Effect of serotonin transporter genotype on carbon dioxide-induced fear-related behavior in mice

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Abstract

Background: Inhaling 35% carbon dioxide induces an emotional and symptomatic state in humans closely resembling naturally occurring panic attacks, the core symptom of panic disorder. Previous research has suggested a role of the serotonin system in the individual sensitivity to carbon dioxide. In line with this, we previously showed that a variant in the *SLC6A4* gene, encoding the serotonin transporter, moderates the fear response to carbon dioxide in humans. To study the etiological basis of carbon dioxide-reactivity and panic attacks in more detail, we recently established a translational mouse model.
Aim: The purpose of this study was to investigate whether decreased expression of the serotonin transporter affects the sensitivity to carbon dioxide.
Methods: Based on our previous work, wildtype and serotonin transporter deficient ($+/-$, $-/-$) mice were monitored while being exposed to carbon dioxide-enriched air. In wildtype and serotonin transporter $+/-$ mice, also cardio-respiration was assessed.
Results: For most behavioral measures under air exposure, wildtype and serotonin transporter $+/-$ mice did not differ, while serotonin transporter $-/-$ mice showed more fear-related behavior. Carbon dioxide exposure evoked a marked increase in fear-related behaviors, independent of genotype, with the exception of time serotonin transporter $-/-$ mice spent in the center zone of the modified open field test and freezing in the two-chamber test. On the physiological level, when inhaling carbon dioxide, the respiratory system was strongly activated and heart rate decreased independent of genotype.
Conclusion: Carbon dioxide is a robust fear-inducing stimulus. It evokes inhibitory behavioral responses such as decreased exploration and is associated with a clear respiratory profile independent of serotonin transporter genotype.

Keywords

Panic attacks, panic disorder, serotonin transporter, carbon dioxide

Introduction

Panic attacks (PAs) are highly prevalent in many psychiatric disorders but are most commonly associated with panic disorder (PD). PAs are sudden surges of intense fear or discomfort peaking within minutes. During this period, four or more specified symptoms such as palpitations, a feeling of choking, or dizziness develop (American Psychiatric Association, 2013).

In humans, PAs can be reliably provoked in the laboratory by inhaling air enriched with carbon dioxide (CO_2). Various concentrations of CO_2 have been examined over the years. Exposure to a low concentration (5–7%) for up to 20 min elicits symptoms as seen in a state of general anxiety and is therefore nowadays considered to represent a model for generalized anxiety disorder (Ainsworth et al., 2015; Bailey et al., 2005, 2007), while a deep breath of 35% CO_2 provokes the sudden symptoms and intense fear associated with PAs in patients with PD (for extensive review see Leibold et al., 2015). Interestingly, a comparably intense emotional response can be triggered in healthy individuals when using a higher dosage of CO_2 (i.e. taking two vital capacity breaths of 35% CO_2 instead of one as in PD patients) (Griez et al., 2007), suggesting a spectrum of CO_2-sensitivity.

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To study the molecular basis of CO_2-sensitivity in depth, the use of CO_2 as an experimental stimulus in animal models has gained increasing attention in recent years (D'Amato et al., 2011; Johnson et al., 2012; Taugher et al., 2014; Vollmer et al., 2016; Winter et al., 2017; Ziemann et al., 2009). As it is not feasible to apply one or two vital capacity breaths of 35% CO_2 in rodents as it is done in humans, a lower percentage of up to 20% is commonly used for 10–20 min to allow assessment of behavior in a testing box filled with CO_2. Using this methodology, studies showed that CO_2 elicits robust fear-related behavior in rodents. A major aim of such rodent research is the translation of findings to humans. However, it is uncertain how well behavioral assessments in rodents correspond to the self-reported experience in humans. To address this challenge we used CO_2 as the experimental stimulus in both rodents and humans and went beyond the traditional outcome measurements by recording additional physiological parameters (Leibold et al., 2016). Next to the finding that CO_2 induces a strong fear-related behavioral response in mice and humans alike, we showed comparable cardio-respiratory effects. In detail, we calculated and statistically compared effect sizes, taking into account that the frequency per minute varies between both species. This allowed us to perform a quantitative comparison to demonstrate that exposure to a low concentration of CO_2 in rodents corresponds to the response to a 35% CO_2 inhalation in humans. Therefore, while the paradigms are not identical, the comparable effects of the cardio-respiratory system in addition to the behavioral fear response suggests that this rodent model represents a model of panic and is a promising approach to bring closer research in rodents and humans.

The mechanisms of how CO_2 causes fear and exerts effects such as respiratory stimulation are not fully understood. Under physiological conditions the concentration of CO_2 and pH level within the body are maintained within a narrow range. Small fluctuations are not consciously perceived, while larger acute changes typically induce adaptive physiological responses, sensations such as breathlessness, feelings of arousal and panic, and avoidance behavior (Brannan et al., 2001; Diaper et al., 2011; Ziemann et al., 2009). Emotions, in turn, can influence breathing (Homma and Masaoka, 2008) and thereby affect the respiratory response to CO_2. Chemoreceptors that detect changes in CO_2/pH are located in the peripheral carotid bodies and throughout the entire brain. For instance, Mitchell et al. did groundbreaking work on identifying the location of CO_2-chemosensitivity in the medulla (1963b) and showed that increased CO_2 in this region mediates an increase in breathing (1963a). Later experiments showed that also the medullary retrotrapezoid nucleus (Guyenet and Bayliss, 2015), and subsets of serotonergic neurons in the medullary raphe nucleus and the dorsal raphe nucleus are pH-sensitive (Richerson, 2004). The retrotrapezoid nucleus receives excitatory serotonergic innervation from both of these raphe nuclei, which can shift the CO_2 threshold and stimulate respiration (Guyenet and Bayliss, 2015; Mulkey et al., 2007). Particularly serotonin (5-HT) neurons of the medullary raphe nucleus have been associated with driving the respiratory response to CO_2. In contrast, midbrain 5-HT neurons have been associated with arousal and anxiety (Richerson, 2004).

In line with these basic research findings, many studies in humans provided support for a role of the 5-HT system in emotional sensitivity to CO_2. For instance, tryptophan depletion, a method to reduce brain 5-HT levels, increased the fear response

to CO_2 inhalation in PD patients and healthy individuals (Miller et al., 2000; Schruers et al., 2000; Schruers and Griez, 2003), whereas administration of a 5-HT precursor, increasing brain 5-HT levels, reduced the response (Schruers et al., 2002). Blocking the 5-HT transporter (5-HTT) and thereby increasing the extracellular availability of 5-HT also reduced the response to CO_2 (e.g. Bertani et al., 1997; Perna et al., 2002; Schruers and Griez, 2004). Further, our group showed that the fear reaction to CO_2 is moderated by a functional polymorphism in the transcriptional control region of the gene encoding the 5-HTT (polymorphism referred to as 5-HTT gene-linked polymorphic region, 5-HTTLPR). In brief, subjects homozygously carrying the long (L)-allele reported more fear than short (S)-allele carriers (Schruers et al., 2011). In line with this, we also reported a negative association between DNA methylation in the 5-HTT regulatory region and emotional CO_2-sensitivity (Leibold et al., 2020).

Making use of our rodent model and as translational follow-up to our previous studies in humans, we here aimed to explore whether partial or complete loss of 5-HTT moderates the response to CO_2. For this purpose, wildtype (WT), hetero- (+/–), and homozygous (–/–) 5-HTT knockout mice were exposed to CO_2, while the response was examined in terms of fear-related behavior and by monitoring cardio-respiration. Based on the findings of our previous studies, we hypothesized that wildtype mice would show the strongest sensitivity to CO_2.

Materials and methods

For overview see Figure 1.

Fear-related behavior

Animals. Male 5-HTT deficient (Slc6a4[tm1Kpl]; 5-HTT^{+/–} (n=28); 5-HTT[–/–](n=28)) mice and their WT littermates (n=28) were housed individually in ventilated cages within a temperature-controlled environment (21±1°C) and under a reversed day/night cycle (12 h light/12 h dark cycle). Standard rodent chow and water were provided *ad libitum.* From postnatal day 90 onwards, mice were subjected to fear-related behavioral tasks. For this purpose, per genotype 14 mice were assigned randomly to the experimental group (10% CO_2) and 14 mice to the control group (air, 0% CO_2): (a) WT mice exposed to air, (b) 5-HTT +/– mice exposed to air, (c) 5-HTT –/– mice exposed to air, (d) WT mice exposed to CO_2, (e) 5-HTT +/– mice exposed to CO_2, (f) 5-HTT –/– mice exposed to CO_2. All procedures of this study were executed according to protocols approved by the Animal Ethics Board of Maastricht University, the Netherlands.

Behavioral testing

Modified open field test (OFT). All animals were first tested in the modified OFT. The OFT chamber consisted of a transparent Plexiglas square (50×50×40 cm), covered with a clear lid, allowing live monitoring of behavior during gas exposure. Gas was infused using an infusion port on the upper part of the box. Overpressure was prevented by two holes at the opposite site. In addition, two computer fans (5×5 cm, 26 dB), fixed to the lid, were used to ensure a homogenous concentration of the infused gas throughout the entire chamber. The CO_2
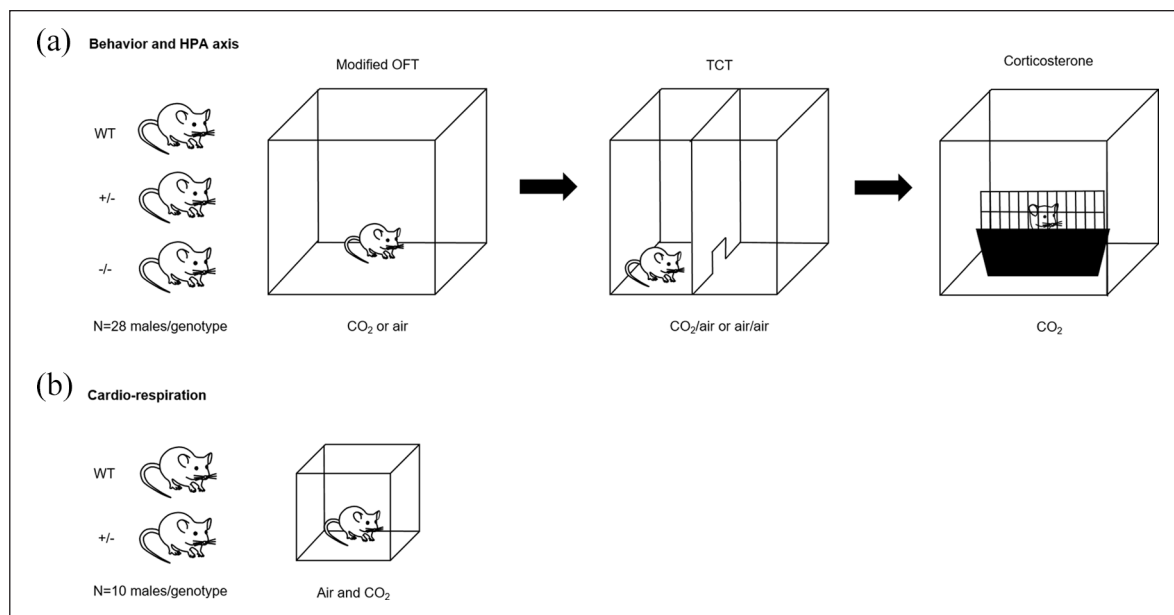
Figure 1. Overview of experimental design.

(a) Male wildtype (WT), serotonin transporter (5-HTT) heterozygous (+/−) and 5-HTT homozygous (−/−) knockout mice were first behaviorally tested in the modified open field test (OFT), while being exposed to either carbon dioxide (CO_2) (*n*=14/genotype) or air (*n*=14/genotype), followed by a two-chamber test (TCT) in which a chamber was filled with CO_2 and the other one with air or both chambers were filled with air. Then CO_2-induced plasma corticosterone levels were determined. (b) Based on the results of these experiments, cardio-respiration was assessed in a new cohort of male WT and 5-HTT +/− mice. HPA: hypothalamo-pituitary-adrenal.

concentration was constantly controlled using a 30% CO_2 Sampling Data Logger (CO_2 meter, Ormond Beach, Florida, USA).

Mice were placed in the center of the chamber that was pre-filled with either compressed air or 10% CO_2 (premixed gas tanks, SOL Nederland B.V., Landgraaf, The Netherlands) depending on the experimental group. Movements were scored automatically with a computerized system (Ethovision Color Pro, Noldus, The Netherlands) for 20 min. For analysis purposes, the floor was subdivided into a 30×30 cm central zone, 10×10 cm corners, and 30×10 cm walls. Time spent in the different zones and total distance moved were analyzed. At the end of the trial, the boxes were cleaned thoroughly.

Behavioral testing was performed under low light conditions during the animals' dark phase and by an investigator blind to genotype.

Two-chamber test (TCT). After completing the modified OFT, mice were tested in the TCT. The apparatus consisted of two Plexiglas chambers (each 50×25 cm with 40 cm high walls) that were connected by an open door (3.5×3.5 cm) to allow free crossing, whilst limiting mixing of the gasses between chambers. Each chamber had a separate gas infusion port, a hole to prevent overpressure, and a computer fan (5×5 cm, 26 dB) to ensure a homogenous gas concentration throughout the chamber.

For mice assigned to air exposure, both chambers were pre-filled with air. For mice subjected to CO_2 exposure, a chamber was pre-filled with 10% CO_2 and the other one with air. The chamber that was filled with CO_2 and the chamber in which mice were placed at the beginning of the test were randomized. The concentration of CO_2 was continuously controlled using a 30% CO_2 Sampling Data Logger (CO_2 meter, Ormond Beach, Florida, USA; a steady state of 9% and 2% was reached in case of CO_2

infusion). Movements, number of crossings, and time spent in each chamber were automatically scored with a computerized system (Ethovision Color Pro, Noldus, The Netherlands) for a period of 10 min. At the end of each trial, the box was thoroughly cleaned. As in the modified OFT, testing was done under low light conditions during the animals' dark phase and by an investigator blind to genotype.

CO_2-evoked freezing. In both behavioral tests, freezing was scored by a trained observer blind to exposure and genotype. Freezing was defined as absence of any movements apart from respiration and is considered to reflect fear-related behavior in rodents (Mongeluzi et al., 2003).

CO_2-induced plasma corticosterone secretion

To assess the hypothalamo-pituitary-adrenal (HPA) axis responsiveness to CO_2, plasma corticosterone levels were determined. Immediately after taking the mice from their home cage, the first blood sample was drawn via saphenous vein puncture (pre-CO_2 level) using heparinized blood collection tubes (Microvette CB300, Sarstedt, Germany). Then, mice were returned to their home cage and exposed to 10% CO_2 for 20 min, after which a second blood sample was taken (post-CO_2 level). Blood samples were kept on ice and centrifuged at 2000 g for 10 min at 4°C. Plasma was collected and stored at −80°C until further processing. Blood corticosterone levels were analyzed using radioimmunoassay. An ImmunuChem Double antibody corticosterone ^{125}I RIA Kit for rodents (MP Biomedicals, Orangeburg, New York, USA) was used according to the manufacturer's instructions. Per sample 5 μL plasma was diluted in steroid diluents (1:100 for pre-CO_2 and 1:200 for post-CO_2 samples). Fifty μL of

corticosterone ^{125}I and 100 µL anti-corticosterone were added per 50 µL of diluted sample, after which the tubes were incubated for 2 h at room temperature. Then, 250 µL precipitant solution was added to each sample and subsequently centrifuged at 2300 rpm for 15 min. After removing the supernatant the precipitation was counted in a Wizard gamma counter 2470 (PerkinElmer, Waltham, Massachusetts, USA).

Cardio-respiratory response

In a next step, separate groups of WT and 5-HTT +/– knockout mice (background C57BL/6, $n=20$/genotype, all male) were tested to assess the cardio-respiratory response to CO_2. 5-HTT +/– mice might be considered the best model for the human 5-HTTLPR, because the S-allele is associated with a reduced expression of 5-HTT and not a complete deficiency as seen in 5-HTT –/– mice. Mice were housed in pairs of the same genotype within a temperature-controlled environment ($25\pm1°C$) and under reversed day/night cycle (12 h light/12 h dark cycle). Animals had access to standard rodent chow and water *ad libitum*. All procedures were approved by the Institutional Animal Care and Use Committee at Yale University, New Haven, Connecticut, USA. All assessments were made under low-light conditions during the dark phase. Mice were weighted on testing days. Breathing and heart rate were recorded simultaneously in a custom-made whole-body Plexiglas chamber (350 cm³). After habituation to the chamber for 30 min (with air infusion, all tanks from Airgas, Cheshire, Connecticut, USA), mice were exposed to air for 20 min, followed by 9% CO_2 for 10 min. Flow rates were controlled using a digital flowmeter (0.4 L/min; WU-32446-33, Cole-Parmer, Inc., Hoffman Estates, Illinois, USA). Breathing was measured using a low pressure transducer (DC002NDR5; Honeywell International, Inc.; Minneapolis, USA) that was fitted to the recording chamber. Pressure oscillations induced by breathing were calibrated with 150 pulses/min (300 µL). Heart rate was measured non-invasively using two electrodes placed on the sides of the shaved thorax. The electrodes were connected to an amplifier (Model 440 Instrumentation Amplifier, Brownlee Precision Co., San Jose, California, USA). Ambient temperature (BAT-12 microprobe, Physiotemp Instruments, Inc., Clifton, New Jersey, USA) and relative humidity (HIH-4602-A sensor; Honeywell International, Inc., Minneapolis, Minnesota, USA) within the chamber were recorded throughout the entire experiment. Immediately after the experiment, the animal's temperature was measured rectally (BAT-12 microprobe, Physiotemp Instruments, Inc., Clifton, New Jersey, USA). Signals were digitized (PCI-6221 or USB-6008 National Instruments Corp., Austin, Texas, USA) and displayed in Matlab (version R2011b, Mathworks Co., Natick, Massachusetts, USA) with a custom-written acquisition program.

Statistical analysis

All analyses were done using the Statistical Package for the Social Sciences (SPSS 25, Inc., Chicago, Illinois, USA). Statistical significance was considered as $p<0.05$. Data are presented as mean + standard error of the mean (SEM). Behavioral data were analyzed using 3×2-way analysis of variance (ANOVA) to assess interaction effects of genotype (WT, 5-HTT +/– or 5-HTT –/–) and exposure (air or CO_2), which were

followed by separate ANOVA and *post-hoc* Bonferroni tests. For the TCT, additionally univariate analyses were used to assess genotype effects in the CO_2 or air chamber (separate analyses). Corticosterone levels were ln-transformed before being analyzed using repeated measures ANOVA and separate ANOVAs for distinct time points. Physiological data of WT mice was published previously (Leibold et al., 2016), and here analyzed using repeated measures ANOVA on ln-transformed values. About 400 breathing-induced pressure changes of the second half of each gas exposure were analyzed. Artifacts due to moving, coughing, sniffing, and sighs were excluded. Animal temperature and body weight were considered to calculate tidal volume (amplitude) and ventilation. Heart rate was analyzed in terms of 30 s of the second half of each gas exposure and using the quick peaks gadget in Origin 9.0 (Origin Lab Corp., USA).

Results

Fear-related behavior

Modified OFT. In the modified OFT, significant overall effects of genotype ($F_{(2,78)}=14.081$, $p<0.001$), exposure ($F_{(1,78)}=552.442$, $p<0.001$), and a genotype × exposure interaction ($F_{(2,78)}=18.996$, $p<0.001$) were observed on total distance moved (Figure 2(a)). *Post-hoc* analysis showed that within all three genotypes, CO_2 exposure strongly reduced the distance covered compared to air exposure ($p<0.001$). In addition, when exposed to air, 5-HTT –/– mice covered less distance in comparison with both WT and 5-HTT +/– mice (both $p<0.001$). This effect was not present during CO_2 exposure (both $p=1.000$).

With regard to time spent in center (Figure 2(b)), there was no overall genotype effect ($F_{(2,78)}=1.953$, $p=0.149$), but a significant exposure effect ($F_{(1,78)}=1.953$, $p=0.008$) and a genotype × exposure interaction ($F_{(2,78)}=4.731$, $p=0.011$). *Post-hoc* follow-up revealed that CO_2 exposure decreased the time spent in the center zone within WT mice only ($p=0.003$). Furthermore, the duration spent was shorter in 5-HTT –/– air-exposed mice compared to WT air-exposed mice ($p=0.028$), indicative of relatively more fear-related behavior in 5-HTT –/– mice. However, under CO_2 exposure, 5-HTT –/– and 5-HTT +/– spent a comparable amount of time in the center as they did during air exposure (both $p=1.000$), while WT mice spent less time in the center during CO_2 exposure ($p=0.003$). Comparing 5-HTT –/– and 5-HTT +/– mice with WT mice during CO_2 exposure did not lead to a statistically relevant difference (both $p=1.000$).

Moreover, regarding freezing (Figure 2(c)), overall analysis showed that there was no main effect of genotype ($F_{(2,78)}=0.069$, $p=0.933$), while CO_2 exposure was associated with alterations in freezing behavior ($F_{(1,78)}=88.88$, $p<0.001$). The interaction genotype × exposure was not significant ($F_{(2,78)}=1.503$, $p=0.229$). When exposed to CO_2, mice showed a robust freezing response in comparison with air exposure (WT and 5-HTT +/–: $p<0.001$, 5-HTT –/–: $p=0.002$), reflecting fear-related behavior.

TCT. In the TCT, with one chamber filled with CO_2 and one with air, or both filled with air, overall effects of genotype ($F_{(2,78)}=5.647$, $p=0.005$), exposure ($F_{(1,78)}=319.772$, $p<0.001$) and an interaction of genotype × exposure ($F_{(2,78)}=3.266$, $p=0.043$) on total distance moved were present (Figure 3(a)). *Post-hoc* comparisons showed that in line with the
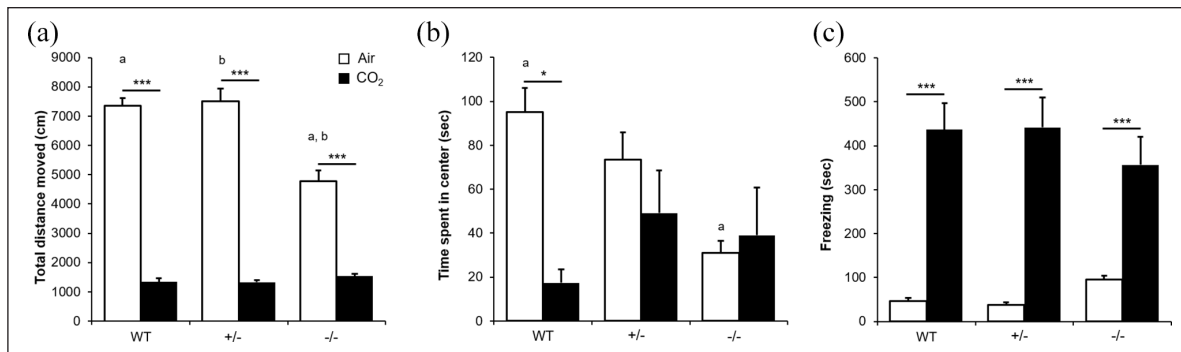
Figure 2. Assessment of the behavioral performance in the modified open field test. (a) Under air exposure serotonin transporter (5-HTT) –/– mice covered less distance than mice of other genotypes. Exposure to carbon dioxide (CO_2) significantly reduced the total distance moved in all genotypes. (b) During air exposure 5-HTT –/– mice spent less time in the center zone in comparison with wildtype (WT) mice. When exposed to CO_2, only WT mice spent less time in the center compared to air exposure, no difference was found in 5-HTT +/– and 5-HTT –/– mice. (c) In all genotypes, CO_2 exposure resulted in a robust freezing response compared to air exposure. Bars represent mean + standard error of the mean (SEM). +/–: heterozygous 5-HTT knockout mice; –/–: homozygous 5-HTT knockout mice. *$p < 0.05$, ***$p < 0.001$; same letters indicate a group difference of $p < 0.05$.
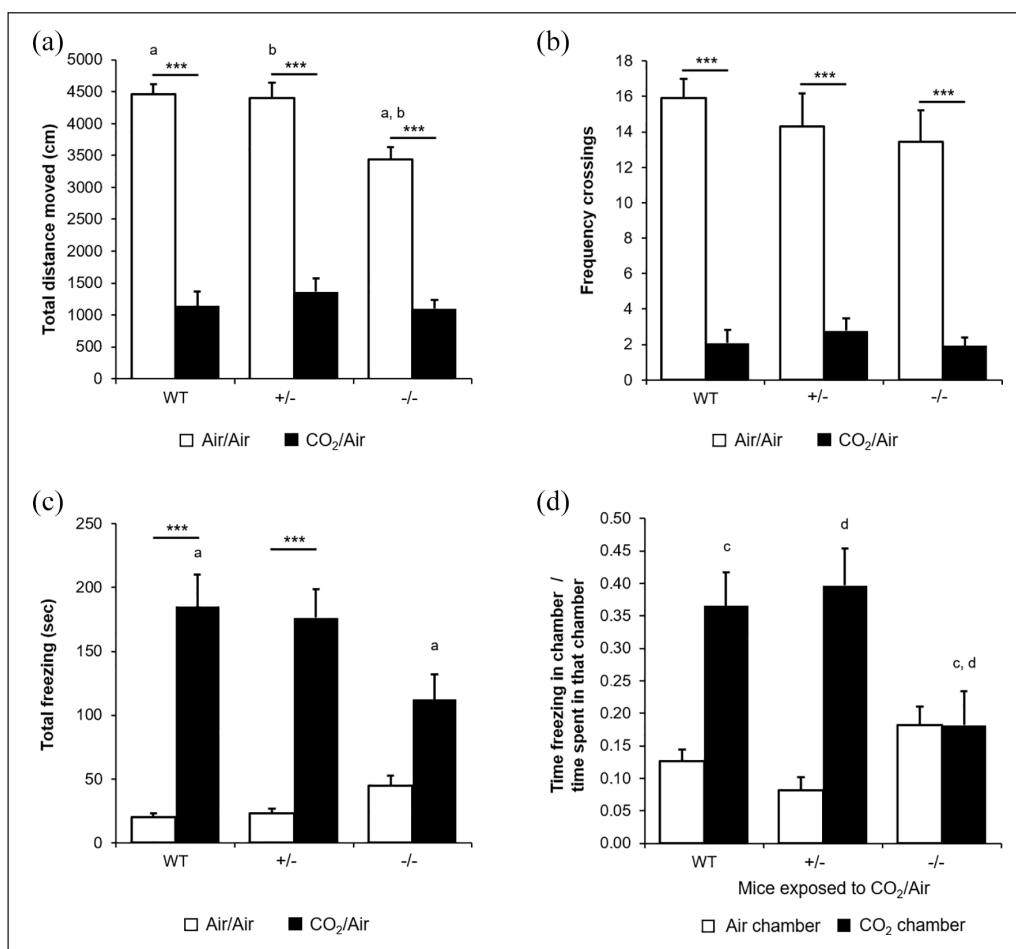
Figure 3. Assessment of the behavioral performance in the two-chamber test. (a) The total distance moved was strongly reduced under carbon dioxide (CO_2) exposure in all genotypes. Serotonin transporter (5-HTT) –/– mice covered less distance than wildtype (WT) and 5-HTT +/– mice during air exposure. (b) Under CO_2 exposure the number of crossings was significantly lower than during air exposure. (c) When exposed to air, no genotype differences were found. When exposed to CO_2, a marked freezing response was observed only in WT and 5-HTT +/– mice compared to air-exposure. 5-HTT –/– mice froze less to CO_2 than WT mice. (d) The ratio of time spent and freezing per chamber indicated that WT and 5-HTT +/– mice froze longer in the chamber filled with CO_2 than 5-HTT –/– mice. No effect was found within the chamber filled with air. Bars indicate mean + standard error of the mean (SEM). +/–: heterozygous 5-HTT knockout mice; –/–: homozygous 5-HTT knockout mice. ***$p < 0.001$; a, b, d same letters indicate a group difference of $p < 0.05$; c, same letters indicate a group difference of 0.05.

modified OFT, when exposed to CO_2, the distance covered was significantly reduced in all genotypes when compared to air exposure ($p < 0.001$). Again, under air exposure, 5-HTT –/– mice travelled less than WT mice ($p = 0.007$) and 5-HTT +/– mice ($p = 0.015$), a difference that was absent when exposed to CO_2 (both $p = 1.000$).

Further, genotype did not significantly affect the number of crossings between the chambers ($F_{(2,78)} = 0.587$, $p = 0.559$; Figure 3(b)). There was an overall exposure effect ($F_{(1,78)} = 147.743$, $p < 0.001$), but not a genotype × exposure interaction ($F_{(2,78)} = 92.967$, $p = 0.549$). Within all genotypes, the number of crossings was reduced under CO_2 exposure compared to air exposure ($p < 0.001$). Regarding time spent in each chamber, when both chambers were filled with air, there was no overall effect of genotype (e.g. left chamber $p = 0.653$). Likewise, when one chamber was filled with CO_2, there was no genotype difference in the duration spent in the CO_2 chamber ($F_{(2,39)} = 1.425$, $p = 0.253$). As in the modified OFT, overall, there was no genotype effect ($F_{(2,789)} = 1.272$, $p = 0.286$), but a significant exposure effect ($F_{(1,78)} = 92.967$, $p < 0.001$). Moreover, we observed a genotype × exposure interaction ($F_{(2,78)} = 5.382$, $p = 0.006$) on the total freezing duration (Figure 3(c)). Post-hoc analyses showed that within genotype, both WT and 5-HTT +/– mice froze significantly more under CO_2 exposure than when exposed to air ($p < 0.001$), which was not the case for 5-HTT –/– mice ($p = 0.072$). Between genotypes, 5-HTT –/– mice froze less to CO_2 than WT mice ($p = 0.034$).

Evidently, the freezing duration influences the time spent in a particular chamber. To correct for this, for mice exposed to CO_2 in one chamber and to air in the other chamber a ratio of time spent freezing relative to the total time spent in that particular chamber was calculated (Figure 3(d)). Within the CO_2 chamber, an overall effect of genotype was found ($F_{(2,37)} = 4.623$, $p = 0.016$). *Post-hoc* analysis revealed that the freezing/time spent ratio tended to be lower in 5-HTT –/– mice compared to WT ($p = 0.067$) and was significantly lower than in 5-HTT +/– mice ($p = 0.022$). No overall genotype effect was found in the other chamber, i.e. the one filled with air ($F_{(2,30)} = 0.776$, $p = 0.469$).

CO_2-induced plasma corticosterone secretion

Repeated measures ANOVA on plasma corticosterone levels showed a significant effect ($F_{(1,78)} = 334.286$, $p < 0.001$) of increased levels after CO_2 exposure for 20 min, but no time × genotype interaction ($F_{(2,78)} = 2.232$, $p = 0.114$) (Figure 4). Analysis of the distinct time points showed no genotype differences in corticosterone levels (pre-CO_2: $F_{(2,78)} = 1.423$, $p = 0.247$; stress: $F_{(2,78)} = 1.841$, $p = 0.166$). However, there was a significant effect of previous CO_2 exposure in the behavioral tasks on pre-CO_2 ($F_{(1,78)} = 5.707$, $p = 0.019$) and post-CO_2 corticosterone levels ($F_{(1,78)} = 17.929$, $p < 0.001$), with higher levels when previously exposed to CO_2.

Breathing and heart rate recordings

In a next step, the cardio-respiratory response was assessed in WT and 5-HTT +/– mice only, i.e. excluding 5-HTT –/– mice, because these genotypes might represent the best model for the human 5-HTTLPR that is associated with a reduced and not complete deficiency of 5-HTT, and because re-analyzing the behavioral
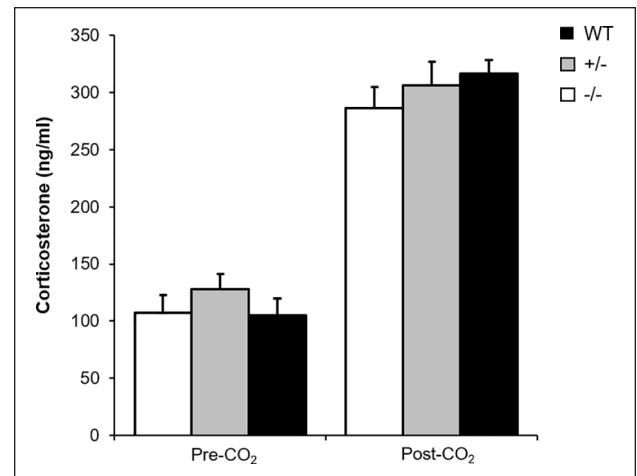

Figure 4. Plasma corticosterone secretion before and after exposure to 10% carbon dioxide (CO_2) for 20 min. CO_2 exposure strongly increased corticosterone levels in mice of all genotypes (exposure effect $p = 0.002$). Bars represent mean + standard error of the mean (SEM). +/–: heterozygous 5-HTT knockout mice; –/–: homozygous 5-HTT knockout mice; 5-HTT: serotonin transporter; WT: wildtype mice.

response of WT and 5-HTT +/– mice retained a significant genotype × exposure interaction ($F_{(1,52)} = 4.156$, $p = 0.047$) on the time spent in the center zone of the modified OFT. WT animals spent less time in the center when exposed to CO_2 compared to air-exposure ($p = 0.001$), while there was no difference in 5-HTT +/– animals ($p = 1.000$).

Analyzing breathing frequency, repeated measures analysis showed no main effect of genotype ($F_{(1,37)} = 1.887$, $p = 0.178$) or genotype × exposure interaction ($F_{(1,37)} = 1.224$, $p = 0.276$) (Figure 5(a)). However, a strong main effect of exposure was observed ($F_{(1,37)} = 123.192$, $p < 0.001$), with increased breathing frequency during CO_2 compared to air exposure. Similarly, overall, tidal volume was not affected by genotype ($F_{(1,37)} = 0.245$, $p = 0.623$), but by exposure ($F_{(1,37)} = 392.665$, $p < 0.001$) and a tendency towards an interaction of both ($F_{(1,37)} = 3.563$, $p = 0.067$). CO_2 appeared to increase tidal volume in comparison with air exposure (see Table 1 for mean + SEM). Furthermore, there was no overall effect of genotype ($F_{(1,37)} = 0.158$, $p = 0.693$) and no genotype × exposure interaction ($F_{(1,37)} = 2.661$, $p = 0.111$) on ventilation. However, a main effect of exposure was present $F_{(1,37)} = 340.193$, $p < 0.001$, with increased ventilation when exposed to CO_2 compared to air. With regard to heart rate, there was no genotype effect ($F_{(1,36)} = 0.508$, $p = 0.481$), but overall, CO_2 exposure decreased the mean heart rate compared to air exposure ($F_{(1,36)} = 54.219$, $p < 0.001$) (Figure 5(b)). No interaction of genotype × exposure was observed ($F_{(1,36)} = 2.241$, $p = 0.143$).

Discussion

As follow-up to our previous study in humans showing a differential self-reported fear response to CO_2 depending on the 5-HTTLPR genotype (Schruers et al., 2011), we further investigated the relationship between chemosensitivity and the 5-HT system by exposing WT, 5-HTT +/–, and 5-HTT –/– mice to
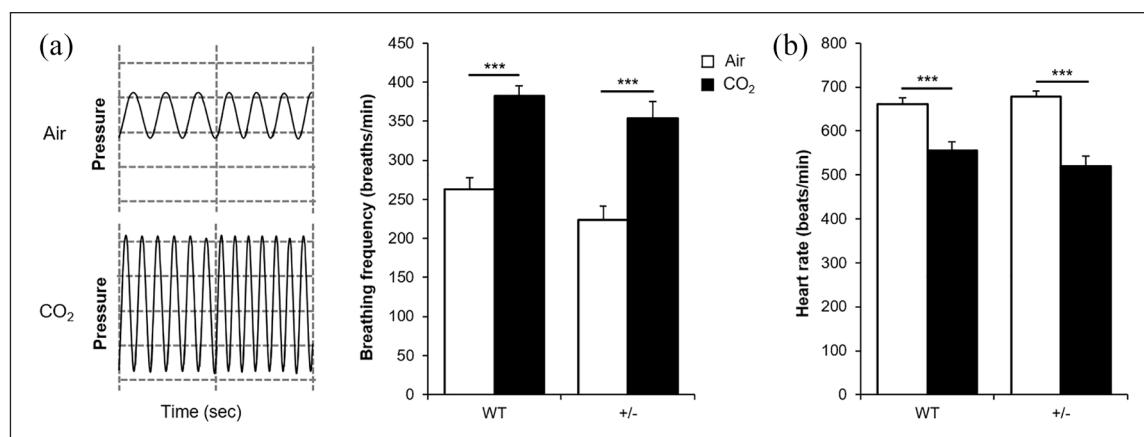
Figure 5. Respiratory and cardiovascular monitoring during exposure to air and carbon dioxide (CO_2). (a) Schematic representation of a 2 s epoch of pressure-induced changes to assess breathing frequency and tidal volume (amplitude) during inhaling air (top) and CO_2 (bottom). CO_2 strongly increased breathing frequency in comparison with air exposure. (b) Heart rate decreased during inhaling CO_2. No genotype effect was present in any outcome measurement. Bars represent mean + standard error of the mean (SEM). +/–: heterozygous 5-HTT knockout mice; 5-HTT: serotonin transporter; WT: wildtype mice. ***$p < 0.001$.

Table 1. Carbon dioxide (CO_2)-induced changes in respiratory and cardiovascular measurements.

Genotype	Breathing frequency (breaths/min)		Tidal volume (mL/g)		Ventilation (mL/min/g)		Heart rate (beats/min)	
	Air	CO_2	Air	CO_2	Air	CO_2	Air	CO_2
WT	263 (15)	382 (14)	0.06 (<0.01)	0.10 (0.01)	13.37 (1.29)	37.97 (2.63)	661 (13)	554 (20)
5-HTT +/–	224 (17)	354 (21)	0.05 (<0.01)	0.11 (0.01)	11.64 (0.94)	40.70 (2.82)	677 (12)	520 (23)

5-HTT: serotonin transporter; 5-HTT +/–: heterozygous 5-HTT knockout mice; WT: wildtype mice.
Values represent mean (standard error of the mean (SEM)).

CO_2. In line with our previous studies in humans (Leibold et al., 2016; Schruers et al., 2011) and mice (Leibold et al., 2016), CO_2 induced robust fear-related behaviors and strongly activated the respiratory system. However, in contrast to our hypothesis, there were only minor effects of differential 5-HTT expression on behavior.

In humans, the use of CO_2 as experimental panic-stimulus is well established in PD patients and, when using a higher dosage, also in healthy individuals (for review see Leibold et al., 2015). This is suggestive of the involvement of fundamental neurobiological mechanisms. Inhaling air enriched with CO_2 is associated with an acute drop in brain pH (Schuchmann et al., 2006; Ziemann et al., 2008, 2009) that can trigger various adaptive responses, as such a pH drop can have life-threatening consequences. In this study, we investigated the responses to CO_2 by using two behavioral tasks (modified OFT and TCT) and by assessing cardio-respiration. Generally, the OFT is considered an anxiety test, in which avoidance of the center zone (open zone) is one of the main outcome measurements. It has to be mentioned that anxiety is distinct from fear/panic and that different brain structures and behaviors are involved (Blanchard and Blanchard, 1990). Anxiety predominates when the distance to a threat is large, i.e. when there is a *potential* threat. This is associated with risk assessment and approach behavior. Fear dominates when the distance to a threat is small, i.e. when there is a *direct* threat. This leads to primitive survival responses. Conceptually, prefilling the OFT testing box with CO_2 that reduces the pH within the body

and thus causes a threat from the smallest distance possible, namely from within body, can be considered to elicit a state of fear/panic rather than anxiety. Further support for this notion is provided by our previous study in mice, in which we showed that that exposure to a low concentration of CO_2 in rodents leads to cardio-respiratory effects corresponding to the ones evoked by a 35% CO_2 inhalation in humans (Leibold et al., 2016). In the TCT, with one chamber being filled with CO_2 and one with air, avoidance to an aversive stimulus is tested. It is assumed that mice spend more time in the chamber filled with air to avoid the effects of CO_2 (Ziemann et al., 2009).

Examining the effects of CO_2 in these tasks showed that CO_2 induced a robust fear-related behavioral response in mice, in line with previous reports (Johnson et al., 2011, 2012; Leibold et al., 2016; Ziemann et al., 2009). However, unexpectedly, there were only minor effects of 5-HTT genotype. More specifically, total distance moved was strongly decreased in both tasks, independent of genotype. Likewise, the total duration of freezing, a correlate of fear and panic in mice (Mongeluzi et al., 2003; Ziemann et al., 2009), in the modified OFT and the number of crossings between compartments in the TCT were similarly affected in all genotypes. Exceptions are the duration spent in the center zone of the modified OFT, an outcome measurement that is relatively independent on locomotor activity, and freezing in the TCT. With regard to the duration spent in the center zone of the modified OFT, 5-HTT –/– mice show no decrease in time spent in the center zone during CO_2 exposure compared to air exposure. This

suggests an altered threat perception and/or altered coping strategy in mice carrying the full knockout. For instance, the fact that 5-HTT –/– mice already spent very little time in the center during air exposure could represent heightened aversion to the OFT itself in this genotype, leading to a ceiling effect. In the TCT, however, 5-HTT –/– mice spent less time freezing over the total time spent in the aversive compartment. This, again, could indicate that mice carrying the full knockout perceive CO_2 differently or have an altered coping strategy. Furthermore, it cannot be excluded that the TCT is less aversive in comparison to the OFT, as the compartments are smaller. These observations are in line with previous work showing, e.g., that mice fully deficient of 5-HTT performed worse in the more stressful water maze test compared to WT and 5-HTT +/– littermates, while no differences were observed in Barnes maze (Karabeg et al., 2013).

Next to direct effects on animal behavior, we aimed to measure avoidance of CO_2 as an aversive stimulus. In the TCT, animals had the option to leave the compartment filled with CO_2. Unexpectedly, we did not observe any avoidance, in contrast to previous studies in mice, in which WT mice spent most of the time in the chamber filled with air (D'Amato et al., 2011; Ziemann et al., 2009). This discrepancy can be explained by the immense freezing response that we observed in the present study when CO_2 was present, which seems to have prevented the animals from leaving the CO_2 chamber. This notion is supported by the strongly reduced crossing frequency between both chambers in the presence of CO_2 Ziemann et al. (2009) used 15% CO_2 in this task in their previous study. In our study, a steady state of 9% CO_2 was reached, which is lower than the previously used concentration of 15% CO_2. In addition, care was taken to use an optimized methodological approach, including fans to homogenize the air, continuous measurement of the CO_2 concentration, and a randomization regarding side of CO_2 administration and side, in which mice were placed at the beginning of the test. Moreover, the chambers of our set-up were bigger compared to the set-up used by Ziemann et al. The combination of a larger box in our study and CO_2 may have been a particularly aversive stimulus. Possibly, this led to a ceiling effect and prevented finding any subtle genotype differences under CO_2 exposure. CO_2 exposure is stressful in nature as shown by the observed increase in plasma corticosterone, which is in line with previous reports (D'Amato et al., 2011). This effect of CO_2 is independent of genotype and suggests that differences in behavioral performance are not primarily dependent on HPA axis reactivity.

In addition, to studying the behavioral effects of CO_2 exposure, also the effects on the cardio-respiratory system were explored. CO_2 strongly activated the respiratory system with an increase in breathing frequency and tidal volume; heart rate decreased in comparison to air exposure. These results are consistent with those previously reported in, for example, freely moving rats (Annerbrink et al., 2003) and phrenic nerve recordings of anesthetized rats under CO_2 exposure (Dumont et al., 2011). In the present study, effects were independent of genotype. To the best of our knowledge, little research has been done regarding examining the respiratory response to CO_2 in mice deficient for 5-HTT or in humans related to the 5-HTTLPR genotype. Li and Nattie (2008) observed in their study that 5-HTT –/– males had a smaller increase in ventilation to CO_2 than WT mice. 5-HTT +/– mice were not tested. The lack of a difference between WT and 5-HTT +/– mice in our study might be explained by 5-HT neurons sufficiently acting as chemosensors

and initiating an adaptive response (Richerson, 2004), independent of 5-HT availability, or by a functionally insufficient difference in extracellular 5-HT availability to exert effects in this regard.

In addition to the potential explanations above that could contribute to the lack of clear differential 5-HTT expression effects in our study, another possibility is that several genetic and environmental vulnerability factors have to interact to lead to an altered functioning in the brain and a phenotype of heightened CO_2-sensitivity. For instance, likewise to 5-HT neurons, many other neurons including noradrenergic neurons in the locus coeruleus (LC) are activated by CO_2/pH (Biancardi et al., 2008; Filosa et al., 2002; Pineda and Aghajanian, 1997). The LC receives major input from the medullary nucleus paragigantocellularis, a key region in cardio-respiratory regulation (Aston-Jones et al., 1986), and was shown to modulate the respiratory response to CO_2 (Biancardi et al., 2008). Furthermore, LC neurons are involved in the behavioral response to stress (Stanford, 1995) and by projecting to nuclei of the limbic system these neurons likely mediate fear. Based on basic research findings like these and other research, it has been suggested that the central noradrenergic system, particularly the LC, is the common site for mediating the increases in fear and physiology to a 35% CO_2 inhalation in humans (Bailey et al., 2003). It is noteworthy that the LC and the dorsal raphe nucleus are connected by extensive reciprocal connections (Kim et al., 2004). CO_2 was shown to stimulate the release of 5-HT in the LC. By exerting inhibitory effects, 5-HT reduces the stimulatory effects of the LC on breathing in response to CO_2 (de Souza Moreno et al., 2010). Contrary to this effect, in general, projections from the LC have excitatory effects on dorsal raphe nucleus neurons (Brown et al., 2002). These connections make it interesting to investigate, e.g. pharmacologically, whether disturbances in both systems might interact to express a phenotype of heightened CO_2-sensitivity. While it is known that both 5-HT and noradrenergic neurons are sensitive to pH/CO_2, it is unknown which neuronal molecule senses these changes and an alteration in that molecule could also contribute to a higher sensitivity to CO_2. An interesting candidate is the acid-sensing ion channel 1a (ASIC1a). Genetic knockout of ASIC1a significantly blunts the CO_2-induced behavioral effects (Ziemann et al., 2009) and antagonism normalizes the hyperreactivity to CO_2 that is seen in a specific mouse model (Battaglia et al., 2019). Further, in addition to genetic factors, also their interaction with environmental stimuli shape behavioral phenotypes. For example, early life adversities such as maternal separation and repeated cross-fostering have been shown to increase the sensitivity to CO_2 in rodents (Battaglia et al., 2019; D'Amato et al., 2011). Therefore, interplays of genetic vulnerabilities and environmental stressors could provide important insights for understanding the pathophysiology in more depth.

Some potential limitations of the present study should be kept in mind: As only male animals were tested no potential sex effects were examined. In humans, a higher prevalence of PD is reported in females (Dick et al., 1994; Gater et al., 1998), while CO_2 studies did not consistently find this effect (Kelly et al., 2006; Leibold et al., 2013; Nardi et al., 2007; Nillni et al., 2012; Poonai et al., 2000). Therefore, more research into the effect of sex and menstrual cycle phase is warranted. Further, cardio-respiration was not assessed in 5-HTT –/– mice. As mice of this genotype were affected differentially in the behavioral tasks, physiological alterations associated with CO_2 exposure should also be assessed in this genotype in future studies.

Conclusion

Overall, under CO_2 exposure there was no effect of differential 5-HTT expression on physiological parameters and only minor interaction effects on behavior. Future research is needed that also addresses the interplays with other systems. These insights could contribute to a better understanding of the pathophysiology underlying CO_2-sensitivity and eventually to develop better treatments.

Declaration of conflicting interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: KPL served as a speaker for Eli Lilly and received research support from Medice, and travel support from Shire, all outside the submitted work. Other authors declare no conflict of interest.

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