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Panic-like escape response elicited in mice by exposure to CO_2, but not hypoxia

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ABSTRACT

Exposure to elevated concentrations of CO_2 or hypoxia has been widely used in psychiatric research as a panic provoking stimulus. However, the use of these respiratory challenges to model panic-like responses in experimental animals has been less straightforward. Little data is available, from behavioral and endocrine perspectives, to support the conclusion that a marked aversive situation, such as that experienced during panic attacks, was evoked in these animals. We here compared the behavioral responses of male CB57BL/6 mice during exposure to 20% CO_2 or 7% O_2 and its consequence on plasma levels of corticosterone. We also evaluated whether clinically-effective panicolytic drugs affect the behavioral responses expressed during CO_2 exposure. The results showed that whereas hypoxia caused a marked reduction in locomotion, inhalation of CO_2-enriched air evoked an active escape response, characterized by bouts of upward leaps directed to the border of the experimental cage, interpreted as escape attempts. Corticosterone levels were increased 30 min after either of the respiratory challenges used, but it was higher in the hypoxia group. Chronic (21 days), but not acute, treatment with fluoxetine or imipramine (5, 10 or 15 mg/kg) or a single injection of alprazolam (0.025, 0.05 or 0.1 mg/kg), but not of the anxiolytic diazepam (0.025, 0.05 or 0.1 and 1 mg/kg) reduced the number of escape attempts, indicating a panicolytic-like effect. Altogether, the results suggest that whereas hypoxia increased anxiety, exposure to 20% CO_2 evoked a panic-like state. The latter condition/test protocol seems to be a simple and validated model for studying in mice pathophysiological mechanisms and the screening of novel drugs for panic disorder.

1. Introduction

Panic attack is an abrupt surge of intense distress, marked by fear, feeling of impending death, and accompanied by cardiorespiratory symptoms, such as sensation of breathlessness, tachycardia and chest pain, which reaches a peak within minutes. The recurrence of unexpected panic attacks combined with persistent anxiety or significant changes in behavior over having further attacks are the criteria for the diagnosis of panic disorder (American Psychiatric Association, 2013).

Although the pathophysiological mechanisms of panic disorder remain unclear, evidence amassed in the last three decades suggests a potential connection between panic disorder and respiratory disturbances (Goodwin et al., 2010; Hasler et al., 2005; Perna et al., 1997; Pollack et al., 1996; Porzelius et al., 1992; Pothirat et al., 2015; Vögele and von Leupoldt, 2008). For example, the prevalence of panic disorder is significantly higher in asthmatics (Goodwin et al., 2010; Hasler et al., 2005; Perna et al., 1997) and in people that have experienced traumatizing suffocation events (Bouwer and Stein, 1997). Equally important, panic attacks can be triggered by respiratory challenges such as breath-holding (Nardi et al., 2003, 2006), hypoxia (Beck et al., 1999, 2000) or inhalation of CO_2-enriched air (Drury, 1918; Gorman et al., 1994).

The association between CO_2 and panic is central to one of the most prominent theories on the etiology of panic attacks, which suggests that panic patients are hypersensitive to this gas. More specifically, Klein (1993) stated that “spontaneous panic attack occurs when the brain's suffocation monitor erroneously signals a lack of useful air, thereby maladaptatively triggering an evolved suffocation alarm”.

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Since suffocation can result from either an increase in CO_2 or a decrease in O_2 partial pressures, distinct respiratory challenge protocols have been used in clinical trials as effective and safe stimuli to provoke panic attack symptoms. For example, Woods et al. (1988) showed that breathing 5% CO_2-enriched air precipitates autonomic and psychological symptoms that resemble naturally occurring panic attacks, such as palpitations, dyspnea, sensation of strong fear, chest pain, breathlessness and dizziness. Similar symptoms have also been triggered by either one or two vital capacity breaths of 35% CO_2-enriched air (Griez et al., 1987; Nardi et al., 2006; Schruers et al., 2004). It is noteworthy that although panic attacks are effectively precipitated by CO_2 in both panic disorder patients and healthy volunteers, recent evidence obtained by Leibold et al. (2016) suggests that the former have a stronger emotional response. Regarding hypoxia, Beck et al. (1999) reported that in panic disorder patients exposure to 12% O_2 causes panic-associated respiratory changes and increase in anxiety similarly as observed after inhalation of 5% CO_2.

The use of respiratory challenges to model panic attacks in experimental animals has been less straightforward, and the results obtained may frequently raise doubts that a panic-like state was indeed evoked in these non-human subjects. Broadly speaking, in these analyses, conducted basically in rats and mice, different parameters, mostly autonomic indexes (i.e., arterial blood pressure and heart and respiratory rates) have been used to infer that an extreme fear response, and hence a panic-like state, was evoked (Borkowski et al., 2011; Cittaro et al., 2016; Dumont et al., 2011; Kinkead et al., 2009; Luchetti et al., 2015).

The analysis of cardio-respiratory changes induced by CO_2 inhalation or hypoxia has, in some cases, also been accompanied by investigation of the behavioral consequences caused by these challenges. Curiously, however, this has been habitually made after, and not during, exposure to respiratory challenges by using exploratory-based tests such as the open-field (Bonaventure et al., 2017; D'Amato et al., 2011; Johnson et al., 2015) and/or standard anxiety (e.g. the elevated plus-maze, social interaction or Vogel tests), but not panic-validated models (Bonaventure et al., 2017; Cuccheddu et al., 1995; Duszczyk et al., 2015; Hickman et al., 2016; Kiray et al., 2014; Nadlewska et al., 2002, 2003; Sherry et al., 2009).

Since the seminal ethoexperimental and pharmacological studies performed by Robert and Caroline Blanchard on the defensive repertoire of rats and mice to predators or cues associated with them (Blanchard et al., 2001; Blanchard and Blanchard, 1988), it has been increasingly recognized that escape or flight behaviors evoked by proximal threats are reliable indexes of panic attacks in non-human animals (Gray and McNaughton, 1983; McNaughton and Corr, 2004; Schenberg et al., 2001; Zangrossi and Graeff, 2014).

In spite of this being state-of-art for the process of modeling panic in animals, there have been few studies until now that systematically addressed how rats or mice behave during exposure to increasing concentrations of CO_2 or to decreasing levels of O_2. Concerning CO_2 inhalation, it has been shown that rats exposed to either 10% (Winter et al., 2017) or 20% (Johnson et al., 2005) CO_2 remain mostly immobile during the stimulus presentation. In mice, a decrease in locomotion has also been reported during exposure to 9–10% CO_2, an effect that seems to be related to a marked expression of freezing behavior (D'Amato et al., 2011; Leibold et al., 2016; Price et al., 2014; Taugher et al., 2014; Vollmer et al., 2016; Ziemann et al., 2009).

To the best of our knowledge, attempts to characterize the behavioral consequences of hypoxia have only been made in rats. Casanova and co-workers (2013) reported that Wistar rats exposed to decreasing levels of O_2 exhibit typical escape behavior. In particular, while exposure to 13% O_2 leads to immobility, decreasing O_2 levels to 6% precipitates running and rearing episodes. A similar pattern of escape responses was also described by Schimitel et al. (2012) as a consequence of KCN peripheral administration, which has been shown to induce cytotoxic hypoxia (Salkowski and Penney, 1994).

More recently, we extended the analyses of the translational value of the escape response expressed by rats during hypoxia for the study of panic attacks. We reported that upward jumps to the border of the experimental cage, interpreted as an escape response, were observed when O_2 concentration was decreased to around 7%. We also reported that this behavioral response was efficiently decreased by treatment with standard panicolytic drugs, such as chronic, but not acute, administration of the selective serotonin reuptake inhibitor fluoxetine or after acute injection of the high-potency benzodiazepine alprazolam (Spiacci et al., 2015).

In the current study, we focused our attention on the behavioral responses expressed by mice during exposure to 20% CO_2 or 7% O_2. Besides comparing the behavior of male C57BL/6J mice to these two respiratory challenges, we also evaluated whether plasma corticosterone levels were affected either immediately or 30 min after the test. There is evidence to suggest that under suffocation circumstances, the hypothalamo-pituitary-adrenal (HPA)-axis is inhibited in order to conserve energy, avoiding an increase in catabolic activity in which oxygen demands would be counterproductively triggered (Coplan et al., 2002; Sinha et al., 1999; van Duinen et al., 2004a, 2004b; Woods et al., 1988).

Given the results showing that exposure to a high concentration of CO_2, but not to hypoxia, evoked an active escape behavior, we next investigated whether treatment with standard panicolytic drugs, specifically fluoxetine, imipramine or alprazolam, or the anxiolytic diazepam interferes with this behavior.

2. Methods

2.1. Animals

Male C57BL/6 mice (University of Sao Paulo, Campus of Ribeirao Preto), 9–10 weeks old on the day of the experiment, were housed in groups of 4 per cage maintained under standard laboratory conditions (23 ± 1 °C and 12: 12 h light:dark cycle, lights on at 07:00), with food and water available ad libitum. All procedures were conducted in conformity with the guidelines of the Brazilian Council for the care and use of laboratory animals (COBEA), which comply with international laws and politics, and were approved by our local ethics committee (protocol number: 65/2017).

2.2. Drugs

Fluoxetine hydrochloride (EMS, Brazil), diazepam (Sigma, USA) and alprazolam (EMS, Brazil) were dissolved in a solution containing sterile saline with 2% Tween-80. Imipramine (Sigma, USA) was dissolved in sterile saline.

2.3. Apparatus

The experimental cage consisted of a cylindrical transparent Plexiglas chamber (18 cm diameter × 18 cm height), sealed by a removable cap. The chamber was connected through a flow valve to an air pump and to nitrogen (N_2) and carbon dioxide (CO_2) cylinders. The O_2 and CO_2 concentrations inside the chamber were continuously monitored using a gas analyzer (ML206 Gas Analyzer, AdInstruments, Australia). Since ML206 Gas Analyzer detects CO_2 levels at a maximum level of 10%, air sample from the chamber was collected with a syringe to analyze higher concentrations of this gas by a microelectrode (Microelectrodes, Inc., New Hampshire, USA). The recordings were saved and analyzed using the software PowerLab Chart 5 (AdInstruments, Australia).

2.4. Procedures

2.4.1. Experiment 1 – effects of hypoxia or CO_2 exposure on animal behavior and corticosterone plasma levels

One day before the behavioral tests, mice were placed in the

experimental cage for 12 min. During this habituation session, room air was flushed into the chamber at a flow rate of 1.5 L/min in order to familiarize the animals to gas flow and to the air jet sound, and to reduce neophobic reactions to the cage environment.

Twenty-four hours later, the animals were randomly allocated to the hypoxia (n = 11), CO_2-enriched air (n = 12) or control (exposure to room air, n = 12) groups. In the hypoxia group, the mice were placed into the chamber and acclimated to it under room air (21% O_2; 0.03% CO_2) for 5 min. For this, room air was flushed into the chamber at a flow rate of 1.5 L/min. Subsequently, pure N_2 was flushed into the chamber (flow rate of 1.5 L/min) in order to decrease oxygen concentration from 21% to 7% in 2 min. After reaching 7% O_2, N_2 infusion was suspended and this low O_2 concentration was maintained over the next 5 min. In the hypercarbic gas group, the mice were acclimated to the chamber under room air condition for 5 min, as previously mentioned. Then pure CO_2 was flushed (flow rate of 1.5 L/min) in order to elevate the CO_2 concentration from 0.03% to 20% in 2 min. After reaching 20%, CO_2 infusion was suspended and CO_2 concentration was maintained at this level over the next 5 min. In the control group, the animals were kept in the experimental cage under normoxic air for 12 min, with room air being flushed into the chamber at a flow rate of 1.5 L/min in the first 7 min of the test.

Immediately (n = 5–6) or 30 (n = 6 for each group) minutes after the test, the animals were decapitated and their trunk blood was collected in chilled heparinized tubes (10 μL of heparin per mL of blood). Plasma was obtained by centrifugation (3000 rpm for 15 min at 4 °C), and aliquots were stored at − 20 °C. Plasma corticosterone level was determined after ethanol extraction by a radioimmunoassay method, as previously described in detail by Ruginsk et al. (2013).

2.4.2. Experiment 2 - effects of acute or chronic antidepressant administration

In Experiment 2A, independent groups of mice (n = 8, for each group) were intraperitoneally injected with fluoxetine (5, 10, or 15 mg/kg) or vehicle, either acutely or daily throughout 21 days (n = 8). In chronic treated mice, on the 20th day, the injection was done immediately after the habituation session.

In Experiment 2B, independent groups of mice (n = 8 for each group) were intraperitoneally injected with imipramine (5, 10, or 15 mg/kg) or vehicle, either acutely or daily throughout 21 days. Similarly as in experiment 2A, in chronic treated animals, on the 20th day, the injections were made immediately after the habituation session.

In all these experiments, animals were submitted to the CO_2 challenge, as described in experiment 1, 30 min after the last injection.

2.4.3. Experiment 3 - effects of benzodiazepine administration

In experiment 3, independent groups of animals received a single intraperitoneal injection of alprazolam (0.025, 0.05 or 0.1 mg/kg; n = 6) or vehicle (n = 6; experiment 3A) or diazepam (0.025, 0.05, 0.1 or 1 mg/kg; n = 5–6) or vehicle solution (n = 6; experiment 3B) and were submitted to the CO_2 challenge, as described in experiment 1, 30 min after the injection.

In all these pharmacological analyses, the doses of drugs were selected based on previous studies with the mouse defense test battery and the elevated T maze (Griebel et al., 1995a, 1995b; Pulga et al., 2012).

The experimental sessions were recorded using a video camera connected to a DVD recorder. The distance traveled, the immobility time and the maximum speed achieved over continuous 1 min time bins were analyzed by a behavioral tracking system (ANY-maze; Stoelting Co, USA). The number of rearings and jumps over continuous 1 min time bins was scored by an investigator blinded to the assignment of treatment groups. Given the results of experiment 1, in experiments 2 and 3 only the total number of jumps occuring during CO_2 exposure and the total distance traveled under exposure to room air in the 5 min that preceded this respiratory challenge were measured. The latter parameter was used to infer whether the drugs caused non-specific effects on locomotion.

2.5. Statistical analysis

In experiment 1, repeated measures ANOVA was used to analyze the distance traveled, maximum speed, immobility time, number of rearings and jumps, with the type of respiratory challenge as the independent factor and time as the repeated measure. Corticosterone levels were analyzed by one-way ANOVA. Pearson analysis was performed in order to evaluate possible correlations between corticosterone levels and the behavioral responses measured during exposure to the respiratory challenges.

In experiments 2 and 3, one-way ANOVA was used to analyze the total number of jumps or the total distance traveled.

In all experiments, when appropriate, post-hoc comparisons were performed with Tukey's test. Statistical analyses were performed using the software SPSS Statistics 20.0 (IBM SPSS Statistical, USA). The level of significance was set at $p \leq 0.05$.

3. Results

3.1. Effects of respiratory challenges on animal behavior and corticosterone levels

Fig. 1 shows the behavioral effects caused in mice by exposure to respiratory challenges. It is noteworthy that none of the groups tested differed in the behavioral parameters analyzed with room air inhalation in the period that preceded exposure to the respiratory challenges.

Repeated-measures ANOVA revealed that during exposure to the respiratory challenges the number of jumps and rearings was significantly affected [$F(2,32) = 110.23$; $p < 0.001$ and $F(2,32) = 194.29$; $p < 0.001$, respectively]. There were significant time effects [$F(11,352) = 15.28$; $p < 0.001$ and $F(6192) = 44.11$; $p < 0.001$] and procedure by time interactions [$F(22,352) = 15.52$; $p < 0.001$ and $F(12,192) = 9.90$; $p < 0.001$]. As can be seen in Fig. 1A, CO_2 inhalation, but not hypoxia, evoked active escape behavior from the seventh to the twelfth minute of the test. This was characterized by bouts of upward leaps directed to the border of the chamber. Both hypoxia and CO_2 exposure decreased the number of rearings performed by the animals during the test (Fig. 1B).

Respiratory challenges also significantly affected the maximum speed [$F(2,32) = 4.91$; $p < 0.05$], the time spent immobile [$F(2,32) = 5.63$; $p < 0.01$] and the distance traveled [$F(2,32) = 4.39$; $p < 0.05$] by the animals. There were significant time effects [$F(6192) = 2.86$; $p = 0.05$; $F(6192) = 6.59$; $p < 0.001$ and $F(6192) = 2.25$; $p = 0.05$; respectively], but not procedure by time interactions [$F(12,192) = 1.22$; $F(12,192) = 1.04$ and $F(12,192) = 0.48$]. Tukey's *post-hoc* test revealed that whereas CO_2 exposure increased the maximum speed (Fig. 1C), hypoxia raised the time of immobility during the test (Fig. 1D), when compared to the control group. The distance traveled was higher in the CO_2 exposed group, when compared to animals submitted to hypoxia (Fig. 1E).

Regarding corticosterone levels, statistical analysis showed that the concentration of this hormone was significantly changed 30 min [$F(2,15) = 27.75$; $p < 0.001$; Fig. 1G], but not immediately [$F(2,14) = 0.88$; Fig. 1F], after exposure to the respiratory challenges. The *post-hoc* analysis showed that although both CO_2 exposure and hypoxia significantly increased corticosterone levels, this effect was even higher in the latter group.

Correlation analysis by Pearson's test revealed that corticosterone levels measured 30 min after the test were positively correlated with the total number of jumps expressed by the mice during the CO_2 challenge ($r = 0.79$, $p < 0.05$). Regarding hypoxia, whereas the total time spent in immobility tended to correlate positively ($r = 0.76$,

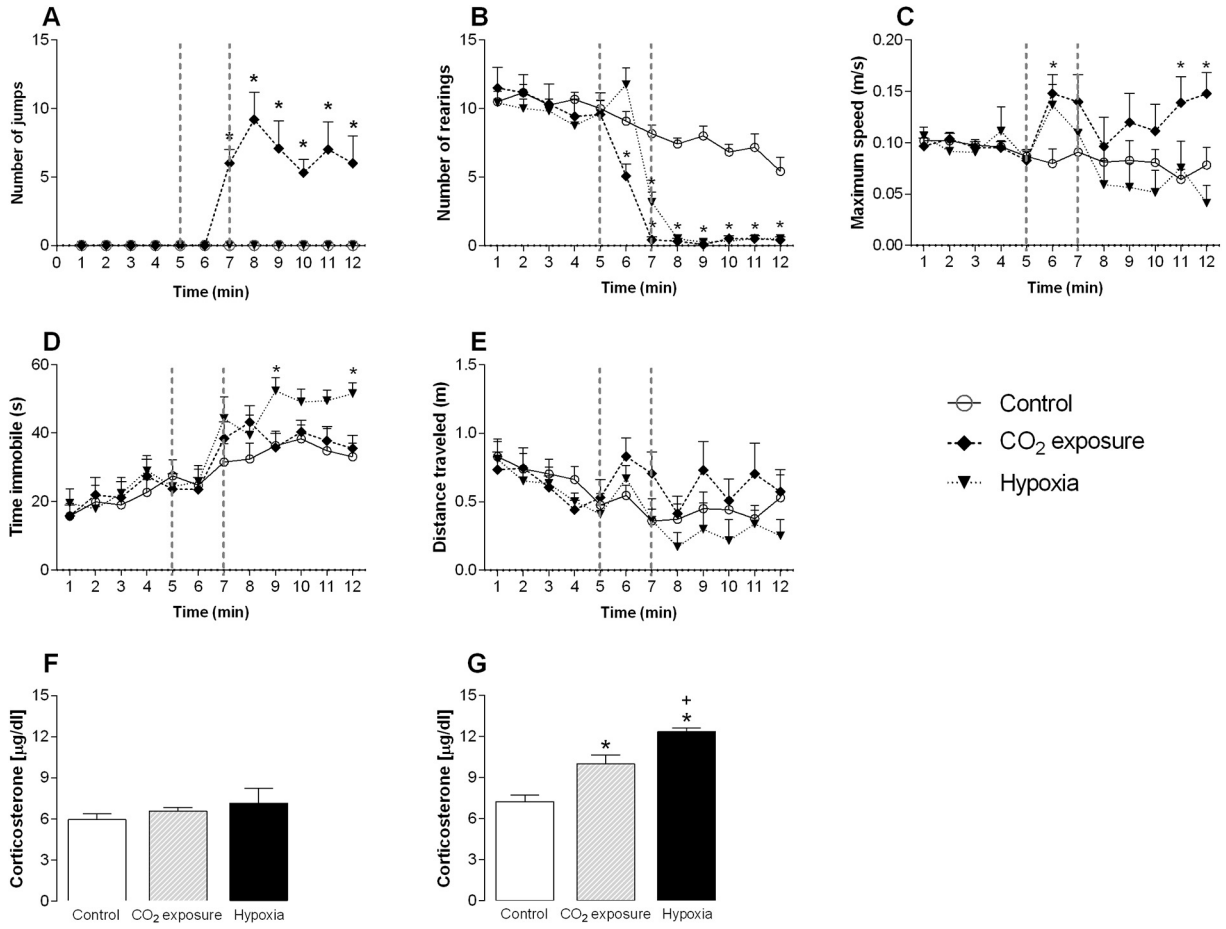

Fig. 1. Effects (mean ± SEM) of room air (control), hypoxia or CO_2 exposure on: (A) number of jumps, (B) number of rearings, (C) maximum speed, (D) time spent immobile and (E) distance traveled over continuous 1 min time bins. In (F) and (G) are shown the plasma corticosterone levels measured immediately or 30 min after exposure to these respiratory challenges, respectively. Between dashed lines are indicated the period when gas inflow(CO_2)/removal(O_2) was started and when the final concentration was reached. *$p < 0.05$ compared to the control group. $^{+}p < 0.05$ compared to the CO_2-exposed group.

$p = 0.08$) with the corticosterone concentration measured 30 min after the test, the number of rearings tended to correlate negatively ($r = -0.76$, $p = 0.08$) with this measure.

3.2. Effects of acute or chronic antidepressant administration

As can be seen in fig. 2, one-way ANOVA revealed that both acute and chronic systemic treatments with fluoxetine significantly changed the total number of jumps expressed during the CO_2 challenge [$F(3,22) = 3.97$; $p = 0.01$ and $F(3,22) = 4.73$; $p < 0.05$, respectively]. Whereas acute administration (upper panel) of fluoxetine (5 mg/kg) significantly increased the number of jumps, chronic injection of three doses of this antidepressant reduced this behavioral index (lower panel).

Regarding imipramine, as shown in Fig. 3, one-way ANOVA revealed that the total number of jumps was significantly affected by chronic [$F(3,26) = 3.63$; $p < 0.05$, lower panel], but not acute treatment [$F(3,21) = 0.61$; upper panel] with this drug. The *post-hoc* analysis showed that chronic administration of imipramine (15 mg/kg) significantly reduced the escape response during CO_2 exposure.

As shown in Table 1, neither acute nor chronic systemic treatment with fluoxetine or imipramine changed the locomotor activity evaluated before exposure to the respiratory challenge.

3.3. Effects of benzodiazepine administration

Acute systemic administration of alprazolam [$F(3,23) = 5.16$; $p < 0.01$], but not of diazepam [$F(4,26) = 1.43$] significantly interfered with jump expression (see Fig. 4). The *post-hoc* analysis showed that acute administration of alprazolam (0.1 mg/kg) significantly reduced the expression of this behavior.

As shown in Table 1, neither alprazolam nor diazepam affected locomotion before exposure to the respiratory challenge.

4. Discussion

In this study we compared the behavioral responses of mice exposed to two panic-evoking stimuli, exposure to high concentrations of CO_2 or to low levels of O_2, and their effects on plasma corticosterone levels. We also investigated the effects of standard panicolytic drugs on the escape behavior evoked by CO_2 inhalation.

The results showed that a decrease of O_2 levels to 7% increased the time spent by mice immobile and decreased the expression of rearing, suggesting a reduction in the locomotor activity of these animals. On the other hand, raising CO_2 concentration to 20% evoked a typical repertoire of behaviors interpreted as an active escape response. More specifically, CO_2 inhalation, though causing a marked reduction in rearing episodes, significantly increased the maximum speed achieved by the animals and triggered bouts of upward leaps directed to the border of the chamber, presumably performed in order to flee from the experimental cage.

To our knowledge, this is the first evidence that CO_2 exposure evokes an active escape response in mice. It is noteworthy that previous results revealed that exposure of C57BL/6 mice to lower levels of CO_2

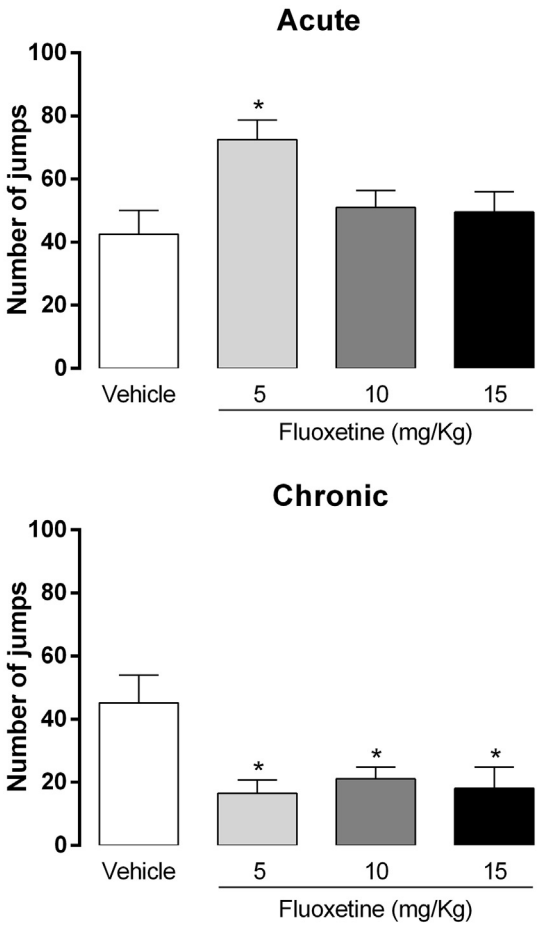

Fig. 2. Effects (mean ± SEM) of acute or chronic (21 days) treatment with fluoxetine (5; 10 or 15 mg/kg, i.p.) or vehicle solution on the number of jumps expressed during CO_2 exposure. *p < 0.05 compared with the control group.

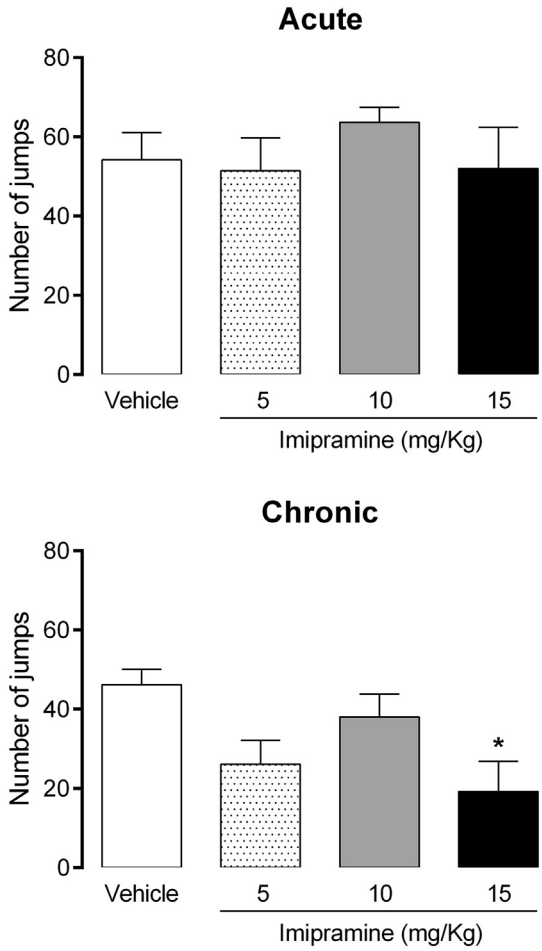

Fig. 3. Effects (mean ± SEM) of acute or chronic (21 days) treatment with imipramine (5; 10 or 15 mg/kg, i.p.) or vehicle solution on the number of jumps expressed during CO_2 exposure. *p < 0.05 compared with the control group.

(up to 10%) evoked freezing behavior (Leibold et al., 2016; Price et al., 2014; Taugher et al., 2014; Vollmer et al., 2016). Curiously, a study by Ziemann et al. (2009) using a gas sealed square open-field showed that whereas exposure of the same mice strain used here to 10% CO_2 increased freezing and reduced the time spent in the arena center, inhalation of 20% CO_2 tended to only decrease locomotion in the central region, indicating in both cases an increase in anxiety. No mention was made of the expression of jumps during the CO_2 challenge at either concentration. It should be noted that, different from our test protocol, besides the features of the experimental cages, in their study the animals were introduced into an open-field that was already pre-filled with these concentrations of CO_2. Therefore, it is conceivable that besides a high gas level, a gradual increase in CO_2 may be required to evoke a more discernible behavioral panic-like state in this strain.

In contrast to what was observed here with mice, hypoxia exposure is reported to evoke an active escape behavior in rats. For instance, Casanova et al. (2013) showed that whereas exposure of Wistar rats to 13% O_2 leads to immobility, a decrease of O_2 levels to 6% precipitates running and rearing episodes. A similar response was also reported by Schimitel et al. (2012) after the induction of chemical hypoxia by intravenous administration of KCN. Finally, in a recent study from our group, it was observed that 7% O_2, but not higher concentrations, markedly evoked vertically-oriented active escape responses, similar to those observed here with mice during the CO_2 challenge.

As such, it comes as a surprise that in the present study severe hypoxia in mice did not equally induce escape attempts. However, there is evidence to suggest that evolutionary adaptations have rendered mice more resistant than rats to the life-threatening effects of hypoxia. For instance, in a study comparing physiological parameters between mice and rats raised for more than 30 generations at 3600 m above sea level (La Paz, Bolivia), Jochmans-Lemoine et al. (2015) reported that rats display, among other characteristics, elevated right ventricular hypertrophy (a sign of higher pulmonary hypertension) and a reduced metabolic rate, compared with mice. As protection against elevated pulmonary hypertension is common in species adapted to high altitude, and reduced O_2 consumption rate is a key strategy to adaptation to hypoxia (Bickler and Buck, 2007; Jochmans-Lemoine et al., 2016), mice seem to have innate traits that favor adaptation to live under low levels of O_2. As mentioned by Jochmans-Lemoine et al. (2015), these physiological differences are in line with ecological reports showing that mice are found living at altitudes as high as 4000 m, while rats, under natural conditions, have not been able to establish stable colonies in such elevated areas (Anderson, 1997; Storz et al., 2007).

Regarding corticosterone, our results showed that exposure to either CO_2 or hypoxia elevated the concentration of this hormone when measured 30 min, but not immediately after the test. However, animals tested under hypoxia had higher corticosterone levels than those exposed to CO_2. Moreover, in the former group, corticosterone levels tended to correlate positively with the time spent immobile and negatively with the number of rearings expressed during the test. On the other hand, in CO_2 exposed mice, corticosterone levels were correlated positively with the number of jumps.

In an attempt to interpret these results, one intriguing possibility is that exposure to these respiratory challenges generated different emotional states in these animals. Whereas hypoxia may have evoked

Table 1
Distance traveled (mean ± SEM) analyzed at the first 5 min that preceded exposure to the respiratory challenges.

Experiment	Distance traveled (m) Mean ± SEM
Acute Fluoxetine (i.p)	
Vehicle	5.08 ± 0.54
5 mg/Kg	4.88 ± 0.46
10 mg/Kg	4.18 ± 0.36
15 mg/Kg	5.62 ± 0.62
Chronic Fluoxetine (i.p)	
Vehicle	4.26 ± 0.43
5 mg/Kg	4.21 ± 0.38
10 mg/Kg	5.10 ± 0.32
15 mg/Kg	4.41 ± 0.36
Acute Imipramine (i.p)	
Vehicle	4.01 ± 0.41
5 mg/Kg	4.13 ± 0.30
10 mg/Kg	4.87 ± 0.52
15 mg/Kg	4.23 ± 0.37
Chronic Imipramine (i.p)	
Vehicle	5.12 ± 0.33
5 mg/Kg	5.00 ± 0.24
10 mg/Kg	4.78 ± 0.39
15 mg/Kg	5.64 ± 0.39
Acute Alprazolam (i.p)	
Vehicle	4.67 ± 0.81
0.025 mg/Kg	5.00 ± 0.69
0.05 mg/Kg	4.89 ± 0.44
0.1 mg/Kg	5.33 ± 0.49
Acute Diazepam (i.p)	
Vehicle	5.24 ± 0.32
0.025 mg/Kg	5.22 ± 0.31
0.05 mg/Kg	5.74 ± 0.45
0.1 mg/Kg	4.40 ± 0.50
1 mg/Kg	4.01 ± 0.37

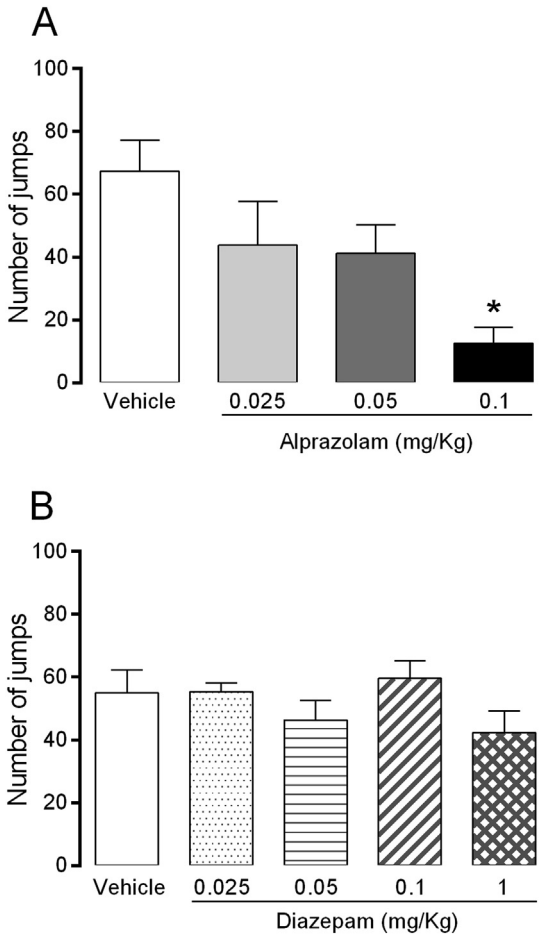

Fig. 4. Effects (mean ± SEM) of acute injection of alprazolam (0.025; 0.05 or 0.1 mg/kg, i.p.), diazepam (0.025; 0.05; 0.1 or 1 mg/kg, i.p) or their respective vehicle solutions on the number of jumps expressed during CO_2 exposure. *p < 0.05 compared with the control group.

anxiety, CO_2 inhalation induced a panic-like state. In support of this idea, evidence in the literature indicates that the HPA-axis is little affected by a panic attack (for a review see Leibold et al., 2015 and Graeff et al., 2005), despite this being overwhelming and, therefore, a very stressful situation for a person. This is also true for experimentally induced panic attacks by means of CO_2 inhalation either with healthy volunteers or panic disorder patients. It is noteworthy, however, as reviewed recently by Leibold et al. (2015), that although exposure to low concentrations of CO_2 (up to 5%) has yielded mostly negative results in terms of cortisol release, the main corticosteroid hormone produced by the human adrenals, conflicting results have been reported after inhalation concentrations as high as 35% of this gas, with some studies showing moderate increases in cortisol levels. These findings are compatible with the moderate elevation in corticosterone levels reported here after CO_2 inhalation at a reasonably high concentration. On the other hand, the more pronounced increase in corticosterone levels observed after hypoxia is consistent with a wealth of evidence that anticipatory anxiety-related stimuli or anxiety-inducing drugs commonly recruit the HPA-axis (Graeff, 2007; Guijarro et al., 2007; Hestermann et al., 2014; Johnston et al., 1988; Muñoz-Abellán et al., 2010; Nicholson, 1989; Pellow and File, 1985).

A concurrent interpretation to the above for the observed increase in corticosterone concentration in CO_2 exposed mice should also be considered. In light of the fact that plasma corticosterone levels were positively correlated with the number of jumps expressed during the CO_2 challenge, it is also possible that the exhausting and energy demanding exertion of this active escape response led to the endocrine change observed.

In support of this idea are the results of a recent study by de Souza Armini et al. (2015) on the behavioral and neuroendocrine consequences in rats of electrical stimulation of the dorsal periaqueductal gray (DPAG), a key structure in the pathophysiology of panic disorder (Canteras and Graeff, 2014; Graeff and Del-Ben, 2008; Schenberg et al., 2001, 2014). These authors showed that this procedure, besides evoking a full-blown escape/flight response (characterized by trotting, galloping and/or jumps), also increased plasma levels of ACTH and lactate, which were used as markers for HPA-axis activation and muscular fatigue, respectively. Importantly, there was a highly significant positive correlation between the levels of these two substances, indirectly supporting a major influence of exercise. But, of greater importance, neither the levels of ACTH nor lactate were altered after DPAG stimulation with the escape/flight threshold intensity of rats confined in a small compartment that prevented the expression of this behavioral response.

Future studies are needed to determine whether physical exertion also accounted for the change in corticosterone levels found here in the CO_2 exposed group. It should be noted, however, that there is a report showing that mice exposed to 6% CO_2 for 20 min, which does not induce escape, had significantly higher corticosterone levels detected immediately after the test (D'Amato et al., 2011). Thus, it would also be of interest to evaluate, for instance using pharmacological tools, whether at this low CO_2 concentration the endocrine change observed reflects the triggering of an anxiety- and not a panic-like state in the animals.

The results of the pharmacological exploitation of the escape response expressed under CO_2 exposure (experiments 2 and 3) give further support to the idea that a panic-like state was generated in the animals. Thus, it was observed that chronic, but not acute, administration of the antidepressants fluoxetine and imipramine or acute administration of the high-potency benzodiazepine alprazolam

significantly decreased the number of jumps during the test. These drugs are widely used in the treatment of panic disorder patients and are reported to decrease the reactivity to CO_2 inhalation in panic disorder patients (Bertani et al., 1997; Bocola et al., 1998; Gorman et al., 1997; Perna et al., 2002; Sanderson et al., 1994). The anti-escape effect caused by these three drugs was observed at doses that did not non-specifically interfere with locomotion, as measured before the CO_2 exposure. In addition, acute treatment with the lowest dose of fluoxetine facilitated the expression of this behavior. Overall, the results are consistent with clinical findings showing that prolonged and continuous treatment with antidepressants are required in order to achieve their full antipanic effect. At the beginning of the treatment, these drugs may even cause the opposite effect, i.e., exacerbation of panic- and/or anxiety-related symptoms (Giesecke, 1990; Gorman et al., 1987; Michelson et al., 1999; Sinclair et al., 2009).

They also corroborate evidence obtained with the same drugs and doses in other experimental models that associated escape/flight responses with panic attacks, such as the mouse defense test battery (Griebel et al., 1995a, 1995b) and the elevated T-maze (Pulga et al., 2012). It should be noted that chronic treatment with fluoxetine or a single administration of alprazolam or of another clinically-effective panicolytic drug, clonazepam, also reduced the escape response expressed by rats under different hypoxia challenges (Schimitel et al., 2014; Spiacci et al., 2015). Therefore, whereas a panic-like state is evoked in mice by exposure to high CO_2 concentrations, in rats this is induced by hypoxia.

Curiously, our results also revealed that a single injection of the anxiolytic diazepam did not significantly affect the number of jumps during the test. This is further evidence that this drug is not effective in changing escape/flight responses both in mice and rats. More specifically, in the dose range used here, this benzodiazepine failed to change panic-associated escape behaviors in the mouse defense test battery (Griebel et al., 1996) and in the rat or mouse elevated T-mazes (Pulga et al., 2012; Zangrossi and Graeff, 2014). Despite diazepam being far less used than high-potency benzodiazepines such as alprazolam or clonazepam for the treatment of panic disorder (Bakker et al., 2005; Moylan et al., 2011), some controlled studies have shown clinical effectiveness at high, mostly sedative, doses (Noyes et al., 1984, 1996). It is interesting to note that to our knowledge no study has investigated the effects of this drug on volunteers or panic disorder patients submitted to CO_2 challenges. Therefore, it is conceivable that higher doses are necessary to affect escape performance in these two rodent species, but this would also add difficulties in the interpretation of the results given the occurrence of motor deficits.

In conclusion, our findings indicate that exposure of mice to 20% CO_2, but not to severe hypoxia, evokes defensive behaviors and endocrine changes that are suggestive that the animals are experiencing a highly aversive situation, that resembles the triggering of a panic attack. In agreement, drugs used in the treatment of panic disorder patients reduced the escape behavior expressed during the CO_2 challenge. Given the lack of behavioral and/or pharmacologically well-validated panic models in mice, the model presented here can serve as simple tool for studying the pathophysiological mechanisms behind this psychiatric condition and for the screening of novel panicolytic drugs.

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