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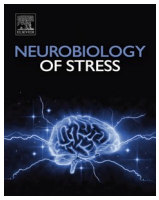

Sex-specific threat responding and neuronal engagement in carbon dioxide associated fear and extinction: Noradrenergic involvement in female mice

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ABSTRACT

Difficulty in appropriately responding to threats is a key feature of psychiatric disorders, especially fear-related conditions such as panic disorder (PD) and posttraumatic stress disorder (PTSD). Most prior work on threat and fear regulation involves exposure to external threatful cues. However, fear can also be triggered by aversive, within-the-body, sensations. This interoceptive signaling of fear is highly relevant to PD and PTSD but is not well understood, especially in the context of sex. Using female and male mice, the current study investigated fear-associated spontaneous and conditioned behaviors to carbon dioxide (CO_2) inhalation, a potent interoceptive threat that induces fear and panic. We also investigated whether behavioral sensitivity to CO_2 is associated with delayed PTSD-relevant behaviors. CO_2 evoked heterogenous freezing behaviors in both male and female animals. However, active, rearing behavior was significantly reduced in CO_2-exposed male but not female mice. Interestingly, behavioral sensitivity to CO_2 was associated with compromised fear extinction, independent of sex. However, in comparison to CO_2-exposed males, females elicited less freezing and higher rearing during extinction suggesting an engagement of active versus passive defensive coping. Persistent neuronal activation marker ΔFosB immuno-mapping revealed attenuated engagement of infralimbic-prefrontal areas in both sexes but higher activation of brain stem locus coeruleus (LC) area in females. Inter-regional co-activation mapping revealed sex-independent disruptions in the infralimbic-amygdala associations but altered LC associations only in CO_2-exposed female mice. Lastly, dopamine β hydroxylase positive ($D\beta H^{+ve}$) noradrenergic neuronal cell counts in the LC correlated with freezing and rearing behaviors during CO_2 inhalation and extinction only in female but not male mice. Collectively, these data provide evidence for higher active defensive responding to interoceptive threat CO_2-associated fear in females that may stem from increased recruitment of the brainstem noradrenergic system. Our findings reveal distinct contributory mechanisms that may promote sex differences in fear and panic associated pathologies.

1. Introduction

Our biological understanding of fear genesis has mostly focused on exposure to exteroceptive aversive stimuli such as predator exposure or pain (Gross and Canteras, 2012). However, homeostatic threats and aversive bodily sensations that signal an imminent danger to survival also evoke fear. This interoceptive signaling of fear is relevant to psychiatric disorders such as panic disorder (PD) (Van Diest, 2019; Yoris et al., 2015) and posttraumatic stress disorder (PTSD) (Harricharan et al., 2021; Joshi et al., 2023), highly prevalent conditions that are often comorbid. Currently, fear evoked by interoceptive cues is not well understood. Furthermore, recent studies have shown discrete interoceptive processing and emotional regulation between males and females (Longarzo et al., 2020; Robinson et al., 2021). There is a significant gap in understanding how sex impacts interoceptive threat responding and associated mechanisms, as previous studies have largely focused on

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male subjects.

Carbon dioxide (CO_2) inhalation, an interoceptive threat to survival, is a pathologic marker in panic disorder and evokes intense fear and panic attacks in individuals with PD and PTSD (Gorman et al., 1994; Kellner et al., 2018; Leibold et al., 2016; Muhtz et al., 2012; Vollmer et al., 2015). CO_2 inhalation has been used as a biological challenge in the laboratory as it reliably induces unpleasant interoceptive reactions and self-reported symptoms that closely match panic attacks (Colasanti et al., 2008; Forsyth et al., 2000). Emotional responsivity to CO_2 inhalation is associated with the later development of PTSD symptoms in veterans (Telch et al., 2012), suggesting a potential intersection of interoceptive signaling with subsequent stress and fear memory regulation. Given its translational relevance, the CO_2 paradigm has been used in our lab (McMurray et al., 2019, 2020; Vollmer et al., 2016; Winter et al., 2017) and others (Leibold et al., 2016; Ziemann et al., 2009) as a valuable tool for mechanistic insights on interoceptive signaling of fear and panic. A recent study from our lab reported heterogeneity in the magnitude of CO_2-evoked fear in mice (McMurray et al., 2020) relevant to the variations in CO_2-sensitivity reported in humans (Battaglia, 2017; Coryell et al., 2001). Importantly, "CO_2-sensitive mice" that elicit significantly higher freezing during CO_2 inhalation demonstrated impaired extinction of contextual conditioned fear a week later, as well as disruptions in regional forebrain fear circuit activation. This suggests an association of behavioral sensitivity to interoceptive threats such as CO_2 with altered fear responding to subsequent exteroceptive threats. Although our previous data provides valuable information on fear regulation by interoceptive threats, it was confined to male subjects, a limitation that requires further investigation.

With this background and the paucity of knowledge regarding interoceptive threat responding and associated fear behaviors in females, the current study was undertaken with the following objectives: a) to assess fear-relevant passive and active defensive behaviors to CO_2 inhalation in female mice in comparison to male animals, b) to determine whether females exhibit heterogeneity in CO_2 responding that predicts the development of exaggerated contextual fear and impaired extinction as observed by us previously in male mice. To further determine potential mechanistic contributions in divergent, sex-based behaviors, we used persistent neuronal activation marker delta (Δ) FosB mapping and interregional correlations between multiple brain areas regulating defensive responding and fear. Lastly, we also assessed dopamine β hydroxylase (DβH)-positive noradrenergic neurons in the locus coeruleus based on their role in central CO_2-chemosensitivity (Biancardi et al., 2008; Gargaglioni et al., 2010), and the regulation of fear and arousal (Giustino and Maren, 2018; Ross and Van Bockstaele, 2020). Our data reveal differential sex-dependent behavioral responding to CO_2 with females engaging more active defensive behaviors as well as discrete fear circuitry and brain stem noradrenergic system recruitment as compared to males.

2. Methods

2.1. Animals

C57Bl/6J mice (female n = 23, male n = 24) were obtained from Jackson Laboratories (Bar Harbor, Maine, USA) at 8 weeks of age. Although BALBc mice have been used by us in previous CO_2 studies (Vollmer et al., 2016; McMurray et al., 2019, 2020, 2022, Winter et al., 2019), C57Bl/6J mice were chosen for the current study as they are commonly used for rodent behavioral assessments and most transgenic lines are available in this strain. Mice were pair-housed in a climate-controlled vivarium (temperature averages 23 ± 4 °C, humidity averages 30 ± 6 %) and acclimated for two weeks before the start of behavioral testing. All behavioral tests were performed during the 14h light cycle between 9am and 2pm. Study protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Cincinnati VA Medical Center and University of Cincinnati, in a vivarium accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

2.2. Behavioral testing

To investigate the effects of interoceptive threat, CO_2 on fear-associated defensive behaviors, we used a CO_2-startle-fear conditioning-extinction paradigm recently developed by our lab (McMurray et al., 2020). Passive and active defensive behaviors (freezing and rearing) to CO_2 inhalation, as well as the delayed effects of prior CO_2 inhalation on defensive responding to discrete exteroceptive cues (acoustic startle, foot shock contextual fear conditioning-extinction) are investigated. Briefly male and female mice underwent a single exposure to 10 % CO_2 or air inhalation. Acoustic startle and foot shock contextual fear conditioning-extinction-reinstatement, testing was conducted one week later under ambient room air conditions (see Fig. 1 for experimental layout). Male and female cohorts were tested separately.

2.2.1. CO_2 context conditioning paradigm

The CO_2 inhalation context conditioning paradigm (see Fig. 2a) has been used by our group previously (McMurray et al., 2022; Vollmer et al., 2016; Winter et al., 2019). The setup was a dual vertical Plexiglas chamber (25.5 cm × 29 cm x 28 cm per chamber). Breathing air or CO_2 (10 %; custom industrial mix in breathing air, Linde Gas & Equipment Inc., Cincinnati, OH) was infused in the upper chamber while the mice were placed in the lower compartment to avoid direct blowing of the gas which is highly aversive to rodents. A flow meter with a steady infusion rate of 10 L/min was used for all animals. Ambient concentration of CO_2 within the lower chamber was verified (10.0 ± 0.5 %) by the CARBOCAP® GM70 carbon dioxide meter (GMP221 probe with accuracy specification ± 0.5 %) (Vaisala, Helsinki, Finland). This concentration of CO_2 falls within a range that reliably induces fear in healthy volunteers and freezing in C57Bl/6 mice (Colasanti et al., 2008; Ziemann et al., 2009). As shown in Fig. 2 schematic, mice were habituated to the CO_2 chamber for 7min on Day 0, the day prior to air or CO_2 exposure. On Day 1, mice were placed back in the chamber and exposed to air or 10 % CO_2 for 10min. The following day, Day 2, animals were re-exposed to CO_2 context for 5min in the absence of CO_2 for assessment of context-conditioned behaviors. Mice were video recorded during habituation, CO_2 inhalation and context exposure. Freezing, the complete lack of movement except for respiration, was analyzed using FreezeScan software (CleverSys Inc., Reston, VA). Rearing, standing on hind legs, was also counted for each session by a rater blinded to the experimental condition.

2.2.2. Acoustic startle

One week following the CO_2 exposure, startle response to an unexpected acoustic stimulus was measured using the SR-LAB startle response system (San Diego Instruments, San Diego, CA) as previously described with modifications (Schmeltzer et al., 2015). The enclosure was of sufficient size to restrict but not restrain the animal and allowed it to turn around. The chambers were calibrated using the SR-LAB standardization unit (San Diego Instruments, San Diego, CA), prior to testing. Background noise in the chamber was maintained at 68 dB. After a 5-min acclimatization period, mice were exposed to 30 acoustic trials randomly generated at 0, 95, 110, and 120 db stimuli over background (40 ms duration; 30–38s inter-trial interval). Movement inside the tube was detected by a piezoelectric accelerometer below the frame. For each trial, measurements were taken at 1 ms intervals for a response window of 150 ms following the startle stimulus using National Instruments Data Acquisition Software (San Diego Instruments, San Diego, CA). The maximum response amplitude (Vmax; mV) within the recording window was used for data.

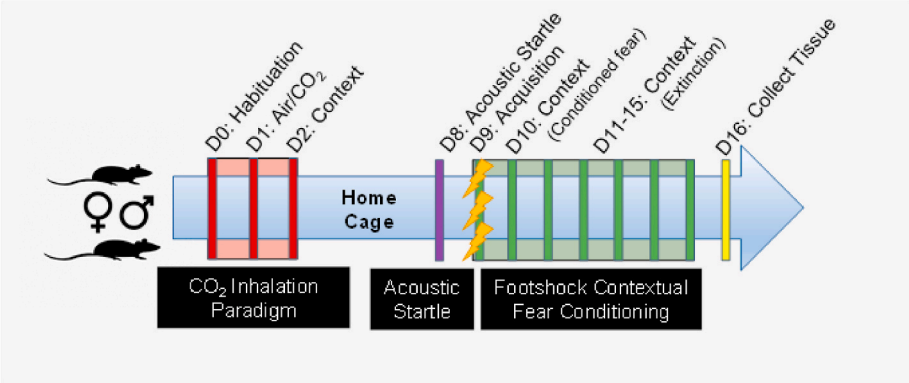

Fig. 1. Timeline of study: Male and female mice were habituated to the CO_2 chamber (Day 0, D0). On Day 1 (D1) they were exposed to CO_2 or Air inhalation for 10 min and followed by re-exposure to context on Day 2 (D2). After a 1-week rest period in the home cage, mice were tested for reactivity to exteroceptive stimuli in the form of acoustic startle (Day 8) followed by a contextual fear conditioning paradigm (Day 9–15). Mice were administered 3-foot shocks for fear acquisition (Day 9), then exposed to context after 24h for testing conditioned fear expression (Day 10), followed by repeated context exposure for extinction (Days 11–15). Mice were sacrificed 24h post behavior (Day 16) and brains collected for delta FosB (ΔFosB) immunostaining as a readout of persistent neuronal activation.

2.2.3. *Contextual fear conditioning and extinction*

We selected a contextual fear conditioning paradigm for our study (see Fig. 4a schematic) as reported previously by our group (McMurray et al., 2020, 2022; Schubert et al., 2018) with modifications. Fear acquisition, conditioned fear, and extinction were investigated. Briefly, operant chambers housed in sound attenuated isolation cabinets were used (San Diego Instr.). The floors of the chambers consisted of stainless-steel grid bars that delivered scrambled electric shocks. The grid, floor trays and chamber walls were wiped with 10 % ethanol and allowed to dry completely. For acquisition, each animal was acclimated to the chamber for 5min, then received 3 shocks of 0.5 mA intensity, 1s duration administered 1min apart. The animals were placed in the chamber the next 6 days and recorded for 5min without shocks to measure conditioned fear and extinction. Freezing, defined as complete lack of movement except respiration was measured using the Freeze Scan software (Clever Sys Inc.). Rearing counts were also obtained during acquisition, conditioned fear and extinction to assess treatment and sex effects on active defensive responding.

2.3. *Immunohistochemistry*

Mice were perfused transcardially with 4 % paraformaldehyde 24 h after the last day of extinction (post-behavior). Brains were post-fixed in 4 % paraformaldehyde overnight and then placed in 30 % sucrose. Brains were sectioned at 30 μm on a sliding microtome and the resulting sections were stored in cryoprotectant (0.1 M phosphate buffer, 30 % sucrose, 1 % polyvinylpyrrolidone, and 30 % ethylene glycol) at −20 °C until processed for immunohistochemistry. Tissue was stained for ΔFosB (1:20,000, Abcam, ab184938) to assess persistent neuronal activation and dopamine β hydroxylase (DβH) (1:2,000, Abcam, ab209487) to assess activation of noradrenergic cells in the locus coeruleus. Slices were transferred to 50 mM PBS (pH 7.4; 40 mM potassium phosphate diabasic, 10 mM potassium phosphate monobasic, and 0.9 % sodium chloride) and rinsed five times for 5 min at RT. Sections were transferred to 0.3 % H_2O_2 in PBS and incubated for 10 min at RT. Slices were rinsed five times for 5 min in PBS and transferred to blocking solution [50 mM PBS, 0.5 % bovine serum albumin (BSA), and 0.4 % Triton X-100] for 1h at RT. Slices were incubated overnight at 4 °C in primary antibody diluted in blocking solution. The following day, sections stained for DβH were rinsed (five times for 5 min) in PBS and incubated in secondary antibody (Cy-3 anti-rabbit; Jackson Immunoresearch) diluted (1:500) in 50 mM PBS plus 0.5 % BSA for 1h at RT on a shaker in the dark. Tissue was rinsed five times for 5 min in PB, mounted onto Fisherbrand Superfrost plus microscope slides (Fisher Scientific, cat. 12-550-15) and cover-slipped with Gelvatol (Sigma, cat. 10981, Milwaukee, WI). Sections stained for FosB were incubated in biotinylated anti-rabbit secondary antibody (Vector Laboratories, Inc., Burlingame, CA; 1:400 in blocking buffer for 1h). Sections were washed again five times for 5min in PBS and incubated in avidin-biotin complex using ABC Vectastain kit, diluted 1:800 for 1h. Following washes, sections were incubated in diaminobenzadine (DAB, Pierce, Rockford, IL) for 10 min. Sections were washed again in PB and mounted onto microscope slides followed by dehydration in xylene solutions. Finally, slides were cover-slipped using DPX (Sigma, cat. 44581).

2.4. *Imaging, quantification and analysis*

Immunolabeled sections were imaged using the AxioImager ZI microscope (Zeiss) equipped with apotome (z-stack) imaging capability (Axiocam MRm camera and AxioVision Release 4.6 software; Zeiss). Processing for DβH and FosB imaging and quantification was performed following previously published procedures from our lab. Images were analyzed using Image J software (NIH open access). Briefly, for DβH labeled tissue, Z-stacks were acquired using the 20× air objective lens at 568 nm. DβH positive noradrenergic cell counts within the locus coeruleus, LC (bregma −5.34 to −5.68) were counted throughout the Z-stack images using the ImageJ software "multi-point" tool and "cell counter". For FosB analyses, images were acquired in fear regulatory regions demonstrating immunopositive cells. The regions were delineated using characteristics of each nucleus taken from the atlas of Paxinos and Watson (Paxinos, G and Watson, 1998) as follows: infralimbic (IL)/prelimbic (PL) cortex (bregma +1.94 to +1.54), amygdala nuclei basolateral (BLA)/central (CeA)/intercalated cells (ITC) (bregma −1.46 to −2.06), dentate gyrus (DG) (bregma −1.70 to −2.30), ventrolateral/dorsolateral periaqueductal grey (vlPAG/dlPAG) (bregma −4.16 to −4.72), dorsal/ventral bed nucleus of stria terminalis (dBNST/vBNST) (bregma +0.50 to +0.02), dorsomedial hypothalamus/perifornical area (DMH/PeF) (bregma −1.46 to −2.06) and LC (bregma −5.34 to −5.68). To quantify the number of immunoreactive nuclei the Image-J "cell counter" tool was used by an investigator blind to experimental group. Regions were quantified at a similar distance from bregma within all animals. At least, four images per region of interest per mouse were collected. Cell counts for each section were averaged for each animal and individual means averaged to derive group means.

2.5. *Data analysis and statistics*

Data are represented as mean ± SEM and were analyzed by two-factorial ANOVA, or student t-test. Normality was tested formally for all behavior data and met assumptions of the statistical tests being used.

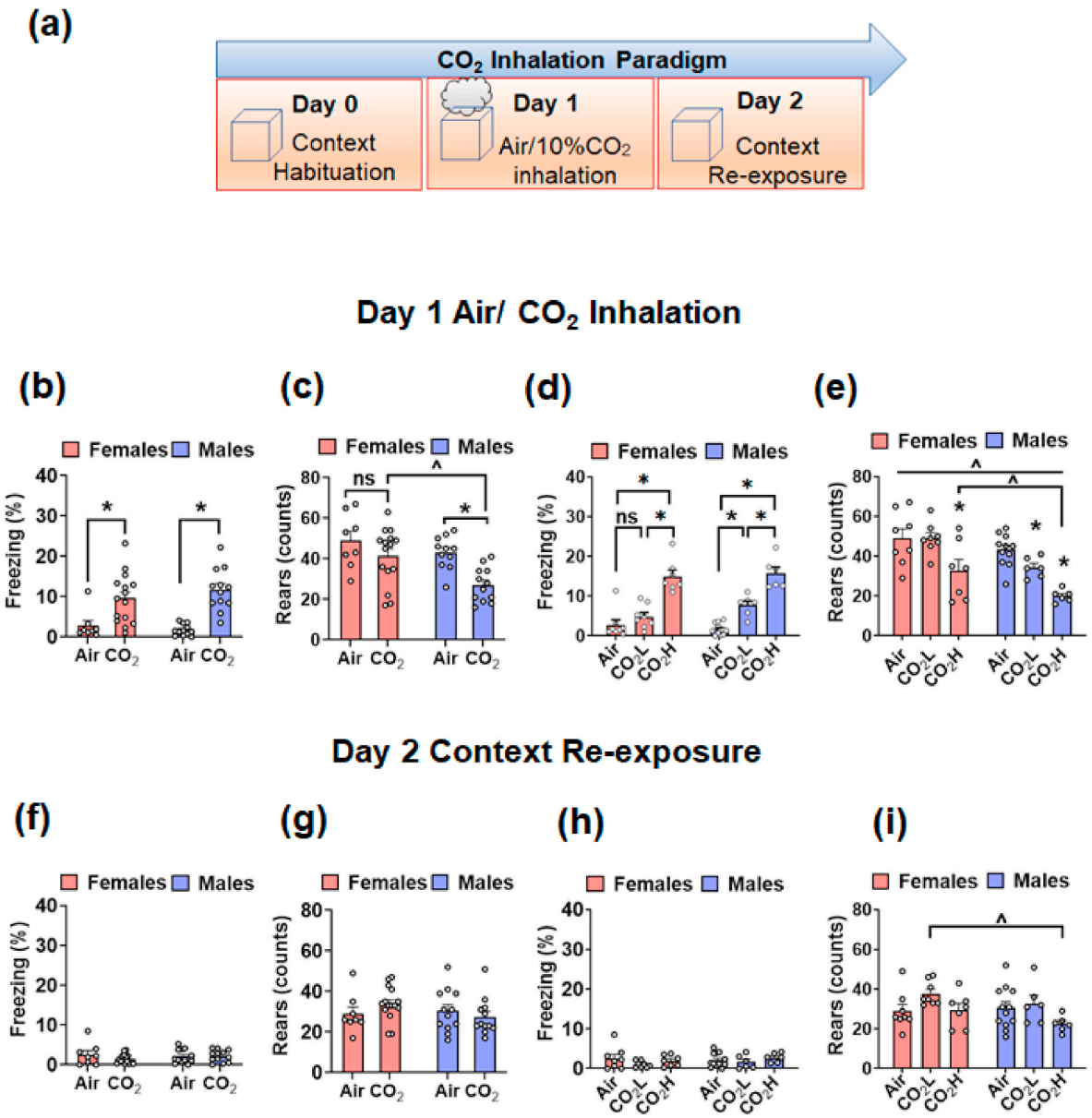

Fig. 2. CO_2 inhalation evokes fear-relevant defensive behaviors: similar passive (freezing) but distinct active (rearing) behaviors in females compared to males. Female and male mice were exposed to the CO_2 inhalation paradigm (see layout). CO_2 inhalation evoked freezing (panels b, d) and rearing (panels c, e). Significantly higher freezing in both sexes was observed during CO_2 exposure in comparison with the air cohort on Day 1 (b). Interestingly, CO_2 inhalation significantly reduced rearing only in males but not females and CO_2 exposed female mice reared significantly higher than CO_2 exposed males (c) Dichotomization of data using a median split showed that during air or CO_2 inhalation, a subset of male and female mice, CO_2 high responders (CO_2–H) showed significantly higher freezing in comparison to both air and CO_2-L groups (d) and no significant difference between air and CO_2-L cohorts was observed. Panel (e) shows significantly higher rearing in females versus males and notably CO_2–H females reared more than CO_2–H males. Exposure to context on Day 2 did not reveal significant group differences in freezing (panels f, h) or rearing without split (g), however, dichotomization revealed lower rearing in CO_2–H males while CO2–H females were not different from other groups Data are represented as mean ± SEM; *$p < 0.05$ within sex group differences as indicated or versus air, ^ $p < 0.05$ between sex group differences as indicated (N = 6–12 mice per group).

Grubbs' tests were performed to determine and remove any outliers. Welch's correction was used when variances were unequal. Behavior in the CO_2 inhalation paradigm expressed as percent freezing or rearing counts was analyzed using two-way ANOVA with inhalation and sex as variables. For further analysis of the CO2 behavioral response, CO_2-exposed mice were divided into high and low freezers based on a median split of the percent freezing duration during CO_2 inhalation, as described previously by us (McMurray et al 2020). Males and females were analyzed separately. For fear conditioning and startle data we assessed within sex effects using repeated measures (RM) two-way ANOVA for treatment and time effects. Selected outcomes were further analyzed for between sex differences by two-way ANOVA using inhalation and sex as variables. Where main effects were significant, post-hoc analysis was performed using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli's with false discovery rate (FDR) correction analysis. ΔFosB data were analyzed for each region by two-way ANOVA using inhalation and sex as variables. For functional connectivity mapping, pairwise Pearson correlation coefficients were determined for interregional ΔFosB signals in all groups (e.g., air, CO_2-low (CO_2-L), CO_2-high (CO_2–H), as described by us previously (McMurray et al., 2020). To evaluate interregional correlations across conditions, correlation matrices were generated and plotted as heatmaps for each

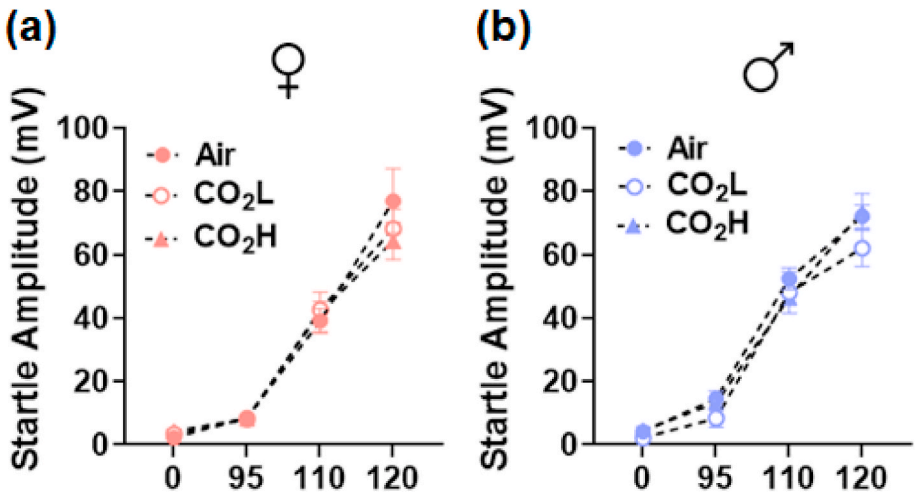

Fig. 3. Mean startle amplitude in air, CO_2-L and CO_2–H mice to a range of acoustic stimuli ranging across 95, 100, 110 and 120 db is presented in female (a) and male mice (b). Significant effects of decibel but no significant effects of inhalation were noted.

treatment group. For all data, statistical significance was set as $p < 0.05$. Prism 8.0 software was used for statistical analysis (GraphPad Software, Inc., La Jolla, CA).

3. Results

3.1. CO_2 inhalation evokes fear-relevant defensive behaviors in females: similar passive (freezing) but distinct active (rearing) behaviors compared to males

As there are limited studies in female mice on behavioral responding to CO_2 inhalation, we investigated passive (freezing) and active (rearing) behaviors at pre (day 0), during CO_2 exposure (day 1) and on context re-exposure in the absence of CO_2 (day 2) (Fig. 2a). Previous studies by our group in male mice revealed significantly higher freezing and reduced rearing behavior in CO_2-exposed animals (McMurray et al., 2020, 2022). Furthermore, there is heterogeneity in behavioral responsivity to CO_2, with high (CO_2-high, CO_2–H) and low (CO_2-low, CO_2L) responders. As shown in Fig. 2b, CO_2 exposed female mice showed significantly higher freezing than air and the magnitude was comparable to male mice. A 2-way ANOVA revealed a significant effect of inhalation [$F_{(1, 43)} = 35.35$; $p < 0.0001$] but no effect of sex ($p > 0.05$) or a sex x inhalation effect ($p > 0.05$). Post hoc analysis revealed significant differences between air exposed and CO_2 exposed cohorts ($p < 0.05$), but no sex differences for air and CO_2 inhalations. Interestingly, in contrast to similarities in passive freezing behavior, CO_2-evoked rearing showed a distinction between sexes (Fig. 2c). While we replicated the previously observed reduction in exploratory rears in CO_2-exposed male mice, this drop in rearing frequency by CO_2 was not observed in female animals. A 2-way ANOVA showed a significant effect of inhalation [$F_{(1, 42)} = 11.63$; $p = 0.0014$] and sex [$F_{(1, 42)} = 8.51$; $p = 0.0056$], but no sex $\times$ inhalation interaction ($p > 0.05$). Post hoc analysis revealed significantly reduced rearing in CO_2-exposed male than air group, an effect not observed in female cohorts ($p > 0.05$). Importantly, rearing in CO_2-exposed female mice was significantly higher than CO_2 exposed males ($p < 0.05$) suggesting active defensive coping or maintenance of exploratory behaviors during exposure to interoceptive threat. Based on the observed heterogenous CO_2 response and our previous report on differential CO_2-sensitivity in mice (McMurray et al., 2020), dichotomization into CO_2–H and CO_2-L subgroups using a median split was performed and group differences were analyzed by ANOVA (Fig. 2d). A two-way ANOVA showed significant effect of inhalation on freezing behavior [$F_{(2, 41)} = 72.29$; $p < 0.0001$] but no effects of sex ($p > 0.05$) or a sex $\times$ inhalation interaction ($p > 0.05$). Interestingly, significant sex and inhalation effects were observed for rearing behavior (Fig. 2e) [sex; $F_{(1,40)} = 14.18$; $p = 0.0005$], [inhalation; $F_{(2,40)} = 16.15$; $p < 0.0001$] but no sex $\times$ inhalation interaction ($p > 0.05$). Importantly, post hoc analysis revealed significantly higher rearing in female CO_2–H mice versus CO_2–H males, suggesting that despite similar passive freezing behaviors, females elicit more exploratory/active defensive behaviors during CO_2 inhalation. Baseline behavioral assessments during the acclimation to CO_2 chamber (context) on day 0 were also assessed. Minimal freezing to context exposure was observed in both female and male animals (Supplementary Figure 1a). Investigation of rearing counts revealed significantly higher rearing in females versus males (Supplementary Figure 1b). A two-way ANOVA revealed an effect of sex [$F_{(1, 40)} = 20.39$; $p < 0.0001$]. Post hoc analysis revealed significantly higher rearing in Air and CO_2-L female mice compared to all male groups ($p < 0.05$). CO_2–H females showed higher rearing than CO_2–H males during habituation although statistical significance was not achieved ($p = 0.06$). Thus, even prior to CO_2 exposure, females exhibit more exploration in novel context compared to males, as well as more exploratory behavior while inhaling CO_2.

In contrast to our previous observations of conditioned freezing to context on day 2 in CO_2-exposed BALBc mice, we observed no significant differences based on inhalation or sex for freezing behavior between C57Bl/6J mice during context re-exposure (Fig. 2f–i), suggesting strain differences in contextual conditioned freezing to the CO_2 inhalation. A significant inhalation effect was observed for rearing behavior [$F_{(2, 40)} = 3.695$; $p = 0.033$]. Post hoc analysis revealed significantly reduced rearing in male CO_2–H mice versus CO_2-L female ($p < 0.05$) and CO_2-L male ($p = 0.051$) groups, while CO_2–H females were not significantly different compared to other groups.

3.2. Unconditioned Acoustic startle responding is not impacted by prior CO_2 inhalation

Female and male mice with prior exposure to air and CO_2 inhalation were exposed to a variable range of acoustic stimuli one week following inhalation (Fig. 3a and b). Within-sex analyses revealed a significant overall effect of decibel on startle amplitude in both females ($F_{(3, 60)} = 169.6$, $p < 0.0001$) and males ($F_{(3, 63)} = 285.5$, $p < 0.0001$). No significant overall effects of inhalation ($p > 0.05$) or an inhalation $\times$ decibel interaction ($p > 0.05$) was observed in females or males. We also conducted a between-sex group analyses at each decibel (Supplementary Figure 2). Two-way ANOVA revealed a significant effect of inhalation [$F_{(2, 41)} = 3.8$, $p = 0.031$] and an inhalation $\times$ sex interaction [$F_{(2, 41)} = 3.76$, $p = 0.032$] at baseline (0 dB) (Supplementary Figure 2a), but no

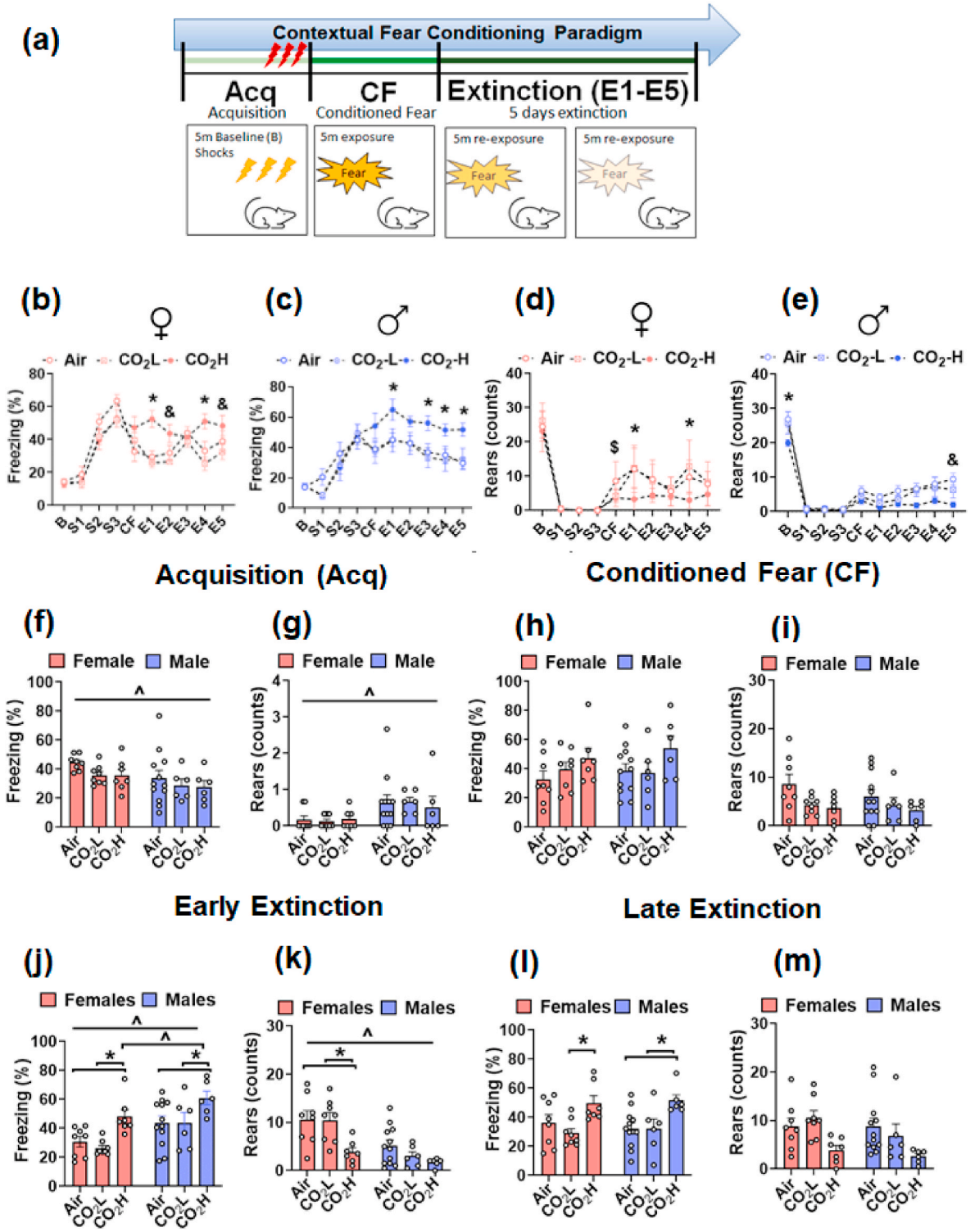

Fig. 4. Behavioral sensitivity to CO_2 selectively impacts performance during fear extinction: divergence in passive and active behaviors between female and male mice. One week following Air/CO_2 inhalation mice were exposed to a contextual fear conditioning paradigm (see layout, a). Following exposure to 3 foot shocks (acquisition) and conditioned fear 24h later, mice were re-exposed to the acquisition context for extinction of fear for 5 days (E1-E5). Female and male CO_2–H mice elicited significantly higher freezing (b, c) and reduced rearing (d,e) specifically during the extinction phase. No significant group differences in freezing were observed during the context acclimation baseline period (B), foot shocks (shock 1 through 3, S1–S3) and conditioned fear (CF). Between sex analyses for each phase of fear conditioning revealed significant differences in freezing and rearing behaviors. Females elicited significantly higher freezing and reduced reared during acquisition compared to males (f,g). During early extinction (mean of E1-E2 (panels j. k), female mice showed significantly less freezing (j) and higher rearing (k) compared to males. No significant sex differences in freezing or rearing were observed during conditioned fear (h, i) and late extinction. Data are mean ± SEM. For within sex data (graphs b,c,d,e) *$p < 0.05$ CO_2–H versus air and CO_2-L; & $p < 0.05$ CO_2–H versus either air or CO_2-L group, \$ $p < 0.05$ CO_2–H and CO_2-L mice versus air. For between sex data (graphs f,g,h,i,j,k,l,m): ^ $p < 0.05$ indicates significant between sex group differences * $p < 0.05$ indicates significant within sex inhalation group differences (N = 6–12 mice per group).

effect of sex ($p > 0.05$). Post-hoc analyses revealed higher startle in CO_2–H versus air in females ($p = 0.06$) and lower startle in CO_2-L males versus air ($p = 0.06$), possibly an outcome of stress or novelty. Furthermore, female cohorts showed significantly lower startle compared to males at 95 dB [$F_{(1, 41)} = 5.28$, $p = 0.027$] and 110 dB [$F_{(1, 41)} = 4.71$, $p = 0.036$] (Supplementary Figure 2b and c) but no group differences were identified by post hoc analysis ($p > 0.05$). Lastly, no sex differences were observed at 120 dB (Supplementary Figure 2d).

3.3. High behavioral sensitivity to CO_2 inhalation impacts defensive behaviors to subsequent foot shock fear conditioning specifically during fear extinction: differential engagement of higher active (rearing) behaviors in females

We hypothesized that behavioral sensitivity to CO_2 will associate with subsequent responding to exteroceptive threats based on our previous data in male mice (McMurray et al., 2020) showing compromised fear extinction in mice that previously showed high freezing to CO_2 (CO_2–H) and investigated whether this phenomenon extended to females. Cohorts exposed to air and CO_2 underwent a contextual fear conditioning-extinction paradigm a week after CO_2 exposure (see Fig. 4a). Assessment of passive (freezing) and active (rearing) behaviors was conducted during all phases of the paradigm: acquisition, conditioned fear and extinction. A within-sex group comparison revealed that both female and male CO_2–H mice showed significantly higher freezing compared to air and CO_2-L groups only during the extinction phase (Fig. 4b and c). A two-way RM ANOVA for females revealed a significant effect of inhalation [$F_{(2,20)} = 3.57$, $p = 0.047$], time [$F_{(9, 180)} = 32.39$, $p < 0.0001$], and an inhalation × time interaction [$F_{(18,180)} = 3.24$, $p < 0.0001$]. Post-hoc analysis revealed significantly higher freezing in female CO_2–H mice on extinction days E1, E2, E4 and E5 versus air and CO_2-L groups. No significant differences were noted during habituation baseline, acquisition shocks or conditioned fear. A two-way RM ANOVA in males revealed a significant effect of time [$F_{(9,189)} = 25.29$, $p < 0.0001$] and a significant inhalation × time interaction [$F_{(18,189)} = 2.23$, $p = 0.004$] but no significant effect of inhalation ($p > 0.05$). Post-hoc analysis revealed significantly higher freezing in male CO_2–H mice on extinction days E3 and E5 ($p < 0.05$) and non-significant trends on E1 ($p = 0.0502$) and E4 ($p = 0.06$) versus air and CO_2-L groups. Notably, no significant differences were evident during habituation/baseline, acquisition shocks or conditioned fear. Analysis of active rearing behavior also revealed significant between group differences in females (Fig. 4d) and males (Fig. 4e), however, at different phases of the paradigm. A two-way RM ANOVA for females revealed a significant effect of inhalation [$F_{(2,20)} = 6.13$, $p = 0.0084$], time [$F_{(9, 180)} = 89.87$, $p < 0.0001$], and an inhalation × time interaction [$F_{(18,180)} = 2.53$, $p = 0.001$]. Post hoc analysis revealed significantly reduced rearing in CO_2-exposed mice compared to air on conditioned fear (CF) day. Notably, CO_2-L mice increased their rearing behavior during the extinction phase similar to air mice while CO_2–H mice reared significantly less than both groups during extinction ($p < 0.05$). A two-way RM ANOVA for male rearing behavior revealed a significant effect of time [$F_{(9,189)} = 89.59$, $p < 0.0001$], and an effect of inhalation that did not reach statistical significance [$F_{(2, 21)} = 3.15$, $p = 0.06$], but no inhalation × time interaction ($p > 0.05$). Post hoc analysis revealed significantly reduced rearing in male CO_2–H mice during the pre-shock habituation baseline compared to air and CO2-L groups ($p < 0.05$) and versus air during extinction (E5). Between-sex group analyses were also performed for freezing and rearing behaviors during acquisition (Fig. 4f,g), CF (Fig. 4h,i), early (E1-2) extinction (Fig. 4j,k) and late (E3-5) extinction (Fig. 4l,m). Consistent with extinction-selective inhalation effects, differential sex-dependent defensive responding was only observed during extinction, particularly in the early phase. A two-way ANOVA for early extinction freezing revealed a significant effect of sex [$F_{(1,41)} = 13.81$, $p = 0.0006$] and inhalation [$F_{(2,41)} = 9.33$, $p = 0.0005$], but no sex × inhalation interaction ($p > 0.05$). Similarly, early extinction rearing revealed significant sex [$F_{(1,41)} = 20.33$, $p < 0.0001$] and inhalation [$F_{(2,41)} = 7.64$, $p = 0.0015$] effects but no sex × inhalation interaction ($p > 0.05$). Overall, females elicited significantly less freezing and higher rearing than males ($p < 0.05$) (Fig. 4j and k). CO_2–H females elicited reduced freezing ($p = 0.055$) compared to CO_2–H males. During late extinction only significant effects of inhalation were observed for both freezing [$F_{(2,41)} = 8.90$, $p = 0.006$] and rearing [$F_{(2, 41)} = 6.18$, $p = 0.0045$] but no sex or sex x inhalation effects were noted for both behaviors ($p > 0.05$). Significant sex differences were also noted during acquisition with females showing significantly higher freezing [two-way ANOVA, $F_{(1,41)} = 5.47$, $p = 0.024$]; and lower rearing [two-way ANOVA, $F_{(1,41)} = 8.15$, $p = 0.007$] as compared to males, suggesting that the intensity of threat may influence the choice of defensive response between passive and active behaviors. Lastly, no sex or treatment effects were observed during conditioned fear (CF) (Fig. 4h and i). Overall, our data revealed an association of CO_2 inhalation with extinction of learned fear to an aversive exteroceptive footshock stimulus and highlights that females may adapt more active defensive behaviors than males during extinction.

3.4. Differential CO_2 and sex-dependent recruitment of cortico-amygdala-LC regions and altered forebrain-hindbrain co-activation patterns

To get insights on brain areas that may contribute to sex- and CO_2-dependent behaviors we conducted an expansive delta FosB analysis that included regions relevant for the regulation of defensive behaviors and/or CO_2 chemosensing. Our initial analysis comprised of within-sex, inhalation group comparisons for each region (Supplementary Figures 3 and 4). Furthermore, we conducted between sex comparisons on selected regions (Fig. 5) that either showed significant treatment effects within sex or have been reported to be sexually dimorphic. Additionally, to further identify neural networks and map interregional co-activation patterns, correlation matrices of FosB cell counts were visualized across regions for each group (Fig. 6). Significant effects of sex and inhalation were observed in a region-specific manner in several forebrain-hindbrain areas. In agreement with our previous data (McMurray et al., 2020, 2022) our within-sex analysis revealed significant effects of inhalation within amygdala nuclei (basolateral amygdala BLA, central nucleus CeA and intercalated nuclei ITCs), infralimbic cortex (IL), ventrolateral periaqueductal grey (vlPAG) and the locus coeruleus (LC) (see supplementary Figures 3 and 4). Both males (Supplementary Figure 3a) and females (Supplementary Figure 4a) showed reduced cell counts within the IL of CO_2–H mice suggesting that the IL dysregulation may be key to the observed effects on extinction. However, effects on amygdala subnuclei were in opposite directions, as for the BLA, males showed increased while females showed decreased FosB cell counts (Supplementary Figures 3e, 4e). Additionally, females showed significantly higher CeA counts in CO_2 exposed mice. (Supplementary Figure 4f). Reduced counts in the ITC cell cluster were observed for male CO_2-L mice (Supplementary Figure 3g) and female CO_2–H mice (Supplementary Figure 4g). In addition to forebrain regions, mid brain-hindbrain areas showed altered cell counts in a sex-dependent manner. The cell counts within the ventrolateral periaqueductal grey (vlPAG) of CO_2-exposed female mice were reduced compared to air (Supplementary Figure 4h). In males, significantly lower cell counts were observed within the LC of CO_2–H mice (Supplementary Figure 3c). To further understand sex and treatment effects a two-way regional analysis was conducted. Significant effect of treatment but not sex was observed in the mPFC IL subdivision [two-way ANOVA, $F_{(2, 40)} = 7.973$, $p = 0.0012$]. Post hoc analysis revealed significantly reduced IL cell counts in CO_2–H female and male mice as compared to air and CO_2-L groups (Fig. 5a). Females had significantly lower cells counts within the PL compared to males (Fig. 5b) [two-way ANOVA, $F_{(1, 40)} = 10.97$, $p = 0.0020$] but no effect of treatment was observed ($p > 0.05$). A subregional analysis within the amygdala revealed for the BLA a significant effect of treatment [$F_{(2, 41)} = 4.602$, $p = 0.0157$] and a treatment × sex

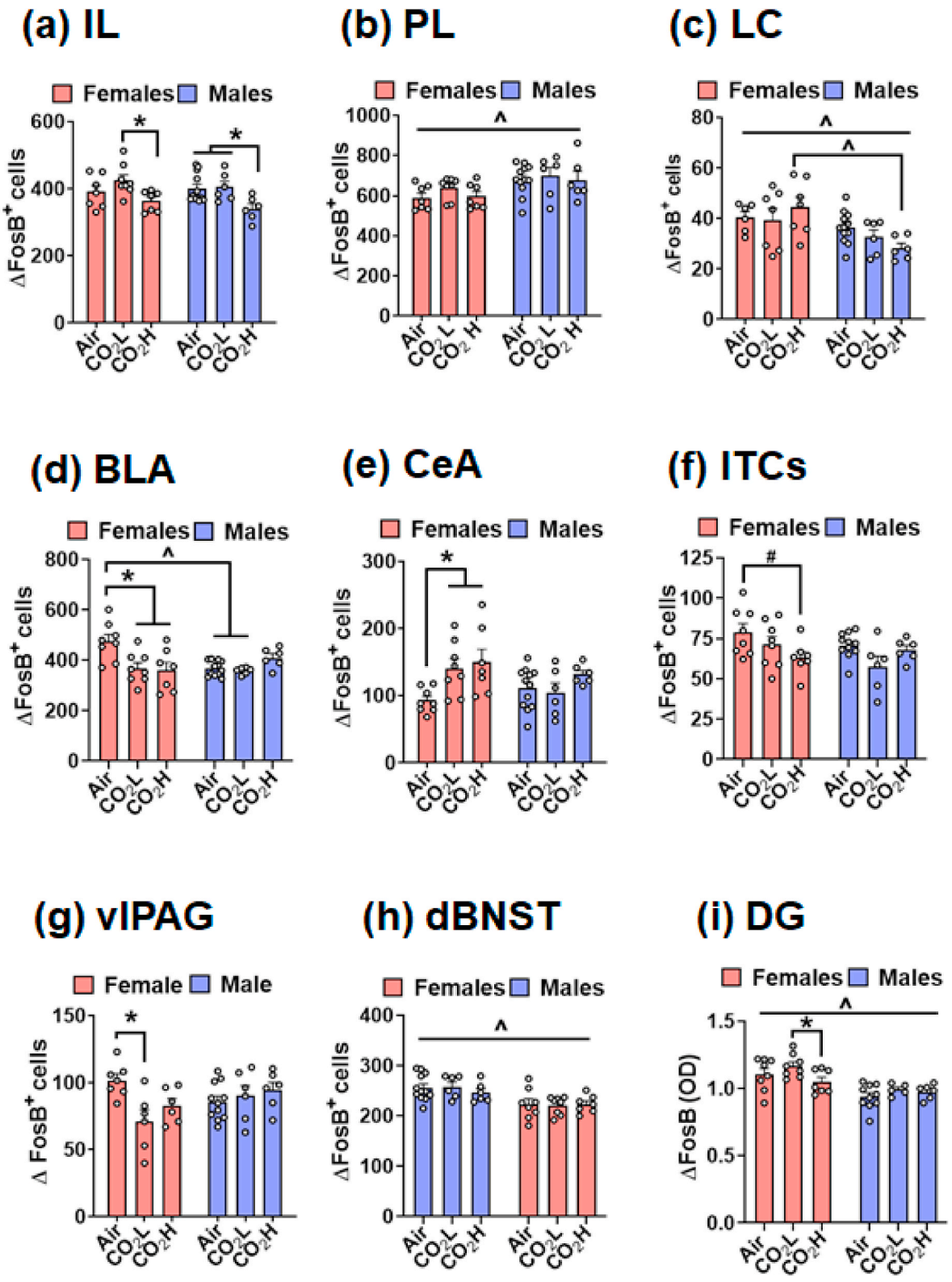

Fig. 5. Sex differences in post behavior regional ΔFosB + cell counts in CO_2- or air-exposed cohorts. (a) Within infralimbic cortex (IL), CO_2–H mice had significantly reduced FosB + cell counts. (b) Males had significantly higher FosB + cell counts in the prelimbic cortex (PL) compared to females. (c) Females elicited significantly higher FosB + cells in the locus coeruleus (LC) and female CO_2–H group was significantly higher than male CO_2–H. (d) Within the basolateral amygdala (BLA) female air-exposed mice had significantly higher cell counts than CO_2-L and CO_2–H groups as well as male air and CO_2-L groups. (e) CO_2 inhalation increased central amygdala (CeA) FosB + cell counts in females but not males (f) Reduced FosB + cell counts were observed within the intercalated cells of the amygdala (ITC) in females. (g) Reduced cell counts in CO_2 exposed female mice in the ventrolateral periaqueductal grey (vlPAG). (h) Female cohorts had significantly lower FosB + cell counts compared to males within the dorsal bed nucleus of stria terminalis (BNST). (i) Females elicited significantly higher FosB + cell counts in the dentate gyrus of the hippocampus (DG). Data are mean ± SEM. Within sex differences are indicated as $*p < 0.05$ and between sex differences as ^ $p < 0.05$ (n = 6–8 mice per group).

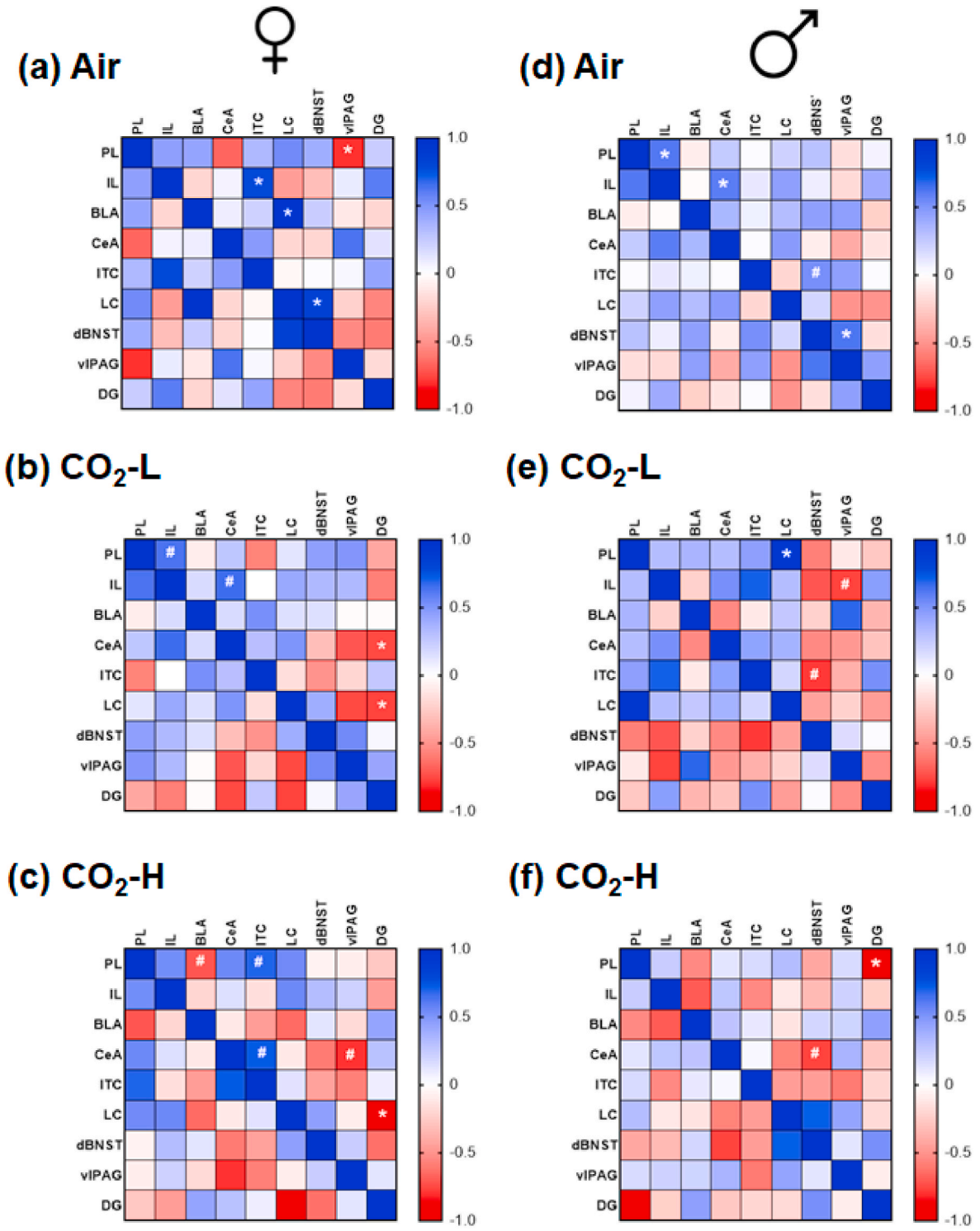

Fig. 6. ΔFosB interregional correlation matrices reveal differential patterns of co-activation in males and females between (a) air, (b) CO_2-L and (c) CO_2–H. Dark blue represents strong positive correlations while dark red represents strong negative correlations. Positive co-activation patterns of IL with amygdala subnuclei, ITC and CeA observed in air (a,d) and CO_2-L mice (b) were disrupted in CO_2–H mice (c,f) irrespective of sex. Differential co-activation pattern of the IL in CO_2-L mice between females (IL-CeA, panel b) and males (IL-vlPAG, panel e). In females only, positive LC correlation with dBNST is disrupted into negative LC-DG co-activation patterns in CO_2-L (b) and CO_2–H mice (c). Other areas that showed differential engagement between inhalation groups are the PL, and the vlPAG. Abbreviations: IL, infralimbic cortex; PL, prelimbic cortex; locus coeruleus (LC); BLA, basolateral amygdala; CeA, central nucleus of the amygdala, intercalated cells amygdala (ITC); dBNST, dorsal bed nucleus of stria terminalis, DG, dorsal dentate gyrus. *$p < 0.05$; #$p = 0.06$–0.08. ($N = 6$–8/group). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

interaction [$F_{(2, 41)} = 8.087$, $p = 0.0011$] but no effect of sex ($p > 0.05$). Post-hoc analysis revealed significantly lower BLA FosB counts in female CO_2–H and CO_2-L groups compared to air ($p < 0.05$). Male air and CO_2-L groups also had significantly lower cell counts compared to female air group ($p < 0.05$). A significant effect of treatment was also observed for the central nucleus (CeA) [$F_{(2, 41)} = 5.288$, $p = 0.009$] and the intercalated nuclei (ITC) [$F_{(2, 41)} = 5.288$, $p = 0.009$] cell cluster but no sex or a sex × treatment interaction was observed ($p > 0.05$). Post-hoc analysis revealed significantly higher FosB cell counts in the CeA of female CO_2–H and CO_2-L groups compared to air ($p < 0.05$) and reduced cell counts in the ITCs in CO_2–H mice versus air ($p = 0.08$). Interestingly, and consistent with the active threat responding observed in females during CO_2 inhalation and fear extinction, significantly higher FosB counts were observed in the LC of females versus males (Fig. 5c) [two-way ANOVA, $F(1, 38) = 12.60$, $p = 0.001$]. Furthermore, post hoc analysis revealed significantly higher LC FosB + cell counts in female CO_2–H mice compared with the male CO_2–H group. An interesting sex × inhalation interaction [$F_{(2, 36)} = 5.255$, $p = 0.009$] and a non-significant trend for inhalation [$F_{(2, 36)} = 2.52$, $p = 0.09$] was observed for the vlPAG, with post hoc analysis revealing significantly lower cell counts in

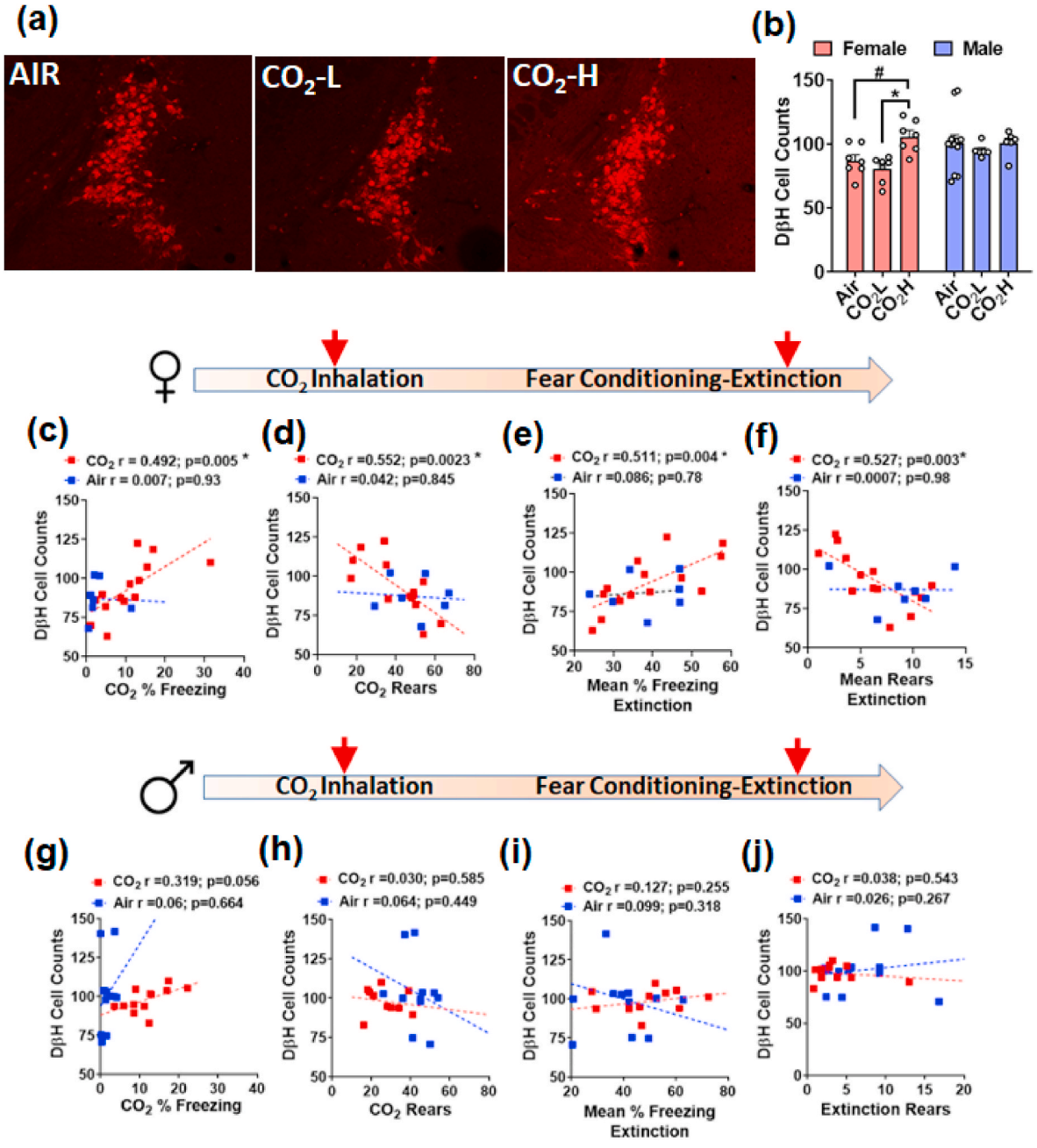

Fig. 7. Engagement of locus coeruleus (LC) noradrenergic (dopamine beta hydroxylase, DβH $^{+ve}$) cells in behaviors during CO_2 inhalation and fear extinction in females but not males. (a) Panels show representative images of (DβH) immunopositive cells from female mice exposed to air or CO_2 low responder (CO_2-L) and CO_2-high responder (CO_2–H) groups. (b) Quantification of LC DβH $^{+ve}$ cells revealed higher cell counts in female CO2–H mice compared to CO2-L and Air groups. Male cohorts indicated no group differences. Linear correlation plots between LC DβH $^{+ve}$ cell counts and freezing or rearing during CO_2 inhalation in females (c,d) and males (g,h). LC DβH $^{+ve}$ cell counts show significant positive correlation with freezing (c) and negative correlation with rearing (d) during CO_2 inhalation but not air. These correlations were not significant in males. Linear correlation plots between LC DβH $^{+ve}$ cell counts and mean freezing or rearing during fear extinction in females (e,f) and males (i,j) revealed significant correlation that was positive for freezing (e) and negative for rearing (f) only in females but not males (i,j). Data are expressed as mean ± SEM for 6–12 mice. *$p < 0.05$.

CO_2-L female mice versus air ($p < 0.05$). Significant sex-differences were also observed in other areas such as the prelimbic cortex (PL) (Fig. 5b), dorsal bed nucleus of stria terminalis (dBNST) (Fig. 5h), with females eliciting significantly lower FosB + cell counts compared to males [PL, two-way ANOVA, $F_{(2, 40)} = 10.07$, $p = 0.002$, dBNST, two-way ANOVA, $F_{(1, 41)} = 20.30$, $p < 0.0001$] and the dorsal hippocampal dentate (DG) where significantly higher cell counts were observed in females versus males [two-way ANOVA, $F\ (1, 38) = 25.5$, $p < 0.0001$].

To further identify neural networks and inter-regional co-activation patterns that may be engaged within this paradigm in female and male cohorts, correlation matrices of ΔFosB cell counts were visualized across regions for each group and examined as a proxy of functional connectivity between brain regions (Fig. 6a–f). These maps revealed differential patterns of co-activation between inhalation groups in females and males. Notably, compared to air controls, significant disruption of IL correlations with amygdala subnuclei (ITC, CeA) was observed in CO_2–H mice, independent of sex (Fig. 6c,f). Interestingly, the IL was differentially engaged in CO_2-L group as females showed positive IL-CeA co-activation patterns (Fig. 6b) while males elicited inverse IL-vlPAG associations (Fig. 6e). Importantly, LC-coactivation patterns were significantly altered between air and CO_2 groups only for females but not males, suggesting engagement of the LC in CO_2-associated behaviors in females. Specifically, significant inverse LC-DG correlations were observed only in CO_2-exposed females (Fig. 6b and c). Interestingly, distinct patterns in co-activation are also apparent in CO_2-L cohorts such as a strong positive PL-IL association in females and PL-LC association in males that were not observed in air or CO_2–H groups.

3.5. Association of locus coeruleus noradrenergic neurons with behaviors during CO_2-inhalation and fear extinction in females

Given our data showing significant sex differences in LC FosB cell counts and co-activation patterns, the established role of the LC as a CO_2 chemosensory locus and its relevance as a sexually dimorphic area regulating stress and arousal, we assessed dopamine β hydroxylase positive ($D\beta H^{+ve}$) noradrenergic neurons within the LC (Fig. 7a). Alterations in $D\beta H^{+ve}$ cell counts were observed in female but not male mice. A two-way ANOVA analysis revealed a significant effect of treatment [$F_{(2, 39)} = 3.676$, $p = 0.034$] and non-significant trend for sex [$F_{(2, 39)} = 2.94$, $p = 0.08$], but no sex × treatment interaction ($p > 0.05$). Post-hoc analysis revealed significantly higher $D\beta H^{+ve}$ cell counts in female CO_2–H mice as compared to CO_2-L mice ($p < 0.05$) and higher counts than the air group though not statistically significant ($p = 0.07$). No group differences were noted in males.

Furthermore, to determine whether female $D\beta H^{+ve}$ counts associated with observed behaviors, a correlational analysis was performed between $D\beta H^{+ve}$ cell counts with freezing and rearing behavior during CO_2 inhalation (Fig. 7c and d) as well as extinction (Fig. 7e and f). A significant positive correlation was revealed between LC $D\beta H^{+ve}$ counts and freezing behavior during CO_2 inhalation ($r = 0.492$, $p = 0.005$) (see Fig. 7c) and fear extinction ($r = 0.511$, $p = 0.004$) (Fig. 7e). Interestingly, rearing behavior showed a negative correlation with LC $D\beta H^{+ve}$ counts for CO_2 inhalation ($r = 0.552$, $p = 0.023$) and fear extinction ($r = 0.527$, $p = 0.003$). Importantly these correlations were not observed in air-exposed female mice (Fig. 7c–f) suggesting an association with the behavioral responses to CO_2 inhalation. Strikingly, no significant correlations were observed for males between LC $D\beta H^{+ve}$ cell counts and freezing or rearing behaviors during CO_2 inhalation or extinction freezing, although a statistical trend was noted for a positive correlation between LC $D\beta H^{+ve}$ counts and freezing during CO_2 inhalation ($r = 0.319$, $p = 0.056$). Overall, these correlations suggest a potential contribution of the LC noradrenergic system in regulating CO_2-associated defensive behaviors specifically in females both during inhalation and in delayed effects of CO_2 on fear extinction.

4. Discussion

Behaviors are shaped by the internal homeostatic state of an animal as well as individual behavioral biases, in a sex-dependent manner. Using a paradigm designed to test behavioral responses to both interoceptive and exteroceptive threatful cues, we report distinct engagement of active versus passive defensive behaviors in female mice as compared to males. Consistent with our previous (McMurray et al., 2020) and current male data we observed an association of behavioral sensitivity to CO_2 inhalation with altered extinction of contextual fear in females. Interestingly, CO_2-associated disruption of forebrain-hindbrain neuronal activation patterns showed significant sex differences. Furthermore, a selective recruitment of the brain stem noradrenergic system was observed in females that is consistent with their choice of active defensive coping to interoceptive and exteroceptive threats. This sex-specific engagement of threat response systems may explain differences between males and females in the development of fear and threat-associated disorders, panic and PTSD.

Currently, there is a growing interest in how the body's "interoceptive state", the internal homeostatic milieu modulates the brain and behavior (Furman, 2021). Interoception is commonly referred to as the process by which the nervous system senses and integrates information about the inner state of the body. Although it is well understood how homeostatic perturbations in the body drive motivated behaviors to help restore homeostasis (Damasio and Carvalho, 2013), our current understanding of how aversive interoceptive signals within the body regulate emotional responses is limited. It is also unclear how unpleasant interoceptive experiences impact the processing of other threats and conditioned behaviors that may be exteroceptive in nature. Importantly, even less is known about the impact of sex on interoceptive threat responding.

Experiments undertaken in this preclinical study attempted to address this knowledge gap. We utilized a paradigm recently developed in mice by our group (McMurray et al., 2020) that uses two distinct threat modalities: carbon dioxide (CO_2) inhalation (a potent interoceptive stimulus) followed a week later by a foot shock contextual conditioning (exteroceptive threat with a memory component) to capture (and associate) defensive behaviors to these experiences. The physiologic effects of CO_2 result from increased acidosis (H^+), that constitutes a homeostatic threat to survival. In humans, CO_2 produces heterogenous, dose-dependent responses that lie on a continuum (Colasanti et al., 2008), and individuals on the high spectrum of CO_2 sensitivity are at risk for subsequent psychopathology (Battaglia, 2017). Accordingly, individuals with panic disorder and PTSD have increased emotional and physiological reactivity to CO_2 (Gorman et al., 1994; Kellner et al., 2018; Leibold et al., 2016; Muhtz et al., 2012) resulting in fear, panic attacks and intrusive flashbacks. Interestingly, pre-deployment studies in veterans reported that soldiers with high emotional reactivity to CO_2 inhalation later exhibited significantly higher PTSD symptoms during deployment (Telch et al., 2012), suggesting that high CO_2 sensitivity may confer risk and vulnerability to subsequent trauma. Although CO_2 potently stimulates respiration and autonomic functions in most individuals, the remarkable feature is the behavioral vulnerability to CO_2, shared solely by those who are prone to develop panic attack (Griez and Schruers, 2003), highlighting the usefulness of assessing behavioral measures of CO_2 sensitivity.

Our current study in female and male C57Bl/6J mice replicates previously observed heterogeneity in CO_2-evoked behaviors in BALBc mice (McMurray et al., 2020) suggesting that variance in CO_2 sensitivity is independent of strain and sex. Although translationally relevant (see above), the cause for these differential responses is not evident since pre-CO_2 assessments were not undertaken. Recent work has revealed individual-specific patterns of behavior that is governed by stochastic aspects of gene expression and development as well as individual differences in experience, which collectively impinge upon neural circuit structure and function resulting in adopted behavioral strategies and biases (Levy et al., 2023). Uncontrollable environmental factors, such as

early life stressors, intrauterine position of the embryo and feeding hierarchy in newborns may also contribute to behavioral differences (Lathe, 2004; Loos et al., 2015). Individual differences in CO_2 variance could also be attributed by differential expression and functioning of CO_2 chemosensory mechanisms. In this regard, early life stress altered the expression of acid sensing ion channels (ASICs) and was associated with CO_2 hypersensitivity (Battaglia and Khan, 2018; Cittaro et al., 2016). The acid-sensing G protein-coupled receptor, TDAG8 that regulates CO_2-associated fear in mice (Vollmer et al., 2016) may also be relevant as a large variance in TDAG8 expression is observed in humans (Strawn et al., 2018). Neuroimmune factors may also contribute to variable CO_2 sensitivity as we previously reported a contribution of microglia and pro-inflammatory cytokines in CO_2-evoked fear (Vollmer et al., 2016; McMurray et al., 2022). CO_2 evoked a lower magnitude of freezing during inhalation and minimal context-conditioned freezing in C57Bl/6J mice in the current study as compared to BALBc mice in previous studies (Vollmer et al., 2016; McMurray et al., 2020). It is possible that 10 % CO_2 was not as aversive to C57Bl/6J mice under our conditions. However, it was sufficient to generate heterogenous behavioral responsivity and impact long term effects on fear conditioning and regional neuronal activation. Previously, we reported differential behavioral sensitivity to CO_2 inhalation between rat strains (Winter et al., 2017) that showed differences in the expression of hindbrain serotonergic and noradrenergic neurons that have been associated with CO_2 chemosensing, fear and anxiety. It is possible that these and/or other CO_2 regulatory systems contributed to differential magnitude of CO_2 response between mice strains.

An important finding of our study was the choice of active versus passive behaviors in females versus males that was apparent during CO_2 exposure and the early extinction phase of fear conditioning. In addition to the traditional fear-relevant, passive freezing behavior we also assessed rearing, an active defensive response that represents exploration of the context and reported to occur as a prelude or "look out" for escape in threatful contexts (Biagioni et al., 2016). Rearing frequency is reported to be reduced and replaced by immobility when the threat becomes more severe (Lever et al., 2006). In males, attenuated rearing and increased immobility evoked by CO_2 likely represents a tendency for passive defense responses rather than active escape behavior. Others have reported an engagement of active "escape-oriented" behaviors versus freezing in female animals in a conditioned fear paradigm compared to males (Gruene et al., 2015). Our current data further expands this observation to interoceptive threat, CO_2. Furthermore, in a subset, the CO_2 high responders (CO_2–H), a shift towards reduced rearing and increased freezing was observed, compared to air and CO_2-L, suggesting individual variation in CO_2-responsivity that favors decreased exploration in CO_2-sensitive mice. Interestingly, behaviors during the preCO_2 novel context habituation revealed significantly higher rearing in female air and CO_2-L mice (but not CO_2–H) mice as compared to males suggesting an innate predisposition to active behavioral responding that may extend to threatful situations.

Mice elicited higher freezing during fear conditioning in comparison to CO_2 exposure. Inhalation of CO_2 (interoceptive) and exposure to foot shocks (exteroceptive) are very different experiences that are expected to generate significantly different magnitudes of fear (freezing) and other defensive behaviors. Our objective for the CO_2 exposure was to generate a heterogenous behavioral response with high and low responders to a translationally relevant 10 % CO_2 concentration and not to make the CO_2 experience highly stressful or comparable to fear experienced to foot shocks. High freezing observed during the pre-acquisition baseline prior to foot shocks may have been an outcome of prior manipulations such as inhalation exposure and acoustic startle testing.

There are two major take always from our fear conditioning-extinction data collected a week following CO_2 inhalation exposure: 1) high behavioral sensitivity to CO_2 was specifically associated with altered defensive behaviors during extinction, an effect observed in both females and males; and, 2) choice of defensive behaviors (active vs passive) was distinct between sexes only during early extinction with females exhibiting higher rearing and reduced freezing strategies compared to males. The selectivity and association of prior CO_2 sensitivity with fear extinction is intriguing and is consistent with a previous study by us (McMurray et al., 2020) and others (Monfils et al., 2018). The reduction in extinction freezing was modest in all groups. Prior handling and manipulations (inhalation exposure and acoustic startle testing) likely impacted the magnitude of fear conditioning and efficacy of extinction. However, male and female CO_2–H mice demonstrated significantly higher extinction freezing compared to air and CO_2-L groups suggesting altered fear regulatory mechanisms in these animals. The lack of significant group differences in acoustic startle, freezing during pre-shock habituation period, fear acquisition and conditioned fear suggests that prior CO_2 exposure does not result in a generalized fear sensitization effect. Association of CO_2 sensitivity and fear extinction may suggest a convergence of threat response regulatory systems and circuits that process interoceptive and exteroceptive experiences. CO_2-chemosensory targets such as acid sensing ion channel 1 (ASIC1) and T cell death associated gene-8 (TDAG8) receptor may be important. ASICs have been reported to regulate fear extinction circuits (Wang et al., 2018) and TDAG8-enriched circumventricular organ (CVO), subfornical organ (SFO), sends direct projections to the infralimbic cortex, a key extinction regulatory area, although the role of this circuit remains to be investigated. Interestingly, sex differences in behavioral responding were only evident during early extinction phase. Early extinction learning involves risk assessment of the threatful context to gauge safety. Sex differences in the risk assessment behaviors with female rodents exhibiting more active responses than males in ambiguous and fear-related situations have been reported (Arakawa, 2019a, 2019b) Discrete neurobiological substrates and neurocircuits may underlie adopted behavioral choices between sexes (see Velasco et al., 2019; Clark et al., 2019 and references within).

Our post behavior ΔFosB mapping and inter-regional correlations provide important insights on potential regional/circuit alterations that may have contributed to convergent or divergent behavioral effects in males and females. Sampling of several fear-regulatory, CO_2-chemosensory and sexually dimorphic regions revealed inhalation and sex-dependent alterations. In agreement with our previous CO_2-inhalation studies (McMurray et al., 2019, 2020, 2022), reduced activation of the infralimbic cortex in CO_2–H males and females highlights a key role of this area in CO_2-associated fear and compromised extinction in these animals. The IL is well-recognized for the regulation of extinction (Do-Monte et al., 2015; Sierra-Mercado et al., 2011), and IL dysfunction is associated with extinction deficits (Arruda-Carvalho and Clem, 2015; Milad et al., 2007). However, it's identification as a key node for CO_2-associated fear is a novel observation that needs future exploration. In conjunction with IL-PFC alterations, the CeA, BLA and ITC subregions of the amygdala showed inhalation effects that appeared to be more pronounced in female CO_2 mice. Previous studies have reported cortical-amygdala interactions in regulating fear learning and extinction (Marek et al., 2013; Ng et al., 2023; Strobel et al., 2015). Reduced activation in the IL and ITCs has been associated with increased fear and compromised extinction (Do-Monte et al., 2015; Likhtik et al., 2008; Sierra-Mercado et al., 2011). Interestingly, the BLA showed opposite CO_2 effects in female (reduced) versus males (increased) suggesting an engagement of distinct inhibitory-excitatory cellular mechanisms. Previous work has reported segregated cell populations within the BLA that regulate "high" and "low" fear states (McCullough et al., 2016; Zhang et al., 2020), however, their contribution to CO_2-evoked fear or differential engagement by sex is currently unknown. Reduced ventrolateral PAG activation was noted in female CO_2 exposed mice versus air. The vlPAG is recognized as a key site regulating the balance between freezing and panic/escape-oriented behaviors (Paul et al., 2014) and recent studies highlight it's role in assessing threat probability to produce optimal fear responses (Wright and McDannald, 2019). Consistent with the current study, we previously reported reduced serotonergic cell

counts in the vlPAG of CO_2-sensitive rats (Winter et al., 2017), suggesting that CO_2-associated dampening of the vlPAG contributes to defensive behaviors Significant sex differences were also evident in several threat response regulatory areas such as the LC, prelimbic cortex, BNST, and dentate gyrus, some of which are well known as sexually dimorphic areas (Bangasser et al., 2016). Higher activation in the LC and DG in females in contrast to higher activation in PL and BNST in males is indicative of distinct pathways and a recruitment of brain stem versus forebrain mechanisms in females and males, respectively. A role of LC noradrenergic to hippocampal dentate circuit in aversive contextual fear processing was recently reported (Seo et al., 2021). The prelimbic cortex and BNST are well established nodes for threat and fear regulation (Goode and Maren, 2017; Sotres-Bayon et al., 2012) and are reported to mediate sexually dimorphic expression of fear (Bauer, 2023; Fenton et al., 2016).

Our interregional co-activation mapping corroborated region-specific alterations and further highlighted contributory neurocircuits. Overall disrupted cortico-amygdala co-activation patterns in CO_2–H mice reveal an aberrant top-down control of the amygdala by prefrontal areas that may have resulted in deficits in extinction learning in these animals, independent of sex. Co-activation patterns revealed sex differences in LC recruitment with inverse LC-hippocampal associations in CO_2 mice. The contributory role of these circuits in CO_2-associated fear remains to be established in a sex-specific manner.

We further investigated the potential involvement of the locus noradrenergic system in observed behavioral differences between sexes. The LC coordinates threat-associated adaptive/maladaptive arousal, and fight-or flight-or freeze defensive responses (Aston-Jones et al., 1999; Aston-Jones and Cohen, 2005). LC is a major source of norepinephrine (NE) to the entire forebrain axis and is a sexually dimorphic structure in morphology, gene expression, and stress responsiveness (Bangasser et al., 2016; Curtis et al., 2006; Mulvey et al., 2018). Importantly, and pertinent to our paradigm the LC is a key chemosensory site as hypercapnia and focal acidosis, such as during CO_2 inhalation, causes a rapid increase in the firing rate of LC neurons (Elam et al., 1981), leading to increased ventilation and arousal (Gargaglioni et al., 2010), thus serving as a chemo-alarm system in the brain. Increased noradrenergic activity and turnover has been reported in patients with PTSD and panic disorder, implicating the relevance of the LC noradrenergic system in threat and fear-associated pathologies (Morris et al., 2020; Southwick et al., 1999; Sullivan et al., 1999). Selective association of LC DβH counts with freezing and rearing behaviors in females during CO_2 inhalation and fear extinction strongly supports the involvement of brain stem noradrenergic mechanisms in these behaviors, an association that was absent in males. Other mechanisms may have contributed to observed behaviors, as previous work in male mice from our lab (Vollmer et al., 2016) and others (Ziemann et al., 2009) reported the recruitment of forebrain CO_2 chemosensory sites such as the amygdala and circumventricular organ, subfornical organ (SFO) in regulating CO_2-evoked freezing.

The ascending LC projections to the limbic system and cortex mediate fear (Giustino and Maren, 2018). LC NE signaling to the mPFC is involved in fear after an initial threat exposure as well as remote fear associated with the initial threat (Fan et al., 2022). We report for the first time that the LC NE system may also regulate and integrate fear associated with discrete threats that are interoceptive or exteroceptive in nature. What downstream circuits may regulate these behaviors? Previous work and our FosB data support mPFC, hippocampus and BLA as likely downstream target areas. Collectively, CO_2 threat sensing and associated fear appear to engage divergent hindbrain (females) versus forebrain (males) mechanisms and circuitry that needs to be investigated in future studies.

While our study provided important information there are limitations. Our observations were restricted to behavioral assessments. As CO_2 and fear conditioning also promote physiological responses such as elevated breathing (Colasanti et al., 2008) and autonomic activation (Savulich et al., 2019), it would be important to determine these outcomes in a sex-dependent manner. We did not assess the effects of sex hormones or the female estrous cycle in the current study or their association with CO_2 sensitivity and fear extinction deficits. Lastly, our tissue analysis provided interesting and significant effects, however, these data are correlational in nature. ΔFosB analysis represents broad neuronal plasticity and does not provide information on specific behaviors during the paradigm. Targeted interventions at a regional, circuit level are required to identify regions/cell types associated with specific behaviors as well as overlapping or distinct contributory mechanisms.

In conclusion, our data provide novel insights on differential, sex-dependent behavioral responses to interoceptive and exteroceptive threat exposures with higher active defensive behaviors in females, a possible outcome of discrete cell-circuit engagement of brain stem mechanisms. Current findings are highly relevant in the context of gender differences in fear-associated pathologies related to abnormal interoceptive sensitivity and sensitized threat responding (Boettcher et al., 2016; Longarzo et al., 2020) such as panic and posttraumatic stress disorder (PTSD).

CRediT authorship contribution statement

Rebecca Ahlbrand: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Allison Wilson:** Validation, Methodology, Investigation. **Patrick Woller:** Methodology, Investigation, Formal analysis. **Yuv Sachdeva:** Methodology, Investigation. **Jayden Lai:** Methodology, Investigation. **Nikki Davis:** Methodology, Investigation. **James Wiggins:** Methodology, Investigation. **Renu Sah:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors have no conflicts of interest to declare.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ynstr.2024.100617.

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