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Predicting susceptibility to a PTSD-like phenotype in female rats

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Abstract

Post-Traumatic Stress Disorder (PTSD) is a complex mental health disorder that impacts women twice as often as men. This sexual dimorphism makes it imperative to understand the underlying causes. We previously developed the RISP (Revealing Individual Susceptibility to a PTSD-like Phenotype) model to evaluate male rats (RISP-M) for susceptibility to a PTSD-like phenotype before trauma. Here, we hypothesized that the pre-established RISP-M model would be able to predict a PTSD-like phenotype in female rats. Female Sprague-Dawley rats (n= 107) were classified twice: once with RISP-M and a second time with the RISP-F model, reported here for the first time. RISP-M assesses susceptibility with startle and anxiety-like behavior after exposure to a mild stressor (cat hair). RISP-F assessed susceptibility with response to cat hair and anxiety-like behavior in the elevated plus maze. PTSD-like phenotype, after contextual-fear conditioning, was assessed in both models with fear extinction, anxiety-like behavior, and startle. RISP-F included evaluation of persistent avoidance. RISP-M had a poor predictive power for fear extinction in female rats- Positive Predictive Value (PPV)=0.65, while RISP-F had a PPV=0.85. Most importantly, the RISP-F model had face and predictive validity across multiple PTSD-associated behaviors. The brief exposure to cat hair was indeed a mild stressor that did not induce conditioned fear in female rats. Importantly, anxiety-like behavior used for classification was induced by the mild stressor, lasted for at least 4 days, and was not phenotypic anxiety. The RISP-F model described here can successfully predict susceptibility to a PTSD-like phenotype in female rats.

Introduction

Post-Traumatic Stress Disorder (PTSD) is a mental health condition induced by intense trauma, with or without physical injury. Notable symptomology includes intrusive trauma-associated thoughts, heightened reactivity, and avoidance behaviors.[35] An important feature of PTSD is that it is sexually dimorphic: it has twice the lifetime prevalence in women compared to men.[19] Additionally, not everyone who experiences a trauma will develop PTSD.[11] Thus, sex and individual differences may contribute to PTSD development.

Being able to predict individual susceptibility to PTSD will allow us to generate therapeutic strategies that boost resilience against developing PTSD. To understand risk factors impacting PTSD development, we utilize a RISP (Revealing Individual Susceptibility to a PTSD-like Phenotype) model that reflects a PTSD-like phenotype in rats. The RISP model reflects multiple components of Diagnostic and Statistical of Mental Disorders, Fifth Edition (DSM-5) criteria for PTSD diagnosis[35] as it utilizes quantifiable rodent behaviors that translate to human experiences as it relates to trauma and post-trauma behaviors.[1] Additionally, the RISP model evaluates sustained symptomology after the trauma. This is an important feature as PTSD diagnosis requires symptoms to be present for at least a month following a trauma.

Our lab developed the RISP model in male rats (RISP-M) and demonstrated that it has good face and predictive validity. When male rats were exposed to a mild stressor and evaluated four days later only some showed elevated startle responses and anxiety-like behaviors and were classified as 'susceptible'. After subsequent trauma, contextual fear

conditioning (CFC), only susceptible rats showed impaired fear extinction, elevated startle response, and sustained anxiety-like behavior.[16]

It is unknown if RISP-M has sufficient face and predictive validity for female rats, given existing sex differences in fear and anxiety-like behaviors.[28,26,3] Rats show sex differences in behavioral phenotypes in anxiogenic conditions: after CFC, female rats avoid the Shock Arm similarly to males but show low freezing and engage in anxioescapic behavior.[26] This indicates that female rats have similar fear memory to males but tend to express fear avoidance differently.

In this study, we demonstrate that the RISP-M model does not have sufficient face and predictive validity for female rats. Therefore, we developed a female rat-specific model (RISP-F) that succeeds in predicting susceptibility. We also show that cat hair is necessary to reveal susceptibility in female rats, similar to male rats. This work offers a novel model with excellent face and predictive validity for identifying susceptibility to a PTSD-like phenotype in female rats before trauma.

Methods

Subjects

Young adult female Sprague-Dawley rats (170–300 g, Charles River Laboratories Inc, MA), n= 107 across six cohorts, were housed in pairs on a 12 hr light/dark cycle (lights on at 7:00 am) with food and water freely available. Five cohorts were used for RISP classification and one for evaluating fear conditioning to cat hair and foot-shock. Experiments occurred during the light phase (8 am-5 pm). The number of rats per group

is listed in each figure. Prior to the start of behavioral testing, each animal was handled for 2–3 minutes for three consecutive days, starting three days after arrival. All behavioral procedures were approved by the IACUC at the CNVAMC.

Behavioral Testing

All behavior was recorded for analysis using Noldus Ethovision XT 18.

Cat hair (CH) exposure: Animals were exposed to a ball of cat hair (~10 cm) for 3 minutes obtained from a pathogen-free male cat and infused with ~ 150ul of cat urine in a 32 cm × 32 cm × 50 cm box. Cat hair interactions were scored when the animal's head was within 1 cm of, or front paws contacted, the ball of cat hair. The number of entries into each quadrant of the box was recorded to assess locomotor activity. The number of fecal boli and the presence of urine were recorded as an indicator of autonomic fear response.

One cohort was evaluated for conditioned fear to the CH context 24 hours after CH exposure. Rats were placed in an identical empty box for 5 minutes the day after CH exposure. Freezing was defined as cessation of all body movements except for those required for respiration.

Light-dark open field (LDOF): The LDOF apparatus was a circular arena measuring 142 cm diameter x 60 cm height.[25] The floor of the apparatus was dark blue to contrast with white rats for the purpose of accurate measurements using Noldus Ethovision XT 18. The center region was ~25% of the total arena area, the dark zone was~20% of the total arena area, and the light zone was ~20% of the total arena area.

Elevated plus maze (EPM): Anxiety-like behavior was measured in the EPM 4 days after CH exposure. The EPM was plus-shaped with four 50 cm × 12 cm arms, elevated 84 cm

above the floor. Two opposing arms were surrounded by 46 cm tall black walls on three sides; the other two arms were open, except for a 1 cm high ledge (Kinder Scientific, San Diego, CA). Each animal was introduced into the center area (10 cm × 10 cm) facing an open arm and allowed to explore freely for 5 minutes. An arm entry was scored when all four paws and the base of the animal's tail entered an arm.

Acoustic startle response (ASR) protocol: Rats were placed in clear acrylic restraining tubes with accelerometers in sound-attenuated startle chambers (SR-LAB, San Diego Instruments, San Diego, CA). After a 3-min habituation period, background noise of 68 dB, rats experienced 15, 120dB acoustic bursts, 40 ms each, delivered at random intervals (between 30–45 seconds). ASR, expressed in arbitrary units, was recorded. Each chamber was calibrated prior to testing each animal.

Pre-pulse Inhibition (PPI) protocol: *Habituation to background*-180 seconds in the startle chamber (SR-LAB, San Diego Instruments) with a background noise of 65dB. *Habituation to stimulus* (“pulse” tone) - 4 acoustic bursts of 120dB (40 ms each with 10 ms intervals). *Pre-pluse:* A “pre-pulse” (20 ms) preceded the “pulse” tone (120dB, 20 ms) at a lower decibel (70, 75, and 80dB) by 100 ms. A total of 60 trials were performed which randomly cycled between five measurement groups (NoStim, Pre_{70}+Pulse, Pre_{75}+Pulse, Pre_{80}+Pulse, Stimulus) with intertrial intervals of 5-25 seconds.

Startle responses within groups were either tested with ASR (cohorts 1-3) or PPI (cohorts 4-5) protocols because: 1) RISP-M utilizes the ASR protocol as one classification criterion; 2) we found that the PPI was a better predicter of susceptibility in female rats than the ASR protocol.

Y-maze apparatus and Habituation: Contextual fear conditioning (CFC) and extinction were conducted in a custom Y-maze as previously described.[37,26] One arm of the Y-maze (Shock Arm) was fitted with stainless steel walls and floor plates separated by a 1 cm gap in the floor through which a foot-shock can be administered. A removable wall isolated the Shock Arm from the other arms (Safe Arms). All arms had distinct visual and tactile cues. On Habituation day, each rat was introduced to the apparatus in the Shock Arm facing the short wall and allowed to freely explore for 5 minutes. Arm entries and time in each arm were assessed.

Contextual fear conditioning (CFC) training: CFC was done in the closed off Shock Arm of the Y-maze apparatus a day after Habituation. After 1 minute of free exploration, animals received two inescapable foot-shocks (1 mA ac, 1 sec) delivered with a 30-second intertrial interval. Ninety seconds after the second foot-shock the rats were returned to their home cages. Number of crossings across the center line of the Shock Arm before and after foot-shock delivery were scored.

Avoidance CFC extinction: Fear extinction was evaluated in the Y-maze with access to all arms and no foot-shocks, starting on the day after CFC. Rats were reintroduced into one of the Safe Arms of the Y-maze (alternating Safe Arms each day) for 5 min per day for four consecutive days. Successful extinction was defined as 40% or more decrease in latency to enter the Shock Arm on Day 4 compared to Day 1.

CFC test within the Shock Arm: One cohort was evaluated for contextual fear conditioning while placed in the Shock Arm separated from the rest of the Y-maze 24 hours after CFC training.

Froot Loops Persistent Avoidance Trial: After extinction, rats were tested for persistent avoidance of the Shock Arm despite the presence of a reward (Froot Loops). First, rats were introduced to Froot Loops by being allowed to eat one Froot Loop each in their home cage. Then, rats experienced *Froot Loops in Box*: they were placed for 3 minutes in a novel box (42 cm x 42 cm x 31 cm) that contained a small container (12.5 cm x 8.5 cm x 2 cm) filled with bedding material and two Froot Loops of different colors. Froot Loops in Box was repeated for a minimum of two consecutive days, or until all animals were readily eating the Froot Loops. The following day, rats were tested for persistent avoidance of the Shock Arm: two Froot Loops of different colors were placed at the end of the Shock Arm of the Y-Maze. Rats were placed in a safe arm of the Y-Maze and allowed to explore for 5 minutes. Color of Froot Loops consumed, and the time when they were consumed were recorded for both the Froot Loops in Box and Froot Loops Persistent Avoidance trials.

RISP Classification Criteria

RISP-M

Resilient (Res): ≥1 open arm entries in the EPM and lower than average startle (with more than 6 individual startles lower than the average)

Susceptible (Sus): zero open arm entries in the EPM and higher than average startle (with more than 5 individual startles higher than the average)

RISP-F

Res: >4 interactions with the ball of cat hair and spent <200 seconds in the closed arm of the EPM

Sus: <4 interactions with the ball of cat hair and spent >200 seconds in the closed arm of the EPM

Rats that did not fit these criteria were classified as intermediate and not evaluated further.

Statistics

All data sets passed Kolmogorov-Smirnov Test of Normality. Unpaired t-tests and mixed design RM-ANOVA with factors Susceptibility and Time were used where appropriate. Fisher's post hoc test was used for individual group comparisons after overall effects were significant. Positive predictive values (PPVs) were generated to assess the predictability of the RISP-M and RISP-F models. Receiver operating characteristic (ROC) curves were generated to further evaluate the predictability of the RISP-F model. Mixed model ANOVA was used to analyze ASR1 and ASR2 between Res and Sus rats. Differences were considered statistically significant at $p < 0.05$ (GraphPad Prism version 10.0.0 for Windows, GraphPad Software, San Diego, CA).

Results

The hypothesis that RISP-M[16] can successfully predict susceptibility in female rats was tested with the experimental design illustrated in Figure 1A.

RISP-M reveals an anxiogenic phenotype in female rats prior to trauma

During Cat Hair (CH) exposure, rats that were later classified as Susceptible (Sus) and Resilient (Res) had similar number of interactions with the CH in the first [$t(9.418)=1.231$,

p=0.2483], and entire 3 minutes [t(8.840)=0.04189, p=0.9675], of exposure (Figure 2A&B), CH quadrant entries [t(16.81)=2.672, p=0.0162] (Figure 2C), and total quadrant entries [t(13.09)=1.904, p=0.0792] (Figure 2D). Sus rats had significantly higher startle measurements during ASR1 [t(5.190)=3.944, p=0.0101] (Figure 2E) and significantly fewer number of open arm entries on the EPM when compared to Res rats [t(9.418)=1.231, p=0.2483] (Figure 2F), because these two measures were the RISP-M classification criteria. Sus rats also had decreased total arm entries [t(9.418)=1.231, p=0.2483] (Figure 2G), percent time spent in the open arms [t(13.00)=3.865, p=0.0020] (Figure 2H), ratio of open arm and total entries [t(13.00)=6.590, p<0.0001] (Figure 2J), and spent more time in the closed arms [t(13.19)=3.070, p=0.0088] (Figure 2I). Combined, the data reveal Sus rats have normal responses to a mild stressor but an overall higher anxiogenic phenotype.

RISP-M fails to predict successful fear extinction and sustained startle responses in female rats

To evaluate if RISP-M classification can successfully predict fear extinction and sustained startle behavior, we evaluated these measures after fear conditioning. There were no differences between Sus and Res female rats across any measures of avoidance fear extinction (latency to enter ShA (Susceptibility [F(1,18)=1.641, p=0.2165], Time [F(1.881,33.86)=0.8685, p=0.4227], Sus x Time [F(1.881,33.86)=0.7772, p=0.4605]), percent time spent in ShA (Susceptibility [F(1,18)=2.814, p=0.1107], Time [F(2.132,38.38)=1.389, p=0.2621], Sus x Time [F(2.132,38.38)=0.4803, p=0.6343]), number of ShA entries (Susceptibility [F(1,18)=2.546, p=0.1280], Time [F(2.698,48.56)=1.916, p=0.1448], Sus x Time [F(2.698,48.56)=0.9555, p=0.4139]), and

total entries during extinction (Susceptibility [F(1,18)=4.382, p=0.0508], Time [F(2.810,50.59)=3.219, p=0.0330], Sus x Time [F(2.810,50.59)=0.5919, p=0.6125]) (Figure 3A-D). While Sus rats had a significantly higher startle than Res rats during ASR2 [t(6.150)=3.252, p=0.0168] (Figure 3E), they showed decreased startle between ASR1 and ASR2 (Susceptibility [F(1,18)=32.44, p<0.0001], Time [F(1,10)=6.437, p=0.0295]) (Figure 3F), which is inconsistent with a PTSD-like phenotype.

RISP-M classification resulted in 29.2% Res and 12.5% Sus (Figure 3G) with a Positive Predictive Value (PPV) of 0.65 for extinction (Figure 3H) and 0.33 (not shown) for sustained startle (ASR2≥ASR1), which demonstrate low predictive validity.

Together, these data suggest that RISP-M fails to predict susceptibility in female rats, therefore we generated a new model for use in female rats.

Predicting susceptibility in females (RISP-F)

The experimental design for predicting susceptibility in female rats (RISP-F) is illustrated in Figure 1B. We hypothesized, based on pilot data from different cohorts, that the initial response to a mild stressor and sustained anxiety would adequately predict PTSD-like susceptibility. RISP-F classification criteria were determined by the number of interactions with CH in the first minute of exposure and the amount of time spent in the closed arms of the EPM.

RISP-F reveals suppressed activity in the presence of a mild stressor and anxiogenic behavior prior to trauma

CH interactions in the first minute of exposure and time in the closed arms of the EPM were used for RISP-F classification. Per classification, Sus rats had significantly fewer

CH interactions [t(25.20)=11.31, p<0.0001] (Figure 4A) and spent more time in the closed arms [t(32.94)=11.68, p<0.001] (Figure 4H).

Additionally, Sus rats had overall suppressed activity during CH exposure (CH interactions across the three minutes of exposure [t(26.23)= 7.231, p<0.0001], CH quadrant entries [t(15.98)= 4.740, p=0.0002], total quadrant entries [t(25.65)=5.346, p<0.0001]) (Figure 4B-D). Importantly, four days later Sus rats had an elevated anxiogenic phenotype (open arm entries [t(17.87)=2.917, p=0.0092], total entries [t(24.58)=5.554, p<0.0001], and percent time spent in the open arms [t(20.36)=2.421, p=0.0249]) (Figure 4E-G), when compared to Res rats. This data suggests that female rats classified as Sus using the new RISP-F criteria, demonstrate higher unconditioned fear in the presence of a mild stressor and elevated anxiety-like behavior four days later.

RISP-F successfully predicts a PTSD-like phenotype in female rats

A PTSD-like phenotype was defined as impaired fear extinction, sustained anxiety-like behavior, and elevated startle after trauma. Sus rats had impaired fear extinction as evidenced by having a longer latency to enter the Shock Arm (Susceptibility [F(1,33)=25.87, p<0.0001], Time [F(2.417,79.76)=1.610, p=0.2014], Sus x Time [F(2.417,79.76)=6.796, p=0.0010]) (Figure 5A), lower percent time spent in the Shock Arm (Susceptibility [F(1,33)=15.45, p=0.0004], Time [F(2.908,95.97)=4.889, p=0.0036], Sus x Time [F(2.908,95.97)=7.698, p=0.0001]) (Figure 5B), lower number of Shock Arm entries (Susceptibility [F(1,33)=23.81, p<0.0001], Time [F(2.377,78.44)=8.748, p=0.0002], Sus x Time [F(2.377,78.44)=10.09, p<0.0001]) (Figure 5C), and fewer total entries during extinction (Susceptibility [F(1,33)=25.27, p<0.0001], Time

[F(2.662,87.85)=2.656, p=0.0600], Sus x Time [F(2.662,87.85)=4.002, p=0.0130]) (Figure 5D) when compared to Res rats.

Sus rats also had sustained anxiety-like behavior evidenced by a trend for spending a longer time in the dark zone during LDOF3 [t(12.54)=2.059, p=0.0609] (Figure 5E). This was not due to phenotypic anxiety, because there were no differences between the two groups in LDOF1 (t(9.745)=1.485, p=0.1692) (Figure 5F). Additionally, both groups showed increased anxiety-like behavior after the mild stressor, as shown in LDOF2 (t(11.08)=1.389, p=0.1920) (Figure 5F). However, only Sus animals maintained high anxiety-like behaviors after trauma in LDOF3 when evaluating performance across time (Susceptibility [F(1,14)=7.330, p=0.0170], Time [F(2,28)=5.675, p=0.0085], Sus x Time [F(2,28)=0.6118, p=0.5495]) (Figure 5F). The lack of statistically significant interaction reflects the fact that the two groups were similar during LDOF1 and LDOF2.

Finally, Sus rats had elevated startle and impaired ability to suppress startle responses using the PPI protocol. Successful PPI is suppression of a startle response when a stimulus tone is immediately preceded by a lower intensity pre-tone. Sus rats had impaired PPI at all pre-tone levels (Pre70+Stim [t(5.00)=2.993, p=0.0303], Pre75+Stim [t(5.00)=3.114, p=0.0264], Pre80+Stim [t(5.00)=2.810, p=0.0375]) (Figure 5G), and a higher startle when examining stimulus only trials (Stim [t(5.00)=3.864, p=0.0118]) (Figure 5G) compared to Res rats. Baseline reactivity, when there was no stimulus, was comparable in both groups (NoStim [t(5.00)=0.7018, p=0.5141]) (Figure 5G).

To evaluate persistent avoidance, another aspect of a PTSD-like phenotype, we assessed two cohorts of Res and Sus rats on their willingness to retrieve a known reward (two Froot Loops) from the Shock Arm. Sus rats retrieved significantly fewer Froot Loops

(t(4.00)=3.207, p=0.0327) (Figure 5H) and had a significantly higher latency to retrieve the first Froot Loop, when compared to Res rats (t(4.00)=3.323, p=0.0293) (Figure 5I). Notably, all rats eagerly ate Froot Loops during habituation and most entered the Shock Arm on the test day with only a trend for a group difference (t(5.361)=2.313, p=0.0651, data not shown). These data suggest that Sus rats have persistent avoidance of cues associated with the trauma despite the presence of a reward incentive.

RISP-F has high face and predictive validity

RISP-F classification resulted in 21.6% Res and 27% Sus (Figure 5J). PPV for extinction was 0.85 (Figure 5K). This PPV is substantially higher than the PPV when applying the RISP-M model to the same cohorts (Figure 3J). Additionally, when performing ROC analysis of RISP-F for latency to enter the Shock Arm on day four of extinction and percent time spent in the dark zone during LDOF3, we observed an area under the curve of 0.8503 and 0.7813, respectively (Figure 5L&M). Taken together, these data suggest that the RISP-F model has good face and predictive value in female rats.

Neither RISP-F classification criterion alone can predict susceptibility to a PTSD-like phenotype in female rats

We tested the hypothesis that RISP-F, which has two classification criteria, would be a better predictive model than either criterion alone. To evaluate classification based on EPM alone, we used the time spent in the closed arms of the EPM to classify the same cohorts of animals. This criterion alone resulted in 56.6% Res and 43.4% Sus (Figure 6A). Furthermore, the PPV was 0.64 (Figure 6B). Similarly, the CH criterion alone resulted in 38.4% Res and 46.6% Sus (Figure 6C) and a PPV of 0.66 (Figure 6D). Both PPVs for

the individual criterion were lower than the PPV for RISP-F (0.85), demonstrating that the combination of both criteria in the RISP-F model is sufficient to produce a model with satisfactory face and predictive validity.

A mild stressor is necessary to reveal susceptibility to a PTSD-like phenotype in female rats

We further assessed if exposure to a mild stressor was necessary to reveal susceptibility. If susceptibility can be elucidated without a mild stressor, then the RISP-F model would produce similar results if EPM is administered prior to or after CH exposure (Figure 6E). The results reject that hypothesis. When EPM occurred prior to CH exposure, there was no Sus group (Figure 6F), which is not representative of female rat behavior post-trauma. When EPM occurred after CH exposure, all three classification groups were represented (Figure 6G). These data suggest that exposure to a mild stressor is necessary to reveal susceptibility to a PTSD-like phenotype in female rats. We allowed four days between CH exposure and EPM to ensure that stress responses to CH exposure return to baseline.

Although these data are based on a small n, when the RISP-F model with EPM post-CH is applied to a larger sample size and across multiple cohorts, the model reliably predicts PTSD susceptibility in female rats, as demonstrated in Figure 5.

CH is a mild stressor that does not induce trauma in female rats

Previously we published that CH is a stressor for female rats.[27] Given that Sus rats have sustained anxiety after CH exposure (Figure 4E-H, Figure 5F), it is possible that this is a traumatic event for female rats. The results rejected this hypothesis. Female rats exposed to CH showed no fear conditioning to the CH context, demonstrated by low percent

freezing during context re-exposure 24 hours later without CH present (Figure 6H). Importantly, these freezing levels were significantly lower than freezing in the CFC context ($t(27.74)=5.205$, $p<0.0001$) (Figure 6H). Combined, these data demonstrate that CH exposure is a mild stressor and does not induce trauma, unlike CFC.

Discussion

This study tested the hypothesis that a previously described model for predicting susceptibility to a PTSD-like phenotype in male rats (RISP-M) has high face and predictive validity in female rats. The presented data reject this hypothesis, as RISP-M had low face and predictive validity when applied to five cohorts of female rats. Furthermore, we describe and validate a RISP-F model and demonstrate that it has high face and predictive validity. While susceptibility in RISP-M is evaluated using startle and anxiety-like behavior four days after exposure to a mild stressor, RISP-F assessed susceptibility with response to cat hair and anxiety-like behavior in the elevated plus maze four days later. We also demonstrate that the combination of the two criteria used in the RISP-F model is necessary and sufficient to reveal susceptibility in female rats. Lastly, we report that cat hair exposure, which is a necessary element of the RISP-F model, is a non-traumatic mild stressor for female rats.

The RISP-M model does not predict susceptibility to a PTSD-like phenotype in female rats

We previously demonstrated that the RISP-M model had high face and predictive validity in male rats.[16] Here we demonstrated that RISP-M criteria were inadequate to classify

female rats. RISP-M had low face validity when applied to female rats: the rate of Res was more than twice that of Sus rats (Figure 3G). This is inconsistent with the reported rates of lifetime prevalence of PTSD in women, known to be double that of men.[35] The RISP-M model demonstrated that ~20% of male rats had PTSD-like phenotypes[16], therefore our model should have had ~30-40% Sus females, in contrast to the observed 12.5%.

RISP-M also had inadequate predictive validity, indicated by Sus rats having similar to Res rats avoidance fear extinction (Figure 3A-D) with a low PPV (Figure 3H) and lacking a sustained startle phenotype (Figure 3F).

RISP-F is the first model, to our knowledge, that identifies susceptibility before trauma.[5,6]

The RISP-F model has face and predictive validity for a PTSD phenotype in female rats

Unlike the results from the RISP-M model, the RISP-F model applied to the same cohorts of female rats had better face and predictive validity. Sus female rats, classified with RISP-F, had a phenotype consistent with Diagnostic and Statistical of Mental Disorders, Fifth Edition, Text Revision (DSM-5) PTSD criteria. Sus rats had impaired fear extinction and PPI (Figure 5A-D, DSM-5 PTSD criterion B), as well as higher startle (Figure 5G, DSM-5 PTSD criterion E), sustained anxiety-like behavior (Figure 5E-F, DSM-5 PTSD criterion D), and sustained avoidance of trauma cues even in the presence of a desirable reward (Figure 5H-I, DSM-5 PTSD criteria C&D). This was further supported by a PPV of 0.86 for fear extinction (Figure 5K), which is a reliable indicator of predictability. Additionally, the percent of Sus female rats identified with the RISP-F classification criteria

was 27% (Figure 5J), which is an adequate reflection of PTSD lifetime prevalence, demonstrating that the RISP-F model has both face and predictive validity that is clinically relevant to PTSD.

Additional considerations of RISP-F

Both RISP-F classification criteria are necessary

RISP-F uses two mandatory classification criteria. We demonstrated that classification based only on the EPM criterion or only on the CH criterion produced low PPVs (Figure 6B&D). While the two criteria alone had reasonable face validity, each lacked predicative validity.

Cat hair is necessary to reveal susceptibility

CH exposure is necessary to reveal susceptibility, indicated by sustained anxiety in some (Sus), but not all animals. CH exposure occurring after EPM failed to sufficiently reveal susceptibility (Figure 6F), which may be caused by CH exposure inducing lasting anxiety.[27] The necessity of CH exposure in revealing susceptibility to a PTSD-like phenotype in female rats is similar to that reported in male rats.[16]

The current results extend these findings to demonstrate that the animals disproportionately affected by CH exposure are susceptible to a PTSD-like phenotype. The mild stressor in our rat model is comparable to a stressful life event, which has been shown to increase the likelihood of PTSD development when occurring prior to a traumatic experience.[18,23]

Cat hair is a mild stressor, not a traumatic experience

CH exposure with the current parameters functions as a mild, but not traumatic stressor, unlike CFC (Figure 6H) or protocols in which a prolonged exposure to a cat is a traumatic experience.[38,39] We hypothesize that CH exposure primes the hypothalamic-pituitary-adrenal (HPA) axis through activation and elevated levels of corticosterone[10], similar to the dysregulation observed in PTSD patients.[12,24] We are currently testing this hypothesis.

Clinical Relevance

In addition to RISP-F having relevance to DSM-5 PTSD criteria, the model offers the opportunity to evaluate brain functionality in Res and Sus rats, both before and after trauma. Some of the brain regions involved in the behaviors evaluated with RISP-F have been shown to have altered functionality in human PTSD patients.[4,14,17,34] CH exposure, EPM, LDOF, fear extinction, and PPI all involve the activation of the medial prefrontal cortex (mPFC), hippocampus, and regions of the amygdala.[7,2,33,35,9,30] Some of these tests also share activation within the nucleus accumbens and thalamus.[2,30] Importantly, the amygdala, hippocampus, and PFC have altered functionality in PTSD patients[20,29], which is a pathological hallmark of the disorder. We are currently testing if altered function of these brain regions is present in Sus female rats after trauma, which will demonstrate construct validity for the RISP-F model. Furthermore, we are examining if these regions have impaired function before trauma in Sus rats and whether functional improvement will result in increased resilience against a PTSD-like phenotype. Combined with already published data on male rats, this will provide the ability to investigate sex differences in a pre-clinical model and will suggest ways to build resilience against developing PTSD in the human population.

Methodological Considerations

- The two criteria of the model must be applied as: CH exposure first, EPM second. This order was validated by the finding that if testing was reversed, RISP-F lost face validity (Figure 6G).
- EPM was tested four days after CH exposure. This timing ensures that the measures reflect long lasting anxiety-like behavior rather than initial stress response from CH exposure. Within the four-day rest period, corticosterone levels return to baseline.[21]
- LDOF was evaluated at three different timepoints to assess different types of anxiety: phenotypic anxiety (LDOF1), anxiety after a mild stressor (LDOF2), and sustained anxiety after a trauma (LDOF3). Unlike EPM, LDOF can be reliably administered multiple times.[25]
- ASR involves the activation of the bed nucleus of the stria terminalus (BNST) and central amygdala, which regulate emotional and startle responses, and are sexually dimorphic.[31,13] The ASR protocol used to classify male rats is unreliable when classifying female rats, which may be due to the sexual dimorphisms in these brain regions.
- Changing the EPM criteria to time spent in the closed arms, instead of open arm entries, is based on data that demonstrated that female rats tend to make more entries into the open arm of the EPM when compared to male rats[22,26], and female rats engage in axioescapic behavior in anxiogenic conditions.[26] Therefore, using the percent of time spent in the closed arms, as opposed to number of open arm entries, better reflects the anxiety-like state of female rats.

Limitations

- Estrous cycle was not evaluated for this report. We are currently testing how the estrous cycle and procedures used to monitor the estrous cycle impact female rat behavior across these tests.
- This study only uses one strain of rat and one age range. There could be strain differences that are not accounted for in this work.

Despite the limitations, to our knowledge, this work is the first model that can successfully predict susceptibility in female rats before trauma. RISP-F and RISP-M are reliable models for revealing susceptibility in female and male rats, respectively. The combination of these models will allow the identification and mediation of susceptibility factors before trauma, which may be different for the two sexes.

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Conflict of interest

The authors declare no conflict of interest.

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Figures

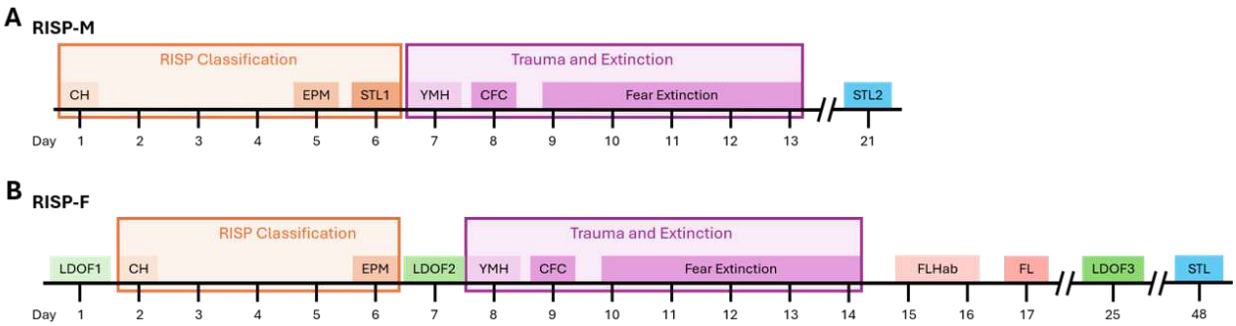


Figure 1. Timeline of behavioral tests within the RISP paradigms. A) RISP-M. B) RISP-F. Acronyms: LDOF = light-dark open field, CH = cat hair, EPM = elevated plus maze, STL = startle, YMH = Y-maze habituation, CFC = contextual fear conditioning, FLHab = Froot Loop habituation, FL = Froot Loop test.

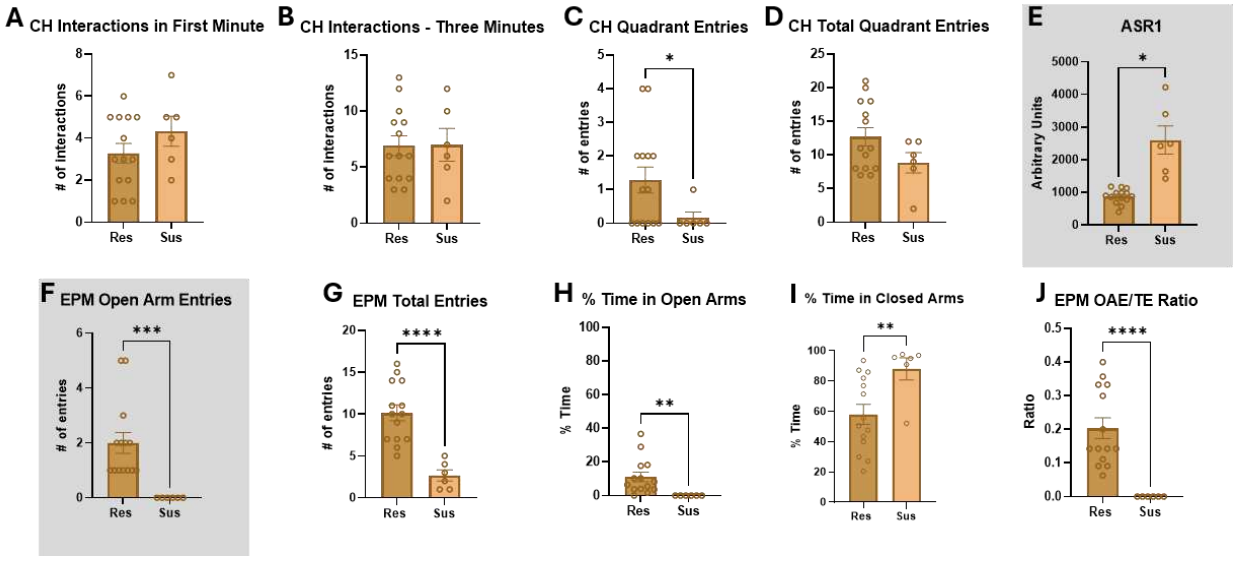


Figure 2. Behavior of Res and Sus female rats classified with the RISP-M model in the CH, ASR, and EPM tests. A) Number of interactions with the ball of CH within the first minute of CH exposure. B) Number of interactions with the ball of CH across all three

minutes of CH exposure. C) Number of entries into the CH quadrant. D) Total quadrant entries during CH exposure. E) Average startle during ASR1. F) Number of entries into the open arms of EPM. G) Total arm entries during EPM. H) Percent of time spent in the open arms of EPM. I) Percent of time spent in the closed arms of EPM. J) Ratio of open arm entries and total arm entries of EPM. Grey boxes indicated measures used in the RISP-M classification. *p<0.0332, **p<0.0021, ***p<0.0002, ****p<0.0001. Res n = 14, Sus n = 6.

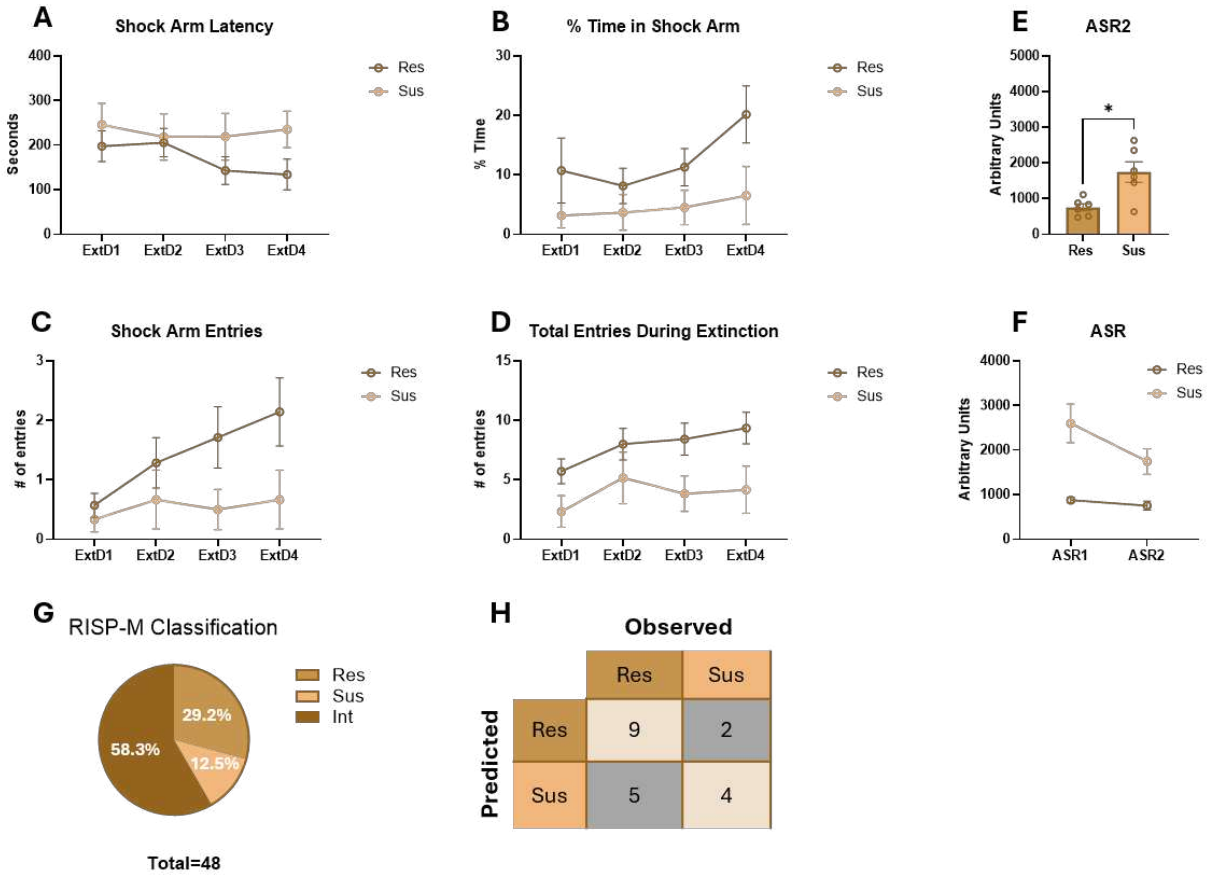


Figure 3. Res and Sus female rats evaluated for fear extinction and startle behaviors following classification using the RISP-M model. A) Latency to enter the

Shock Arm (Res n = 14, Sus n = 6). B) Percent time spent in the Shock Arm (Res n = 14, Sus n = 6). C) Number of entries into the Shock Arm (Res n = 14, Sus n = 6). D) Total arm entries on the Y-maze during extinction (Res n = 14, Sus n = 6). E) Average startle during ASR2 (Res n = 6, Sus n = 6). F) Average startle during ASR before trauma and after extinction (ASR1 Res n = 14, ASR1 Sus n = 6, ASR2 Res n = 6, ASR2 Sus n = 6). G) Percent of total animals in Res, Int, or Sus groups after classification. H) PPV chart of RISP-M classified female rats (PPV = 0.65). *p<0.0332.

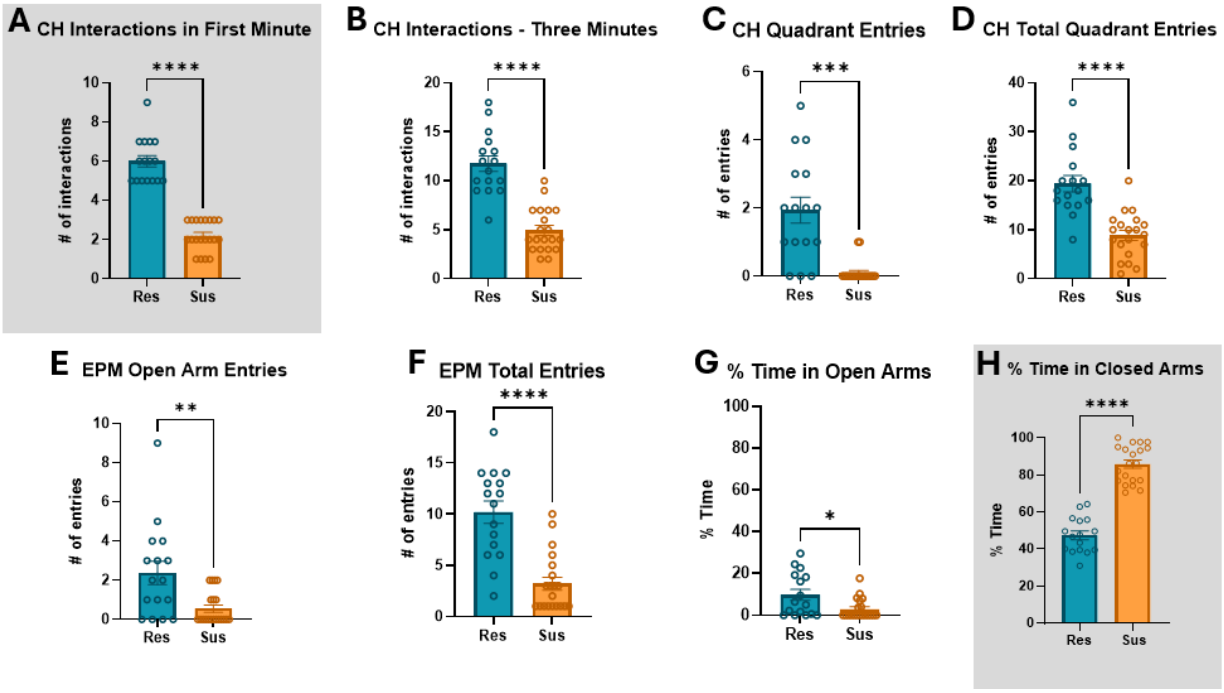


Figure 4. Res and Sus female rats evaluated for CH, and EPM behaviors following classification using the RISP-F model. A) Number of interactions with the ball of CH within the first minute of CH exposure. B) Number of interactions with the ball of CH across all three minutes of CH exposure. C) Number of entries into the CH quadrant. D) Total quadrant entries during CH exposure. E) Number of entries into the open arms of

EPM. F) Total arm entries during EPM. G) Percent of time spent in the open arms of EPM. H) Percent of time spent in the closed arms of EPM. Grey boxes indicated measures used in the RISP-F classification. *p<0.0332, **p<0.0021, ***p<0.0002, ****p<0.0001. Res n = 16, Sus n = 20.

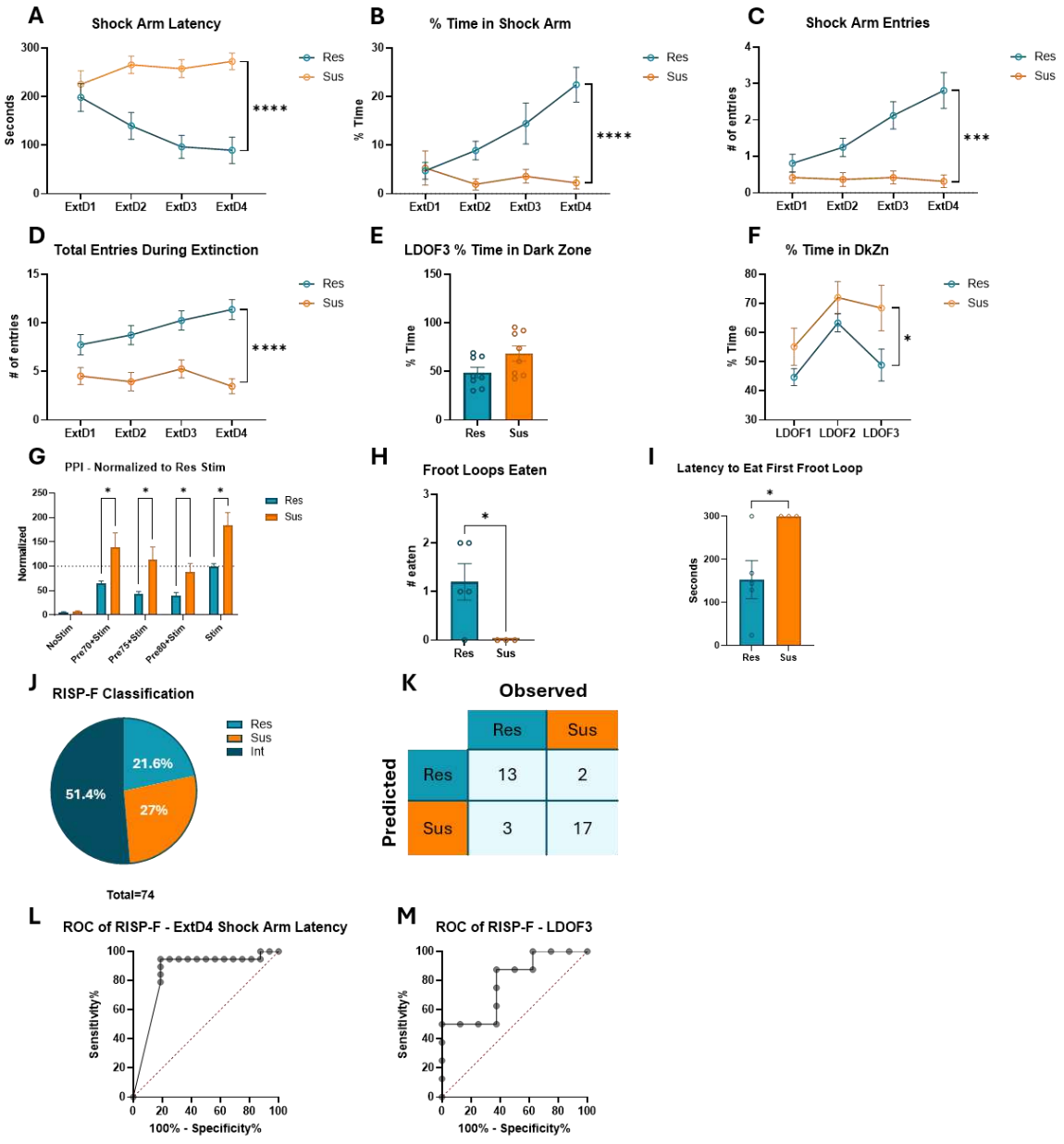


Figure 5. Res and Sus female rats evaluated for fear extinction and startle behaviors following classification using the RISP-F model. A) Latency to enter the Shock Arm (Res n = 16, Sus n = 19). B) Percent time spent in the Shock Arm (Res n = 16, Sus n = 19). C) Number of entries into the Shock Arm (Res n = 16, Sus n = 19). D) Total arm entries on the Y-maze during extinction (Res n = 16, Sus n = 19). E) Percent time spent in the dark zone during LDOF3 (Res n = 8, Sus n = 8). F) Percent time spent in the dark zone of LDOF apparatus before CH exposure, before trauma, and after extinction (Res n = 8, Sus n = 8). G) Average startle measurements during PPI for NoStim, Pre_{70}+Pulse, Pre_{75}+Pulse, Pre_{80}+Pulse, and Stimulus (Res n = 4, Sus n = 3). H) Number of Froot Loops eaten during persistent avoidance trial (Res n = 5, Sus n = 3). I) Latency to eat first Froot Loop during persistent avoidance trial (Res n = 5, Sus n = 3). J) Percent of total animals in Res, Int, or Sus groups after classification. K) PPV chart of RISP-F classified female rats (PPV = 0.85). L) ROC analysis of predictability on Shock Arm latency during extinction day 4 (Res n = 16, Sus n = 19). M) ROC analysis of predictability on percent time spent in the dark zone during LDOF3 (Res n = 8, Sus n = 8). *p<0.0332, ***p<0.0002, ****p<0.0001.

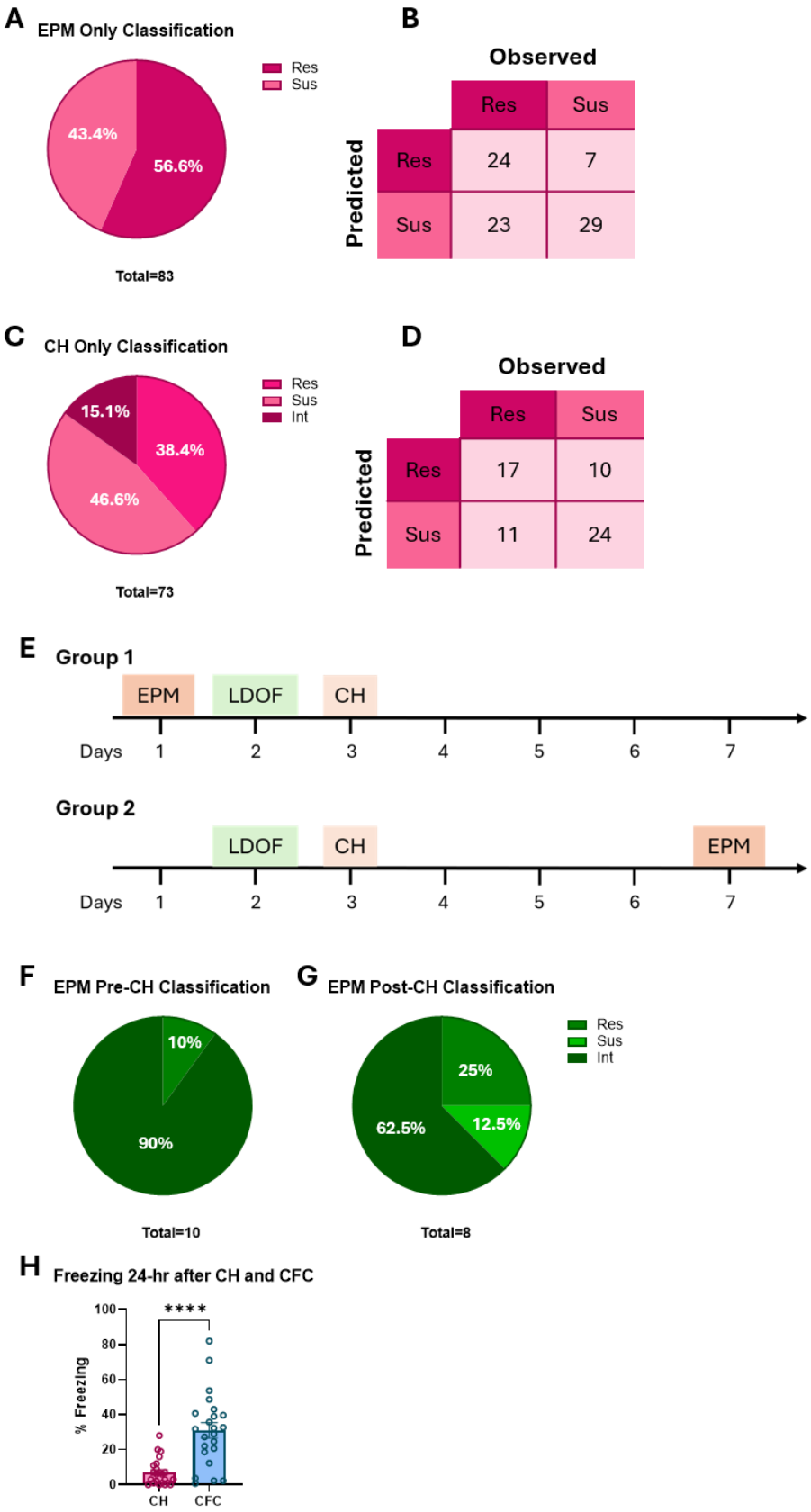

A
EPM Only Classification
Res
Sus
43.4%
56.6%
Total=83
B
Observed
Predicted
Res
Sus
Res
Sus
24
7
23
29
C
CH Only Classification
Res
Sus
Int
15.1%
38.4%
46.6%
Total=73
D
Observed
Predicted
Res
Sus
Res
Sus
17
10
11
24
E
Group 1
EPM
LDOF
CH
Days
1
2
3
4
5
6
7
Group 2
LDOF
CH
EPM
Days
1
2
3
4
5
6
7
F
EPM Pre-CH Classification
10%
90%
Total=10
G
EPM Post-CH Classification
Res
Sus
Int
25%
12.5%
62.5%
Total=8
H
Freezing 24-hr after CH and CFC
****
% Freezing
100
80
60
40
20
0
CH
CFC

Figure 6. Successful classification of females requires exposure to a mild stressor prior to evaluating anxiety-like behavior. A) Percent of total animals in Res, Int, or Sus groups after classification using only time spent in the closed arms. B) PPV chart of EPM only classified female rats. C) Percent of total animals in Res, Int, or Sus groups after classification using only CH interactions within the first minute of CH exposure. D) PPV chart of CH only classified female rats. E) Experimental design. F) Percent of total animals in Res, Int, or Sus groups after classification using EPM when EPM was performed prior to CH exposure. G) Percent of total animals in Res, Int, or Sus groups after classification using EPM when EPM was performed after CH exposure. H) The percentage of time freezing 24 hours after CH exposure and CFC to evaluate recall of fear. CH n = 23, CFC n = 23. ****p<0.0001.