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Assessing sex differences in threat responding to posttraumatic stress disorder (PTSD)–relevant challenges in mice

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Introduction

- Post-traumatic stress disorder (PTSD) is a fear-associated disorder that afflicts approximately 6 million individuals annually with higher prevalence in war veterans and females. PTSD is characterized by maladaptive threat responding and persisting trauma-associated fear memories.
- There is an interest in identifying intrinsic factors that may promote PTSD phenotype.
- Previous studies: PTSD patients have increased sensitivity to CO₂ inhalation (Muhtz et al., 2011)
- Pre-deployment study found that soldiers more sensitive to CO₂ later were more at risk to symptoms of PTSD and anxiety. (Telch et al., 2012).
- CO₂ inhalation produced fear extinction deficits in male mice (McMurray et al., 2020). High sensitivity to CO₂ correlated with passive coping to stress and fear.
- McMurray et al. found increase expressions of ΔFosB+ in male mice more sensitive to CO₂ in the basal lateral amygdala (BLA) and the central nucleus of the amygdala (CeA) (2020).
- BLA: role in the learning and expression of fear conditioning (Muller et al., 1997) and fear extinction (Sotres-Bayon et al., 2007).
- CeA: both fear learning and fear memory (Wilensky et al., 2006)
- Previous studies of Male and Female Differences
- Females and males: not different in regards to freezing behavior during conditioning, fear extinction, and extinction memory retrieval. Males: different structures in the IL-BLA projection for the high freezers, which relates to fear suppression when experiencing fear extinction and memory of extinction. Females did not display this difference (Grüne et al., 2015)
- Females froze more than males during fear conditioning and had more cFos activation in the amygdala than males (Keiser et al., 2017).
- The purpose of this study:** to assess differences in threat responding to CO₂ homeostatic stressor and contextual fear conditioning and extinction in male and female mice
- Fear Response Behavior: Freezing (passive coping) & Rearing (active coping)
- Brain Activity: ΔFosB+ immunostaining in basal lateral amygdala (BLA) and central nucleus of the amygdala (CeA)

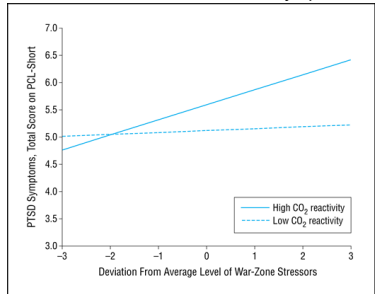


Fig. 1. Pre-deployment study from Telch et al.

Hypothesis
Females and males will display differences in behavioral response to fear and fear circuit recruitment

Methods

Subjects: Male and female C57Bl/6J mice (Jackson Laboratories). Mice were 8 weeks old on arrival, pair housed and acclimated for 2 weeks before behavioral studies began.

CO₂ Inhalation: On Day 1, mice habituated to a CO₂ exposure chamber for 7 min. On Day 2, mice were exposed to 10% CO₂ or air for 10 min. On Day 3, mice were returned to the context for 5 min. Animals were videotaped. Freezing behavior, defined as a lack of movement, and rearing, defined as standing on hind legs and stretching up, were scored by an observer blind to the treatment.

Contextual Fear Conditioning: One week later, on day 11, mice habituated to the fear conditioning chamber for 5 min after which they received 3 shocks (0.5mA), 1 min apart. On day 12, mice were returned to the chamber for 5 min to assess conditioned fear. Mice were then returned to the chambers daily for 5 minutes for fear extinction. **Data Analysis:** Group data was run with ANOVA and t-tests with post-Hoc analysis only if significance was found initially (Prizm).

ΔFosB+: Mice were euthanized and transcardially perfused with 4% paraformaldehyde. Brains were sectioned and treated with Rb x FosB (1:20,000) and stained with DAB. Images for BLA-CeA were captured with a AxioImager Z1 microscope and cells were counted using ImageJ.

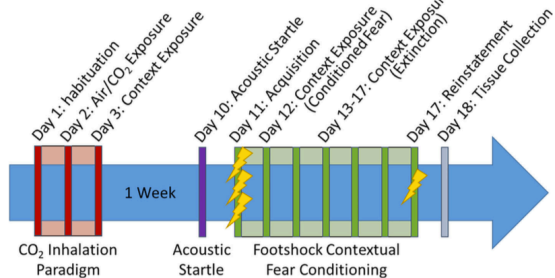


Fig. 2. Experimental Approach

Experimental timeline: Mice underwent the CO₂ inhalation paradigm. After a week rest, mice underwent the footshock contextual fear conditioning paradigm (see methods for details).

Results

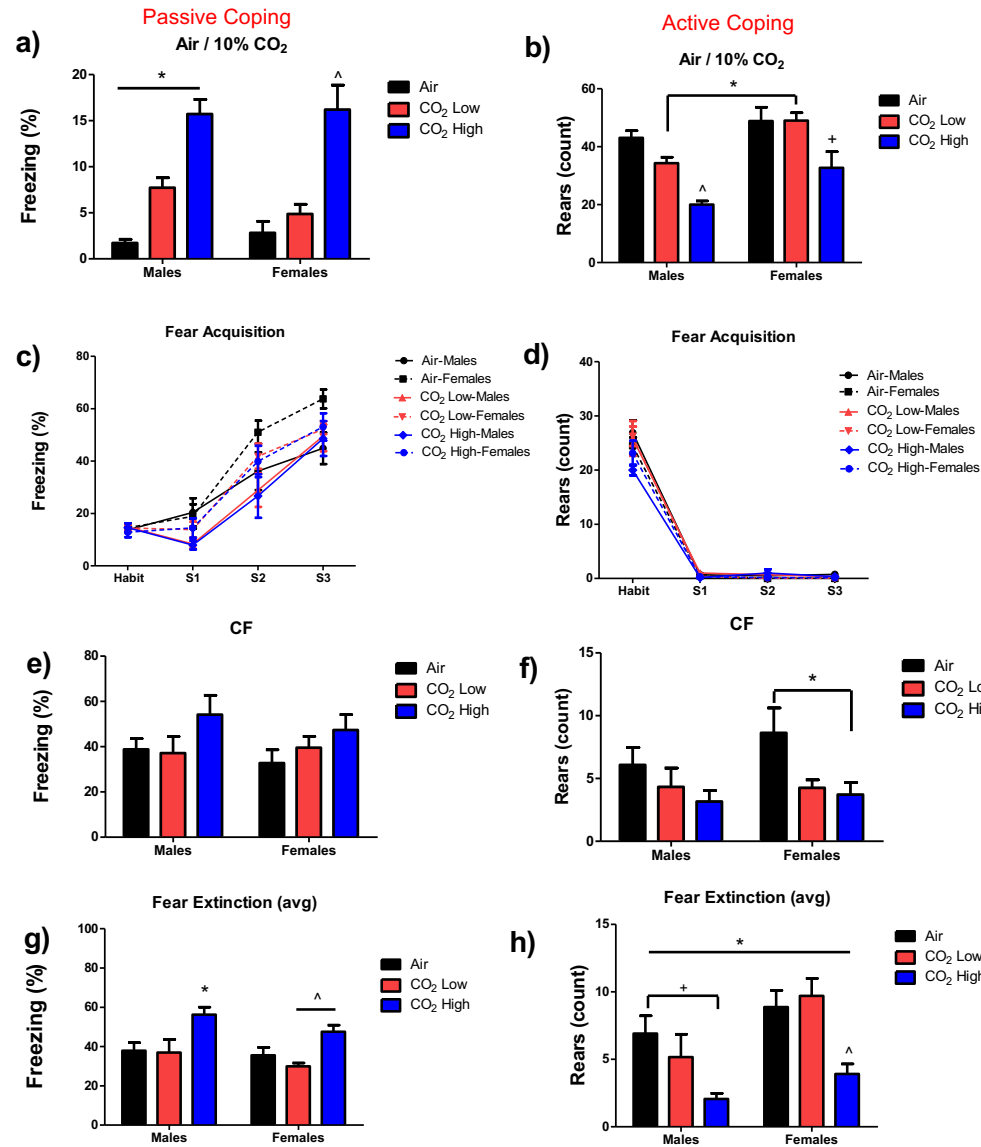


Fig. 3. CO₂ inhalation evokes sex differences in active (rearing) but not passive (freezing) behaviors. (a) & (b) High CO₂ responders showed increased freezing and decreased rearing in both males and females, respectively. Male groups show significance between all three groups ($p < 0.05$); female CO₂ high responders froze significantly more than CO₂ low and air ($p < 0.01$). (c) & (d): No significant differences across groups shown in fear acquisition. (e) & (f): Conditioned fear following fear acquisition: no effect of CO₂ but sex differences in freezing are trending ($p = 0.054$) (f) Females reared more in air than in CO₂H responders ($p < 0.05$). (g) During fear extinction, male and female CO₂ high responders had increased freezing. $p < .05$ M CO₂ H v M CO₂ L and Air, $p < .05$ F CO₂ H v F CO₂ L. (h) Overall, females reared more than males during fear extinction. Male CO₂ high responders reared less than air. $p < .05$ M CO₂ H v M Air; female CO₂ high responders reared less than CO₂ low and air. $p < .05$ F CO₂ H v F CO₂ L and F Air.

Results (cont.)

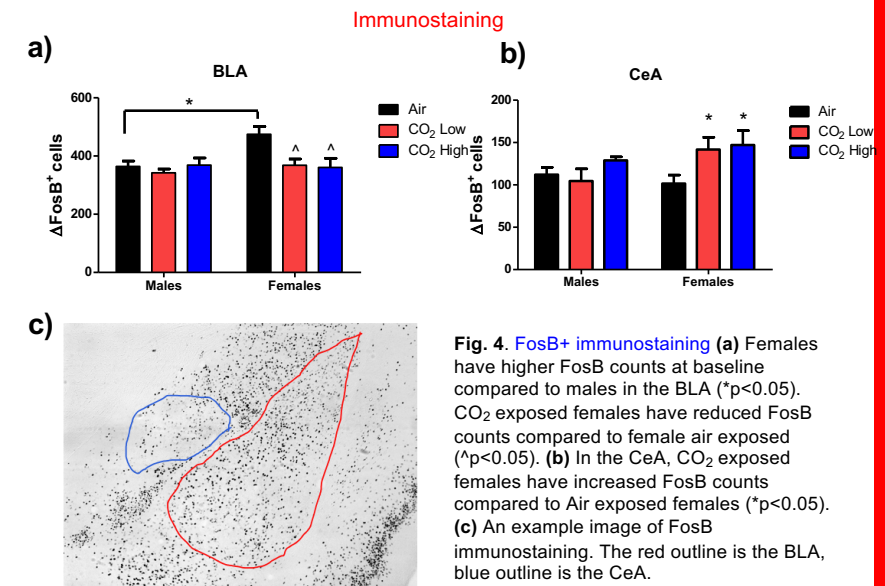


Fig. 4. FosB+ immunostaining (a) Females have higher FosB counts at baseline compared to males in the BLA ($p < 0.05$). CO₂ exposed females have reduced FosB counts compared to female air exposed ($p < 0.05$). (b) In the CeA, CO₂ exposed females have increased FosB counts compared to Air exposed females ($p < 0.05$). (c) An example image of FosB immunostaining. The red outline is the BLA, blue outline is the CeA.

Conclusions/Future Directions

- We used a CO₂ inhalation-PTSD paradigm to show how an interoceptive threat, namely CO₂, modulates PTSD risk in males and females separately.
- Our data revealed heterogeneous freezing and rearing behaviors to CO₂ in male and female mice similar to CO₂ variance observed in humans.
- Those more sensitive to CO₂ froze more and reared less.
- Females demonstrated significantly more active coping behavior compared to males during CO₂ exposure and fear extinction.
- Females also had alterations in FosB cell counts in BLA / CeA when exposed to CO₂, which was not observed in male mice (consistent with Keiser et al. 2017 study).
- FosB is merely an indication of neuron recruitment and thus does not indicate if the neurons were activated or inhibited. Further research can delve into this phenomenon.
- Future direction could include investigating the different circuitry that male mice may recruit that females may or may not also recruit, including the prefrontal cortex, hippocampus and locus coeruleus of the brain stem.
- Future direction could also include evaluating male and female differences with the female estrogen cycle as an independent variable.

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