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RESEARCH ARTICLE

Anxiety-Like Behaviors in Mice Unmasked: Revealing Sex Differences in Anxiety Using a Novel Light-Heat Conflict Test

Sydney E. Lee[1,2] | Sung-Hoon Park[1,2,3] | John C. Aldrich[1,2] | Laura K. Fonken[4] | Andrew D. Gaudet[1,2]

[1]Department of Psychology, College of Liberal Arts, University of Texas at Austin, Austin, Texas, USA | [2]Department of Neurology, Dell Medical School, University of Texas at Austin, Austin, Texas, USA | [3]McGovern Medical School, The University of Texas Health Science Center at Houston, Houston, Texas, USA | [4]Division of Pharmacology and Toxicology, College of Pharmacy, University of Texas at Austin, Austin, Texas, USA

Correspondence: Andrew D. Gaudet (andrew.gaudet@utexas.edu)

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ABSTRACT

Anxiety and chronic pain afflict hundreds of millions worldwide. Anxiety and pain are more prevalent in females compared to males. Unfortunately, robust sex differences in human anxiety are not recapitulated in rodent tests, and results from rodent pain studies frequently fail to translate clinically. Therefore, there is a need to develop tests that reflect the differential salience of anxiety or pain-related stimuli between the sexes. Accordingly, here we introduce the Thermal Increments Dark–Light (TIDAL) conflict test. The TIDAL test places an anxiety-relevant stimulus (dark vs. illuminated chamber) in conflict with a heat-related stimulus (incrementally heated vs. isothermic chamber); mice freely explore both apparatus chambers. Here, we aim to determine whether the TIDAL conflict test reveals in mice underappreciated sex differences in anxiety and/or heat sensitivity. We establish in four distinct experiments that females on the TIDAL conflict test persist substantially longer on the dark-heated plate, suggesting that female mice exhibit elevated anxiety-like behavior. Mice more strongly prefer the heated-dark plate on the TIDAL conflict test compared to control thermal place preference with both chambers illuminated. We also reveal that an anxiety-relieving drug, paroxetine, reduces mouse preference for the heating dark plate, supporting the validity of the TIDAL test. Therefore, our new TIDAL conflict test reliably unmasks the relative salience of anxiety (vs. heat sensitivity): mice that are female exhibit robust anxiety-like behaviors not consistently observed in classical tests. Future studies should incorporate TIDAL and other conflict tests to better understand rodent behavior and to identify mechanisms underlying anxiety and pain.

1 | Introduction

Anxiety disorders cost the United States approximately $49 billion annually (Trautmann, Rehm, and Wittchen 2016). Anxiety consists of intense feelings of worry or unease, as well as changes in physical parameters such as blood pressure, and can develop into a debilitating disorder (Dieleman et al. 2016). The 12-month prevalence of anxiety disorders is 11%–17%, with a nearly 2× higher prevalence in females (Baxter et al. 2013; McLean et al. 2011; Somers et al. 2006). Females show increased

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Summary

- Anxiety is twice as prevalent in women, yet sex differences for anxiety-like behaviors are not detected in rodents using commonly used tests.
- Here, we develop a new test with two chambers that places an anxiety-inducing stimulus—light—in conflict with heat.
- The dark chamber floor heats to uncomfortable temperatures, whereas the illuminated chamber temperature remains comfortable.
- Our data reveal that females remain on the dark-heating plate for longer than males.
- These anxiety-like behaviors were reduced using an anxiety-relieving drug.
- Therefore, the new TIDAL conflict test unmasks sex differences in mouse anxiety-like behavior, and could identify new mechanisms and treatments for anxiety.

prevalence of separation anxiety, phobias, generalized anxiety, and panic disorders beginning at childhood or adolescence (Altemus, Sarvaiya, and Neill Epperson 2014; Bekhbat and Neigh 2018; Donner and Lowry 2013). Given the high prevalence and burden of these disorders, studying underlying mechanisms and related behaviors could help identify therapies that alleviate maladaptive anxiety.

Anxiety-like behavior is assessed in rodents using validated behavioral assays. For example, the elevated plus maze and the open field test induce a stress response through an aversive event or anticipated aversive event (Bailey and Crawley 2009), which results in a predictable behavioral output (e.g., thigmotaxis) that is modified based on the rodents prior experience (e.g., predator odor increases thigmotaxis). However, there are several limitations to available test of anxiety-related behavior. First, tests of anxiety-like behaviors in rodents were primarily validated in males to investigate pharmacologic treatments. Current anxiety-related assays detect some sex differences, but results are inconsistent across tests and often diverge from findings in humans (see Börchers et al. 2022; Donner and Lowry 2013). In rats, females as compared to males travel increased distances and reduce anxiety-like behaviors in the open field test and the elevated plus maze (e.g., Börchers et al. 2022; Johnston and File 1991; Knight et al. 2021; Scholl et al. 2019). In addition, outcomes regarding rodent sex differences in these anxiety-related tests are inconsistent and fail to recapitulate human sex differences (An et al. 2011; Võikar et al. 2001). A second limitation is that existing tests often evaluate a single variable—e.g., light/dark or enclosed/open space—and therefore might underestimate differences in anxiety-like behavior that would occur in more complex environments. Anxiety-like symptoms in mice of different sexes might appear minimal under baseline conditions, but could be unmasked by placing the anxiety-inducing stimulus in conflict with another factor.

Here, we explore the salience of anxiety versus heat avoidance in mice using a place preference conflict test: the Thermal Increments Dark–Light (TIDAL) conflict test. The conflict test occurs in a place preference apparatus with two chambers connected by a walkway; the conflict test is created by placing a strong "anxiety"-salient stimulus—dark (preferred) versus illuminated—in conflict with a weaker but increasingly aversive "pain"-relevant thermal choice—increasing heat versus maintained isothermic temperature. We aim to discover whether the relative salience of anxiety versus heat sensitivity better reflects anxiety-related sex differences reported in ethological and clinical settings. Both anxiety-like behaviors and heat sensitivity are expected to be increased in female compared to male mice, so it is unclear which stimulus would be more salient to mice in our TIDAL conflict test. The TIDAL conflict test is used here to test whether sex affects the salience of anxiety versus heat avoidance. We find that the TIDAL conflict test does not clearly expose group differences in pain-related heat sensitivity; rather, the TIDAL conflict test is a compelling, reliable, and valid tool for unmasking previously underappreciated differences in anxiety-like behavior.

2 | Methods and Materials

2.1 | Animals and Housing

All housing, care, and testing were approved by The University of Texas at Austin Institutional Animal Care and Use Committee. All animals were fed standard chow and filtered tap water *ad libitum* and maintained on a 12:12 light/dark cycle. Adult (8–12 weeks old) male and female C57BL/6J mice (Jackson stock 000664) were tested during the light cycle. Mice were housed in pairs. Mice in all treatment groups were numbered randomly to ensure researchers were blind to group. At the experimental endpoint, mice were injected with an overdose of Pentobarbital (200–270 mg/kg, MWI Animal Health 011355) and tissue was collected for potential later analyses.

2.2 | Behavioral Tests for Anxiety-Like Behavior

2.2.1 | Thermal Increments Dark–Light (TIDAL) Conflict Test

The TIDAL conflict apparatus is a modified thermal place preference (TPP) apparatus (Ugo Basile, Cat. No. 35250), which consists of two cylinders (20 cm diameter × 25 cm high) connected by a narrow center walkway (Figure 1). For TIDAL testing, one cylinder (the "light chamber") is kept in constant light and at a temperature of 31°C, which is an isothermic temperature for mice; in contrast, the other cylinder ("dark chamber") is covered with a fitted opaque lid and a flexible opaque outside cover to maintain darkness inside the cylinder and the temperature is manipulated from 31°C to 44°C (Figure 1A,D). Room illumination levels were 1200 lx, light chamber illumination levels were 1000 lx, and dark chamber illumination levels were 8 lx. The Ugo Basile device is costly; other laboratories considering similar studies could use the Ugo Basile device (which has plates that distribute temperature evenly); explore other commercially available options; or create custom thermal preference chambers using hot plates. In addition, the center walkway was covered with a clear plastic film "roof" to limit mouse interest in escaping through the open space. Mice are not acclimated to the apparatus prior to testing.

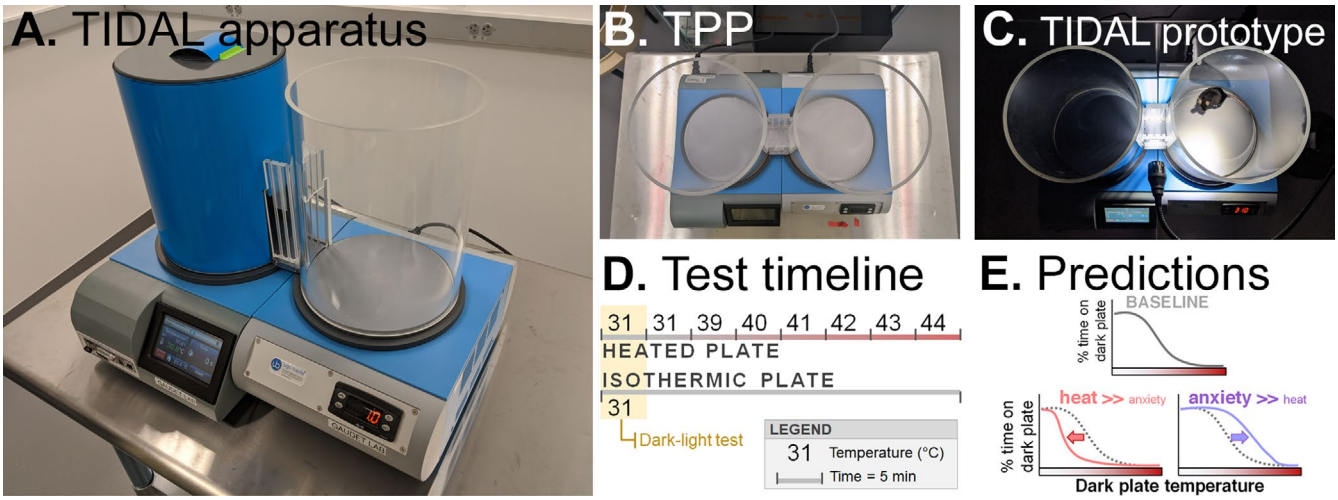

FIGURE 1 | The Thermal Increments Dark–Light (TIDAL) conflict test for exploring anxiety-like behavior and thermal sensitivity in mice: Design, controls, timeline, and predictions. (A) Final, optimized setup of the TIDAL apparatus. Note that the overhead room lights are on, and the dark-heated plate is covered with a lid and opaque film. (B) Setup of the Thermal Place Preference (TPP) apparatus, a control condition that removes the light–dark (anxiety-related) aspect of the TIDAL conflict test to isolate the effects of shifting heat on one plate. (C) TIDAL prototype setup for data described in Figures S1 and S2. Note lighting using focal lamps (rather than overhead lights) and lack of a lid on the top of the dark plate. (D) Optimized TIDAL/TPP test timeline. In TIDAL, the heat-shifting plate is dark and the isothermic plate is lighted, whereas in TPP both plates are lighted. Mice are placed in the apparatus with both sides set to 31°C. The first 5 min are recorded as an initial dark–light test, a control condition that reveals baseline preference for the dark (a measure of anxiety-like behavior). Mice remain in the apparatus as the heating plate continues for 5 min at 31°C, then 39°C, then increases incrementally 1°C every 5 min through 44°C. The lighted isothermic side is maintained throughout at 31°C. (E) Using the TIDAL conflict test, mice are expected to initially prefer the dark side, but will increasingly avoid the heated-dark plate as its temperature increases. We predict that sex and neurotrauma will alter the salience of heat discomfort versus anxiety-related sensitivity, manifesting as shifted heated-dark plate preferences over time.

Pilot studies used a wide range of temperatures (heated-dark plate incremental temperatures: 31°C, 42°C, 44°C, 46°C, 49°C, 52°C) and times at each temperature (5–10 min) (prototype setup: Figure 1C). A pilot study with this setup revealed females had increased heated-dark plate preference at 42°C–46°C; however, mice of both sexes had low heated-dark plate preference at 49°C and 52°C, suggesting that maintaining heated-dark plate temperatures in the low-to-mid 40°C range would be ideal for detecting differences in anxiety-like behavior (Figures S1 and S2).

Based on these pilot studies, to optimally detect the salience of anxiety versus thermal avoidance we defined the following parameters: Prior to testing, mice are brought into the behavioral testing room and allowed to acclimate in their homecage for ~30 min. Following room acclimation, mice are placed on the illuminated side in TIDAL or the equivalent side in the TPP, and initially allowed to explore the apparatus for 5 min with both plates at 31°C (exploratory phase; initial light–dark test); next, an additional five minutes is spent with both plates at 31°C; then, the temperature on the dark plate is raised to 39°C and increased by 1°C every five minutes to a maximum temperature of 44°C (with the light plate maintained at an isothermic 31°C). Thus, a single mouse completes the TIDAL conflict test (or control thermal place preference) assay in 40 min (Figure 1D).

2.2.2 | Thermal Place Preference (TPP) Assay

The TPP assay is used as a control to isolate thermal sensitivity from the anxiety-like portion of the TIDAL conflict assay. The TPP setup is the same as the TIDAL setup (two cylinders connected by a center walkway), except that both the heated side and the side maintained at 31°C are exposed to room lighting (Figure 1B)—that is, the heated side is lighted, not dark as in the TIDAL conflict assay. Next, the same incremental temperature increases are applied.

2.2.3 | TIDAL and TPP—Testing, Automated Video Recording, and Analysis

Mice tested on TPP and TIDAL assays were interspersed throughout the day (i.e., during the light phase—Zeitgeber time 1–11). Unless otherwise noted, distinct mice were used for these tests to avoid effects of learning observed in repeated testing. The percent time spent in the dark cylinder ((dark cylinder time)/(dark + illuminated time); center walkway time excluded), distance traveled, and dark crossings were automatically recorded and scored using an overhead video camera and EthoVision software. EthoVision is an applied video tracking software capable of real-time analysis of mouse behavior, movement, and activity at the pixel-level. Time in the center walkway was excluded from analyses in the main manuscript for two reasons: (1) the surroundings in the center zone differed from the test chambers; and (2) analyzing behavior in the identically shaped illuminated chamber versus dark-heating chamber enabled a two-chamber preference comparison with equal preference clearly defined at 50% time in each chamber. Including the center zone in analysis had little effect on heated-dark plate preference differences between groups. The arena was cleaned with 70% ethanol between trials.

2.2.4 | Paroxetine Administration

Paroxetine hydrochloride hemihydrate was obtained from Sigma-Aldrich and diluted in vehicle (mixture of 10% DMSO and 5% TWEEN 80 in saline) before experiments. Mice were restrained and administered 10 mg/kg I.P. paroxetine 1 h prior to TIDAL conflict testing. After paroxetine administration, mice were placed into a novel holding cage for 1 h to mitigate injection stress.

2.3 | Experiments, Mouse Numbers, and Sex as a Biological Variable

Unless noted otherwise, all mice were 8–12 weeks old at time of testing. In Experiment 1 (initial characterization of TIDAL conflict test with broad temperature range), groups included female TIDAL ($n = 13$) and male TIDAL ($n = 15$). In Experiment 2 (sex comparison on optimized TIDAL vs. TPP), groups included female TPP ($n = 12$), female TIDAL ($n = 14$), male-TPP ($n = 13$), and male TIDAL ($n = 15$). In Experiment 3 (sex differences in TIDAL over two sessions), groups included female ($n = 6$; 1 excluded from session 2 [EthoVision analysis detection issues]) and male ($n = 6$) mice. In Experiment 4 (TIDAL with paroxetine) groups included female-vehicle ($n = 8$), male-vehicle ($n = 8$), female-paroxetine ($n = 9$; 1 excluded [EthoVision analysis detection issues]), and male paroxetine ($n = 11$).

2.4 | Statistics

Mouse TIDAL and TPP behavior was analyzed using two-way Repeated Measures Analysis of Variance (RM ANOVA). For most analyses, the dependent variable was heated-dark plate preference (i.e., % time spent on the dark, heated plate at a given temperature) while the within-subjects factor was dark plate temperature and the between-subjects factor was either sex (male, female; Figure S1B,C), test type (TIDAL, TPP; Figures 2C–F and 3C–E), or drug treatment (paroxetine, vehicle, Figure 4B–E). We also analyzed sex differences in the < 50% heated-dark plate preference threshold (i.e., the temperature at which a mouse's preference for the heated-dark plate dropped below 50%) via standard two-way ANOVA using sex and either test type (Figure 2B) or session number (1, 2; Figure 3B) as factors. Behavior in the Dark–Light test over multiple sessions (Figure 3A) was analyzed using a two-way RM ANOVA with heated-dark plate preference as the dependent variable and sex and session number as the between- and within-subjects factors, respectively. The effect of paroxetine treatment on behavior in the Dark–Light test (Figure 4A) was assessed using a standard two-way ANOVA with sex and drug treatment as factors. Full ANOVA tables are provided in Table S1. When appropriate, post hoc pairwise comparisons were made using two-tailed, unpaired, Bonferroni-corrected *t*-tests. Student's *t*-test (Figures S1A and 2A) and the Mann–Whitney Rank Sum test (Figure S1D) were used in experiments with only two groups. Prism 9 (GraphPad) was used for visualizing data and SigmaPlot 14 (SPSS) was used for statistical analyses.

3 | Results

3.1 | Placing Anxiety in Conflict With Incrementally Increasing Temperature Unmasks Sex Differences in the Salience of Anxiety (vs. Heat Sensitivity)

Women have increased susceptibility to anxiety compared to men, yet mouse models of anxiety show mixed results between the sexes. Women also withdraw more quickly from painful heat stimuli (Bartley and Fillingim 2013; Bragdon et al. 2002; Feine et al. 1991; Reddan et al. 2020; Rhudy and Meagher 2001). To explore whether differences in anxiety- versus pain-like behavior in mice could be uncovered by incorporating conflicting stimuli, we developed and evaluated the TIDAL conflict test.

In our first experiment, individual female or male mice were placed on the TIDAL apparatus (trial setup as in Figure 1A). Temperature on the illuminated plate was maintained at an isothermic 31°C, whereas the temperature on the dark plate was increased incrementally: dark plate temperature started at 31°C (acclimation—dark–light test), then maintained at 31°C for another five minutes, then raised incrementally through 39°C, 40°C, 41°C, 42°C, 43°C, and 44°C (five minutes at each temperature) (Figure 1D). If females as compared to males had increased salience of heat hypersensitivity, we would expect them to avoid the heated-dark plate at lower temperatures (curve shifted left); conversely, if females had increased salience of anxiety, we predict they would persist on the heated-dark plate to higher temperatures (curve shifted right) (Figure 1E).

On the dark–light test, females and males undergoing TIDAL both preferred the heated-dark plate versus the illuminated plate (Figure 2A) (70 ± 5% vs. 61 ± 3%; $t_{27} = 1.61$; $p = 0.12$), suggesting that our apparatus refinements improved light–dark contrast between the two plates. As expected, female and male mice in the control TPP assay—with both plates identically illuminated—had no significant difference in preference for the equivalent-but-illuminated plate (females: 47 ± 3%, males: 55 ± 3%; $t_{23} = -1.871$; $p = 0.07$). Here, the dark–light test did not expose significant differences in heated-dark plate preference between females and males.

Next, behavior on the TPP (control) versus TIDAL conflict test was assessed in female versus male mice. Female TIDAL mice remained in the dark portion of the apparatus at higher temperatures than males, suggesting that females exhibit amplified anxiety-like behavior (Figure 2B–F). Indeed, females as compared to males preferred the heated plate to higher temperatures in both tests (Figure 2B), and mice of both sexes remained on the heated plate longer under TIDAL conditions (with the heated plate also being dark) (Figure 2C,D).

Female mice in TIDAL displayed a stronger preference for the heated plate as temperatures increased compared to TPP, with a significant main effect of the test type ($F_{1,144} = 11.31$; $p < 0.005$) and temperature ($F_{6,144} = 21.27$; $p < 0.001$) (Figure 2C). Male mice in the TIDAL test tended to stay on the heated plate at higher temperatures compared to those in the TPP test. The

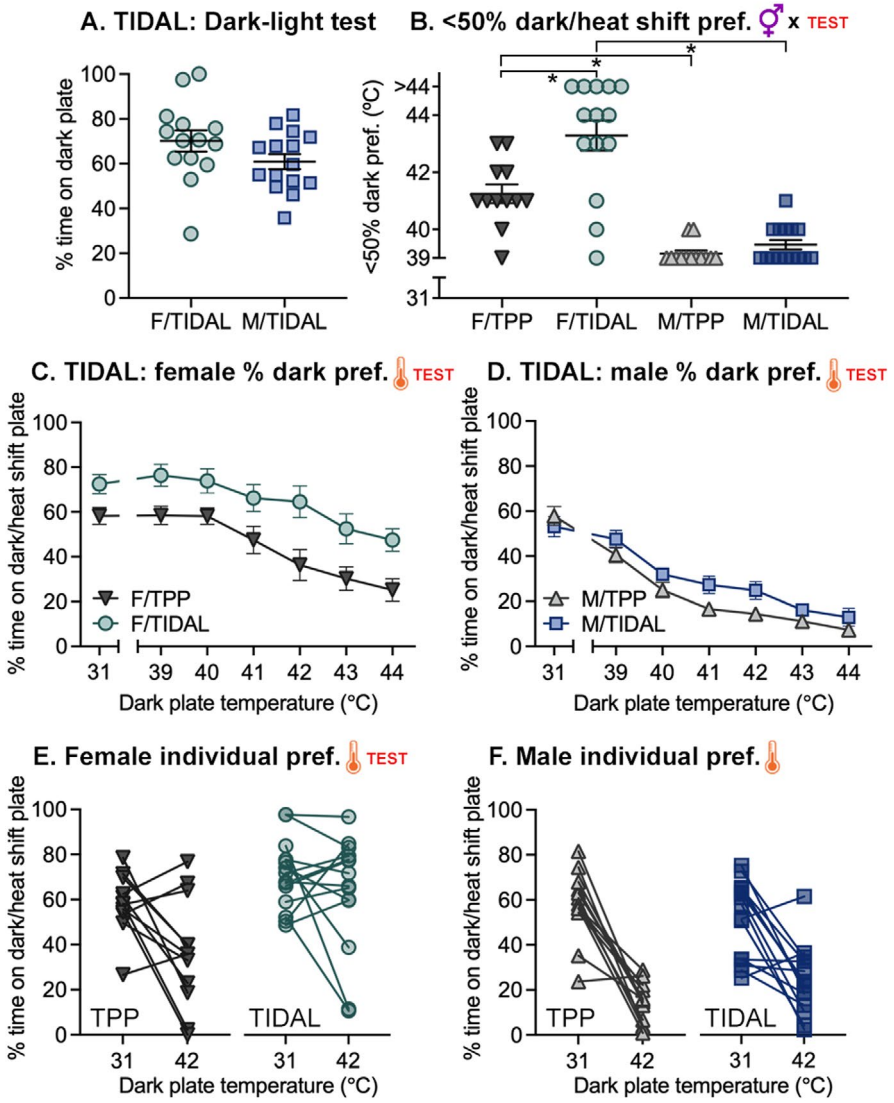

FIGURE 2 | The TIDAL conflict test exposes differences in anxiety-like behavior with the dark-heated plate increasing incrementally from 39°C to 44°C. Adult female and male mice were tested on the TIDAL conflict test (heated side dark; isothermic side lighted) or on the thermal place preference (TPP) test (both sides lighted). (A) In the dark–light test with both plates at 31°C, females and males showed similar heated-dark plate preferences. (B) Threshold at which female and male mice showed less than 50% preference for the dark or heat shift plate in the TIDAL conflict test and TPP test with increasing temperature on the dark/heat shift plate only. Female/TIDAL mice increased <50% threshold temperature compared to both female/TPP and male/TIDAL mice. (C, D) Female (C) and male (D) TIDAL/TPP test with increasing temperature on the dark/heat shift plate only. Compared to TPP mice, female and male mice tested on the TIDAL conflict test had heightened preference for the heating dark plate versus the constant 31°C lighted plate. (E, F) Dark/heat shift plate preferences of individual mice with the dark/heat shift plate at 31°C and 42°C. At 42°C, TPP females averaged 36% time on the lighted-heated plate, whereas TIDAL females averaged 65% preference for the dark-heated plate (E). At 42°C, TPP males averaged 14% time on the lighted-heated plate, whereas TIDAL males averaged 25% preference for the dark-heated plate (F). $n = 12$ TPP female, $n = 14$ TIDAL female, $n = 13$ TPP male, $n = 15$ TIDAL male mice; * indicates $p < 0.05$ between female and male mice; "sex × TEST" symbol indicates significant sex × test interaction; thermometer or TEST symbols alone indicate significant main effects of temperature and TPP/TIDAL, respectively; * indicates $p < 0.05$ between the indicated groups; two-way RM ANOVA (B–F) with *post hoc* Bonferroni *t*-test (B).

statistical analysis revealed a significant effect due to temperature ($F_{6,156} = 61.71$, $p < 0.001$) but only a trend towards significance in the test type ($F_{1,156} = 4.097$, $p = 0.053$) (Figure 3D). Focusing on a pivotal temperature, we found at 42°C that females in particular preferred the heated-dark TIDAL plate versus the illuminated TPP heated plate (65 ± 6% vs. 36 ± 6%), while the difference in heated-dark plate preference at 42°C between TIDAL and TPP in male mice was much less pronounced (25 ± 4% vs. 14 ± 4%; male TIDAL) (Figure 2E,F). Overall, when comparing between sexes, female TPP and TIDAL mice showed higher heat-shift plate preference than males on those same tests (Figure 2A–D). Furthermore, female TIDAL mice traveled further per minute in the illuminated area and crossed into the dark chamber more frequently compared to male TIDAL mice (Figure S3). These data suggest that this temperature range is well-suited to assess salience of anxiety versus heat, and that the test effectively unveils anxiety-like behavior (TIDAL vs. TPP results). In addition, females as compared to males strongly

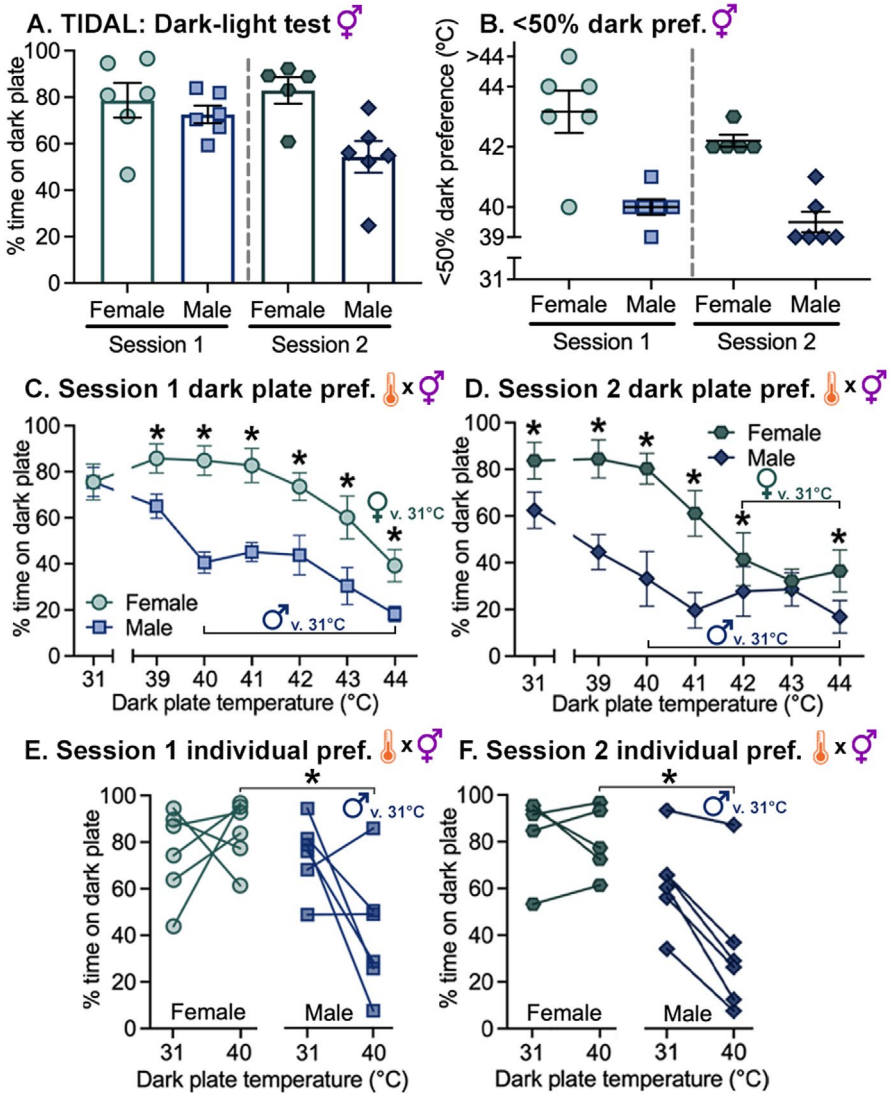

FIGURE 3 | Sex differences in TIDAL conflict test behavior persist across two repeat sessions. Female and male mice completed the TIDAL conflict test twice, two weeks apart. (A) During dark–light tests with both plates at 31°C, females had increased heated-dark plate preference relative to male mice (main effect of sex). (B) Threshold at which mice showed less than 50% preference for the heated-dark plate; females persisted on the heated-dark plate longer than males for both sessions. (C) In the Session 1 TIDAL conflict test, mice of both sexes reduced time spent on the heated-dark plate as temperatures increased. Further, female as compared to males mice preferred the heated-dark plate at all hyperthermic temperatures tested (39°C–44°C). (D) In the Session 2 TIDAL conflict test, mice of both sexes reduced time spent on the heated-dark plate as temperatures increased more quickly than in Session 1. Female as compared to males mice preferred the heated-dark plate at all hyperthermic temperatures tested except 43°C. (E) Session 1 heated-dark plate preferences of individual mice with the heated-dark plate at 31°C and 40°C. At 40°C, females had higher heated-dark plate preference than males (F: 85%; M: 42%). (F) Session 2 heated-dark plate preferences of individual mice with the heated-dark plate at 31°C and 40°C. At 40°C, females had higher heated-dark plate preference than males (F: 80%; M: 33%). Session 1: $n = 6$ female mice, $n = 6$ male mice; Session 2: $n = 5$ female mice, $n = 6$ male mice. * indicates $p < 0.05$ between female and male mice; "thermometer × sex" symbol indicates significant temperature × sex interaction; sex symbol alone indicates significant main effect of sex. Two-way RM ANOVA with Bonferroni *post hoc* test.

increased anxiety-like behavior in TIDAL to an extent that is not consistently observed in a simple dark–light test.

Overall, these data suggest that this temperature range is well-suited to assess salience of anxiety versus heat, and that the test effectively unveils anxiety-like behavior (TIDAL vs. TPP results). In addition, females as compared to males strongly increased anxiety-like behavior in TIDAL to an extent that is not consistently observed in a simple dark–light test.

3.2 | Over Two Repeated Sessions, Females as Compared to Males Maintain Prolonged Heated-Dark Plate Preference Under Hyperthermic Conditions

Next, we aimed to replicate our sex differences in TIDAL conflict test behavior in a separate cohort to establish reproducibility of our results, and we sought to determine whether mice with prior exposure to TIDAL show evidence of learning.

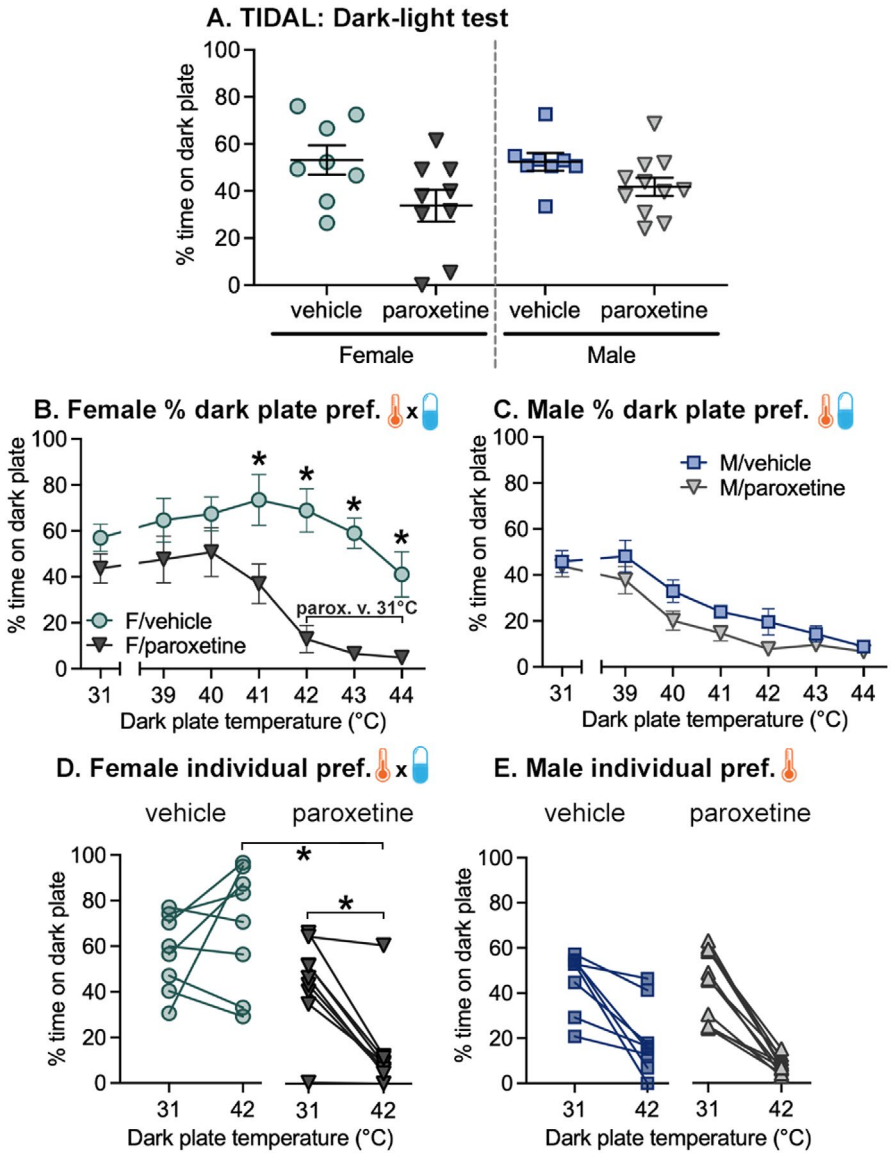

FIGURE 4 | Paroxetine reduces anxiety-like behavior in female mice in the TIDAL conflict test. (A) Mice that received paroxetine prior to TIDAL conflict testing showed reduced dark cylinder preference relative to vehicle controls with both plates at 31°C. (B) During the TIDAL conflict test, all mice reduced time spent on the heated-dark plate as temperatures increased. Further, female mice that received paroxetine exhibited reduced heated-dark plate preference relative to vehicle controls from (31°C to 44°C). (C) Vehicle and paroxetine male mice showed similar preference for the heated-dark plate as temperatures increased. (D, E) Heated-dark plate preferences of individual mice with the dark plate at 31°C and 42°C. At 42°C, vehicle females had higher heated-dark plate preference than paroxetine females (F/vehicle: 68.9%; F/paroxetine: 12.9%). Additionally, paroxetine females showed a significant reduction in heated-dark plate preference from 31°C to 42°C (31°C: 43.6%; 42°C: 12.9%). F/vehicle: $n = 8$ female mice, F/paroxetine $n = 9$ female mice; M/vehicle: $n = 8$ male mice, * indicates $p < 0.05$ between paroxetine and vehicle-treated mice; "thermometer × pill" symbol indicates significant temperature × drug interaction; temperature symbol alone indicates significant main effect of temperature. Two-way RM ANOVA with *post hoc* Bonferroni test.

To address this, a cohort of female and male mice completed two identical TIDAL conflict test sessions separated by two weeks. In the dark–light test over two sessions, females as compared to males had higher preference for the heated-dark plate (two-way RM ANOVA, main effect of sex $F_{1,9} = 14.58$, $p < 0.005$) (Figure 3A). This sex difference was particularly notable in Session 2, when females exhibited 83% heated-dark plate preference versus males' 54% dark preference. Together, these dark–light test data suggest that females on the dark–light test exhibit anxiety-like behavior, and that the sex difference in anxiety-like behavior is exaggerated in a second exposure to the dark–light apparatus.

Next, we assessed TIDAL conflict test behavior in both sexes over two sessions. First, we compare TIDAL results by sex: TIDAL females as compared to males had increased preference for the heated-dark plate in both Sessions 1 and 2 (Figures 3, S4, and S5), thereby closely recapitulating results from our previous study (Figure 2). In both TIDAL sessions, females spent < 50% of their time on the heated-dark plate at higher temperatures compared to males (main effect of sex, $F_{1,19} = 44.21$, $p < 0.001$) (Figure 3B). In TIDAL Sessions 1 and 2, female mice showed a greater preference for the heat shift-dark plate compared to males. Specifically, during Session

1, there was a significant interaction between sex and temperature ($F_{6,60} = 6.22$, $p < 0.001$) with *post hoc* tests revealing that females showed significantly higher preference than males at all temperatures between 39°C and 44°C ($p < 0.05$) (Figure 3C). In Session 2, a similar sex × temperature interaction was observed ($F_{6,54} = 4.04$, $p < 0.005$). *Post hoc* tests indicated that females had a significantly greater preference than males between 31°C and 42°C ($p < 0.01$) and at 44°C ($p < 0.05$) (Figure 3D).

One key temperature that exposed sex differences was 40°C. In Session 1, 85% of females and 41% of males preferred the heated-dark plate (sex × temperature interaction: $F_{1,10} = 16.15$; $p < 0.005$; Bonferroni-corrected t-test at 40°C: $t = 5.21$; $p < 0.001$) (Figure 3E). In Session 2, the preference was 80% for females and 33% for males, with a significant sex × temperature interaction ($F_{1,9} = 11.41$; $p < 0.01$) and post hoc comparison ($t = 6.22$; $p = 0.001$) (Figure 3F). Males (but not females) showed a reduced preference for the heated-dark plate at 40°C compared to 31°C in both sessions ($t = 4.59$ and 5.91, respectively; $p < 0.001$). In addition, females more frequently crossed into the dark chamber (Figure S4). These results bolster our previous findings, showing that females as compared to males exhibit robust anxiety-like behavior in the TIDAL conflict assay with enhanced willingness to remain on an aversive temperature stimulus to avoid an illuminated chamber. Further, these data reveal that sex differences in the salience of anxiety persist in a second session of the TIDAL conflict test.

Therefore, mice completing a second session of TIDAL exhibited signs of learning by increasing heated-dark plate avoidance: male mice in Session 2 already showed place avoidance from the heated-dark plate at isothermic temperature at test start, whereas females in Session 2 showed accelerated avoidance as the dark plate temperature increased (Figures 3 and S5). Mice exhibit learning in the TIDAL conflict test, which manifests differently in female and male mice. Overall, our data suggest that the TIDAL conflict test is reliable and reproducible in showing that female as compared to male mice exhibit heightened anxiety-like behavior.

3.3 | An Anxiolytic, Paroxetine, Reduces Anxiety-Like Behavior in the TIDAL Conflict Test

Finally, we used a pharmacologic compound to validate the TIDAL conflict test as a viable strategy for assessing anxiety-like behaviors in mice (Figures 4 and S6). We aimed to relieve anxiety-like behavior in mice using the selective serotonin reuptake inhibitor (SSRI) paroxetine; paroxetine reduces anxiety in humans (Nemeroff and Owens 2003; Sheehan and Mao 2003) and mice (Bentefour et al. 2015). If paroxetine-treated mice show reduced time in the dark cylinder relative to vehicle controls, this suggests that TIDAL is valid for detecting anxiety-like behavior.

To establish whether anxiolytic treatment shifted behavior in the TIDAL conflict test, mice were administered intraperitoneal paroxetine (10 mg/kg) or saline one hour prior to TIDAL testing. In the dark–light test, mice that received paroxetine showed decreased heated-dark plate preference compared to vehicle controls (two-way ANOVA; main effect of drug $F_{1,32} = 8.13$, $p = 0.008$) (Figure 4A). During TIDAL conflict testing, female-paroxetine (vs. female-vehicle) mice decreased preference for the heated-dark plate with a significant drug × temperature interaction ($F_{6,90} = 3.80$; $p = 0.002$) and significant *post hoc* results for all temperatures between 41°C and 44°C ($t \geq 3.21$; $p < 0.005$) (Figure 4B) controls with significant main effects of drug ($F_{1,102} = 4.62$, $p = 0.046$) and temperature ($F_{6,102} = 35.53$, $p < 0.001$) (Figure 4C). In using paroxetine in the TIDAL conflict test, 42°C robustly uncovered differences in anxiety-like behavior between treatment groups: in mice at 42°C, heated-dark plate preference was 69% for female-vehicle and 13% for female-paroxetine mice, and male heated-dark plate preference was 20% for male-vehicle and 8% for male-paroxetine mice, respectively (Figure 4D,E). Female-vehicle mice preferred the heated-dark plate significantly more than female-paroxetine mice at 42°C ($t = 5.66$ $p < 0.001$ following a significant drug × temperature interaction: $F_{1,15} = 15.09$; $p = 0.001$). Additionally, whereas female-vehicle mice showed similar heated-dark plate preference at 31°C (57%) and 42°C (69%), female-paroxetine mice reduced heated-dark plate preference from 44% at baseline 31°C to 8% at 42°C ($t = 4.08$, $p < 0.001$). All male mice regardless of drug treatment exhibited decreased heated-dark plate preference from 31°C to 44°C (main effect of temperature, $F_{1,17} = 69.74$; $p < 0.001$).

Averaging from 42°C to 44°C, female-paroxetine mice showed 48% reduced heated-dark plate preference compared to female-vehicle mice. From 39°C to 42°C, male-paroxetine mice had 11% decreased heated-dark plate preference compared to male-vehicle mice. These results reveal that paroxetine reduces heated-dark plate preference as temperatures rise in the TIDAL conflict test, suggesting that the TIDAL conflict test for mice is valid for assessing anxiety-like behavior.

4 | Discussion

This study explored anxiety-like behavior in mice using the TIDAL conflict test, a behavioral assay integrating a conflicting dark–light dilemma with incremental increases on a heating plate. The TIDAL conflict test unmasked robust and reproducible sex differences in anxiety: female as compared to male mice maintained prolonged heated-dark plate preference under increasingly aversive hyperthermic conditions, suggesting increased anxiety-like behaviors. When mice completed a second session of TIDAL, females showed increased anxiety-like symptoms in the dark–light test (compared to the dark–light test prior to the first session). Further, mice of both sexes in a second session had accelerated place avoidance of the heating dark plate, implying that they had learned from prior exposure to the test; thus, to avoid confounds of learning, distinct cohorts should be used for testing manipulations or timecourses. Anxiety-related shifts in TIDAL behavior were not simply a preference for the heated plate, since mice exhibited prolonged preference for the heated plate in the TIDAL conflict test (heated plate dark; isothermic plate illuminated) compared to the TPP test (both sides illuminated). Finally, we validated the TIDAL conflict test using an anxiety-relieving drug, paroxetine, which decreased mouse preference for the dark-heating plate. Therefore, compared to one-dimensional,

commonly used tests of anxiety behaviors, our newly established TIDAL conflict test reveals tangible differences in anxiety-like symptoms in mice due to sex.

Differences in human anxiety are not consistently replicated in rodent models (Donner and Lowry 2013; Scholl et al. 2019). Most tests for anxiety-like behavior were developed > 20 years ago and were validated in male rodents only (Börchers et al. 2022; Donner and Lowry 2013). In the elevated plus maze, female mice show decreased anxiety-like behavior (defined as increased time in open arms and open arm entries) when compared to male mice (Rodgers and Cole 1993; Võikar et al. 2001). However, in the light–dark test, mouse anxiety-like behavior is variable between sexes across different mouse strains (Võikar et al. 2001). Accordingly, we found that the light–dark test—completed in the first five minutes in the apparatus with both plates at 31°C—showed little or no difference between sexes; sex differences in anxiety-like behavior were only unmasked as the anxiety-driving stimulus was placed in conflict with an aversive temperature stimulus. This corroborates previous studies described above and underscores a need to develop more refined tests that model anxiety in mice.

Here, we sought to develop a conflict test in mice that better uncovered differences in anxiety-like behavior. We placed an anxiety-relevant dilemma (light vs. dark) in conflict with increasing temperature (on the dark side only). Our optimized TIDAL conflict test incorporates dark plate temperatures initially at 31°C (10 min), then incrementally increasing temperatures of 39°C–44°C (5 min each). Our optimized temperature range is notable, because the heat-activated ion channel involved in sensing low-level noxious heat, TRPV1, is activated at 43°C (Willis 2009). The TIDAL conflict test revealed robust, reproducible increases in anxiety-like behavior in females versus males.

Another notable aspect of our optimized TIDAL conflict parameters is the use of five minutes at each temperature increment. We performed pilot experiments with three minutes per temperature, but found that this shorter time was insufficient for mice to delineate differences across temperatures. Our data suggest that a combination of "temperature history," habituation to the light–dark chambers, and the temperatures chosen for incremental heated-dark plate increases are important for unveiling differences in anxiety-like behavior in mice. It is possible that sex differences in temperature and light habituation could affect sex differences in behavior observed in the TIDAL conflict test. Our time interval is consistent with other discrimination-based assays—for instance, the three-chamber social approach task includes 5–10 min increments of exposure (Arakawa 2023; Fonken et al. 2016; Kaidanovich-Beilin et al. 2011). Another consideration is our TIDAL conflict design using incremental increases in temperature—although it was not possible to rapidly shift temperatures up and down several degrees using our hot-cold plate apparatus, future studies could test altering or randomizing the order of temperature shifts to control for temperature history. Further, future studies should test additional temperature durations (e.g., optimize conditions to shorten the test duration) or other temperature combinations (warm or cool) to explore how these parameters affect anxiety-like behavior between sexes or in neuropathic conditions.

Female rodents exhibit increased pain symptoms on reflexive tests (Hargreaves test or hot plate) (Gaudet et al. 2017; Gioiosa et al. 2008; Mogil 2020) but also prefer warmer ambient temperatures (Kaikaew et al. 2017)—these sex differences in temperature preferences could influence TIDAL outcomes. A recent meta-analysis assessed the extent that research literature reveals sex differences in pain-like behavior—in 85% of studies with significant sex differences in pain-like behavior, female rodents displayed increased pain sensitivity versus males (Mogil 2020). This parallels sex differences observed in humans, with women on average displaying increased heat sensitivity compared to men (Racine et al. 2012). In contrast, females prefer warmer ambient temperatures. Adult female C57BL/6J mice prefer warmer cage temperatures than males, and this is independent of the post-pubertal presence of gonadal hormones (Kaikaew et al. 2017). Similarly, cool temperatures are more aversive to women compared to men (Kaikaew et al. 2018; Kim et al. 2013; Schellen et al. 2012). Sex differences in thermal preference likely relate to females having a higher body surface area to body mass ratio, and to sex-specific body compositions (Kaikaew et al. 2018). In our study, crucial control conditions (thermal place preference assay with no light–dark conflict) confirmed that the observed sex differences were not simply due to enhanced preference for the heated plate for females; females on TIDAL displayed stronger preference for the heated (also dark) plate compared to females on TPP (with the heated plated lighted). Therefore, although sex differences in thermal sensation may influence TIDAL outcomes, our data suggest that the TIDAL conflict test is an effective approach in mice for uncovering sex differences in anxiety-like behavior.

Mice of both sexes exhibited learning on the TIDAL conflict test. Mice were tested in two sessions separated by two weeks using identical TIDAL conflict testing protocols. Learning was apparent in the second session in the initial dark–light test: female mice in the second dark–light test newly showed increased heated-dark plate preference compared to males. In TIDAL Sessions 1 and 2, female as compared to male mice showed increased preference for the heat shift-dark plate from 39°C throughout the remainder of the test. Further, in the second TIDAL session, both females and males expedited exit from the heat shift-dark plate, implying that they anticipated the increasing temperatures and proactively avoided this side. These results extend our previous findings that females as compared to male exhibit robust anxiety-like behavior in the TIDAL conflict assay—in particular, females showed heightened anxiety-like behavior in the Session 2 dark–light test. Further, our results show that mice learn the test, and that sex differences in the salience of anxiety persist through repeated TIDAL sessions. Similarly, rodent learning occurs in other tests of anxiety-like behavior (Bailey and Crawley 2009; File 1993, 2001; Roesler et al. 1999). The fact that rodents exhibit learning on the TIDAL conflict test is important, because this suggests that behavioral timecourses or tests using different treatments must account for this learning effect or experiments must be completed on distinct cohorts.

Our TIDAL conflict test uncovered sex differences in mice that align well with sex differences in anxiety observed in humans. In the human population, the lifetime prevalence of anxiety disorders is up to 60% higher in women (versus men) (Kessler et al. 2005; McLean and Anderson 2009). Symptom progression, treatment response, and average age of onset are also affected by sex in humans (Pigott 2003). In our TIDAL conflict test, female mice showed increased anxiety-like behavior; however, the basic light–dark test—which is frequently used to assay anxiety-like behavior—failed to reliably detect sex differences. This suggests that a more complex test is required to identify behavioral differences in mice of different sexes, which is supported by the differences in clinical presentation observed across sexes in humans. Anxiety disorders often present comorbidly with other health conditions such as depression, hypertension, epilepsy, chronic pain, and neurotrauma (Dickerson et al. 2021; Hingray et al. 2019; Johnson 2019; Tiller 2013). We did not assess estrous cycle, which is accordance with guidelines for studying sex differences in rodents (Shansky 2019; Shansky and Murphy 2021) since gonadal hormones were not the main question studied here. Future studies could further explore the role of hormones in anxiety-like behavior using the TIDAL conflict test. Overall, it is important to identify mechanistic differences between sexes that underlie susceptibility to anxiety and comorbid conditions.

We used the TIDAL conflict test previously to evaluate anxiety-like behavior of mice with spinal cord injury, which revealed that mice with spinal cord injury exhibited increased anxiety-like behavior relative to sham surgery controls (Lee et al. 2023). Here, we sought to further validate the TIDAL conflict test using known pharmacologic agents that ameliorate anxiety-like behavior. Initially, we attempted validation using diazepam, a commonly used benzodiazepine that modulates GABA influx (Campo-Soria, Chang, and Weiss 2006; Lavoie and Twyman 1996; Masiulis et al. 2019). In C57BL/6J mice, however, diazepam can cause strong sedative effects that mask potential anxiolytic effects (Pádua-Reis et al. 2021). In our pilot studies delivering diazepam prior to the TIDAL conflict test, all diazepam-treated mice substantially decreased overall exploration and reduced time spent in the dark cylinder. Therefore, acute diazepam induces hypolocomotion that impedes ability to assess anxiety-like behavior in our place preference tests.

Next, we tested the SSRI paroxetine. SSRIs can treat clinical anxiety without producing severe locomotor side effects (Jakubovski et al. 2019; Sheehan and Mao 2003). Following acute paroxetine administration, mice exhibited reduced overall exploration and time spent in the dark cylinder during the TIDAL conflict test. This result was more pronounced in female mice, suggesting underlying sex differences play a role in anxiety-like behavioral state. It is important to note that in humans, paroxetine treatment is typically a long-term intervention, often eliciting noticeable behavioral changes within 1–3 months (Perna et al. 2016). In contrast, here, mice received a single dose of paroxetine one hour prior to TIDAL conflict testing, which replicates acute anxiolytic effects in mice observed previously (Pádua-Reis et al. 2021). Future studies could assess anxiolytic effectiveness of paroxetine as a long-term intervention in male and female mice. Overall, these results using a non-sedative anxiolytic confirm the validity and reliability of the TIDAL conflict test for assessing anxiety-like behavior.

Paroxetine can relieve anxiety, but this SSRI can also have antinociceptive effects in rodents and humans (Kesim et al. 2005; Matsuzawa-Yanagida et al. 2008; Patetsos and Horjales-Araujo 2016; Sindrup et al. 1990). For example, Duman et al. (2006) found that one 10 mg/kg dose of paroxetine increased thermal withdrawal latency on a 55°C hot plate in both male and female mice one hour after administration. In contrast, our TIDAL results show the opposite effect: paroxetine-treated mice spend less time on the heated-dark plate compared to vehicle-treated control mice (Figure 4). Although we cannot completely rule out off-target effects, with TIDAL, we observe that paroxetine treatment effectively eliminates the conflict introduced by the dark plate—that is, paroxetine-treated mice behave more like mice in a standard TPP assay, reflecting their natural temperature preference. Taken together, these observations suggest that—in the context of TIDAL—paroxetine is acting predominantly as an anxiolytic drug (rather than an analgesic), and that the TIDAL conflict test is valid for assessing anxiety-like behavior.

As mentioned, the TIDAL conflict test more effectively unmasks clinically relevant sex differences in anxiety-like behavior than other tests, and this is reflected in effect sizes. To contextualize the observed sex difference in TIDAL, we calculated the standardized mean difference score (d) (Cohen 2013) based on the 50% threshold temperature values from Figure 2B. The resulting value of $d = 2.68$, with females more strongly preferring the dark, heating chamber compared to males, suggests a relatively "large" effect size (Sullivan and Feinn 2012). In contrast, in the open field test, little-to-no sex differences are observed in percent time in the center zone (Fritz, Amrein, and Wolfer 2017; Vošlajerová Bímová et al. 2016). In the elevated plus maze, an examination of reported sex differences reveals the following: Rodgers and Cole (1993) demonstrate that females spend significantly less time in the closed arms relative to males, yielding a difference score of 0.798, and more time in the open arms ($d = 0.53$), though the difference is non-significant; Hendershott et al. (Hendershott et al. 2016) report that females exhibited an increased preference for the open arms with a maximum difference score of 0.868; and Painsipp et al. (2007) similarly note females' heightened preference for the open arms, with a difference score of $d = 1.54$. The significant sex difference reported in these studies fall near or within the "large" range (≥ 0.8) (Cohen 2013; Sullivan and Feinn 2012); however, these results are in the opposite direction of our finding that females exhibit more anxiety-like behavior than males. Our larger effect size implies less overlap between females in males in the TIDAL conflict test compared to the elevated plus maze, and our TIDAL sex differences are in a direction that parallels human sex differences in anxiety prevalence. Ultimately, this suggests that the sex differences in anxiety-like behavior detected by TIDAL are pronounced and clinically relevant, highlighting unique aspects of anxiety-related responses in this test compared to commonly used tests.

Our results highlight the importance of conflict tests in uncovering anxiety-like behavioral differences in mice of different groups. Conflict tests produce differing motivational states through the introduction of approach-avoidance situations. These tests offer an unconditioned approach to observing anxiety-like behavior, resulting in high ethological

validity (Campos et al. 2013). Previous conflict tests have assayed anxiety-like behavior, including the Vogel test (Basso et al. 2011; Johnston and File 1991; Vogel, Beer, and Clody 1971), the four-plate assay (Boissier, Simon, and Aron 1968), the mechanical conflict-avoidance assay (Chhaya et al. 2019; Ferland et al. 2024; Gaffney et al. 2022; Harte et al. 2016; Li et al. 2020; Richards, Freeman, and Detloff 2024), and the defensive burying test (Fucich and Morilak 2018; Pinel and Treit 1978). There are key considerations and limitations related to these existing conflict tests (Lapiz-Bluhm et al. 2008): (1) many of the tests use shock as a punishment, which may cause pain and assess fear (rather than anxiety); (2) several tests have not been rigorously validated in female versus male rodents (four-plate test) or do not show clearly interpretable sex differences (defensive burying; Arakawa 2007; Castillo et al. 2022); (3) the four-plate test elicits many false positives (File 2001); and (4) the Vogel and probe burying tests were optimized for study in rats, rather than mice. Thus, benefits of the TIDAL conflict test include that it does not require deprivation or training or induce pain; it is validated for use in mice; and the test mouse is free to explore the aversive chamber or not, thereby limiting stressful effects of the test.

One test that has garnered recent attention is the mechanical conflict-avoidance system, which operates on similar principles to the TIDAL conflict test. Mechanical conflict provides an operant method of pain testing with rodents intended to supplement reflexive methods by addressing cognitive and motivational processing through multi-layered conflict (Chhaya et al. 2019; Ferland et al. 2024). The mechanical conflict-avoidance system consists of a light and dark chamber connected by a walkway modified to deliver mechanical stimulation. Mechanical conflict is a useful assay to assess operant principles of chronic mechanical pain-like behavior; in contrast, the TIDAL conflict test places a light stimulus in conflict with heat to unveil differences in anxiety-like behavior. Thus, the mechanical conflict-avoidance system and TIDAL are complementary conflict tests that can help reveal affective behaviors related to pain and anxiety, respectively.

5 | Future Directions and Conclusions

Here, we developed and validated a new assay—the thermal increments dark–light (TIDAL) conflict test—that exposes in mice previously underappreciated differences in anxiety-like behavior. Future studies could use this test to explore neural circuitry related to anxiety or avoidance behaviors (Bangasser and Cuarenta 2021); e.g., to test whether manipulating specific neural pathways alters anxiety-like behavior in TIDAL. Further, TIDAL experiments could explore anxiety-like behavior in other contexts, including stress, early-life adversity, injury, or sickness/neuroinflammation (Bolton et al. 2018; Bourke, Harrell, and Neigh 2012; Fonken, Weil, and Nelson 2013; Fonken et al. 2018; Grace et al. 2021).

In summary, we explored anxiety-like behavior in mice using the TIDAL conflict test. Our data reveal that the TIDAL conflict test reliably unmasks amplified anxiety symptoms in mice that are female compared to males; the test was validated using an anxiety-relieving drug, which reduced mouse preference for the dark-heating plate. Incrementally increasing the magnitude of one of the conflicting factors (here, heat), while maintaining constant the other factor (dark vs. light), enabled deciphering robust differences that might have been overlooked if only a single temperature was used. More broadly, these results suggest that rodent studies should incorporate conflicting stimuli to illuminate potential differences in anxiety-like behavior. Therefore, future preclinical studies should prioritize assays that detect behavioral differences not apparent in commonly used anxiety-like behavioral assays to identify circuits and therapies that benefit health outcomes, emotive state, and well-being.

Declaration of Transparency

The authors, reviewers and editors affirm that in accordance to the policies set by the *Journal of Neuroscience Research*, this manuscript presents an accurate and transparent account of the study being reported and that all critical details describing the methods and results are present.

Author Contributions

S.E.L., S.-H.P., L.K.F., and A.D.G. designed experiments. S.E.L. and S.-H.P. performed experiments. S.E.L., J.C.A., S.-H.P., and A.D.G. analyzed data. S.E.L., J.C.A., S.-H.P., L.K.F., and A.D.G. wrote and edited the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon request.

Peer Review

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.