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Differential behavioral sensitivity to carbon dioxide (CO_2) inhalation in rats

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Abstract

Inhalation of carbon dioxide (CO_2) is frequently employed as a biological challenge to evoke intense fear and anxiety. In individuals with panic disorder, CO_2 reliably evokes panic attacks. Sensitivity to CO_2 is highly heterogeneous among individuals, and although a genetic component is implicated, underlying mechanisms are not clear. Preclinical models that can simulate differential responsivity to CO_2 are therefore relevant. In the current study we investigated CO_2-evoked behavioral responses in four different rat strains: Sprague-Dawley (SD), Wistar (W), Long Evans (LE) and Wistar-Kyoto, (WK) rats. We also assessed tryptophan hydroxylase 2 (TPH-2)-positive serotonergic neurons in anxiety/panic regulatory subdivisions of the dorsal raphe nucleus (DR), as well as dopamine β hydroxylase (DβH)-positive noradrenergic neurons in the locus coeruleus, implicated in central CO_2-chemosensitivity. Behavioral responsivity to CO_2 inhalation varied between strains. CO_2-evoked immobility was significantly higher in LE and WK rats as compared with W and SD cohorts. Differences were also observed in CO_2-evoked rearing and grooming behaviors. Exposure to CO_2 did not produce conditioned behavioral responses upon re-exposure to CO_2 context in any strain. Reduced TPH-2 positive cell counts were observed specifically in the panic-regulatory dorsal raphe ventrolateral (DRVL)-ventrolateral periaqueductal grey (VLPAG) subdivision in CO_2-sensitive strains. Conversely, DβH positive cell counts within the LC were significantly higher in CO_2-sensitive strains. Collectively, our data provide evidence for strain dependent, differential CO_2-sensitivity and potential differences in monoaminergic systems regulating panic and anxiety. Comparative studies between CO_2-vulnerable and resistant strains may facilitate the mechanistic understanding of differential CO_2-sensitivity in the development of panic and anxiety disorders.

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Keywords

panic; CO_2 sensitivity; serotonergic; noradrenergic; dorsal raphe; locus coeruleus

Introduction

Inhalation of CO_2-enriched air produces psychological and physiological responses that can promote anxiety and fear-like behavior. In humans CO_2 sensitivity lies on a continuum (Colasanti et al., 2008), as the composition, frequency, and severity of CO_2-evoked phenotypes have been found to be quite heterogeneous within the population. First described in 1951 by Cohen and White (Cohen and White, 1951), CO_2 inhalation is an established biological challenge, as individuals with a heightened risk for panic disorder (PD) elicit CO_2-hypersensitivity indexed by exaggerated emotional and respiratory responses (Papp et al., 1993; Rassovsky and Kushner, 2003; Leibold et al., 2013). In the extracellular fluid, CO_2 is hydrolyzed to carbonic acid (H_2CO_3) by carbonic anhydrase which readily dissociates into bicarbonate (HCO_3^-) and H^+ (Huckstepp and Dale 2011). The resulting acidosis is thought to be the trigger for the panic and fear symptoms, and neuroimaging studies on PD patients support a role of homeostatic pH disturbances in panic physiology (Maddock et al, 2009).

Evidence from genetically informed studies support risk factors that influence liability to heightened sensitivity to CO_2, an endophenotype to PD (Battaglia et al., 2014). Heightened sensitivity to CO_2 has been associated with both childhood separation anxiety and adult panic disorder (PD) with predispositions to either disorder founded largely on genetic factors (Battaglia et al., 2009). Additionally, twin studies have shown significant association with shared genetic components to CO_2-sensitivity (Bellodi et al., 1998; Battaglia et al., 2007), and the degree of familial relationships to panic disorder patients has been shown to be associated with CO_2 sensitivity (Perna et al., 1996; Coryell et al., 2001). Collectively, these observations strongly support CO_2-hypersensitivity as a valid biological risk and trait marker for panic and anxiety disorders. Currently, biological underpinnings of individual variance in CO_2 sensitivity are not well understood. Rodent and human studies provide evidence for a role of gene x environment interactions towards heightened CO_2 reactivity (D'Amato et al, 2011; Spatola et al, 2011). Although a strong genetic predisposition and gene x environment contributions to CO_2 hypersensitivity has been proposed, contributory mechanisms are not clear. Development of preclinical models that can simulate differential responsivity to CO_2 inhalation is relevant and can facilitate mechanistic understanding of this phenomenon.

Rodent models of CO_2 inhalation-evoked behavior and physiological responses have been studied previously (see Battaglia et al, 2014; Johnson et al, 2014; Vollmer et al, 2015 and references therein), however, studies on variation in CO_2 responsivity have been limited. Previously, strain-dependent variation in CO_2-evoked ventilation was reported in mice (Tankersley et al, 1994). The current study assessed differential behavioral sensitivity to CO_2 inhalation in four rat strains with distinct genetic backgrounds. Our primary objective was to determine whether CO_2 inhalation evoked distinct strain-dependent responses in rats with the purpose to develop a rodent model of susceptibility/resistance to CO_2 inhalation. We

selected three commonly used outbred strains (Sprague-Dawley (SD), Wistar (W) and Long Evans (LE) rats) and one inbred (Wistar-Kyoto, WK) strain. In this initial study, our objective was to capture the wide genetic diversity and phenotypic variation between these commonly used rat strains as there are no studies available comparing CO_2-inhalation responses between these strains. We anticipated greater CO_2 response variation within outbred strains compared with the inbred WK animals where genetic homogeneity and lower variability may be useful for mechanistic studies. In addition to behavioral measurements, we also investigated tryptophan hydroxylase 2 (TPH-2)-positive serotonergic neurons in anxiety and panic regulatory subdivisions of the dorsal raphe nucleus (DR), as well as dopamine β hydroxylase (DβH)-positive noradrenergic neurons in the locus coeruleus (LC). The DR houses topographically organized subsets of serotonergic neurons. These include subpopulations in the dorsal region (DRD) that project to forebrain circuits modulating anxiety-related behaviors, while neurons within the dorsal raphe ventrolateral (DRVL)-ventrolateral periaqueductal grey (VLPAG) division provide inhibitory input to the dorsal PAG to attenuate panic-relevant responses (Hale and Lowry, 2011). TPH-2 positive serotonergic neurons in the dorsal raphe are CO_2-chemosensitive and therefore have the potential to impact CO_2 evoked behavior and physiology (Severson et al., 2003). Serotonergic neurons within the DRVL are significantly activated by CO_2 inhalation (Johnson et al., 2011) and may represent a ‘sympathomotor control system’ that normally limits autonomic/behavioral responses to interoceptive threats.

The LC is an established central CO_2-chemosensitive site (Gargaglioni et al., 2010) and contains the major group of noradrenaline (NA) synthesizing neurons, the A6 cell group. NA cell bodies are well connected to brain regions regulating arousal, anxiety, autonomic responses, and memory. LC activation may regulate CO_2-sensitivity, as lesioning of rat LC noradrenergic neurons has been associated with attenuated physiological responses to CO_2 inhalation (Biancardi et al., 2008). Neuroimaging studies also reveal increased blood oxygen level dependent (BOLD) signal in brain stem areas including the LC following CO_2 inhalation in humans (Pattinson et al., 2009). In addition to their well characterized regulatory role in panic and anxiety, the LC and the DR have reciprocal interactions, with the DR exerting an inhibitory effect on LC while the locus is reported to exert excitatory action on the DR (Szabo et al, 2001; Vandermaelen and Aghajanian 1983).

We hypothesized a strain-dependent variance in CO_2 responsivity, accompanied by reduced TPH-2 and enhanced DβH immunoreactivity in the DR and LC respectively in CO_2-sensitive strains.

Experimental Procedures

Animals

All experiments reported here were performed on adult rats (300–350g) purchased from Harlan. All rat strains (WI, WK, LE and SD) were housed under constant temperature (23–28°C) with a 12hr light, 12hr dark cycle (lights on at 06:00h). Food and water were provided ad libitum. All behavioral experiments were performed between 8am–1pm during the 12h light cycle. Study protocols were approved by the Institutional Animal Care and Use Committee of University of Cincinnati in a vivarium accredited by the Association for

Assessment and Accreditation of Laboratory Animal Care (AAALAC). A total of 48 animals were used for the study (n=6 rats/group).

CO_2 inhalation

Animals were exposed to a three day paradigm (see Fig 1) consisting of habituation (day 1), air or CO_2 exposure (day 2) and CO_2-context exposure (day 3) in the absence of CO_2 as described in previous studies from our group (Vollmer et al, 2016), with modifications. This enabled an assessment of unconditioned (day 2) and conditioned (day 3) behavioral responses to CO_2 inhalation. Briefly, rats were habituated to a Plexiglas CO_2 chamber (8″x 8″ x 6.5″) for 10 min one day prior to the CO_2 challenge. On the following day, animals were exposed to infused breathing air or 10% CO_2 (in 21% O_2, balance with N_2, Wright Brothers Inc., Cincinnati, OH) for 10 min in a dual, top-bottom chamber during which behavior was videotaped. Air/CO_2 was infused into the top chamber and controlled through a valve in the ceiling of the bottom chamber (8″x8″x 6.5″) to avoid direct blowing, which is aversive to rodents. Prior to placing each animal the bottom chamber was pre-saturated with CO_2, the animal was placed and the infusion continued for the rest of the experiment. This created a steady-state of infused air or 10% CO_2 concentration, controlled by a flow meter to ensure a constant infusion rate of 10L/min for all animals. This CO_2 concentration range is translationally relevant to challenge studies in humans (Rassovsky et al, 2003). Animals were returned to their home cage after the 10 min CO_2 exposure. 24 hr following air/CO_2 inhalation, rats were placed in the CO_2 chamber (context) for 5 min in the absence of infused breathing air or CO_2 and videotaped. Videos were scored by a trained observer blinded to genotype and treatment for total time spent immobile, as well as grooming and rearing frequency. Immobility, defined as the absence of all movement except for respiration, was our primary behavioral measure based on CO_2-evoked fear behavior observed in previous studies from our group and others (Mongeluzi et al., 2003; Esquivel et al., 2010). Grooming included all bouts of facial and body grooming time. Rearing was scored when both front legs were lifted off the ground.

Histology and Immunofluorescence

For histological assessments, air and CO_2-exposed animals were sacrificed on Day 3, 1 hr following CO_2-context exposure (Day 3). Rats were deeply anesthetized via i.p. administration of Fatal Plus (Vortech, USA) and perfused transcardially with ice cold 4% paraformaldehyde (in 0.1M Na_2HPO_4 / NaH_2PO_4 buffer, pH 7.4). Brains were removed, postfixed overnight, and then transferred to 30% sucrose (in KPBS) until ready for sectioning. Tissue was cut at 30 μm on a sliding microtome. 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 immunofluorescence. The following primary antibodies were used for immunofluorescence: tryptophan hydroxylase (TPH) 1:500 (MAB308, Millipore) and dopamine beta hydroxylase (DβH) 1:2500 (T0678, Sigma). Sections were transferred from cryoprotectant to 50 mM potassium PBS (KPBS; pH 7.4; 40 mM potassium phosphate dibasic, 10 mM potassium phosphate monobasic, and 0.9% sodium chloride) at RT. Cryoprotectant was rinsed (five times for 5 min) in KPBS, and the sections were transferred to KPBS plus 0.3% H_2O_2 and incubated for 10 min at RT. Sections were then washed (five times for 5 min) in KPBS at RT and placed in blocking solution [50

mM KPBS, 0.5% bovine serum albumin (BSA), and 0.2% Triton X-100] for 1 hr at RT. Sections were incubated overnight at 4°C in the specified primary antibodies diluted in blocking solution. The following day, sections were rinsed in KPBS (five times for 5 min) and incubated in CY3-conjugated mouse antibody (Jackson ImmunoResearch) diluted 1:500 in KPBS plus 0.5% BSA for 1 hr at RT. Sections were rinsed five times for 5 min in KPB at RT, mounted onto Superfrost Plus slides, and coverslipped with Gelvatol (Fluka).

Imaging and Cell Counting

Brain sections were imaged using an AxioImager Z1 microscope (Zeiss), utilizing apotome imaging capability to create z-stacks (Axiocam MRm camera and AxioVision Release 4.8 software; Zeiss). Cy3 was excited using the 568 nm wavelength. Images were acquired for TPH-2 positive cells from the DRVL/VLPAG and the DRD between rostro-caudal levels (−7.64, −8.0, −8.3) and DβH-positive cells within the locus coeruleus at (−9.68, −9.80, −10.04) levels as Z-stacks (see Fig 4 a–c and 5 a–b). Cell counts were accumulated across 0.8μm z-stacks for each image using the interactive measurement and event tool (AxioVision 4.8) by an observer blinded to experimental groups. Cell counts from rostro-caudal coordinates specified above were averaged for each animal for statistical analysis.

Data Analysis

Behavior scored manually by individuals blinded to groups was used for analysis. Normality was formally tested for all experiments. Data met assumptions of the statistical tests being used. Where appropriate, variance was tested between data sets using the F test or Bartlett's test for equal variances. Variance was found to be similar between most data groups. Data were analyzed by two-way ANOVAs using strain x inhalation as variables. Data was also analyzed using a two-way between strains multivariate analysis of variance for analyzing strain x treatment effects using freezing, rearing and grooming as combined dependent variables. For immunofluorescence, two- way ANOVA was used for analyzing mean cell counts with strain x inhalation as variables. Where main effects were significant, post hoc comparisons were performed using Tukey's analysis. Data are presented as mean ± sem and were considered significant at $p < 0.05$. Prism software was used for two-way ANOVA statistical analysis (GraphPad Software version 6, Inc., La Jolla, CA) and Statistica (version 13 Dell, Round Rock, TX) was used for the multivariate analysis on combined variables.

Results

Behavioral responses to CO_2 are strain-dependent

Inhalation of CO_2 induced an increase in immobility, and reduced rearing and grooming behaviors in rats that was dependent on strain. Significantly higher immobility was observed in WK and LE rats compared with the W and SD strains (Fig 2a). CO_2 exposed WK and LE rats showed significantly higher immobility versus air inhalation suggesting higher responsivity to CO_2 in these strains compared with W and SD rats that showed no significant differences between air and CO_2 exposed groups. A two-way ANOVA revealed significant effect of strain [$F_{(3,40)}=19.75$; $p<0.05$], treatment [$F_{(1,40)}=28.67$; $p<0.05$] and a strain x treatment interaction [$F_{(3,40)}= 4.59$; $p<0.05$]. Post hoc analysis revealed a significantly higher immobility in WKs and LE strains compared with W and SD strains ($p<0.05$). Air

inhalation by itself evoked some immobility in WK rats that was significantly higher than W SD and LE rats ($p<0.05$), suggesting this strain was also sensitive to the aversive effects of blowing gas in addition to CO_2. We also assessed rearing frequency in air and CO_2 exposed animals as a measure for exploration (Fig 2b). A significant effect of strain [$F_{(3,40)}= 34.18$; $p<0.05$] as well as treatment [$F_{(1,40)}=34.41$; $p<0.05$] but no strain x treatment interaction [$F_{(3,40)}=1.508$; $p>0.05$] was observed.

Post hoc analysis revealed a significant reduction in rearing frequency in W and LE rats exposed to CO_2 compared to the air group. Frequency of rearing was significantly lower in both air and CO_2 exposed WK rats compared to all other strains suggestive of low exploratory behavior in this strain. Grooming, a measure of self-directed behavior was also assessed in air and CO_2 exposed animals (Fig 2c). All strains except for SD rats showed reduced grooming time in the presence of CO_2 as compared to air inhalation. Two-way ANOVA revealed a significant effect of treatment [$F_{(1,40)}=12.25$; $p<0.05$] but no strain-dependent [$F_{(3,40)}=1.226$; $p>0.05$] or strain x treatment interaction [$F_{(3,40)}=0.79$; $p>0.05$]. No significant within or between differences were obtained following post hoc analysis.

Multivariate test statistic (Wilks' Lambda) for two-way between strains analysis using freezing, rearing and grooming as combined dependent variables verified independent ANOVA results and showed significant effects of strain ($F_{(9, 92.6)} = 2.39$); $p < 0.05$, Wilks' lambda = 0.60), treatment ($F_{(3, 38)} = 20.32$); $p < 0.05$, Wilks' lambda = 0.38) and a significant strain x treatment interaction ($F_{(9, 92.6)} = 2.39$); $p < 0.05$, Wilks' lambda = 0.60).

To test whether 10% CO_2 acts as an unconditioned stimulus to produce conditioned, associative responses, behavior was assessed in the CO_2 context 24 hr post inhalation in the absence of infused air or CO_2. As shown in Fig 3a–c, previous CO_2 exposure did not impact immobility, rearing, or grooming as compared with the air exposed cohorts. However, significant strain dependent differences independent of CO_2 inhalation were observed between groups. Although immobility scores were low, a two-way ANOVA analysis revealed a significant strain effect [$F_{(3,40)}=5.486$; $p<0.05$] but no treatment [$F_{(1,40)}=0.03$; $p>0.05$] or strain x treatment interaction [$F_{(3,40)}=0.97$; $p>0.05$]. Post hoc analysis revealed significantly higher immobility in air exposed Wistar Kyoto rats as compared with air–exposed Wistar and LE rats ($p<0.05$). Rearing behavior also showed a significant strain-dependent difference but no effect of inhalation or strain x treatment interaction [strain: $F_{(3,40)}=25.39$, $p<0.05$; treatment: $F_{(1,40)}=1.28$, $p>0.05$; interaction: $F_{(3,40)}=0.12$, $p>0.05$]. Post hoc analysis revealed significantly higher rearing in W and LE rats as compared to SD and WK rats ($p<0.05$). Exposure to context also led to strain-dependent differences in grooming behavior [$F_{(3,40)}=10.33$, $p<0.05$], but no treatment [$F_{(1,40)}=1.279$, $p>0.05$] or strain x treatment interaction $F_{(3,40)}=0.46$, $p>0.05$]. CO_2-exposed LE rats showed significantly higher grooming compared with SD, WK and W (air) groups.

Strain dependent differences in TPH positive neurons within the dorsal raphe (DR)

Given the relevance of DR serotonergic neurons in the regulation of panic, anxiety, and central CO_2 chemosensitivity, we quantified TPH-like immunoreactivity within two subdivisions of the raphe nucleus: the DRVL/VLPAG division where 5-HT neurons are implicated in the inhibition of panic-associated responses (Johnson et al., 2004), and the

DRD division that is reported to facilitate anxiety-related responses (Hale et al., 2012). Figure panels 4a–c show representative photomicrographs of TPH-2 positive neurons and the rostro-caudal extent of quantified neurons within these areas. As seen in Fig 4d, the number of TPH2-positive cells within the DRVL/VLPAG showed significant strain-dependent differences, however no significant effect of CO_2 inhalation was observed. Two-way ANOVA revealed a significant effect of strain [$F_{(3, 38)}$ =7.177; $p<0.05$] but no significant effect of inhalation [$F_{(1, 38)}$ = 0.003656; $p>0.05$] or a strain x inhalation interaction [$F_{(3, 38)}$ =1.920; $p>0.05$]. Post hoc analysis revealed a significant reduction in TPH-2 positive cells in LE rats ($p<0.05$) and a trending decrease ($p=0.053$) in WK rats as compared with SD rats. Although no significant differences existed within strains between air and CO_2 inhalation groups there were significant between strain differences in CO_2 exposed cohorts. Post hoc analysis revealed significantly lower number of TPH-2- positive cells in CO_2-exposed Wistar rats compared with SD and WK rats. In contrast to changes within the DRVL/VLPAG, no significant strain- or CO_2-dependent differences were observed in TPH-2 positive cells within the DRD (Fig 4e). Two-way ANOVA revealed no effect of strain [$F_{(3, 38)}$ = 2.50; $p>0.05$], inhalation [$F_{(1, 38)}$ = 0.015; $p>0.05$] or strain x inhalation interaction [$F_{(3, 38)}$ = 1.207; $p>0.05$].

Strain dependent differences in DβH positive noradrenergic neurons within the LC

Figure 5a–b shows representative images illustrating the rostro-caudal extent of *DβH* immunopositive cells that were quantified for the analysis. As seen in Fig 5c, significant differences in cell counts for *DβH* positive cells were observed between strains. Two-way ANOVA revealed a significant effect of strain [$F_{(3, 40)}$ =4.389; $p<0.05$] and a strain x inhalation interaction [$F_{(3, 40)}$ =4.872; $p<0.05$], but no significant effect of inhalation [$F_{(1, 40)}$ = 0.00126; $p>0.05$]. Post hoc analysis revealed a significantly higher number of *DβH*-positive cells in LE and WK rats as compared to SD rats. *DβH* positive cell counts in LE and WK groups also showed a trending ($p=0.06$) increase in comparison with W rats. Although no effect of CO_2 inhalation was observed compared to air groups, a significant reduction in *D*βH immunoreactive cells was observed in CO_2 exposed LE rats as compared with CO_2 exposed WK rats.

Discussion

The current study investigated variability in behavioral responsivity to CO_2 inhalation in rats to explore its utility as a potential rodent model of CO_2 sensitivity, a pathological marker for panic and anxiety in humans. Our data revealed differential CO_2-evoked behavior between strains, as well as strain-dependent differences in serotonergic and noradrenergic immunoreactivity in brain areas regulating panic and anxiety related behaviors.

The use of CO_2-enriched air to provoke anxiety or panic responses, in both patient and healthy volunteers, has a long history in psychiatric research (for review see Esquivel et al., 2009; Vollmer et al., 2015). CO_2 challenge studies in healthy volunteers have been used reliably to evoke acute anxiety while similar doses in panic disorder patients can evoke robust panic attacks, making CO_2 a biological marker for trait anxiety and panic vulnerability. CO_2 hypersensitivity, as indexed by exaggerated emotional and respiratory

responses to CO_2-enriched air mixture, is a useful investigational tool for identifying individuals at risk for later pathophysiology. Studies have used the CO_2 challenge task for the validation of candidate genes for panic disorder (Savage et al., 2015), and treatment efficacy in panic patients (Perna et al., 2002). Recent work has established the CO_2 inhalation paradigm as a translational cross-species model for panic (Leibold et al, 2016).

Exposure to CO_2 produced a different magnitude of behavioral responses between strains. For this study, we chose a concentration of CO_2 (10%) that elicits panic attacks in individuals with panic disorder while producing anxiety and panic in some healthy volunteers (Rassovsky and Kushner, 2003). A previous study reported strain-dependent variation in CO_2-evoked ventilation in mice (Tankersley et al, 1994), however, behavioral responses have not been studied. The most prominent behavioral response evoked by CO_2 was immobility (freezing-like behavior), that was markedly different between strains. Previous reports of CO_2 inhalation in rats have reported freezing behavior using a similar concentration (Mongeluzi et al., 2003). 10% CO_2 also evokes freezing in mice as reported by our group (Vollmer et al, 2016), and others (Ziemann et al., 2009; Taugher et al., 2014, Liebold et al, 2016). Thus CO_2-evoked immobility/freezing behavior appears to be consistent across species and strains and likely represents a fear-associated defensive behavior. The inbred WK strain elicited the highest sensitivity to CO_2-evoked immobility. To our knowledge, this is the first observation of CO_2 inhalation-evoked behavior in this strain and suggests that, in addition to their well-recognized role as an animal model for depression (in comparison with Wistars as controls) (Overstreet, 2012), this strain could also be useful for investigating panic pathology or comorbid panic-depressive phenotypes. Outbred strains used in the study showed a large variation in CO_2 reactivity with LE rats showing significantly higher immobility compared to W and SD rats. The Wistar cohort elicited negligible immobility suggesting resistance to this behavioral effect of CO_2 inhalation. The Wistar strain has been reported to elicit lower anxiety-like behavior and active coping responses in the elevated plus maze in previous studies (Casarrubea et al., 2013; Keeley et al., 2015). Immobility to CO_2 likely represents an acute defensive response to a homeostatic threat that appears to be strikingly different between LE/WK and SD/W strains. No comparison studies exist that have reported strain differences in CO_2-evoked behavior. Previous studies have used SD or W rats in CO_2 inhalation paradigms (Mongeluzi et al., 2003; Dumont et al., 2010; Johnson et al., 2012; Schimitel et al., 2012). In agreement with our data, CO_2 inhalation (13%) did not affect immobility in Wistar rats (Schimitel et al., 2012). Significant anxiogenic, autonomic, and respiratory responses were reported in Wistar rats at high CO_2 concentrations (20%) (Johnson et al., 2012). It is possible that higher CO_2 concentrations are required to evoke CO_2 effects in resistant strains as observed for CO_2-sensitivity studies in humans (Colasanti et al., 2008).

Rearing frequency, representative of vertical exploration, was significantly reduced following CO_2 inhalation in most strains, suggesting attenuation of explorative tendencies of animals. CO_2, an interoceptive threat to survival is expected to reduce context awareness and elicit anxiogenic-like behaviors. Rearing behavior of WK rats was markedly reduced compared to other groups irrespective of treatment in agreement with previous studies reporting high trait anxiety and passive coping style of this strain (Pare, 1993). In this strain, CO_2 elicited no further reduction in rearing possibly due to floor effects. Strains such as WK

with significantly high trait anxiety may not elicit CO_2-evoked anxiogenic behavior. As reported previously CO_2-evoked anxiety and trait anxiety may have distinct genetic underpinnings (Roberson-Nay et al, 2013). Rearing frequency has also been reported to represent escape motivation which is reported to be reduced and replaced by immobility when the threat becomes more severe (Lever et al., 2006). Attenuated rearing and increased immobility evoked by CO_2 in our model likely represents a tendency for passive defense responses rather than active escape behavior.

We also observed a CO_2-evoked reduction in grooming behavior independent of strain. Rodent grooming represents complex repetitive and sequentially patterned behaviors that has aided in simulating phenotypes representing anxiety disorders (Kalueff et al., 2016). In our paradigm, exposure to CO_2 inhalation may have led to a decrease in self-directed behaviors, as opposed to increased grooming, generally reported following exteroceptive stressors (Van Erp et al., 1994). Our data is in agreement with a previous study that reported reduced grooming in rats exposed to CO_2 inhalation (Schimitel et al., 2012).

Exposure to 10% CO_2 did not evoke context-conditioned immobility or conditioned anxiety-like behavior in any strain 24h post inhalation. This is in agreement with a previous study that reported absence of CO_2-associated context conditioned freezing in mice (Ziemann et al., 2009). On the contrary, Mongeluzi et al reported significant freezing and conditioned fear at concentrations ranging from 5 to 100% CO_2 (Mongeluzi et al., 2003). Differences in CO_2 setups may have led to discrepant data between studies. As discussed in the previous study, the potential interaction of the sound of gas delivery, combined with CO_2 itself, may have been a confounding factor that resulted in a more aversive contextual experience. In the current study, a dual chamber saturated with CO_2 was used to avoid direct blowing and sound interaction that is highly aversive to rodents. It is possible that additional CO_2 exposures are required to evoke context conditioned responses as reported in an earlier study in mice (D'Amato et al, 2011).

To study potential contributory transmitter systems that may underlie differential CO_2 sensitivity between strains, we focused on serotonergic and noradrenergic expressing neurons in key panic and anxiety regulatory areas. Multiple lines of evidence support the potential role of midbrain serotonergic neurons in modulating CO_2-evoked behaviors. Serotonin neurons in the raphe are CO_2-chemosensors (Severson et al., 2003). Furthermore, serotonergic neurons in the DRVL/VLPAG provide inhibitory input to the dorsal PAG to modulate panic-associated responses (Johnson et al., 2004). Interestingly, association of polymorphisms within the TPH-2 gene and CO_2 responses is observed, further supporting a role of the serotonergic system in the effects of CO_2 (Abe et al., 2012). We hypothesized that strains that elicit enhanced reactivity to CO_2 would have reduced serotonergic immunoreactivity (represented by TPH-2 positive cell counts) in panic-relevant subdivisions of the DR. Consistent with this, we observed significant reduction in TPH-2 immunopositive cells specifically within the DRVL/VLPAG in strains that elicited higher CO_2-evoked immobility. No differences were observed in the DR subdivision known to modulate anxiogenic behaviors. In support of a functional distinction between these nuclei, homeostatic, panicogenic stimuli such as CO_2 and lactate infusion recruit neurons within the DRVL/VLPAG while inescapable stress, anxiogenic drugs, and avoidance tasks on the

elevated T-maze, considered as a test for generalized anxiety (not panic), activate serotonergic neurons within the DRD (Johnson et al., 2004; Lowry et al., 2008; Spiacci et al., 2012).

CO_2-sensitive LE and WK rats had lower number of TPH-2 positive cells in the DRVL/VLPAG as compared to the more resistant SD rats. While this is not representative of actual transmitter levels, a relative reduction in serotonergic tone within the DRVL/VLPAG may contribute to compromised suppression of panic-relevant circuits that may potentially contribute to differential CO_2-sensitivity observed between these strains. Inhalation of CO_2 did not have a significant within strain effect on TPH-2 positive cells as compared to air groups, which is not surprising as low dose CO_2 may not impact serotoninergic immunoreactivity. Although W rats did not elicit differences from other groups in control air groups, significantly lower TPH-2 positive cell counts as compared to other strains were observed in CO_2-exposed W cohorts. Although the exact explanation for this change is not evident, it may represent altered activity and turnover of serotonin or possible recruitment in CO_2 effects. Notably, this strain was most resistant to CO_2-evoked immobility. A subset of 5-HT neurons within this area have been shown to increase in activity after exposure to a heightened CO_2 gas mixture, which is implicated in the suppression of behavior and sympathetic drive (Johnson et al., 2011). In agreement with our observations, previous studies have also reported selective contribution of the DRVL/VLPAG subdivision in vulnerability to sodium lactate and CO_2 (Johnson et al., 2008, 2011; Hale and Lowry, 2011). In a rat model of panic-like responses to sodium lactate, serotonergic neurons in the DRVL are highly activated in control rats, but fail to activate in panic-prone rats (Johnson et al., 2008). Decreased serotonergic neurotransmission in targets of the DRVL/VLPAG, as would be predicted by decreased TPH-2 expression under baseline conditions, would be expected to result in vulnerability to panicogen responsivity, such as for CO_2. We did not observe active escape representative of “panic-like” behavior in our animals. Examining strain differences in physiological responses such as autonomic activation and ventilation would be important in future studies. Social isolation during adolescence downregulates the TPH-2 gene expression, suggesting regulatory effects of development x environment on this gene (Lukkes et al., 2013). It would be relevant to investigate gene x environment interactions between strains for mechanisms underlying individual variability to CO_2.

In addition to serotonergic neurons in the raphe, we also investigated differences in $D\beta H^-$ positive noradrenergic neurons in the locus coeruleus (LC). It is estimated that ~50% of all the noradrenergic projections in the central nervous system originate in the LC, which are directed toward the forebrain, cerebellum, brainstem and spinal cord (Aston-Jones G et al, 1995). The LC is a key chemosensory site as focal acidosis, such as during CO_2 inhalation, induces neuronal activation leading to increased ventilation and arousal (Gargaglioni, et al., 2010). Increased noradrenergic activity and turnover has been reported in patients with anxiety and panic disorders, implicating the relevance of the LC noradrenergic system (Coplan et al., 1997; Sullivan et al, 1999). In agreement with our hypothesis, we observed significantly higher DβH-positive immunoreactive cell counts in LE and WK rats as compared with the SD and W strains. Consistent with our observations, an augmented basal firing rate and burst activity of LC neurons has been reported in WK as compared to W and SD rats, suggestive of strain-dependent alterations in LC noradrenergic transmission

(Bruzos-Cidón et al., 2015). Interestingly, in the same study, burst activity and sensitivity of serotonergic neurons in the dorsal raphe nucleus was significantly lower in the WK rats as compared with W and SD strains, also consistent with our observations. Interestingly, in addition to higher DβH-positive counts in air exposed LE rats, a significant decrease in DβH immunoreactivity was specifically noted in CO_2 exposed LE rats, possibly suggesting recruitment of this system in CO_2 effects or CO_2- associated alterations in NE activity or turnover. Although not directly assessed in this study, strain-dependent differences in LC noradrenergic tone and activity may result in altered CO_2-chemosensitivity given the key role of this area in central chemosensitivity and CO_2-evoked respiratory drive (Gargaglioni, et al., 2010). Ventilatory responses were not measured in this study, however, alterations in respiration may have contributed to behavioral differences between strains. Collectively, our immunohistochemistry data on diminished serotonergic and increased noradrenergic immunoreactive cell counts is interesting in light of previous studies reporting reciprocal interaction between these two areas (Szabo et al, 2001; Vandermaelen and Aghajanian 1983). However, we used TPH-2 and DβH immunoreactivity as representative readouts for these systems. Our neurochemical cell count data does not reflect functionality per se, but suggests that potential differences in serotonergic and noradrenergic systems may be relevant to differential CO_2 responses.

Comparing strains provides an avenue to investigate how polygenetic vulnerability may interact with acute insults to produce deficits relevant to pathophysiology. It may also aid in the investigation of mechanisms underlying individual variability and facilitate identification of novel targets and development of therapeutic testing paradigms. The rationale for strain selection for our study was based on several criteria. Inbred strains are often preferred over outbred stocks due to their genetic stability and homozygous traits. Rat strains such as the Sprague Dawley and Long Evans are cost-effective and have been widely used for behavioral assessments associated with stress and emotional reactivity. On the other hand, the WK rats, an inbred strain, has been used as a model for depressive-like behavior (Overstreet, 2012). It was interesting to observe robust behavioral differences in CO_2-responsivity between strains and low variability within outbred strains. Significant, strain-dependent differences in serotonergic and noradrenergic tone observed within key CO_2-chemosensitive sites regulating panic-relevant responses is also noteworthy.

Our studies open up several lines of investigation. Although robust strain-dependent differences in behavioral responses were observed, it remains to be investigated whether physiological reactivity to CO_2 inhalation was different. Measures such as cardiovascular activation and ventilatory measurements in different strains would be required to further validate this model and its association with observed morphological changes. Our observations will also facilitate the investigation of gene x environment interaction effects on variable CO_2-sensitivity. Recent studies have shown impact of early adversity and epigenetic modifications in regulating CO_2 hypersensitivity (Cittaro et al., 2016). It will be interesting to investigate these manipulations in our model. In the current study we focused on altered serotonergic and noradrenergic tone between strains, however, it is possible that other mechanisms contribute to differential CO_2 sensitivity between strains. Acid sensing ion channels (ASICs) in the amygdala and the bed nucleus of stria terminalis (BNST) have been reported to contribute to CO_2 evoked freezing and anxiety (Ziemann et al., 2009; Taugher et

al., 2014). Additionally, orexin neurons in the lateral hypothalamic area are CO_2-senstive and are relevant to the behavioral and physiological effects of CO_2 inhalation (Johnson et al, 2012). In recent studies, our lab reported regulation of CO_2-evoked fear by microglial acid sensing mechanisms (Vollmer et al, 2016). It will be important to tease out contributions of these targets to differential CO_2 sensitivity. It is likely that more than one mechanism(s) may determine CO_2 sensitivity. Lastly, correlations between cell counts and behavioral measures, as well as interventions in a larger "n" study are required to directly associate these systems to observed strain differences.

In conclusion, we report differential, strain-dependent behavioral responsivity to carbon dioxide inhalation in rats. Comparative studies between CO_2-vulnerable and resistant rats may lead to mechanistic understanding and pathophysiological basis of CO_2-sensitivity in humans, as well the role of gene x environment interactions and contributions of early life adversity on the development of panic and anxiety disorders.

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Highlights

- CO_2 inhalation, a biological challenge for panic and fear elicits differential behavioral sensitivity in rat strains
- Long Evans and Wistar-Kyoto rats show significantly higher CO_2-evoked immobility than Wistar and Sprague Dawley rats.
- CO_2-sensitive strains have decreased TPH2 –positive serotonergic neurons in panic regulatory raphe subnuclei, DRVL-VLPAG
- CO_2-sensitive strains have increased DβH-positive noradrenergic neurons in the locus coeruleus, a CO_2-chemosensitive site
- Rodent models of CO_2-sensitivity will facilitate understanding of CO_2-hypersensitivity in panic and anxiety disorders

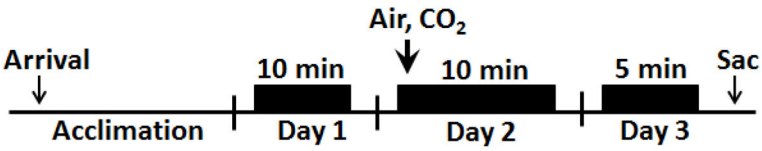

Figure 1.

Schematic showing the experimental layout for measuring CO_2-evoked behavior and conditioned responses to context exposure. Following arrival animals were acclimated to the facility for a week prior to behavioral measurement. The CO_2 inhalation paradigm (Day 1–3) consisted of habituation (Day 1) where animals were exposed to the CO_2 chamber for 10 minutes. On Day 2, animals were exposed to either air or CO_2 (10%) for 10 minutes while being videotaped. 24hr post inhalation animals were returned to the CO_2 context for 5 minutes in the absence of gas inhalation and were videotaped. Following behavior, animals were sacrificed and brain tissue collected for further analyses.

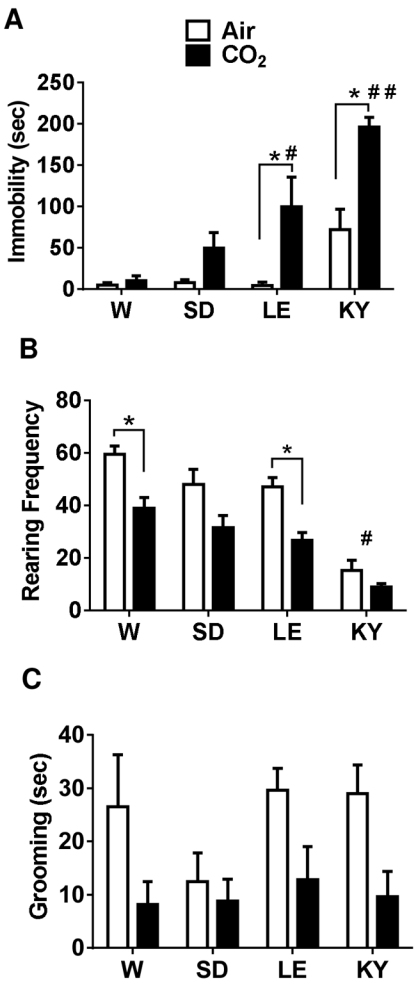

Figure 2.

CO_2 inhalation evokes differential behavioral responses in Wistar (W), Sprague Dawley (SD), Long Evans (LE) and Wistar Kyoto (WK) rats. See schematic (Fig 1) for experimental layout; data from Day 1 during air/CO_2 inhalation is shown here. (A) CO_2-evoked significantly different immobility behavior in strains. CO_2-exposed LE and WK strains elicited significantly higher immobility compared with air inhalation groups within strain (*$p<0.05$) and significant between strain differences for LE rats (# $p<0.05$ versus W and SD air and CO_2 groups) and WK rats (# # $p<0.05$ versus all other groups). (B) CO_2-evoked a significant reduction in rearing behavior in W and LE rats ($p<0.05$ versus air groups within strains). Rearing in air and CO_2-exposed WK rats was significantly lower than all other strains (# $p<0.05$ versus W, SD (air) and LE (air) groups). (C) CO_2-evoked a significant reduction in grooming behavior across strains (significant main effect of treatment but no strain differences). No significant differences were revealed by posthoc analysis. All data are mean ± s.e.m (n=6 animals/air or CO_2 groups)

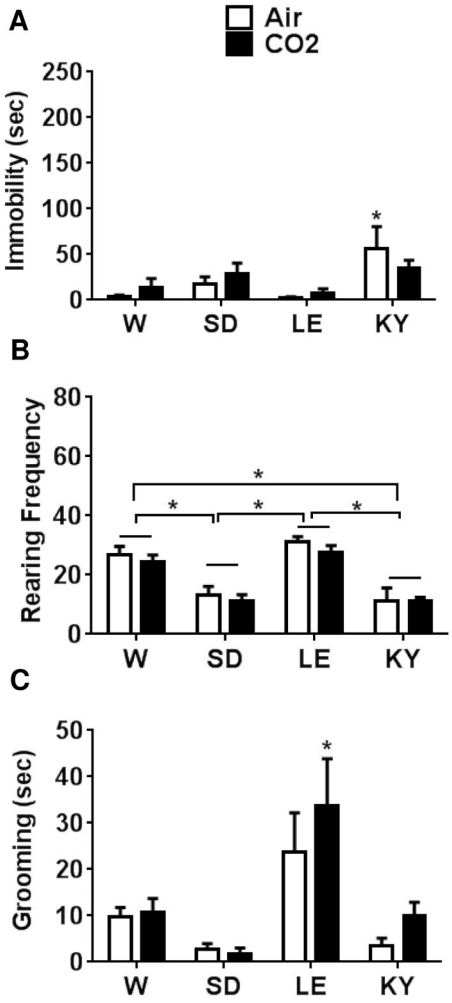

Figure 3.

Prior inhalation of 10% CO_2 does not evoke context conditioned responses in Wistar (W), Sprague Dawley (SD), Long Evans (LE) and Wistar Kyoto (WK) rats. (A) On exposure to context alone, no differences were observed between air and CO_2 groups in immobility in any strain. Air exposed WK rats elicited significantly higher immobility (* $p<0.05$ versus air exposed W and LE rats). (B) Rearing behavior on day 2 showed significant strain but no CO_2-evoked differences. Air and CO_2-exposed SD and WK rats showed significantly lower rearing as compared with W and LE rats (* $p<0.05$). (C) Grooming behavior showed significant differences dependent on strain but not inhalation. CO_2-exposed LE rats showed higher grooming than other strains but were not significantly different from air-exposed LE rats (*$p< 0.05$ versus SD, WK and W groups).

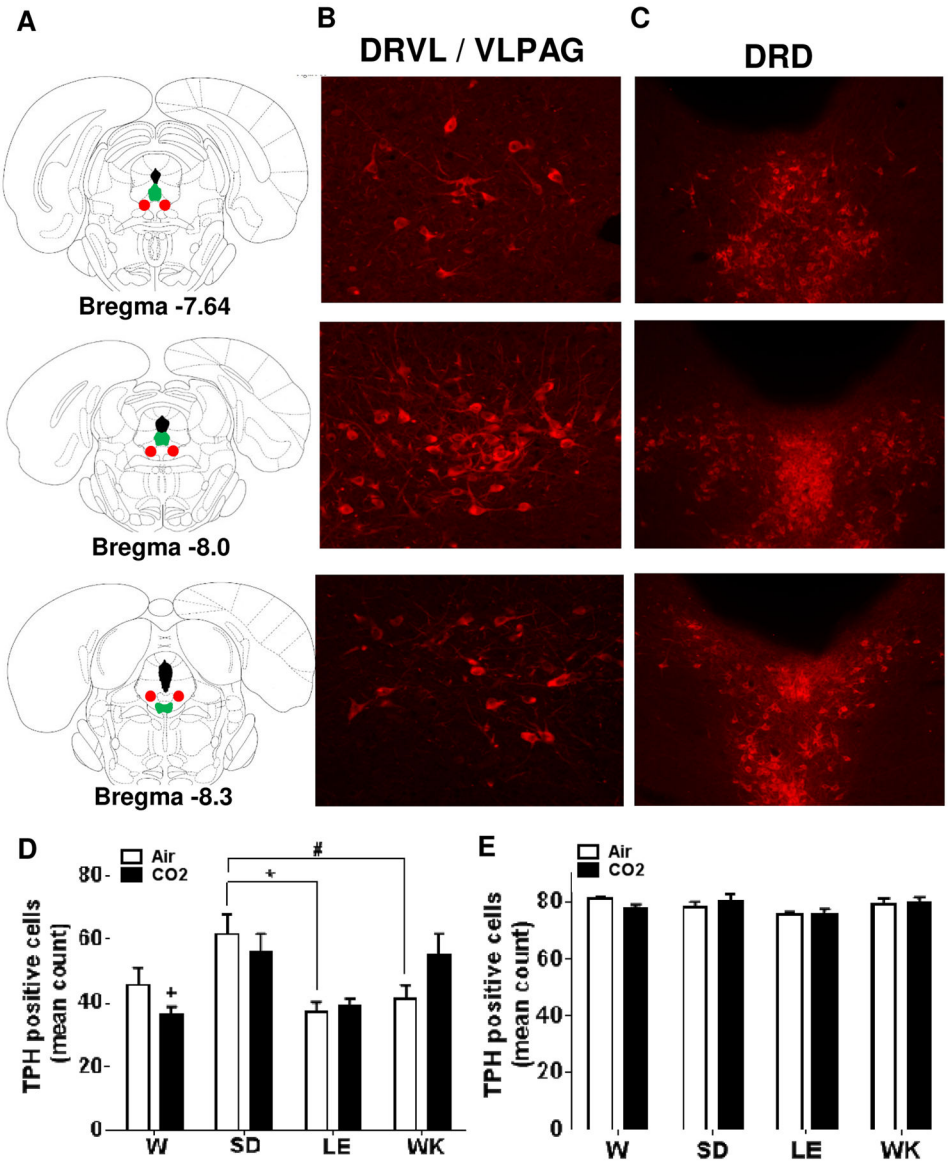

Figure 4.

Alterations in tryptophan hydroxylase-2 (TPH-2) immunoreactivity within dorsal raphe subdivisions in air and CO_2 exposed Wistar (W), Sprague Dawley (SD), Long Evans (LE) and Wistar Kyoto (WK) rats. (A) Panels on the left show stereotaxic illustrations from the atlas of Paxinos & Watson depicting rostral (bregma −7.64) to caudal (bregma −8.3) extent from which cells were quantified. Color represent areas where cells were distributed [red (dorsal raphe ventrolateral -ventrolateral periaqueductal grey; DRVL/VLPAG) and green (dorsal raphe dorsal; DRD)]. Representative images showing (TPH-2) immunopositive cells within the DRVL/VLPAG (panel B) and DRD (panel C). Panel (D) shows TPH-2 cell counts within the DRVL/VLPAG in air and CO_2 exposed animals. Significant strain but no CO_2-dependent differences were observed. (* $p<0.05$; # $p=0.053$; + $p<0.05$ versus SD and WK CO_2 groups) (E) No significant differences were observed in TPH-2 positive cells within the DRD subdivision of the raphe. All data are mean ± s.e.m (n=6 animals/air or CO_2 groups).

Images were acquired at 10X magnification for the DRD and included core and shell areas; and 20X for the DRVL/VLPAG.

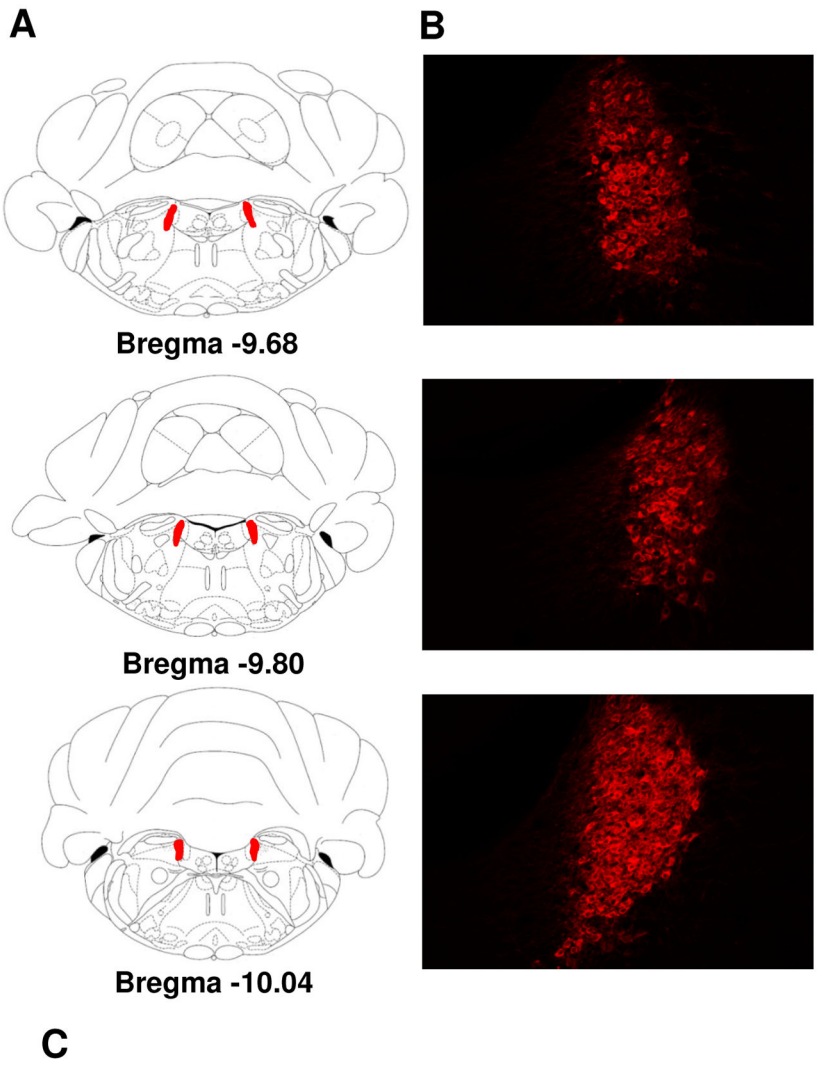

Figure 5.

Alterations in dopamine β hydroxylase (DβH)-positive noradrenergic neurons in the locus coeruleus in air and CO_2 exposed Wistar (W), Sprague Dawley (SD), Long Evans (LE) and Wistar Kyoto (WK) rats. (A) Panels on the left show stereotaxic illustrations from the atlas of Paxinos & Watson depicting rostral (bregma −9.68) to caudal (bregma −10.04) from which cells were quantified (red shows LC area). Panel (B) shows representative images showing (DβH) immunopositive cells within the coordinates shown in (A). Panel (C) shows DβH cell counts in air and CO_2 exposed animals (* $p<0.05$ LE and WK air versus SD group; # $p= 0.06$ LE and WK versus W group; + $p<0.05$ LE versus WK group). All data are mean ± s.e.m (n=6 animals/air or CO_2 groups).