Anxiety-like behaviors in mice unmasked: Revealing sex differences in anxiety using a novel light-heat conflict test

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Abstract

Anxiety and chronic pain afflict hundreds of millions worldwide. Both anxiety and pain are more prevalent in females (vs. 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 the dark-heating and illuminated-isothermic 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 three distinct experiments that females on the TIDAL conflict test persist substantially longer on the dark-heated plate, suggesting that females (vs. males) exhibit elevated anxiety-like behavior. Mice more strongly prefer the dark plate on the TIDAL conflict test compared to control thermal place preference with both chambers illuminated. We also reveal that mice exposed to TIDAL for a second session exhibit signs of learning. 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.

Introduction

Anxiety disorders cost the United States approximately $49 billion annually (Trautmann et al., 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 2x higher prevalence in females (Somers et al., 2006; McLean et al., 2011; Baxter et al., 2013). Females show increased prevalence of separation anxiety, phobias, generalized anxiety, and panic disorders beginning at childhood or adolescence (Donner and Lowry, 2013; Altemus et al., 2014; Bekhbat and Neigh, 2018). 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 currently 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 Donner and Lowry, 2013; Börchers et al., 2022)). In rats, females (vs. males) travel increased distances and reduce anxiety-like behaviors in the open field test and the elevated plus maze (e.g., Johnston and File, 1991; Scholl et al., 2019; Knight et al., 2021; Börchers et al., 2022). In addition, outcomes regarding rodent sex differences in these anxiety-related tests are inconsistent and fail to recapitulate human sex differences (Võikar et al., 2001; An et al., 2011). 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 natural environments. Specifically, 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 vs. heat avoidance in mice using a novel 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) vs. illuminated – in conflict with a weaker but increasingly aversive “pain”-relevant thermal choice – increasing heat vs. maintained isothermic temperature. We aim to discover whether the relative salience of anxiety vs. heat sensitivity better reflects anxiety-related sex differences reported in ethological and clinical settings. As mentioned, both anxiety-like behaviors and heat sensitivity are increased in females (vs. males), so it is unclear which stimulus would be more salient to mice in our newly designed conflict test. The TIDAL conflict test is used here to test whether sex affects the salience of anxiety vs. 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 and reliable tool for unmasking previously underappreciated differences in anxiety-like behavior.

Materials and Methods

Animals and housing:

All housing, surgery, and postoperative care 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.

Behavioral tests for anxiety-like behavior:

<u>Thermal Increments Dark-Light (TIDAL) Conflict Test:</u> 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 x 25 cm high) connected by a narrow center walkway (**Fig. 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 to 44°C (**Fig. 1A,D**). (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. Pilot studies used a wide range of temperatures (31-52°C) and times at each temperature (5-10 min) (**Fig. 1C**). Based on these pilot studies, to optimally detect the salience

of anxiety vs. thermal avoidance we defined the following parameters: Mice are initially allowed to explore the apparatus for 5 minutes 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 minutes (**Fig. 1D**).

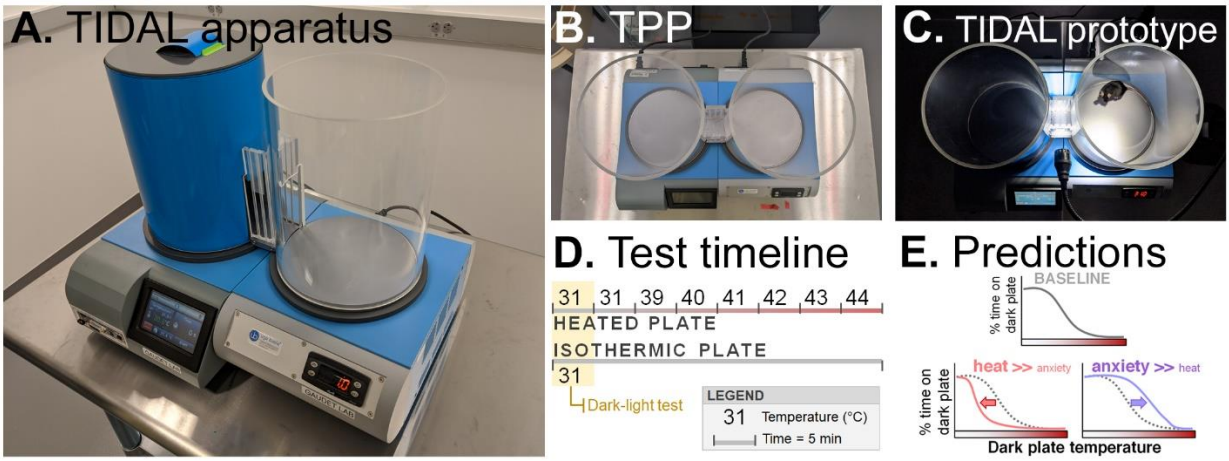

Figure 1. The Thermal Increments Dark-Light (TIDAL) conflict test for exploring anxiety-like behavior and thermal sensitivity in mice: design, controls, timeline, and predictions. **A.** Final, optimized setup of the TIDAL apparatus. Note that the overhead room lights are on, and the dark-heated plate 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 Fig. 2. 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 dark plate as its temperature increases. We predict that sex and neurotrauma will alter the salience of heat discomfort vs. anxiety-related sensitivity, manifesting as shifted dark plate preferences over time.

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 (**Fig. 1B**) – i.e., the heated side is lighted, not dark as in the TIDAL conflict assay. Next, the same incremental temperature increases are applied. Time in the center walkway was excluded from all analyses, because the surroundings there differed from the test chambers, some mice loitered in this zone in a manner that did not relate to the experimental question, and automated mouse tracking was difficult in this zone.

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. The arena was cleaned with 70% ethanol between trials.

Experiments and mouse numbers

Unless noted otherwise, all mice were 6-8 weeks old at time of testing. Each experiment had distinct design and mouse numbers. 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. Experiment 3 data

related to distance traveled was not able to be analyzed, so is excluded from supplemental data herein.

Statistics

Mouse TIDAL behavior (dark plate preference, distance traveled) was analyzed using one-, two-, or three-way ANOVA (repeated measures, when appropriate), followed by Bonferroni *post-hoc* tests. In experiments with two groups, a Student's *t*-test (or nonparametric Mann–Whitney *U* test) was performed. Prism 9 (GraphPad) was used for visualizing data and Sigmaplot 14 (SPSS) was used for statistical analyses.

Results

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 (Feine et al., 1991; Rhudy and Meagher, 2001; Bragdon et al., 2002; Bartley and Fillingim, 2013; Reddan et al., 2020). To explore whether differences in anxiety- vs. 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 **Fig. 1C** – initial test with lighting using directed light (rather than existing room overhead lighting) and no top on the dark plate). 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 42, 44, 46, 48, 50, and 52°C (five minutes at each temperature) (**Fig. 2**). If females (vs. 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) (**Fig. 1E**).

On the dark-light test, females and males spent a similar percentage of time on the dark plate (**Fig. 2A**) (females: 51 ± 2%, males: 49 ± 3%; *t*-test, $p > 0.05$). The dark plate preference on the dark-light test was surprisingly low, so in subsequent studies we refined the dark-light contrast between chambers (see below).

In the TIDAL conflict test, temperature increases caused mice to spend progressively less time

on the heated dark plate. Compared to baseline preference at 31°C, males spent less time on the dark plate beginning at 42°C, and females had reduced dark plate preference beginning at 44°C (males: 31°C: 53 ± 2%, 42°C: 32 ± 2%; $p < 0.001$) (females: 31°C: 47 ± 3%, 44°C: 23 ± 3%, $p < 0.001$) (two-way RM ANOVA with Bonferroni; sex x temperature interaction $F_{5,130} = 5.13$; $p < 0.001$) (**Fig. 2B**). Both females and males spent <10% of time on the dark plate at 49 and 52°C, suggesting that these temperatures were strongly aversive.

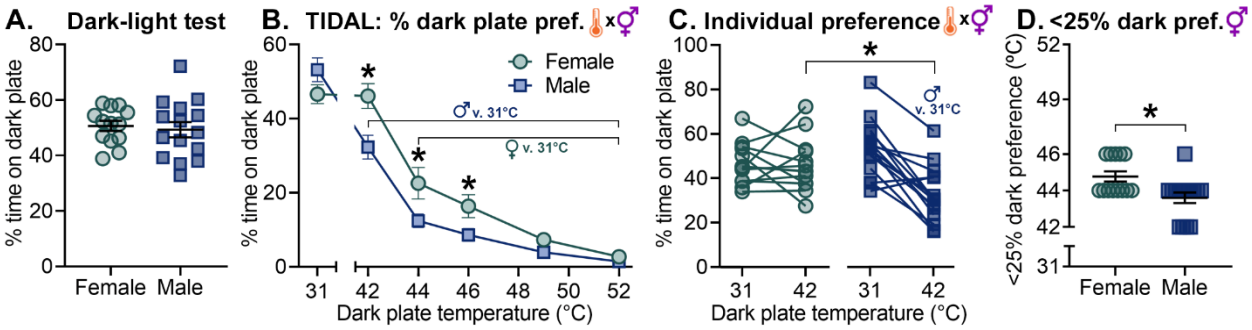

Figure 2. An initial study using the Thermal Increments Dark-Light (TIDAL) conflict test between 42-52°C showed that females (vs. males) displayed increased salience of anxiety. **A.** In the dark-light test with both plates at 31°C, females and males showed no significant difference in dark plate preference. **B.** TIDAL test with increasing temperature on the dark plate only. Compared to males, females showed extended preference for the heating dark plate (vs. the constant 31°C lighted plate). **C.** Dark plate preferences of individual mice with the dark plate at 31°C and 42°C. At 42°C, females averaged 46% time on the dark plate, whereas males averaged 32% dark plate preference. **D.** Threshold at which female and male mice showed less than 25% preference for the dark plate. *n*=13 female, *n*=15 male mice; * indicates $p < 0.05$ between female and male mice; "thermometer x gender" symbol indicates significant temperature x sex interaction; gender symbol alone indicates main effect of sex ;"female/male symbol v. 31°C" indicates within-sex difference vs. baseline 31°C.

This TIDAL conflict test exposed notable sex differences in salience of anxiety: females (vs. males) spent more time on the dark plate at 42, 44, and 46°C (**Fig. 2B**). At 42°C females spent 44% more time than males on the heated dark plate (female 42°C: 46 ± 3%, male 42°C: 32 ± 2%; two-way RM ANOVA, $p < 0.001$), and males, but not females, reduced dark plate preference at 42°C (vs. 31°C) (**Fig. 2C**). Further, females spent <25% of time on the dark plate at a higher temperature than males (Mann-Whitney Rank Sum test, $p < 0.05$) (**Fig. 2D**).

Females and males in the TIDAL test covered a similar distance and females modestly increased percent crossings into the dark plate area (**Fig. S1**). Female and male mice showed surprisingly low baseline dark plate preference (~50%) and a sharp decrease in time spent on the dark plate with these temperature increases; thus, for subsequent experiments, the TIDAL conflict test was further optimized (i.e., improved light-dark contrast between sides and more confined temperature range to better distinguish differences). Overall, this initial study suggests that amplified anxiety-like behavior in female mice can be exposed by placing a known anxiety-related stimulus (dark vs. light) in conflict with increasing temperature.

Refining the TIDAL conflict test temperature window further illuminates that female (vs. male) mice exhibit increased salience of anxiety

To further optimize the TIDAL conflict test, we improved light-dark contrast in the apparatus (by adding a lid and using overhead lights; **Fig. 1A**) and refined the temperature range used to better capture the salience of anxiety vs. heat aversion (31°C for 10 min; then 39-44°C, with 1°C increases every 5 min; **Fig. 1D**). These lighting and temperature range improvements aimed to increase resolution for detecting group differences in TIDAL preferences.

On the dark-light test, females and males undergoing TIDAL both preferred the dark (vs. illuminated) plate (**Fig. 3A**) (females: 70 ± 5%, males: 61 ± 3%; *t*-test, $p > 0.05$), 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: 58 ± 4%, males: 58 ± 4%; *t*-test, $p > 0.05$) (data not shown). Here, the dark-light test did not expose significant differences in dark plate preference between females and males; potential sex differences in anxiety-like behavior may be better delineated using the TIDAL conflict test.

Next, behavior on the TPP (control) vs. TIDAL conflict test was assessed in female vs. 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 (**Fig. 3B-F**). Indeed, females (vs. males) preferred the heated plate to higher temperatures in both tests (**Fig. 3B**), and mice of both sexes remained on the heated plate longer under TIDAL conditions (with the heated plate also being dark) (**Fig. 3C,D**). Female mice in TIDAL (vs. female-TPP) more strongly preferred the heated plate throughout the temperature increases (two-way RM ANOVA; main effect of test [$F_{1,144}$ = 11.31] and temperature [$F_{6,144}$ = 21.27]; both $p < 0.005$) (**Fig. 3C**). Similarly, male mice tested on TIDAL (vs. TPP) persisted on the heated plate to higher temperatures (two-way RM ANOVA; main effect of test [$F_{1,156}$ = 4.097, $p = 0.05$] and temperature [$F_{6,156}$ = 61.71, $p < 0.001$]) (**Fig. 3D**). Male TPP mice reduced preference for the heat shift plate earlier compared to female TPP mice and compared to male TIDAL mice. Focusing on a pivotal temperature, we found at 42°C that females in particular preferred the heated-dark TIDAL plate (vs. illuminated TPP heated plate) (female-TPP 42°C: 36 ± 6%; female-TIDAL 42°C: 65 ± 6%; two-way RM ANOVA, main effects of both temperature and test, $p < 0.05$), whereas male dark plate preference at 42°C was not significantly different between TIDAL and TPP mice (male-TPP 42°C: 14 ± 4%; male-TIDAL 42°C: 25 ± 4%; $p > 0.05$; main effect of temperature only) (**Fig. 3E,F**). Therefore, using TPP vs. TIDAL to establish whether making the heating chamber dark extends the duration of preference for the heated plate, our results suggest that the TIDAL conflict test can be used in female and male mice to assess anxiety-like symptoms.

Comparing between sexes, female TPP and TIDAL mice showed higher heat-shift plate preference than males on those same tests (**Fig. 3B-D, Fig. S2**). Further, females on TIDAL (vs. TPP) increased preference for the heated, dark side compared to males. Females traveled further per minute in the illuminated area and crossed into the dark chamber more frequently

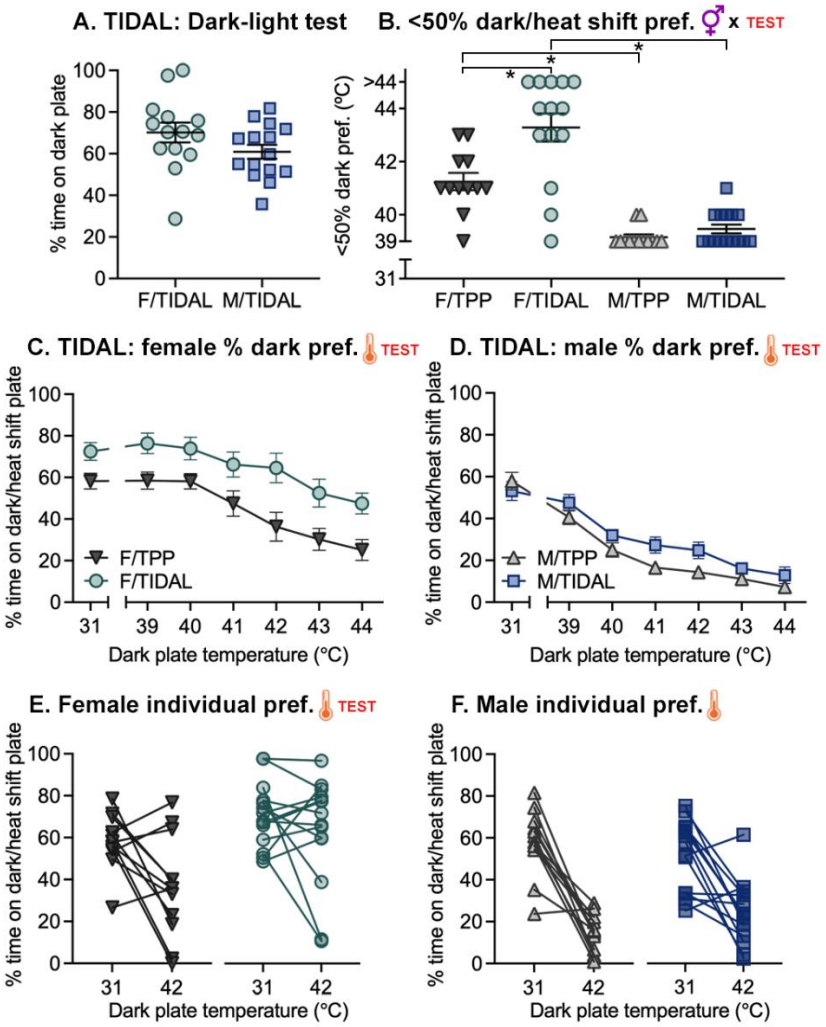

Figure 3. The TIDAL conflict test exposes differences in anxiety-like behavior at optimized incremental heat shift range (39-44°C). Adult female and male mice were tested on the TIDAL conflict test (heated side dark; isothermic side lighted) or on the thermal place preference (TPP) test (both sides lighted). **A.** In the dark-light test with both plates at 31°C, females and males showed similar 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 (vs. 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; “gender x TEST” symbol indicates significant sex x test interaction; thermometer or TEST symbols alone indicate significant main effects of temperature and TPP/TIDAL, respectively.

(**Fig. S2**). Overall, these data suggest that this temperature range is well-suited to assess salience of anxiety vs. heat, and that the test effectively unveils anxiety-like behavior (TIDAL vs. TPP results). In addition, females (vs. males) strongly increased anxiety-like behavior in TIDAL to an extent that is not consistently observed in a simple dark-light test.

Over two repeated sessions, female (vs. male) mice maintain prolonged 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. Repeating the test two weeks apart would be informative for two reasons: (1) it would reveal whether and how mice learn the test and behave differently upon subsequent exposures; and (2) it would inform us about whether the test could be performed multiple times in a cohort without an effect of prior testing (i.e., whether mice could be re-tested with a different drug or time post-treatment/surgery without a learning effect, or whether an unbiased assay would require distinct cohorts of mice to avoid the learning effect).

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 (vs. males) had higher preference for the dark plate (two-way RM ANOVA, main effect of sex $F_{1,9}$=14.58, $p < 0.005$) (**Fig. 4A**). This sex difference was particularly notable in Session 2, when females exhibited 83% dark plate preference vs. 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.

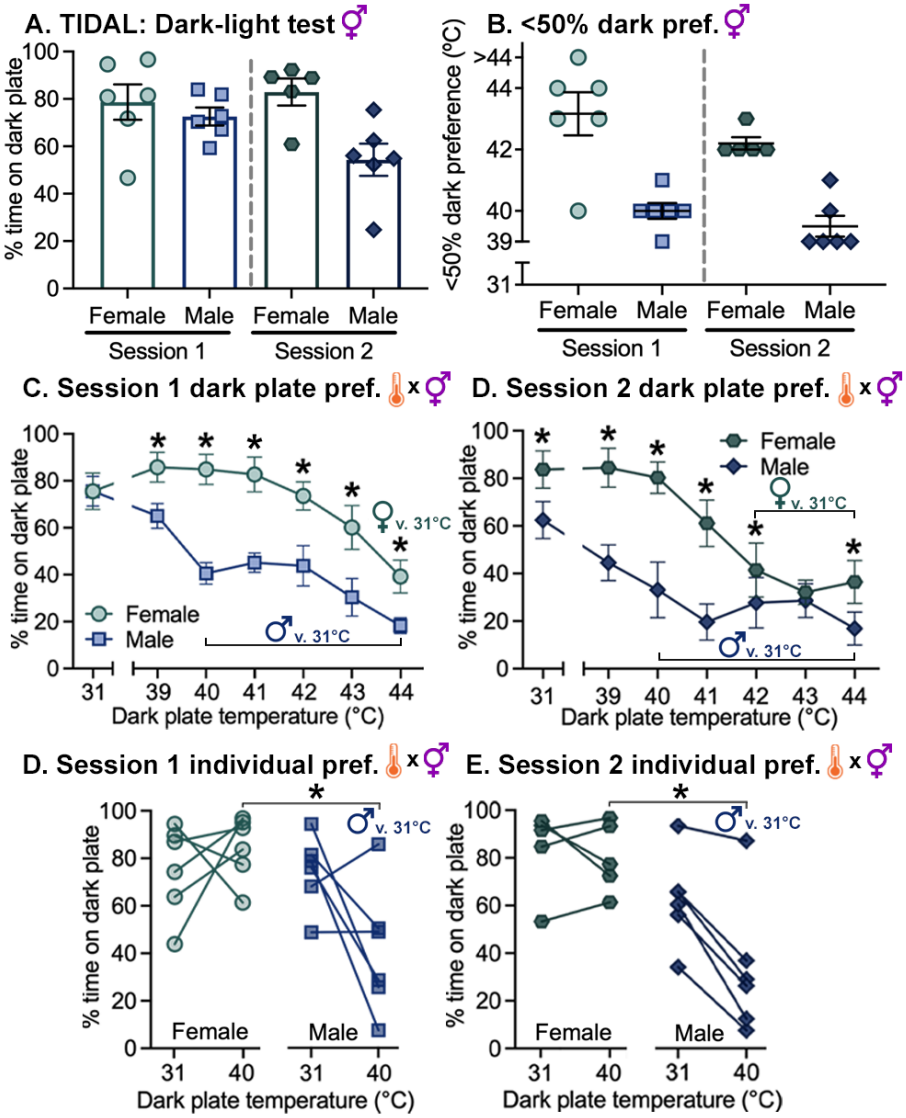

Figure 4. Sex differences in TIDAL conflict test behavior persist across two repeat sessions. Female and male mice completed the TIDAL conflict test twice, two weeks apart. A. In Session 1 and 2 dark-light tests with both plates at 31°C, females had increased dark plate preference. B. Threshold at which mice showed less than 50% preference for the dark plate; females persisted on the heated-dark plate longer than males. C. In the Session 1 TIDAL conflict test, mice of both sexes reduced time spent on the dark plate as temperatures increased. Further, female (vs. male) mice preferred the heated-dark plate at all hyperthermic temperatures tested (39-44°C). D. In the Session 2 TIDAL conflict test, mice of both sexes reduced time spent on the dark plate as temperatures increased more quickly than in Session 1. Female (vs. male) mice preferred the heated-dark plate at all hyperthermic temperatures tested except 43°C. E. Session 1 dark plate preferences of individual mice with the dark plate at 31°C and 40°C. At 40°C, females had higher dark plate preference than males (F: 85%; M: 42%). F. Session 2 dark plate preferences of individual mice with the dark plate at 31°C and 40°C. At 40°C, females had higher 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 x gender” symbol indicates significant temperature x sex interaction; gender symbol alone indicates significant main effect of sex.

Next, we assessed TIDAL conflict test behavior in both sexes over two sessions. First, we compare TIDAL results by sex: TIDAL females (vs. males) had increased preference for the heated-dark plate in both Sessions 1 and 2 (**Fig. 4**), thereby closely recapitulating results from our previous studies (in **Fig. 2, 3**). In both TIDAL sessions, females spent <50% of their time on the dark plate at higher temperatures compared to males. (Sessions 1 and 2: two-way RM ANOVA, main effect of sex, $p < 0.05$) (**Fig. 4B**). In TIDAL Sessions 1 and 2, female (vs. male) mice increased preference for the heat shift-dark plate from 39-44°C (Session 1: two-way RM ANOVA with Bonferroni, sex x temperature interaction, $F_{6,60}=6.22$, $p < 0.001$, female vs. male between 39-44°C) (Session 2: sex x temperature interaction, $F_{6,54}=4.04$, $p < 0.005$, female vs. male between 39-42°C and 44°C) (**Fig. 4C,D**). One key temperature that exposed sex differences was 40°C: in Session 1 at 40°C, dark plate preference was 85% for females and 41% for males, and Session 2 dark plate preference was 80% for females and 33% for males, respectively (**Fig. 4E,F**). Thus males, but not females, reduced their preference for the dark plate at 40°C (vs. same sex at 31°C), and males had lower preference at 40°C vs. females (Sessions 1 and 2: two-way RM ANOVA with Bonferroni, sex x temperature interactions, both $p < 0.05$). In addition, females more frequently crossed into the dark chamber (**Fig. S3**). These results bolster our previous findings, showing that females (vs. males) exhibit robust anxiety-like behavior in the TIDAL conflict assay with enhanced willingness to remain on an aversive temperature stimulus in order 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.

In a second TIDAL session, female and male mice show expedited place avoidance

Based on the data above (see **Fig. 4**), we further explored whether mice exhibited signs of learning in a second TIDAL session. Therefore, data from the two-session TIDAL test in Fig. 4 were re-graphed to better visualize female and male mouse capacity for learning (**Fig. 5**). If

mice exhibit learning, this would be interesting and would help inform the design of future TIDAL studies. As mentioned, the dark-light test revealed that male (but not female) mice showed reduced dark plate preference in Session 2. As temperatures climbed, females in Session 2 showed reduced preference for the dark plate compared to the same mice completing the task in Session 1 (Females: two-way RM ANOVA with Bonferroni, session x temperature interaction, $F_{6,24}$=3.18, $p < 0.05$, Session 1 vs. 2 between 42-43°C). Similarly, males in Session 2 (vs. Session 1) decreased preference for the dark plate (Males: main effect of session [$p = 0.05$] and temperature [$p < 0.001$]) (**Fig. 5A,B**). Focusing on a key temperature, 42°C, female mice in Session 1 showed 79% dark plate preference, which was reduced to 41% preference in Session 2 (two-way RM ANOVA; session x temperature interaction, $p < 0.05$) (**Fig. 5C**). At 42°C, male mice in Sessions 1 and 2 showed 44% and 28% dark plate preference, respectively (no significant differences between sessions, $p = 0.07$; two-way RM ANOVA; main effect of temperature only, $p < 0.001$) (**Fig. 5D**). Therefore, mice completing a second session of TIDAL exhibited signs of learning by increasing dark plate avoidance: male mice in Session 2 already showed place avoidance from the dark plate at isothermic temperature at test start, whereas females in Session 2 showed accelerated avoidance as the dark plate temperature increased. Mice exhibit learning in the TIDAL conflict test, which manifests differently in female and male mice; this suggests that TIDAL conflict test can be used to study learning related to anxiety, and implies that TIDAL studies using different treatments or timecourses should avoid confounds of learning by using distinct cohorts of mice (rather than repeating the test with the same mice). Overall, our data suggest that the TIDAL conflict test is reliable and reproducible in showing that female (vs. male) mice exhibit heightened anxiety-like behavior.

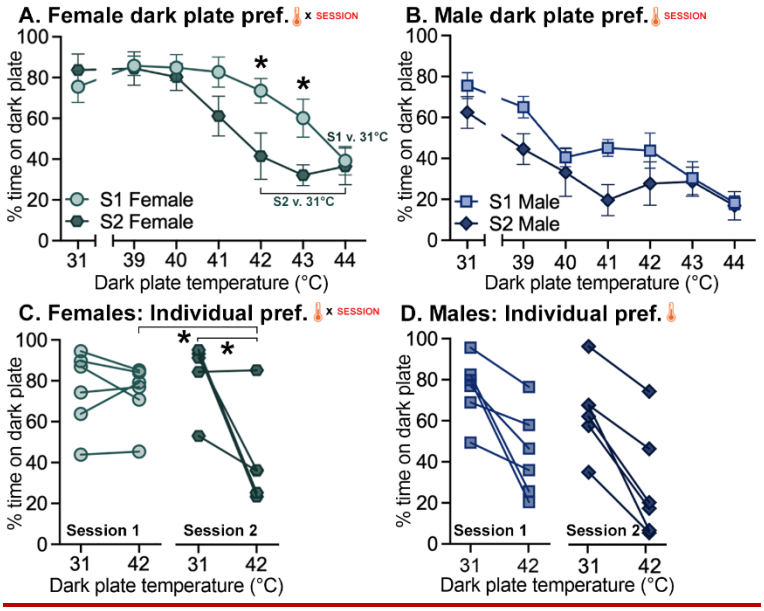

Figure 5. Over two sessions of TIDAL conflict testing, female (left panels) and male (right panels) mice exhibit signs of learning by avoiding the heating-dark side more quickly. **A, B.** In the TIDAL conflict test, mice of both sexes in Session 2 (vs. Session 1) showed expedited decreases in preference for the heated-dark plate. **C, D.** Session 1 and 2 dark plate preferences of individual mice with the dark plate at 31°C and 42°C. * indicates $p < 0.05$ between female and male mice; “thermometer x SESSION” symbol indicates significant temperature x session interaction; thermometer symbol alone indicates significant main effect of temperature.

Discussion

This study explored anxiety-like behavior in mice using the TIDAL conflict test, a novel 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 (vs. male) mice maintained prolonged 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). 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 (Donner and Lowry, 2013; Börchers et al., 2022). 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 suggests there is 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 first test used a temperature range from 42°C-52°C, increasing in increments of 2°C every five minutes. In this preliminary test, females persisted on the dark plate to higher temperatures, but mice of both sexes displayed strong avoidance early in the test (at 44°C). Therefore, we modified the test to better delineate behavioral shifts due to the conflicting stimuli: 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 vs. males. Female rodents exhibit increased pain symptoms on reflexive tests (Hargreaves test or hot plate) (Gioiosa et al., 2008; Gaudet et al., 2017; Mogil, 2020) but also prefer warmer ambient temperatures (Kaikaew et al., 2017) – these sex differences in temperature preferences could influence TIDAL outcomes. Our control conditions 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, the TIDAL conflict test is a novel 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 dark plate preference compared to males. In TIDAL Sessions 1 and 2, female (vs. 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 (vs. males) 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 (e.g., File, 1993; Roesler et al., 1999; Bailey and Crawley, 2009). 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. For instance, TIDAL results from our SCI study at 21 dpo (after completing TIDAL at 7 dpo) must be interpreted with caution due to overlapping effects of learning and different post-injury time.

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 gender (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 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 genders in humans. Anxiety disorders often

present comorbidly with other health conditions such as depression, hypertension, epilepsy, chronic pain, and neurotrauma (Tiller, 2013; Hingray et al., 2019; Johnson, 2019; Dickerson et al., 2021). Therefore, it is important to identify mechanistic differences across genders underlie susceptibility to anxiety and additional comorbid conditions.

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). Early conflict tests for rodents included the Geller-Seifter and Vogel tests, which paired aversive shocks with rewarding food or water, respectively. Although TIDAL is a novel conflict test combining heat avoidance with light-dark preference, other assays have also used thermal place preference to uncover behavioral differences in rodents. Reker et al. paired aversive temperatures with a sucrose reward – female and male mice showed similar sucrose preference at baseline, but females had increased temperature tolerance to obtain reward. Surprisingly, mice with peripheral nerve injury (vs. sham surgery) exhibited increased thermal tolerance (Reker et al., 2020). Caporoso et al. (2020) allowed rats to move freely between an isothemic plate and a cooled plate; neuropathic rats showed reduced time spent on the cooled plate, which was reversible by treatment with analgesics. These past studies mainly explored pain-like symptoms; in contrast, the TIDAL conflict test uniquely combines a light-dark component in parallel with incremental temperature shifts to capture differences in anxiety-like behavior.

Future directions and conclusions

Here, we developed 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 (Bourke et al., 2012; Fonken et al., 2013, 2018; Bolton et al., 2018; Grace et al., 2021). Our TIDAL conflict test has been optimized with progressively increasing warm temperatures. The TIDAL test described here takes 40 minutes per mouse, so future work could optimize an abridged version (e.g., sequential 31→41→44°C) for feasibility with large cohorts or batteries of behavioral tests. Another future direction is to develop a TIDAL version that uses incrementally *decreasing* temperatures in combination with the dark-light chambers, which could reinforce or complement anxiety-related differences in the TIDAL conflict test with heat.

In summary, we explored anxiety-like behavior in mice using the novel heat-light TIDAL conflict test. Our data reveal that the TIDAL conflict test unmasks amplified anxiety symptoms in mice that are female (vs. male). 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.

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Author contributions:

SEL, EKG, LKF, & ADG designed experiments. SEL, EKG, & PO performed experiments. SEL, EKG, PO, & ADG analyzed data. SEL, EKG, LKF, & ADG wrote and edited the manuscript.

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