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Title:

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Date:

2019-11-01

Citation:

Clark, J. W., Drummond, S. P. A., Hoyer, D. & Jacobson, L. H. (2019). Sex differences in mouse models of fear inhibition: Fear extinction, safety learning, and fear–safety discrimination. *British Journal of Pharmacology*, 176 (21), pp.4149-4158. <https://doi.org/10.1111/bph.14600>.

Persistent Link:

<https://hdl.handle.net/11343/285739>

**Sex differences in mouse models of fear inhibition: fear extinction, safety learning, and fear – safety discrimination**

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**Word counts**

Abstract: 245 words

Introduction: 495 words

Results: 1368 words

Discussion: 1476 words

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This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as doi: [10.1111/bph.14600](https://doi.org/10.1111/bph.14600)

**What is already known**

- ❖ Posttraumatic stress disorder (PTSD) is linked with impaired fear inhibition and overly represented in females.

**What this study adds**

- ❖ Female C57Bl/6 mice exhibit decreased fear recall following conditioning but slower fear extinction.
- ❖ The acquisition and recall of safety were not overtly influenced by sex.

**Clinical significance**

- ❖ The processing of fear, but not safety, may be implicated in sex differences underlying PTSD.

## **Abstract**

### **Background and Purpose**

Women are overrepresented in Posttraumatic stress disorder (PTSD); a mental disorder characterised by ineffective inhibition of fear. The use of male animals dominates pre-clinical studies, which may contribute to a lack of understanding as to why this disparity exists. Thus, the current study explores sex differences in three mouse models of fear inhibition.

### **Experimental Approach**

All experiments tested male and female C57Bl/6J mice. Experiment One employed two fear conditioning protocols, in which tones were paired with footshocks of differing intensity (moderate or intense). Fear recall and extinction were tested subsequently. In Experiment Two, safety learning was investigated. Tones were explicitly unpaired with footshocks during safety conditioning. Recall of safety learning was tested 24-hours later. Experiment Three assessed a model of fear-safety discrimination. Cued stimuli were paired or never paired with footshocks during fear and safety conditioning, respectively. Discrimination between stimuli was assessed 24-hours later.

### **Key Results**

In fear extinction, males, compared to females, responded with greater fear in sessions most proximal to conditioning, but subsequently showed a more rapid fear extinction over time. Sex differences were not observed during safety learning. During fear-safety discrimination, both males and females discriminated between stimuli, however, males revealed a greater level of freezing to stimuli.

### **Conclusion and Implications**

The current study provides evidence that sex differences influence fear, but not safety-based behaviour in C57Bl/6J mice. These findings indicate processing of fear, but not safety, may play a greater role in sex differences observed for PTSD.

### **Key words**

Sex, context, cue, fear, safety, extinction, recall, discrimination, posttraumatic stress disorder

### **Abbreviations**

PTSD, Posttraumatic stress disorder; CS+, 'threat' conditioned stimuli; CS-, 'safe' conditioned stimuli; kHz, kilohertz; mA, milli Ampere; ITI, inter-trial interval; dB, decibel; ANOVA, analysis of variance; SEM, standard error of the mean.

### **Introduction**

Posttraumatic stress disorder (PTSD) is a prevalent form of psychiatric illness (Kessler, Chiu, et al., 2005; Kilpatrick, Resnick, et al., 2013). PTSD is associated with vast deficits to health and quality of life (Ramsawh, Fullerton, et al., 2014; Brudey, Park, et al., 2015) as well as significant economic costs to society (Kessler, 2000). Rates of PTSD are disproportionately higher in females, compared to males (Kilpatrick, Resnick, et al., 2013; McLean, Asnaani, et al., 2011). Specifically, women are almost twice as likely to be diagnosed with PTSD in their lifetime (McLean, Asnaani, et al., 2011). However, the origin of sex differences in PTSD are not well understood (McLean, Anderson, et al., 2009). Understanding mechanisms underlying such differences is in part impeded by a bias toward the use of males in preclinical pharmaco-behavioural studies (Beery & Zucker, 2011; Wald & Wu, 2010). Thus, inclusion of both males and females in preclinical neuro-pharmacological

research is important for the contribution of critical insight into mechanisms underlying sex differences in PTSD.

The inability to effectively inhibit fear is a phenotype of PTSD and is implicated in its neurocircuitry and clinical symptoms (Jovanovic, Kazama, et al., 2012; Jovanovic & Norrholm, 2011). Generally, fear inhibition includes three processes: 1) fear extinction refers to learning previous threat cues are no longer dangerous (Milad & Quirk, 2012); 2) safety learning occurs when one learns that environmental stimuli predict safety (Marshall, Archeson, et al., 2014); and 3) fear–safety discrimination is the process of distinguishing between ‘threat’ and ‘safety’ cues when simultaneously presented (Day, Reed, et al., 2016). Animal models of fear inhibition have been developed to advance understanding of these phenomena (Bowers & Ressler, 2015; Pollak, Monje, et al., 2010). For example, attempts to model sex differences in PTSD revealed female rats exhibit fear extinction impairment (Voulo, Parsons, et al., 2017; Graham & Milad, 2013), which is influenced by oestrous phase (Zeidan, Igoe, et al., 2011; Graham, Milad, et al., 2013; Milad, Igoe, et al., 2009). However, to our knowledge, no study has investigated sex differences in safety learning or fear–safety discrimination in C57Bl/6J mice. The C57Bl/6J is the most commonly used mouse-strain in preclinical neuro-pharmacological studies (Palanza & Parmigiani, 2017). Thus, it is important to investigate whether this strain of mice exhibit sex differences across fear inhibition-related processes.

In the current study, we examined whether sex differences exist across several mouse-models of fear inhibition. Specifically, we tested male and female C57Bl/6J mice using three experimental models; 1) fear extinction; 2) safety learning; and 3) fear–safety discrimination.

In fear extinction (Experiment One), two fear conditioning paradigms with differing footshock intensities (moderate and intense) were employed to quantify whether sex differences depend on the intensity of the fear conditioning protocol utilised. In safety learning (Experiment Two), males and females were tested to determine their ability to recognise cued safety in an aversive context. Finally, in fear–safety discrimination (Experiment Three), we assessed whether sex differences influence discrimination between threat- and safety-based cues.

## Methods

### Animals

Experiments were performed on young adult (~4 month) male and female C57Bl/6JArc (Animal Resource Centre, Canning Vale, WA 6970, Australia; RRID:IMSR\_ARC:B6) mice. Following transportation from the breeding facility, male and female mice weighed 20.9 – 30.4 and 17.1 – 21.2 grams, respectively. Separate cohorts were utilised in each experiment. Mice were randomly allocated within experiments. Mice were group housed (2 - 4/cage) in open-top cages under an inverted 12:12-hour light:dark cycle (lights off at 0700), with *ad libitum* access to standard chow and water. Cages contained wood-shaving-based bedding (Tapvei Estonia OÜ) and tissue paper (Austwide Paper Products Pty Ltd, Australia) as nesting material. Mice were handled for three days prior to behavioural testing to decrease anxiety associated with the experimenter (~five minutes/handling session/animal). Testing sessions commenced two-hours following dark-phase onset, unless otherwise described. One experimenter (male) conducted each session of animal handling and behavioural testing. Experiments were approved by the Florey Institute

for Neuroscience and Mental Health's Animal Ethics Committee (application number: 17-058) and conducted in accordance with requirements outlined by the Prevention of Cruelty to Animals Act and NH&MRC Code of Practice for the Use of Animals for Scientific Purposes.

### **Apparatus and behavioural protocols**

A computerised conditioning system (MED Associates, Vermont, USA; RRID:SCR\_016928) was utilised in each experiment. The system consists of eight conditioning chambers, each located within a sound attenuated cubicle. Chambers were not illuminated during testing. The flooring of each chamber consisted of stainless-steel grids connected to a shock unit which delivered electrical stimuli (i.e., footshocks). Chambers were fitted with speakers for the presentation of auditory stimuli (i.e., conditioned stimuli). Two experimental contexts were utilised: 1) square-shaped chamber with stainless steel flooring underlying shock grid; and 2) chamber with curved wall inserts, wood shavings underlying shock grid and an olfactory contextual change (peppermint tea bag). These were counterbalanced as context A and B in experiments where both contexts were used. Chambers were cleaned with water in between sessions. The delivery of stimuli was controlled by the conditioning system's computer software. Digital recordings were utilised to automatically assess animal movement during testing. Freezing behaviour was defined as lack of movement (other than that required for respiration) for a minimum of 0.5 seconds. Jumping behaviour was manually scored from videos by one researcher blinded to the sex of the mice. Jumping was counted when all four of the animal's feet left the shock grid. Figure 1 provides a schematic representation of the experimental protocols utilised in each experiment.

***Experiment One: Fear extinction***

Two paradigms of fear conditioning were employed with differing levels of footshock intensity (n = 10 male, n = 10 female mice per paradigm; Jacobson, Commerford, et al., 2011). On day one, mice habituated to experimental context A for two minutes before presentation of five tone stimuli (CS+; 5 kHz, 80 dB, 10 seconds; ITI = 60 seconds), of which co-terminated with a footshock (moderate paradigm, 0.6mA, 1 second; intense paradigm, 0.8mA, 2 seconds). On day two, mice were placed in experimental context A for the assessment of contextual fear recall for three minutes before being returned to their home cage. Two hours later, mice were placed in experimental context B for the assessment of contextual fear generalisation and cued fear recall. Following a 30 second habituation period, mice were presented with the same tone stimuli utilised during conditioning, however without co-terminating footshock. An identical session was repeated every 24-hours thereafter for the assessment of fear extinction. A total of 10 fear extinction sessions were performed.

***Experiment Two: Safety learning***

Mice (n = 14 males; n = 14 females) were tested consecutively across four days during safety learning (Pollak, Monje, et al., 2010). Only one experimental context was utilised during the experiment (square-shaped chamber with a stainless-steel floor underlying shock grid). On day one, mice were habituated to the experimental context for two minutes before presentation of five tone stimuli (CS-; 80 dB, 30 seconds), which were explicitly unpaired with a footshock (0.6mA, 2 seconds). That is, the footshocks were administered at

pseudorandom timepoints in between two tone stimuli (mean ITI: 120 seconds, range 60 – 180 seconds) but not closer than 30 seconds to a tone. On days two and three, mice were returned to the experimental context and habituated for 30 seconds. The same stimuli (both auditory and footshocks) were presented. Timing of stimuli differed across each conditioning session, however, the ITI mean and range remained the same. Safety learning recall was assessed on day four. Mice were placed in the experimental context and 30 seconds later exposed to the same auditory tones presented during safety learning conditioning (ITI=30 seconds) but without interleaving footshocks.

### ***Experiment Three: Fear-safety discrimination***

Mice (n = 10 males, n = 10 females) were tested consecutively across four days during fear-safety discrimination testing. On day one, mice were habituated to experimental context A for two minutes before presentation of five tone stimuli (5 kHz, 80 dB, 30 seconds) and five white-noise stimuli (80 dB, 30 seconds). One stimulus (fear stimulus, CS+) co-terminated with a footshock (0.6 mA, 2 seconds), whereas the other was never paired with a footshock (safety stimulus, CS-). Stimulus allocation to the CS+ or CS- was counterbalanced across the study. Intervals separating stimuli varied in duration (mean ITI: 120 seconds, range 60 – 180 seconds). On days two and three, mice were returned to experimental context A and habituated for 30 seconds. The same stimuli (both auditory and footshocks) were presented. The timing of stimuli differed across each conditioning session, however, the ITI mean and range remained the same. Fear-safety discrimination was assessed on day four. Mice were placed in context B and after 30 seconds exposed to five CS+ stimuli, which were interleaved by five CS- stimuli (ITI=30 seconds). No footshocks were administered in this session.

### Data and statistical analysis

Data consisted of two dependent variables: 1) percent time freezing (freezing %); or 2) number of jumps counted. Baseline freezing data were analysed using a series of independent measures t-tests. All other data were analysed using a series of one-way repeated measures analysis of variance (ANOVA). Holm-Sidak post-hoc analyses were conducted when the *F*-statistic was significant for ANOVA factors, and the homogeneity of variance parametric assumption was not violated (Wei, Carrol, et al., 2012). Additionally, non-linear regression was employed for the analysis of rates of fear extinction over time. The data were analysed via Graphpad Prism version 7 (RRID:SCR\_002798; California, USA) or Sigmastat Version 4 (California, USA).

For each experiment, between-group differences in baseline data were assessed during the two-minute habituation period of the initial conditioning session. In Experiment One, data during the CS+ were analysed for: 1) fear conditioning, 2) cued fear recall; and 3) initial fear extinction (i.e., the session following cued fear recall). For these analyses, the between-subject factor was defined as 'sex' and the within-subject factor was defined as 'trial'. For analysis of contextual fear recall, data in the first 90 seconds were binned into 30 second epochs (between-subject factor = sex; within-subject factor = epoch). Between-group differences in baseline data during the 30 second baseline period of the cued fear recall session were assessed as a measure of contextual fear generalisation. For the analysis of fear extinction over time (i.e., across ten fear extinction sessions following cued fear recall), data during trials of the CS+ were averaged within session (between-subject factor = sex; within-subject factor = fear extinction session). In Experiment Two, conditioned safety in the first

session was assessed by comparing data in the final CS- with the 30 second period following the CS- (post CS-). For conditioning sessions two and three, as well as safety learning recall, data expressed for each CS- and post CS- were averaged within each session and analysed (between-group factor = sex; within-subject factor = stimulus). In Experiment Three, data during the final presentation of the CS+ and CS- of the first conditioning session were analysed (between-group factor = sex; within-subject factor = stimulus). Conversely, for conditioning sessions two and three, as well as discrimination recall, responses to the CS+ and CS- were averaged within each session and analysed (between-group factor = sex; within-subject factor = stimulus).

Statistical significance was accepted with  $\alpha \leq 0.05$ . Data are presented as mean  $\pm$  standard error of the mean (SEM). Data and statistical analyses comply the recommendations for experimental design and analysis in pharmacology (Curtis, Alexander, et al., 2018).

## Results

In the current study, both freezing and jumping behaviour were assessed as indices of fear. However, jumping behaviour presented only infrequently during all experiments, except during acquisition of fear conditioning in the intense paradigm (Experiment One). Therefore, other than for jumping data collected during fear conditioning in the intense paradigm, freezing data only are reported in the following results sections.

### **Experiment One: Fear extinction**

#### *Acquisition of conditioned fear*

During habituation, for both intensity paradigms, our data illustrate baseline freezing was minimal and not significantly different between groups (moderate,  $t(1, 18) = 1.91$ ,  $p = 0.07$ ; intense,  $t(1, 18) = 1.38$ ,  $p = 0.18$ ; Supplementary Figure S1). During fear conditioning, freezing increased significantly across CS+ trials for both the moderate and intense paradigms (moderate,  $F(4, 72) = 13.82$ ,  $p < 0.001$ ; intense,  $F(4, 72) = 12.21$ ,  $p < 0.001$ ). However, there was no significant effect of sex (moderate,  $F(1, 18) = 1.14$ ,  $p = 0.29$ ; intense,  $F(1, 18) = 0.29$ ,  $p = 0.59$ ) nor was the 'sex' x 'trial' interaction significant (moderate,  $F(4, 72) = 0.32$ ,  $p = 0.86$ ; intense,  $F(4, 72) = 1.19$ ,  $p = 0.32$ ; Supplementary Figure S2). In contrast, jumping data reveal a different pattern across shock intensity levels, with significant increases in jumping illustrated with each CS+ trial for the intense paradigm ( $F(4, 72) = 7.04$ ,  $p < 0.001$ ), in contrast to the moderate paradigm ( $F(4, 72) = 1.49$ ,  $p = 0.22$ ). However, jumping behaviour was not significantly affected by sex (moderate,  $F(1, 72) = 4.24$ ,  $p = 0.054$ ; intense,  $F(1, 72) = 0.04$ ,  $p = 0.84$ ) nor was the 'sex' x 'trial' interaction significant (moderate,  $F(4, 72) = 1.49$ ,  $p = 0.22$ ; intense,  $F(4, 72) = 0.22$ ,  $p = 0.93$ ; Figure 2A).

### ***Contextual fear recall and generalisation***

In the moderate paradigm, freezing across epochs of contextual fear recall were not significantly different ( $F(2, 36) = 2.28$ ,  $p = 0.12$ ). Further, the effects of sex ( $F(1, 36) = 0.09$ ,  $p = 0.76$ ) and the 'sex' x 'epoch' interaction ( $F(2, 36) = 0.36$ ,  $p = 0.70$ ) were non-significant. By contrast, for the intense paradigm, freezing increased significantly across epochs ( $F(2, 36) = 4.56$ ,  $p = 0.01$ ) and was significantly greater among males, when compared to females ( $F(1, 36) = 4.53$ ,  $p = 0.04$ ; Figure 2B). The 'sex' x 'epoch' interaction, however, was not significant ( $F(2, 36) = 0.42$ ,  $p = 0.66$ ). Regarding contextual fear generalisation to an

alternative context, our data demonstrate non-significant differences between males and females for both intensity paradigms (moderate,  $t(1, 18) = 0.29$ ,  $p = 0.79$ ; intense,  $t(1, 18) = 0.63$ ,  $p = 0.54$ ; Supplementary Figure S3).

### ***Recall and extinction of cued fear***

Regarding cued fear recall, freezing during the CS+ significantly increased with trial for both shock intensity paradigms (moderate,  $F(4, 72) = 5.35$ ,  $p < 0.001$ ; intense,  $F(4, 72) = 14.91$ ,  $p < 0.001$ ). Furthermore, the influence of sex trended toward significance for the intense paradigm (males exhibited higher freezing;  $F(1, 72) = 37.5$ ,  $p = 0.07$ ) but not the moderate paradigm ( $F(1, 72) = 0.56$ ,  $p = 0.47$ ). The 'sex' x 'trial' interaction, though, was not significant for both intensity paradigms (moderate,  $F(4, 72) = 0.93$ ,  $p = 0.45$ ; intense,  $F(4, 72) = 0.16$ ,  $p = 0.96$ ; Supplementary Figure S4). In the subsequent testing session (i.e., initial fear extinction), for the moderate intensity paradigm, freezing decreased significantly across CS+ trials ( $F(4, 72) = 2.85$ ,  $p = 0.03$ ). However, the influence of sex ( $F(1, 72) = 0.34$ ,  $p = 0.57$ ) and the 'sex' x 'trial' interaction ( $F(4, 72) = 1.84$ ,  $p = 0.13$ ) were non-significant. By contrast, for the intense paradigm, freezing did not significantly differ across CS+ trials ( $F(4, 72) = 0.84$ ,  $p = 0.51$ ), although it was significantly greater for males, compared to females ( $F(1, 72) = 6.36$ ,  $p = 0.02$ ). Again, however, the 'sex' x 'trial' interaction was non-significant ( $F(4, 72) = 2.12$ ,  $p = 0.09$ ; Figure 2C).

Regarding fear extinction over time, for the moderate paradigm, freezing in response to the CS+ decreased across the ten sessions tested ( $F(9, 162) = 20.92$ ,  $p < 0.001$ ). Though, the influence of sex ( $F(1, 162) = 0.89$ ,  $p = 0.36$ ) and the 'sex' x 'fear extinction session'

interaction ( $F(9, 162) = 0.29, p = 0.97$ ) were non-significant. Non-linear regression did not demonstrate a significant difference in extinction rate for males versus females in the moderate protocol. By contrast, for the intense paradigm, the ‘sex’ x ‘fear extinction session’ interaction on freezing % was significant ( $F(9, 162) = 5.06, p < 0.001$ ), with post-hoc analyses demonstrating between- group differences in fear extinction sessions one and seven. Furthermore, non-linear regression revealed significant differences in the extinction rate between males and females ( $F(2, 196) = 5.95, p = 0.003$ ), such that the curve was steeper for males ( $\alpha = 67.5, \beta = 6.56$ ; where  $y = \alpha + \beta x$ ) when compared to females ( $\alpha = 54.42, \beta = -3.9$ ; Figure 2D).

## **Experiment two: Safety learning**

### *Acquisition of safety learning*

During habituation, our data demonstrate that baseline freezing was minimal and not significantly different between groups ( $t(1, 26) = 1.29, p = 0.20$ ; Supplementary Figure S5). For the first safety conditioning session, analysis revealed significantly less freezing in the presence of the final CS-, compared to the final post CS- ( $F(1, 26) = 5.46, p = 0.03$ ). However, sex differences ( $F(1, 26) = 0.96, p = 0.37$ ) as well as the ‘sex’ x ‘stimulus’ interaction ( $F(1, 26) = 0.004, p = 0.95$ ) were non-significant. For conditioning sessions two and three, freezing % during the CS- was significantly less than during the post CS- when averaged separately within each session (conditioning session two,  $F(1, 26) = 57.8, p < 0.001$ ; conditioning session three,  $F(1, 26) = 27.67, p < 0.001$ ). Again, however, data demonstrate a non-significant effect of sex (conditioning session two,  $F(1, 26) = 0.23, p$

=0.64; conditioning session three,  $F(1, 26) = 0.09, p = 0.77$ ) as well as ‘sex’ x ‘stimulus’ interaction (conditioning session two,  $F(1, 26) = 0.31, p = 0.58$ ; conditioning session three,  $F(1, 26) = 0.96, p = 0.34$ ; Supplementary Figure S6).

### ***Recall of safety learning conditioning***

In safety learning recall, a lower freezing was observed in response to the CS- when compared to the post CS- for both males and females (Figure 3A). Accordingly, the percentage freezing in response to the CS- and post CS- period, when averaged within this session, were significantly different ( $F(1, 26) = 24.36, p < 0.001$ ; Figure 3B). By contrast, sex differences ( $F(1, 26) = 0.45, p = 0.50$ ; Figure 3C) and the ‘sex’ x ‘stimulus’ interaction ( $F(1, 26) = 0.01, p = 0.92$ ) were non-significant.

### **Experiment Three: Fear-safety discrimination**

#### ***Acquisition of fear-safety discrimination***

During habituation, our data demonstrate that baseline freezing was minimal and not significantly different between groups ( $t(1, 18) = 1.98, p = 0.06$ ; Supplementary Figure S7). For fear-safety conditioning session one, freezing in response to the final CS+ and CS- were not significantly different ( $F(1, 18) = 2.52, p = 0.13$ ). Sex differences ( $F(1, 18) = 0.0001, p = 0.99$ ) and the ‘stimulus’ x ‘sex’ interaction ( $F(1, 18) = 0.02, p = 0.89$ ) were also not significant. Moreover, for conditioning sessions two and three, freezing % during the CS+ and CS-, when averaged separately within each session, were not significantly different (conditioning session two,  $F(1, 18) = 1.34, p = 0.26$ ; conditioning session three,  $F(1, 18) = 2.79, p = 0.11$ ). Again, the data illustrate a non-significant effect of sex (conditioning session

two,  $F(1, 18) = 2.75, p = 0.12$ ; conditioning session three,  $F(1, 18) = 0.66, p = 0.43$ ) and 'sex' x 'stimulus' interaction (conditioning session two,  $F(1, 18) = 0.001, p = 0.97$ ; conditioning session three,  $F(1, 18) = 0.41, p = 0.53$ ; Supplementary Figure S7).

### ***Fear-safety discrimination recall***

In fear-safety discrimination recall, freezing during the CS+ was visibly higher than during the CS-, indicating successful discrimination between fear and safety cues (Figure 4A). When averaged separately, freezing during the CS+ was significantly greater than the CS- ( $F(1, 18) = 33, p < 0.001$ ; Figure 4B). Further, freezing was significantly higher for males compared to females ( $F(1, 18) = 5.57, p = 0.03$ ; Figure 4C). Conversely, the 'sex' x 'stimulus' interaction was not significant ( $F(1, 18) = 1.38, p = 0.26$ ).

## **Discussion**

The current study investigated whether sex differences affect performance in specific mouse models of fear inhibition. In the fear extinction model, our data show that sex differences exist during contextual, but not cued, recall of fear. Further, the initial session of fear extinction, as well as fear extinction over time, were also influenced by sex. Our model of safety learning, conversely, did not exhibit differences between male and female mice. By contrast, the fear-safety discrimination model demonstrates freezing responses during recall were influenced by sex. Thus, the current study indicates that sex is a factor which influences specific components of fear inhibition processes in C57Bl/6J mice. Specifically, we provide important evidence to suggest response to cues representing danger, but not safety, are influenced by sex. Therefore, preclinical neuro-pharmacological studies in rodents (more

specifically in C57Bl/6J mice), and particularly those investigating fear-based processes, should be carried out in both sexes.

As indices of natural defensive behaviour, freezing as well as jumping were assessed in the current study. These behaviours represent differing responses to immediate threat stimuli (Blanchard, Griebel, et al., 2001). Specifically, whereas freezing is considered a reliable index of fear, jumping is thought to reflect a more intense, escape-based behaviour (Lang, Davis, et al., 2000; Blanchard, Griebel, et al., 2001). While footshock pain-thresholds are not influenced by sex in C57Bl/6 mice (Podhorna, McCabe, et al., 2002), female rodents, compared to males, are considered more vulnerable to acute stressors (Kokras & Dalla, 2014). Thus, studies have revealed that the prevalence of jumping is highest among female rodents (Pereira-Figueiredo, Castellano, et al., 2017; Day, Reed, et al., 2016). In the current study, jumping was exhibited infrequently by males and females across all fear inhibition models tested. A critical exception, however, was during the fear extinction model, where jumping presented more frequently in the intense paradigm of fear conditioning, compared to the moderate paradigm. This finding supports the definition of these paradigms being moderate versus intense. In contrast, freezing presented consistently across all experiments of the current study and, therefore, the models of behaviour employed are considered predominantly reliable in inducing fear, though not escape-based behaviour.

Our fear extinction model shows sex differences which are dependent on the intensity of footshock administered. Indeed, sex differences were not found to influence any outcome of interest assessed following the moderate paradigm of fear conditioning. Regarding the intense paradigm, however, male mice exhibited greater retention of fear in sessions most

proximal to fear conditioning. These findings align with studies revealing a greater immediate conditioned fear response among males, compared to females (Villasana, Rosenberg, et al., 2010; Kokras & Dalla, 2014). In contrast, we did not observe sex differences in contextual fear generalisation or the recall of cued fear, which is at odds with previous reports (Villasana, Rosenberg, et al., 2010). We also demonstrated males show an initial failure in the acquisition of fear extinction but exhibit more rapid fear extinction when tested over ten sessions. These findings align with studies illustrating male mice extinguish fear sooner than females (Matsuda, Matsuzawa, et al., 2015; Graham & Milad, 2013). Taken together, the current study illustrates sex differences are more prominent following intense fear conditioning paradigms. Moreover, while male mice respond initially with greater fear, female mice exhibit slower fear extinction over time.

Safety learning in the current study revealed males and females exhibit an equal ability to establish learned safety. Mouse models which examine safety learning are relatively novel: therefore, studies utilising these models, particularly to investigate sex differences, are scarce (Pollak, Monje, et al., 2010). To date, only one study has examined safety learning processes in orexin-deficient male and female mice (Khalil & Fendt, 2017). This study similarly revealed safety learning was not influenced by sex. To our knowledge, the current study is the first to investigate safety learning in both male and female C57Bl/6J mice, arguably one of the most commonly utilised preclinical strains in pharmaco-behavioural investigations. Sex differences in behavioural processes are thought to reflect fundamental differences in underlying structure, biology, and chemistry of the brain (Cahill, 2006). Thus, findings of the current study suggest the neurophysiology of safety learning may not differ

substantially between males and females. Therefore, findings of safety learning studies employing male subjects may be generalisable to females, at least with regard to mice.

Finally, the fear-safety discrimination model examined whether sex differences exist in discrimination between 'threat' and 'safe' conditioned stimuli. Importantly, this model tested fear and safety processing simultaneously, rather than independently, which was the case in Experiments One and Two. For both males and females, freezing data suggest initial impairment in discrimination between fear and safety stimuli during conditioning, which aligns with extant studies with rats (Day, Reed, et al., 2016). Our data, however, demonstrate robust fear-safety discrimination during recall. This may potentially be due to the addition of an alternate context which controlled for the contextual fear driving high freezing in response to safety stimuli during conditioning. Moreover, during recall, our data reveal greater general levels of freezing among males, compared to females. This is consistent with previous studies with rats which also illustrate males exhibit greater freezing during discrimination recall (Day, Reed, et al., 2016; Folib, Bals, et al., 2018). These findings are similar to those observed in contextual fear recall as well as the initial fear extinction session of Experiment One, which suggests the conditioning protocol employed in the fear-safety discrimination paradigm was intense. Moreover, our data demonstrate initial cued fear generalisation in male mice, that was not apparent in females, and which may have masked initial safety learning effects. By the second trial of the CS-, however, sex differences in freezing response to the safety cue were no longer exhibited.

Together, our findings demonstrate that fear processes, but not safety processes *per se*, are influenced by sex. Specifically, in C57Bl/6J mice, these differences were illustrated in

the fear extinction and fear-safety discrimination model of fear inhibition. In contrast, the safety learning model, as well as the safety component of the fear-safety discrimination model, did not reveal major sex differences. This may reflect important sex differences in neurocircuitry implicated in fear but not safety-related processes. Specifically, neural structures that mediate response to threat, such as the amygdala and orbitofrontal cortex, demonstrate sex differences (Gruene, Roberts, et al., 2015; McClure, Monk, et al., 2004). Furthermore, previous studies in rodents have shown estrogen levels influence processing of fear memory (Hwang, Zsido, et al., 2015; Milad, Igoe, et al., 2009). We did not assess oestrous phase in the current study, though, our data demonstrate robust sex differences were present in specific fear inhibition processes, irrespective of oestrus phase.

Fear and safety-based processes are preserved across species and, therefore, findings of studies examining these processes are often translatable (VanElzakker, Dahlgren, et al., 2014). Similar to the present study, in a healthy human population, men showed heightened immediate fear response to conditioned stimuli compared to women (Milad, Goldstein, et al., 2006), but extinguished fear more rapidly (Milad, Zeidan, et al., 2010). Further, sex differences between men and naturally cycling women were not demonstrated in objective fear responses to stimuli reflecting safety (Lonsdorf, Haaker, et al., 2015). Mouse paradigms of fear inhibition, thus, may be particularly useful for the development of understanding of mechanisms which underly differential responses to fear versus safety learning between sexes. For example, studies with rats have identified neurocircuitry underlying fear extinction processes and examined ways of manipulating this circuitry (Ter Horst, Carobrez, et al., 2012; Milad & Quirk, 2012). However, the majority of these studies have utilised males

(Beery & Zucker, 2011; Wald & Wu, 2010). Results of the present study suggest it is inappropriate to generalise findings across sexes in studies investigating fear-related processes, and particularly with regard to cued fear conditioning using intense conditioning protocols. Future preclinical pharmaco-behavioural studies should therefore utilise both males and females when examining such processes.

### ***Conclusion and implications***

Here we present three models of fear inhibition in C57Bl6/J mice that may be engaged for testing pharmacological agents targeting fear inhibition processes. Males displayed greater immediate fear response to fear conditioned stimuli, but faster fear extinction when assessed over time. Conversely, contextual safety learning was not influenced by sex. Thus, cued fear inhibition processes in males should not be generalised to females, or *vice versa*, and inclusion of both males and females in related studies is therefore important. Stress-related psychiatric disorders, especially PTSD, are prevalent in woman (McLean, Asnaani, et al., 2011; Kilpatrick, Resnick, et al., 2013) and associated with fear inhibition impairment (Jovanovic, Kazama, et al., 2012). The current study suggests that extinction of cued fear, but not safety learning, may be a dominant contributor to this sex difference.

### **Conflicts of interest**

The authors state no conflicts of interest.

### **Declaration of transparency and scientific rigour**

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for [Design & Analysis](#), and [Animal Experimentation](#), and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

### **Acknowledgements**

The authors are grateful for the expert technical assistance of Brett Purcell (Florey Institute of Neuroscience and Mental Health, Behaviour Facility) and Simon Miller (Florey Institute of Neuroscience and Mental Health, Information Technology). LHJ and DH are supported by the NHMRC Project Grant 1105284 and the Alzheimer's Association NIRG-396905. The Florey Institute of Neuroscience and Mental Health acknowledge the support of the Victorian Government and in particular the funding from the Operational Infrastructure Support Grant.

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


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### Figure legends

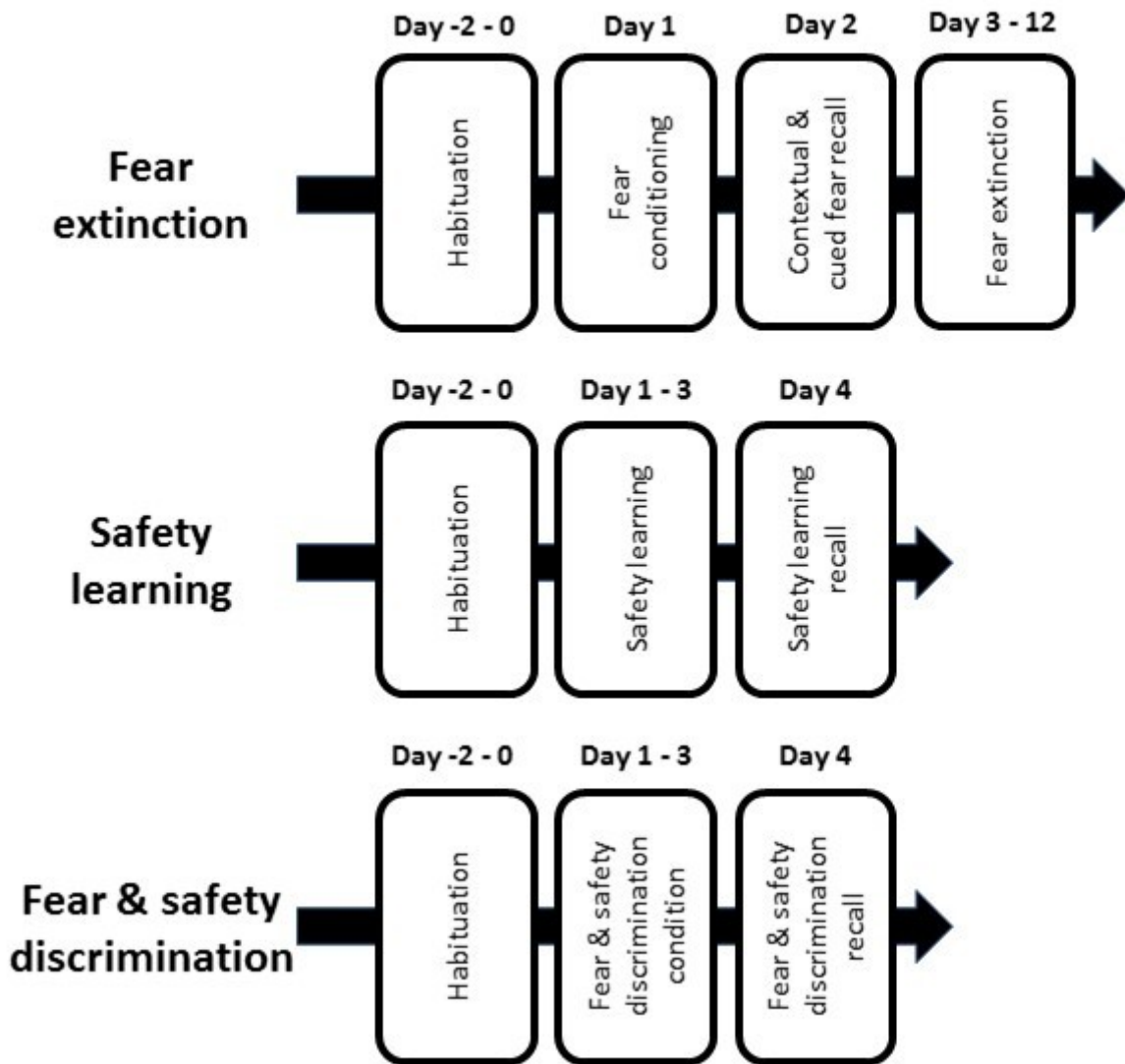
**Fig. 1.** Schematic representations of the fear inhibition paradigms utilised in the current study.

**Fig. 2.** Sex differences in a fear extinction model (per protocol, males,  $n = 10$ ; females,  $n = 10$ ): (A) Jumping behaviour during fear conditioning. Jumping increased significantly among males and female mice allocated to the intense protocol only. (B) Freezing across three epochs of contextual fear recall. For the intense protocol only, males, compared to females, presented with significantly greater freezing. (C) Freezing during the initial fear extinction session. For the intense protocol only, freezing was significantly different between males and females (D) Freezing across fear extinction sessions tested over time (freezing to the CS+ averaged within-session). For the intense protocol only, males, when compared to females, demonstrated a much steeper fear extinction curve. Note. Freezing in the presence of the CS+ during the initial fear extinction session were averaged and included as session one in fear extinction over time data.  Male;  Female; \* with square brackets denote significant main effects, \* without square brackets denote significant differences between sexes within session.

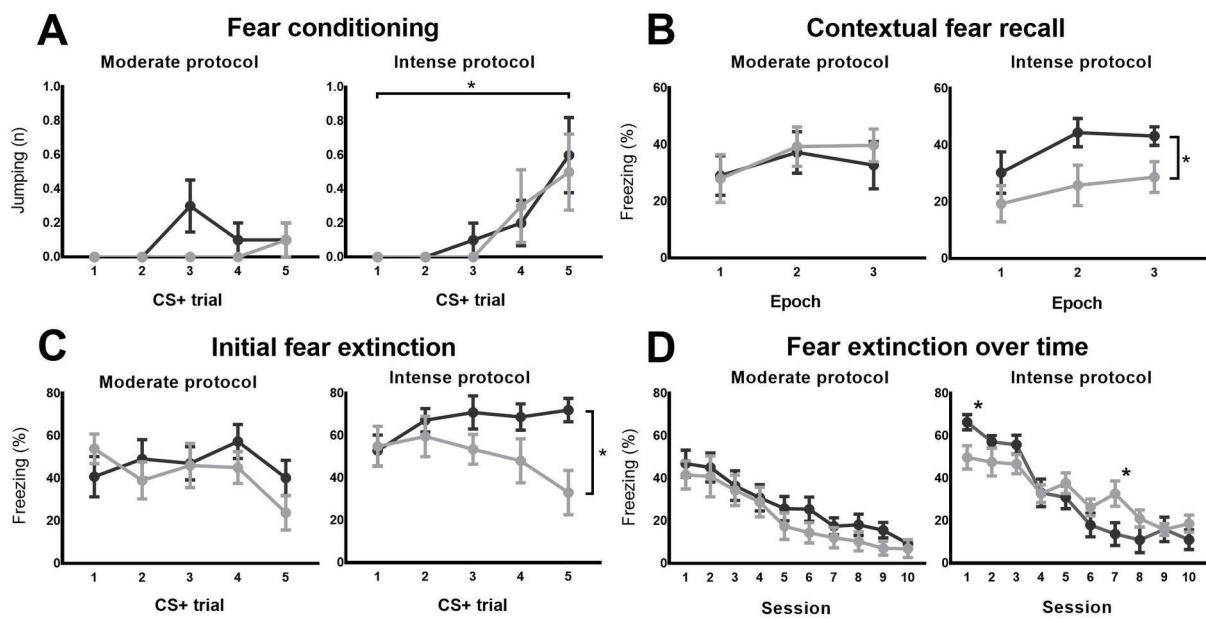
**Fig. 3.** Sex differences in safety learning recall (Males,  $n = 14$ ; Females,  $n = 14$ ): (A) Pattern of discrimination between the CS- and Post CS- illustrated in the safety recall session. Freezing response to stimuli are plotted in the order presented during testing (  Male;  Female). (B) Freezing response to the CS- and Post CS- period averaged within-session. Mice (males and females) demonstrated significant differences in freezing response between stimuli. (  ) *versus* post CS- (  ). (C) Males and females did not differ

in general levels of freezing % ( ■ Male; ■ Female). \* with square brackets denote significant main effect.

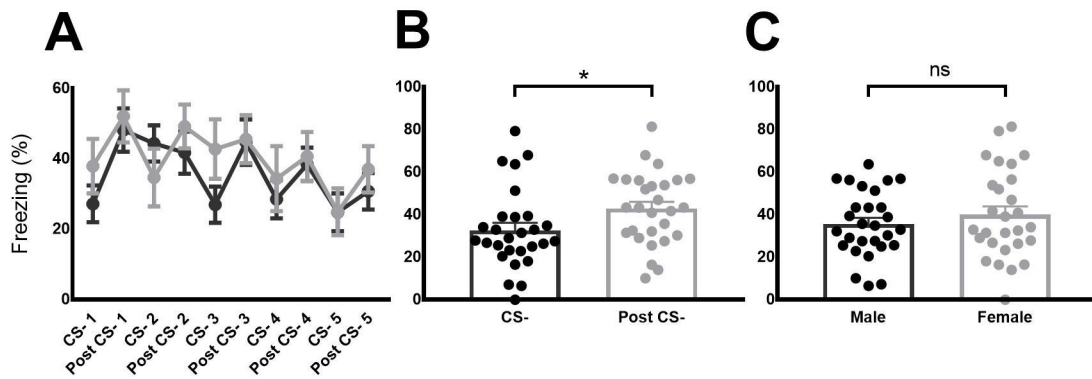
**Fig. 4.;** Sex differences in fear-safety discrimination (Males, n = 10; Females, n = 10): (A) The pattern of discrimination between the CS+ and CS- demonstrated in the fear and safety recall session. Freezing response to stimuli are plotted in the order they were presented during testing ( ■ Male; ■ Female). (B) Freezing, averaged within-session, was greater for the CS+ ( ■ ) than the CS- ( ■ ) and were higher in males than in females (C; ■ Male; ■ Female); \* with square brackets denote significant main effects.



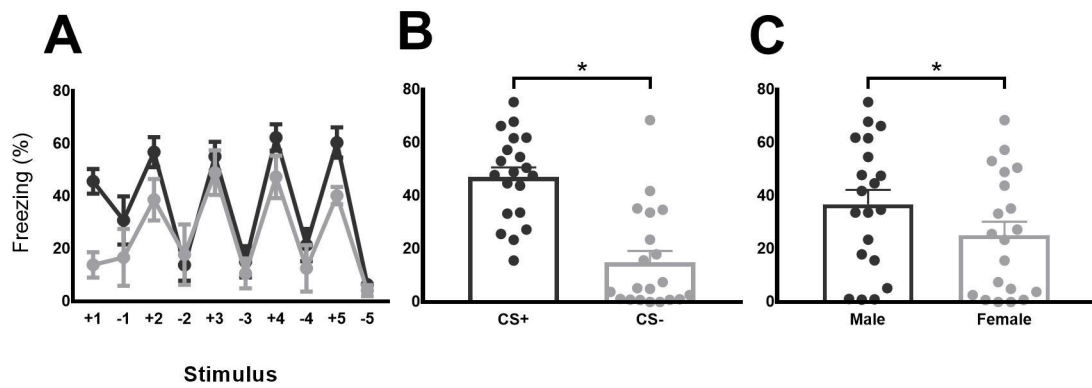
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