and lateral septum) [20,21,26]. In our experiments, half of the males and 26% of the females produced 22 kHz aversive USVs during the avoidance task. In these vocalized subjects, the ascending cholinergic system might be upregulated. The overexpression of acetylcholine receptors in the basal forebrain and limbic areas may be related to a strong emotional reaction that is expressed vocally as 22 kHz calls. Bihari and colleagues [45] showed that when carbachol, a muscarinic acetylcholine receptor (mAChR) agonist, was administered directly into the lateral septal region, it caused 22 kHz calls in males, and similar 22 kHz vocalizations were observed when the cholinergic neurons in the laterodorsal tegmental nucleus were activated.

The metabotropic muscarinic acetylcholine receptors are involved in the modulation of negative emotional responses [46–48]. It is known that activation of mAChR induced long-term anxiogenic-like responses in rats [49]. We found a lower density of 22 kHz calls in females (vs. males), suggesting that the ascending cholinergic system in females might
be downregulated or target neurons in the basal forebrain and limbic areas might be less sensitive to acetylcholine. Avissar and co-authors showed that female rats exhibited lower binding of muscarinic receptor agonists in the preoptic area of the hypothalamus than males [50].

The density of aUSVs in males demonstrated an inverted U-shape distribution: aUSVs were minimal at the beginning and at the end of the test. Similar results were achieved by Cuomo and co-authors, who used the 75-trial active avoidance test in adult male Wistar rats [28]. They demonstrated that the rate of ultrasonic vocalizations (20–30 kHz) decreased during the acquisition of the avoidance response. As expected, vocalized male subjects displayed a reduction in aUSVs when they learned to avoid the footshock, resulting in a decrease in stress levels. However, the aUSV density in females was very low during the avoidance test and eventually reached zero. The intensity of 22 kHz calls provides essentially noninvasive information about the emotional state, as suggested by [51], but only in male rats. Sex and individual differences in rat ultrasonic vocalization must be more thoroughly studied, as they provide unique insight for improved behavioral phenotyping.

3.3. Aversive Ultrasonic Vocalization and Freezing Behavior

Cholinergic neuromodulation has been identified as being involved in fear extinction and in the inhibition of freezing behavior in rats [47–49]. Fear-associated freezing or alert immobility in adult male Long–Evans rats was found to be correlated with an elevated release of acetylcholine in the neocortex and hippocampus [52]. We found freezing in one exceptional female (3.7%) and in twelve males (40%), providing evidence for our suggestion that the cholinergic neuromodulatory system is downregulated in females. Estrogen is known to affect cholinergic receptors [53,54]. Sex differences in freezing observed in the active avoidance test may be due to the poorly understood neuromodulatory effects of the cholinergic system.

Here, we did not observe a well-known correlation between poor performance in the two-way avoidance test and increased levels of ultrasonic vocalization. In the abovementioned study performed by Cuomo and co-authors [28], non-vocalized males (approximately 24%) displayed the most rapid avoidance learning. Contrary to our findings, 53.3% of non-vocalized males successfully learned the task. Moreover, the number of vocalized males and non-vocalized males did not statistically differ, suggesting that males vocalized irrespective of how they acted. Moreover, around 26% of males (seven NEW rats and five WAG/Rij rats) experienced freezing and were not able to perform the task. It was surprising to observe a poor performance in the avoidance test exclusively in males, with a high level of freezing and a greater density of aversive vocalizations, but not in females. This cannot be linked to absence epilepsy because both sexes have a genetic predisposition to absence epilepsy, and NEW rats have a non-epileptic phenotype. Perhaps, WAG/Rij rats and the minor NEW substrain are prone to emotional exhaustion or enhanced emotionally in fear-related tasks. Males exhibited poor outcomes in the avoidance task, frequent freezing behavior, and a high number of aversive vocalizations, indicating that they were in a negative emotional state, rather than having poor cognitive abilities.

Our results have clear translational value, even though active avoidance has been a long-standing technique. Active avoidance, together with the measurement of biologically important information, such as ultrasound, can provide important insights into emotional reactions, fear acquisition, fear reduction, stress reactions, and signs of depression in rat models of neuropsychiatric human diseases.

3.4. Neural Substrates Underlying Active Avoidance

Fear-related learning involves a complex interplay of neural circuits and physiological responses, such as changes in heart rate, increased body temperature, etc. [55–57]. Although consistent and substantial behavioral changes did correlate with physiological responses, the results were not as conclusive as we had hoped.
1. Heart rate: Fear conditioning led to a decrease in heart rate in healthy participants but not in patients with psychiatric disorders [55]. In healthy participants, a transient heart rate deceleration in anticipation of noxious stimuli could be considered a sign of increased parasympathetic (vagal) activity when facing the conditioned stimulus. Similar results were obtained in rats: a transient deceleration of heart rate in fear-conditioning learning [56]. Knippenberg and co-authors [56] used a discriminatory fear-conditioning protocol in which a series of six auditory stimuli was followed by a footshock, unless a safety light had signaled that the shock would not be delivered. The visual safety stimulus evoked a brief phasic deceleration of the heart rate over the first 1 s following light onset. Generally, cardiac responses in rats (both phasic and tonic changes in heart rate) during fear conditioning resembled well-documented responses in humans.

Recently, Battaglia et al. (2023) highlighted fear-induced bradycardia as a powerful tool for assessing fear conditioning in patients with various psychiatric disorders [55]. Future research is needed to expand our understanding of fear-induced bradycardia and its potential as a biomarker to enhance and expedite psychiatric interventions.

2. Body temperature: Stress-induced hyperthermia is a well-known phenomenon [58–60]. Vianna et al. (2005) demonstrated that fear-conditioned rats had an increase in body temperature and marked drop in the temperature of their tail and paws which lasted for the entire duration of the response [60]. Further on, Marks et al. (2009) showed that conditioned fear-induced hyperthermia had a non-shivering origin and was mediated by beta-adrenoceptors [59]. In their most recent review, Battaglia et al. (2023) conducted an in-depth analysis of fear memory and underlying neurochemical synaptic transmission, such as glutamatergic, noradrenergic, and endocannabinoid systems [61]. Novel neuropharmacological manipulations targeting these systems may be useful for treating fear-based and anxiety-related disorders [61].

In general, our research emphasized that employing rat models can assist in gaining a more comprehensive understanding of human mental disorders [14,62–66], particularly those of a negative emotional nature [67]. Recent investigations have focused on elucidating the neural mechanisms underlying fear conditioning and active avoidance. An effective translational approach has the potential to uncover novel biomarkers, elucidate disease mechanisms, and promote targeted therapies for patients.

3.5. WAG/Rij and NEW Rats

Here, we used two inbreed rat strains: WAG/Rij and NEW. WAG/Rij rats have a genetic predisposition to absence epilepsy and spontaneously exhibit spike-wave discharges after 5 months of age [31,32,68]. Epileptic discharges are known to occur during passive behavior, resting state, drowsiness, and light sleep. Absence seizures do not occur when a rat is in a stressful situation or actively engaged in an activity, such as an active avoidance task. We did not examine WAG/Rij rats for epilepsy due to the necessity of invasive surgery and electrode implantation. All of our rats were drug-naïve and intact.

The NEW (non-epileptic WAG/Rij) is a minor rat substrain that we have been selecting since 2016 from non-epileptic WAG/Rij rat subjects. It is a control substrain to epileptic WAG/Rij rats, in which spontaneous spike-wave seizures were absent during the entire life [29,33]. The current study was rather exploratory, focusing on males’ reaction to perform an avoidance task and on aversive ultrasonic calls. In the current experiments, WAG/Rij and NEW rats of both sexes were housed in the same environmental conditions; therefore, we minimized the risk of variability. Unfortunately, the number of subjects was too low to demonstrate many significant results. However, we found significant strain-specific difference between males. WAG/Rij males demonstrated significantly poorer avoidance learning than NEW males. This fits with our earlier findings that epileptic WAG/Rij male rats had a deficit of executive functions that was obtained during the initial stage of active avoidance test [69]. Similarly, Antonio Leo et al. demonstrated "an age-dependent impairment of cognitive performance in WAG/Rij rats, which could be linked to the age-dependent
increase of spike wave discharges” [70]. Our rats were in the symptomatic state at the age of 8.53 ± 1.15 months, when WAG/Rij rats develop fully blown seizures [32,68].

3.6. Limitations and Further Directions

Here, we defined thousands of ultrasonic calls that were automatically detected with Sonotrack Metris software. The inclusion criterion was a call frequency below 30 Hz. We did not analyze the frequency of calls. There might be several frequency-specific classes of aversive calls in males and females. Here, we roughly examined aversive ultrasonic calls and did not differentiate between subjects with low and high localization levels. An analysis of the physical manifestations of stress (heart rate), the endocrine system and hormones associated with stress (corticosterone), and the activity of the hypothalamic–pituitary–adrenal axis would be beneficial for better understanding why some males vocalized during freezing. Further investigation of sex-specific and strain-specific ultrasonic vocalization might help to better understand the outcomes of fear-related cognitive tasks in rats. Ultrasonic vocalization in rats may provide additional strength for preclinical studies in the field of behavioral pharmacology.

The obvious limitation of our study is a small number of subjects, considering that two sexes were used. We used two inbred rat strains that were born and raised in the same environment to minimize the risk of variability.

4. Materials and Methods

The current study was conducted on WAG/Rij rats (n = 25) and the non-epileptic “NEW” rats (n = 32). These rats were bred at our institution (Institute of Higher Nervous Activity and Neurophysiology of RAS, Moscow, IHNA RAS). The experiments were carried out in accordance with EU Directive 2010/63/EU for animal experiments and were approved by the ethics committee of IHNA RAS (Protocol No. 4 was approved on 13 December 2022). Rats were kept in environmentally controlled conditions with a 12-h light cycle (light on at 08:00) and a 12-h dark cycle (light off at 08:00). They were given free access to food and water ad libitum. Rats were housed in same-sex groups (3–4 subjects per cage). In total, 57 rats were used (30 males and 27 females).

4.1. Active Avoidance Test

This test was performed in the standard two-way shuttle box, which consists of two compartments (30 × 26 × 30 cm) separated by an opaque wall with a passage in the middle. A grid floor in the shuttle box was made of 3 mm stainless-steel bars spaced 1 cm apart. There was also a removable tray below the grid floor designed to catch debris and prevent it from entering the shuttle box. A scrambled electric footshock (0.5 mA, 5 Hz) was used as an unconditioned stimulus. A 70dB pure tone was used as a conditioned stimulus. Unconditioned and conditioned stimuli were delivered automatically, using customary computer software. The shuttlebox experiment was monitored by a video camera (JVC GR-DVP7, JVC, Beijing, China) mounted 180 cm above the shuttle box. An experimenter controlled the experiment from another room, using a computer system.

Rats were introduced to the experimental room and kept in small cages for 10 min to get used to their surroundings. Each rat was placed in the shuttlebox for 3 min to acclimate. The experiment did not involve the assessment of epilepsy in rats, because absence seizures do not occur when a rat is in a stressful situation or actively engaged in an activity [31,32,71].

Testing: A footshock was delivered in any compartment, and the opposite compartment was always “safe”. Immediately after the sound was offset, an electric footshock was delivered until the rat moved to the opposite “safe” compartment. The reaction time was recorded automatically. If a rat did not respond to electric stimulation during 40 s, the stimulation was automatically turned off. The test session consisted of 50 trials. The interval between trials was chosen at random between 20 and 50 s. After the end of the training session, the shuttle box was cleaned with a 50% ethanol solution to remove olfactory cues.
4.2. Ultrasound Recording and Analysis

The Sonotrack system (Metris, Hoofddorp, The Netherlands) was used for non-invasive recording and analysis of ultrasonic vocalizations. The system consisted of a Sonotrack Control Unit, ultrasound microphone and the Sonotrack software v.2.6.2.30. Background noise was greatly reduced by software settings and the small opening angle of the applied microphones. With the Sonotrack system, we recorded the low-level ultrasonic vocalizations, performed a full-spectrum analysis (15–100/125 kHz), and semi-automatically detected instant calls.

4.3. The Estrous Cycle

Female rats were tested during diestrus. The phase of the estrous cycle in females was determined in wet smears immediately after collection (direct cytology, unstained slides) [72–74]. Vaginal swabs were obtained using sterile saline and examined on a slide in a drop of saline. A microscopic examination was performed using a Nikon microscope (Eclipse 200). We used a $10\times$ objective to look at the cellular content of a swab, and a $40\times$ objective to identify cell types.

The swabs contained two types of cells: leukocytes and epithelial cells. The latter were (1) small nucleated and non-keratinizing cells, (2) large nucleated epithelial cells, and (3) non-nucleated keratinized epithelial cells or needle-like cells.

Diestrus was characterized by the predominance of leukocytes in vaginal swabs. In the stage of proestrus and estrus, leukocytes were absent. Diestrus was the longest phase of the estrous cycle and lasted, on average, 48–72 h.

4.4. Statistical Analysis

The number of rats was small, and some categories contained less than 5 subjects, therefore, Fisher’s exact test (two-tailed) was used to examine differences between categories. Freeman–Halton extension of Fisher’s exact test was used to analyze $2 \times 3$ contingency tables. The density of aversive vocalizations was analyzed using the non-parametric Mann–Whitney U test for independent variables.

5. Conclusions

Rats are often employed in cognitive studies due to their high capability for learning, but only a small number of studies have determined emotional states through ultrasonic vocalizations. The avoidance test is a stressful and highly fear-inducing situation that requires a high level of motivation to return to safety for a successful outcome. Here, we examined the performance of males and females in a 50-trials active avoidance test. The rats were divided into three groups based on their behavioral responses: successful learners, unsuccessful learners, and those exhibiting freezing behavior and an inability to learn. Males exhibited poorer learning abilities than females and often showed freezing behavior. A high number of aversive vocalizations in males indicated that they were in a negative emotional state, rather than that they had poor cognitive abilities. It is possible that the WAG/Rij rats and the minor NEW rat substrain demonstrated heightened emotionality in fear-related tasks due to their susceptibility to emotional exhaustion or to emotional disorders.

Freezing was found in one exceptional female (3.7%) and in twelve males (40%). Freezing might be classified as helplessness-like behavior; therefore, our data confirm the notion that “females do not express learned helplessness like males do” [13].

Females performed the active avoidance test more successfully than males, a result that is in line with previous studies. Pavlovian fear conditioning and memory retrieval (see the references in Reference [41]). About 50% of males and 26% of females produced 22 kHz aversive ultrasonic vocalizations during the avoidance task, and the rest did not vocalize. The density of aversive ultrasonic vocalizations in females was lower than in males. Sex and individual differences in rat ultrasonic vocalization must be more thoroughly studied, as they provide unique insight for improved behavioral phenotyping.
Given that aversive calls and freezing behavior at 22 kHz are both regulated by the cholinergic neuromodulatory system, we hypothesize that the cholinergic system in female brains may be downregulated. Estrogen might affect cholinergic receptors in the brain structures responsible for fear-related behavior, such as the brainstem tegmentum, basal forebrain, and limbic regions. Sex differences in freezing observed in the active avoidance test might result from the poorly understood neuromodulatory effects of the cholinergic system.

Considering mutually exclusive mechanisms of initiation of 22 kHz and 50 kHz vocalizations, subjects emitting both types of USVs in fear-inducing situations should be given special attention, as they may be exhibiting signs of pathology. The study of ultrasonic vocalizations in rat models of neuropsychiatric diseases may provide insight into the disturbances of cholinergic and dopaminergic control of behavior.

Author Contributions: Conceptualization, E.S.; methodology, E.S. and M.P.; validation, E.S.; formal analysis, E.S.; investigation, M.P. and Z.A.; resources, E.S. and P.A.; data curation, E.S.; writing—original draft preparation, E.S.; writing—review and editing, E.S.; visualization, E.S. and Z.A.; supervision, E.S.; project administration, E.S.; funding acquisition, E.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by Russian Science Foundation, grant number 23-25-00166.

Institutional Review Board Statement: The animal study protocols were approved by the Ethics Committee of Institute of the Higher Nervous Activity and Neurophysiology of the Russian Academy of Sciences (Protocol No. 4 approved on 13 December 2022).

Informed Consent Statement: Not applicable.

Data Availability Statement: All experimental data obtained in the current study are shown in the figures and tables. Primary datasets are available from the corresponding author upon reasonable request.

Acknowledgments: We highly appreciate the technical support from Elizaveta Rutskova.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

- aUSVs—aversive ultrasonic vocalizations; WAG/Rij—Wistar Albino Rats from Rijswijk (a genetic rat model of absence epilepsy); NEW—non-epileptic WAG/Rij (a control minor substrain); USVs—ultrasonic vocalizations.

References


**Disclaimer/Publisher’s Note:** The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.