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The role of 5-HT$_{1A}$ receptors in the anti-aversive effects of cannabidiol on panic attack-like behaviors evoked in the presence of the wild snake *Epicrates cenchria crassus* (Reptilia, Boidae)

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Abstract

The potential anxiolytic and antipanic properties of cannabidiol have been shown; however, its mechanism of action seems to recruit other receptors than those involved in the endocannabinoid-mediated system. It was recently shown that the model of panic-like behaviors elicited by the encounters between mice and snakes is a good tool to investigate innate fear-related responses, and cannabidiol causes a panicolytic-like effect in this model. The aim of the present study was to investigate the 5-hydroxytryptamine (5-HT) co-participation in the panicolytic-like effects of cannabidiol on the innate fear-related behaviors evoked by a prey versus predator interaction-based paradigm. Male Swiss mice were treated with intraperitoneal (i.p.) administrations of cannabidiol (3 mg/kg, i.p.) and its vehicle and the effects of the peripheral pre-treatment with increasing doses of the 5-HT$_{1A}$ receptor antagonist WAY-100635 (0.1, 0.3 and 0.9 mg/kg, i.p.) on instinctive fear-induced responses evoked by the presence of a wild snake were evaluated. The present results showed that the panicolytic-like effects of cannabidiol were blocked by the pre-treatment with WAY-100635 at different doses. These findings demonstrate that cannabidiol modulates the defensive behaviors evoked by the presence of threatening stimuli, and the effects of cannabidiol are at least partially dependent on the recruitment of 5-HT$_{1A}$ receptors.

Keywords

Cannabidiol, panic attacks, serotonin, 5-HT$_{1A}$ receptors, prey versus predator paradigm

Introduction

The understanding of human emotional disorders has increased based on defensive behavior studied in other mammals (Biagioni et al., 2012, 2013; Blanchard and Blanchard, 1984; Darwin, 1872; da Silva et al., 2013b). The investigations of the neural systems involved in the elaboration of emotions also indicate that particular defensive behaviors (freezing, flight, defensive vocalization and defensive attack) can be anatomically differentiated (see Blanchard et al., 1997 for review; Davis et al., 1987; Eichenberger et al., 2002; Gorman et al., 2000; LeDoux, 1986; Mobs et al., 2007), which suggests that each behavioral response should be viewed as related to discrete but intrinsically related neurobehavioral systems (da Silva et al., 2012, 2013a; de Freitas et al., 2013a, 2013b; Lopes et al., 2012; Spiacci et al., 2012).

Fear and anxiety could emerge similarly to the defensive responses of laboratory animals that are exposed to threatening stimuli (Blanchard and Blanchard, 1988; Gray and McNaughton, 2000; McNaughton and Corr, 2004). The ethological manifestations of fear and anxiety are related to the characteristics of the aversive stimulus and seem to be dependent upon the distance between prey and predator (Blanchard and Blanchard 1988; Gray and McNaughton, 2000; McNaughton and Corr, 2004).

Ethological models of innate fear- and anxiety-related responses, such as the prey versus predator confrontation, have been widely used to study these emotional reactions, which allows for the activation of several structures of the limbic system in the

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presence of different kinds of aversive stimuli (Blanchard and Blanchard, 1988; Guimarães-Costa et al., 2007; Lobão-Soares et al., 2008; McNaughton and Corr, 2004; Uribe-Mariño et al., 2012; Weltson et al., 2002). The anti-predatory defense systems have been characterized in terms of the reactions and responses related to threatening stimuli, such as the relationship between the predator, the experimental context and an assessment of the entire environment (Blanchard et al., 2001, 2003; Hubbard et al., 2004). The distinct response pattern of different defensive behaviors modulated by effective drugs used for the treatment of generalized anxiety and panic disorders in humans indicates that the ethological models based on the confrontation between prey and predator are appropriate tools for the preclinical drug testing of these psychiatric conditions (Blanchard et al., 1998; Griebel et al., 1996; Guimarães-Costa, et al., 2007).

Recent attention has focused on the role of the endocannabinoid system in the elaboration and modulation of aversive states in the central nervous system (Fusar-Poli et al., 2009; Mackowiak et al., 2009; Marco and Viveros, 2009; Moreira et al., 2009; Soares et al., 2010; Thiemann et al., 2009). Cannabidiol (CBD) is a major non-psychotomimetic constituent of *Cannabis sativa*. When CBD is systemically administered, it produces several pharmacological effects (Di Marzo et al., 2011; Malfait et al., 2000). CBD also promotes central effects, such as anticonvulsive, neuroprotective, antipsychotic, anxiolytic and antipanic effects (Carlini et al., 1973; Crippa et al., 2010; Guimarães et al., 1990; Mechoulam et al., 2002; Mishima et al., 2005; Moreira et al., 2009; Roser et al., 2008; Soares et al., 2010; Uribe-Mariño et al., 2012; Zuardi et al., 1991). Recent studies have shown that CBD induces antipanic-like effects in several animal models, such as the ethological model based on prey versus predator encounters (Uribe-Mariño et al., 2012), the elevated T-maze (ETM) and electrical stimulation of the dorsal periaqueductal gray matter (dPAG) (Soares et al., 2010).

Nevertheless, the molecular mechanisms responsible for these effects remain ambiguous. Although some studies suggest that CBD could act as a CB1 receptor antagonist or CB2 receptor agonist (Mechoulam et al., 2002; Thomas et al., 1998, 2007), in vitro studies have shown that CBD has a low affinity for these receptors (Bisogno et al., 2001; Thomas et al., 2007). CBD could also facilitate endocannabinoid signaling by inhibiting the cellular uptake and enzymatic hydrolysis of endocannabinoids (Bisogno et al., 2001), which demonstrates the complex actions of CBD on the endocannabinoid-mediated system (Thomas et al., 2007).

In addition to affecting endocannabinoid neurotransmission, CBD was shown to interact with 5-HT_{1A} receptors. There is evidence that CBD could act as an agonist of 5-HT_{1A} receptors in vitro (Russo et al., 2005) and in vivo (Campos and Guimarães, 2008; Mishima et al., 2005) at a micromolar concentration range. Other proposed mechanisms include the inhibition of adenosine uptake or agonism at the vanilloid receptors (TRPV1) (Bisogno et al., 2001; Carrier et al., 2006).

The antipanic-like effect of CBD was recently described by our group using a prey versus predator-based paradigm (Uribe-Mariño et al., 2012). It was shown that mice that were not exposed to a confrontation with a constrictor snake, *Epicrates cenchria crassus*, did not display any defensive-like behavior in the arena. However, when exposed to the predator in an identical context, all mice exhibited defensive behaviors in the presence of the predator. This is clear evidence that the present experimental model of panic attacks can be used to study the effects of different drugs on defensive behavior elaborations. Our results showed that CBD decreased the fear-related responses elicited in mice by the encounter with wild constrictor snakes.

The aim of the present study was to investigate the possible involvement of 5-HT_{1A} receptors as an additional mechanism underlying the antipanic effects of CBD using an ethological model of panic attacks based on predator-related threatening stimuli caused by the presence of rainbow Boidae snakes.

Methods and materials

Animals

Male Swiss mice (25–35 g) from the animal facility of the Ribeirão Preto School of Medicine, University of São Paulo (FMRP-USP), were maintained in a 12-h light/dark cycle (the lights were on from 07:00 to 19:00) in an air-conditioned room (23±2°C) with open access to food and water. Wild constrictor snakes (*Epicrates cenchria crassus*; Reptilia; Boidae) were used as predators and weighed 1000–2500 g. The snakes were collected from southeastern Brazil, in the countryside of Ribeirão Preto and the surrounding districts, and were maintained in captivity in the snake pits of the FMRP-USP animal house. The snakes were maintained in a walled sun-lit field with proper shelter, grass and water sources in the ophidiarium of the Laboratory of Neuroanatomy and Neuropsychobiology of the Ribeirão Preto School of Medicine and Institute for Neuroscience and Behavior (LNN-FMRP-USP/INeC, University of São Paulo, which is licensed by the Brazilian government (IBAMA Committee; processes 3543.6986/2012-SP and 3543.6984/2012-SP). The snake pit of the LNN-FMRP-USP/INeC was illuminated by natural sunlight (and by fluorescent ultraviolet irradiation (reptisun; 20 W; 5 UVB) on rainy days) and had artificial waterfalls and lagoons, natural rocks, and both tropical and artificial plants. The enclosure was maintained under a light/dark cycle of 12/12 h (the lights were on from 07:00 to 19:00) and at a constant room temperature of 25°C ± 1°C (40–70% humidity). The snakes were fed every 15 days, and again 24 h and immediately before the beginning of each experiment with the identical rodent species used in this study (*Mus musculus*). Occasionally, the snakes evoked hunting behaviors and predatory attacks followed by searching responses, prey capture and feeding behavior.

The mice were adapted to the experimental rooms for a minimum of one week before the experiments, and each mouse was handled for 5 min on three consecutive days before the tests. All tests were performed between 19:00 and 22:00. The experiments were performed in accordance with the recommendations of the Commission of Ethics in Animal Experimentation of the FMRP-USP (process 112/2009), which abides by the ethical principles in animal research adopted by the Brazilian College of Animal Experimentation (COBEA) and was approved by the Commission of Ethics in Animal Research (CETEA) on 31 August 2009.

Experimental apparatus

A semi-transparent acrylic enclosure was used for the prey versus predator confrontations. The enclosure consisted of a quadrangular arena (154 cm × 72 cm × 64 cm), and the inner surface of the

walls was covered with a light-reflector film that provided 80% light reflection to minimize the visual contact of the predator with the surrounding experimental area and to focus its attention toward the prey. The floor of the arena was constructed from a transparent acrylic platform and was placed on a rectangular stainless steel plaque beneath the arena. The arena was divided into 20 equal rectangles using a green fluorescent line (4.2 mm width; Pritt mark-it). To minimize vibratory stimuli, the entire apparatus was placed on a granite rock surface (170 cm × 85 cm × 02 cm) elevated 83 cm above the floor of the laboratory. A shelter box (36 cm × 26 cm × 12.5 cm) with black acrylic walls and a complex labyrinth inside was placed in one corner of the arena. This burrow had two entries located on opposite sides, thus allowing the mice to enter and exit from two different positions in the arena. On the day of the experiment, the ceiling of the burrow was replaced with an identical model in translucent acrylic to determine the behavior of the mice inside the burrow.

Procedure

Three days before the experiment, the mice were placed in the arena containing a burrow and were maintained in the enclosure with open access to food and water until the day of the experiment. The burrow was located in a corner, opposite to the food and water. Before the confrontation with the predator, the mice were divided into five groups: 1: saline+vehicle (n=15); 2: saline+CBD (3 mg/kg) (n=11); 3: WAY-100635 (0.1 mg/kg)+CBD (n=14); 4: WAY-100635 (0.3 mg/kg)+CBD (n=10); and 5: WAY-100635 (0.9 mg/kg)+CBD (n=9).

The dose of CBD was chosen based on previous reports from this laboratory that showed that 3 mg/kg of CBD caused antipanic-like behavior in mice submitted to a prey versus constrictor snake-based paradigm (Uribe-Mariño et al., 2012). All mice received treatments intraperitoneally (i.p.). The drugs utilized were CBD (approximately 99.9% pure, THC-Pharm, Frankfurt, Germany and STI-Pharmaceuticals, Brentwood, UK) or its vehicle (saline solution at 0.9% and DMSO (1:1) 0.2 mL/kg) and WAY-100635 (SIGMA-ALDRICH, USA) dissolved in a saline solution at 0.9%.

Thirty minutes after the initial injection (saline or WAY), the mice were treated with a second injection (vehicle or CBD). Thirty additional minutes occurred between the second injection and the exposure of the mice to the constrictor snake. The rainbow Boidae snake was carefully placed in the center of the arena, and each mouse was subsequently placed on the opposite side of the burrow with the snake between the mouse and the burrow, without any physical barrier between prey and predator. The confrontation was recorded during 5 min and after the mouse was removed from the arena with a net the snake was again placed in the center of the polygonal arena. Another mouse was then placed in the enclosure to confront the snake for an identical length of time. After the exposition to predator, the mouse was returned to its home cage. No mouse was used in more than one confrontation. The mice were exposed to the snake randomly between 19:00 and 22:00.

After the experiment, each snake was transferred to the snake pit at the FMRP-USP and was submitted to a 40-day quarantine period. Subsequently, the snakes were placed with other constrictors in the FMRP-USP main ophidiarium. In no case did a snake eat an experimental mouse and in the case of predatory attack the mouse was excluded from the experimental data.

Anti-predatory behavioral recordings

The durations and behavioral indices of defensive and non-defensive behaviors of the mice were recorded by a video-camera and analyzed afterwards by a blinded trained experimenter. Considering the complex behavioral repertoire displayed by rodents in confrontations with wild snakes and other predators in prey versus predator paradigms (Blanchard et al., 2001; Guimarães-Costa et al., 2007; Lobão-Soares et al., 2008; Uribe-Mariño et al., 2012), defensive attention, risk assessment, time spent outside the burrow and interaction with predator were considered as anxiety-related responses. On the other hand, defensive immobility and escape reactions (explosive and oriented to the burrow) were considered panic-like responses.

The defensive attention of the mice was defined as an interruption of on-going behavior to occasionally scan the environment by smelling the surrounding air. A risk assessment was defined as when the mouse stretched its anterior half to monitor behavioral strategies in the potentially dangerous situation (Silva and Brandão, 2000), when the animal stretched to its full length and cautiously moved forward (flat-back approach), or the stretched-attend posture. Direct contact between the mouse and the snake was considered an interaction. Time spent outside the burrow was defined as when the posterior paws of the mouse were outside the burrow. Defensive immobility was registered when the mouse presented immobility followed by autonomic reactions, such as defecation, exophthalmia and/or micturition. An oriented escape was defined as running toward the burrow, whereas an explosive escape was defined as running in a direction in the arena other than toward the burrow.

Statistical analysis

The frequencies of behaviors were proportionally recorded in relation to the time spent by each animal outside or inside the burrow. These data, presented as a 'behavioral index' (BI), were calculated by a previously reported (Uribe-Mariño et al., 2012) formula: BI = (100 × the number of behavioral responses) / (the time in seconds spent outside or inside the burrow). In addition, the duration of each behavior was expressed as the percentage of the total time of the experiment and the time spent in a specific behavioral response exhibited outside or inside the burrow. The data from the outside or inside behaviors were analyzed by one-way ANOVA, and Tukey's test was employed for multiple comparisons. A Bonferroni's multiple comparison test was applied when identical pairs of behaviors outside versus inside the burrow were compared. The data were presented as the mean and standard error of the mean (SEM). The differences were considered significant at the $p<0.05$ level.

Results

Once habituated to the arena for three days, the mice treated with CBD (3 mg/kg, i.p.) or those treated with the vehicle were exposed to the constrictor snake. All of the mice exhibited a series of defensive behaviors characterized by defensive attention, risk assessment, defensive immobility, explosive escape, and oriented escape directed toward the burrow. Interaction with the predator was also observed. Once inside the burrow, the mice showed almost identical patterns of defensive behaviors.

The predators showed moderate exploratory behavior inside the arena interspersed with resting activity periods. Furthermore, after the interaction between the prey and predator, the constrictor snakes evoked defensive reactions characterized by head retrieval or movements of the anterior third of the body to the side of the arena opposite that occupied by the prey. All mice displayed defensive behaviors to the spontaneous exploratory activity and to the defensive reaction of the predator.

With regard to the outside the burrow behaviors, in the mice treated with the dose of 3 mg/kg of CBD ANOVA showed statistical differences in the BI ($F(5,64)= 18.1$; $p<0.001$) and duration ($F(5,64)= 9.27$; $p<0.05$) of defensive attention, and the post hoc test showed increase in responses in the CBD group when compared with the control group (saline + vehicle) ($p<0.001$ to BI; $p<0.05$ to duration of defensive attention) (Figure 1(a) and (b)). However, the post hoc tests did not show any significant differences between the WAY-100635 pre-treated animals and the CBD-treated group in these responses. In addition to anxiety-like behaviors, no significant effects of the CBD treatment were observed for the BI ($F(5,64)= 3.6$; $p>0.05$) and duration ($F(5,64)= 0.5$; $p>0.05$) of risk assessment and duration of interaction ($F(5,64)= 1.3$; $p>0.05$) with the predator outside the burrow when compared with the control group (Figure 1(c)–(e)).

Moreover, ANOVA showed differences in time spent outside of the burrow between groups ($F(5,64)= 5.3$; $p<0.001$) and the post hoc test showed that the CBD-treated mice spent less time outside the burrow when compared with the untreated group ($p<0.05$). Furthermore, animals pre-treated with WAY-100635 showed the reverse of the effect of CBD, increasing the time spent outside of the burrow (0.3 mg/kg and 0.9 mg/kg; $p<0.05$ and $p<0.01$, respectively) (Figure 1(f)).

Regarding the expression of innate fear-related behaviors outside of the burrow, one-way ANOVA revealed significant differences for the BI and duration of following panic attack-related responses: defensive immobility ($F(5,64)= 4.5$; $p<0.01$ and $F(5,64)= 3.6$; $p<0.01$, respectively); explosive escape behavior ($F(5,64)= 3.5$; $p<0.01$ and $F(5,64)= 4.2$; $p<0.001$, respectively); and total escape ($F(5,64)= 5.5$; $p<0.001$ and $F(5,64)= 4.7$; $p<0.001$, respectively) (Figure 2(a)–(h)). A post hoc analysis comparing the CBD-treated group and the control group showed a significant decrease in the BI and duration of these defensive responses: defensive immobility ($p<0.01$ and $p<0.05$, respectively), explosive escape ($p<0.01$ and $p<0.01$, respectively) and total escape ($p<0.05$ and $p<0.001$, respectively) (Figure 2(a)–(h)). Furthermore, the pre-treatment with WAY-100635 prevented a CBD-induced panicolytic-like behavior and these pre-treatments caused a statistically significant increase in the BI ($p<0.01$ to 0.3 mg/kg) and duration ($p<0.05$ to 0.9 mg/kg) of defensive immobility, in the BI ($p<0.05$ to 0.3 mg/kg) and duration ($p<0.05$ to 0.3mg/kg) of explosive escape responses, and also increased the BI ($p<0.001$ to 0.1mg/kg and $p<0.05$ to 0.3mg/kg) and duration ($p<0.05$ to 0.1mg/kg and $p<0.01$ to 0.3mg/kg) of total escape responses, when compared with animals that received saline plus CBD, as shown in Figure 2(a)–(h). However, one-way ANOVA did not show significant differences for the oriented escape behaviors ($F(5,64)= 2.2$; $p>0.05$ to BI and $F(5,64)= 1.3$; $p>0.05$ to percentage of duration), as shown in Figure 2(c) and (d).

No statistically significant difference was detected by the post hoc analysis when comparing the control group (saline + vehicle-treated mice) with the WAY-100635 (0.3 mg/kg)-treated group when considering either anxiety- or panic-like behavior evoked by mice in the presence of the Boidae snake.

Once inside the burrow (Figure 3), all of the threatened mice exhibited defensive behaviors with similar phenomenology to those observed outside of the burrow. However, the pre-treated groups of mice were not significantly different from animals that were not exposed to the snake. One-way ANOVA did not show significant differences in BI and duration of the following fear-induced reactions: defensive attention ($F(5,64)=1.2$ and $F(5,64)=0.60$, for BI and duration, respectively; $p>0.05$ in both cases); risk assessment ($F(5,64)=0.60$ and $F(5,64)=0.63$, for BI and duration, respectively; $p>0.05$ in both cases); defensive immobility ($F(5,64)=0.52$ and $F(5,64)=0.16$, for BI and duration, respectively; $p>0.05$ in both cases); and oriented/explosive escape behaviors ($F(5,64)=1.0$ and $F(5,64)=1.1$, for BI and duration, respectively; $p>0.05$ in both cases).

Distinct behavioral indices and durations were observed for the behaviors elicited inside and outside the burrow. One-way ANOVA showed significant differences in the inside and outside behavioral indices and durations of responses related to anxiety and panic, such as the risk assessment ($F(11,141)= 31.0$, $p<0.001$ for BI and $F(11,141)= 17.4$, $p<0.001$ for duration), the defensive immobility ($F(11,141)= 4.2$, $p<0.01$ for BI and $F(9,141)= 4.6$, $p<0.01$ for duration) and total escape responses ($F(11,141)= 28.7$, $p<0.001$ for BI and $F(11,141)= 31.6$; $p<0.001$ for duration), as shown in Figure 4. A post hoc analysis using Bonferroni's multiple comparison test revealed a significant decrease in the BI ($p<0.001$ to all comparisons) and duration ($p<0.001$ to all comparisons) of risk assessment evoked inside the burrow compared with those behaviors evoked outside the burrow. For comparisons between defensive immobility evoked inside and outside the burrow, a decrease was also observed in the BI ($p<0.01$ compared with the control and with the 0.3 mg/kg WAY-100635-treated group) and duration ($p<0.01$ and $p<0.001$ compared with the control and with the 0.9 mg/kg WAY-100635-treated group, respectively) (Figure 4(c) and (d)). Finally, regarding the escape behavioral responses, a significant decrease was observed in both BI ($p<0.001$ compared with the control, the 0.1, 0.3 and 0.9 mg/kg WAY-100635-treated groups, $p<0.01$ compared with saline+CBD-treated group) and duration ($p<0.001$ compared with the control, 0.1, 0.3 and 0.9 mg/kg WAY-100635-treated groups, $p<0.05$ compared with the saline+CBD-treated groups) of the total escape responses displayed inside the burrow, compared with those responses displayed outside the burrow (Figure 4(e) and (f)).

Discussion

The present results showed that mice pre-treated with CBD had a significant and robust decrease of explosive escapes (flight behavior) and defensive immobility (freezing), which are behavioral responses related to the panic-related emotion. These results corroborate the previous study performed by Uribe-Mariño et al. (2012). However, typical anxiety-related behaviors, such as the risk assessment, were not statistically influenced by the peripheral treatment with CBD. On the other hand, defensive attention, a behavioral response also related to anxiety, increased after the peripheral treatment of mice with CBD. In addition, the animals treated with CBD spent less time outside the burrow, the place in which the snake was present and which exerts a threatening influence on mice. This apparent anxiogenic effect of CBD should be

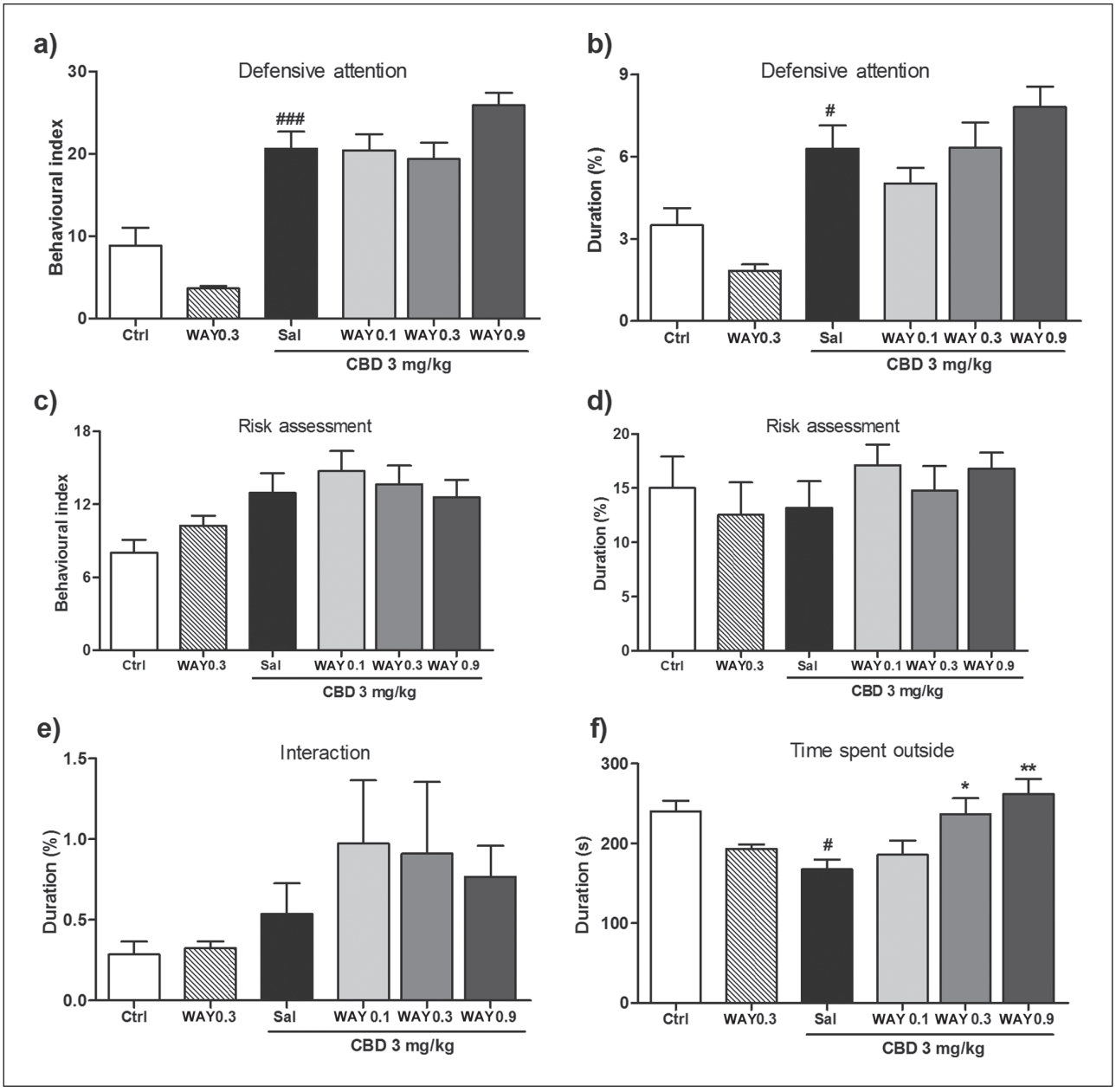

Figure 1. The outside the burrow anxiety-like responses displayed by the mice. The effect of intraperitoneal administration of the vehicle or cannabidiol (CBD) of 3 mg/kg and saline or WAY-100635 at 0.1 mg/kg, 0.3 mg/kg and 0.9 mg/kg on the behavioral index (a) and duration of defensive attention (b), behavioral index (c) and duration of risk assessment (d), duration of the interaction between prey versus predator (e) and duration of the time spent outside of the burrow (f) during the confrontation with the snake after a three-day habituation period in the arena. The columns represent the means, and the bars represent the SEM with n=9–15 mice per group.
$^{\#}p$<0.05 and $^{\#\#\#}p$ <0.001 when compared with the saline+vehicle-treated group (Ctrl), and $^{*}p$ <0.05 and $^{**}p$ <0.01 when compared with the saline+CBD-treated group.

considered with caution, because actually this drug caused a main panicolytic-like effect in the present model, so CBD may not be effective on anxiety-related responses inherent to the snake presence in the experimental environment.

Previous work published by our group (Uribe-Mariño et al., 2012) showed similar findings: a statistical tendency to increase defensive attention induced by CBD injection and impairment of panic-like responses elicited in mice in the presence of rainbow Boidae snakes. All those data suggest that the pharmacological suppression of panic-like responses (defensive immobility and explosive escape responses) in mice, in the presence of threatening stimuli caused by the presence of the wild snake, drives the prey to maintain anxiety-related behavioral responses, such as defensive attention and inhibitory avoidance (expressed by the increase in time spent inside the burrow), in an attempt to facilitate some active defensive behaviors possibly needed in a sudden predatory attack. All these findings support the idea that the present experimental model is more suitable for the study of

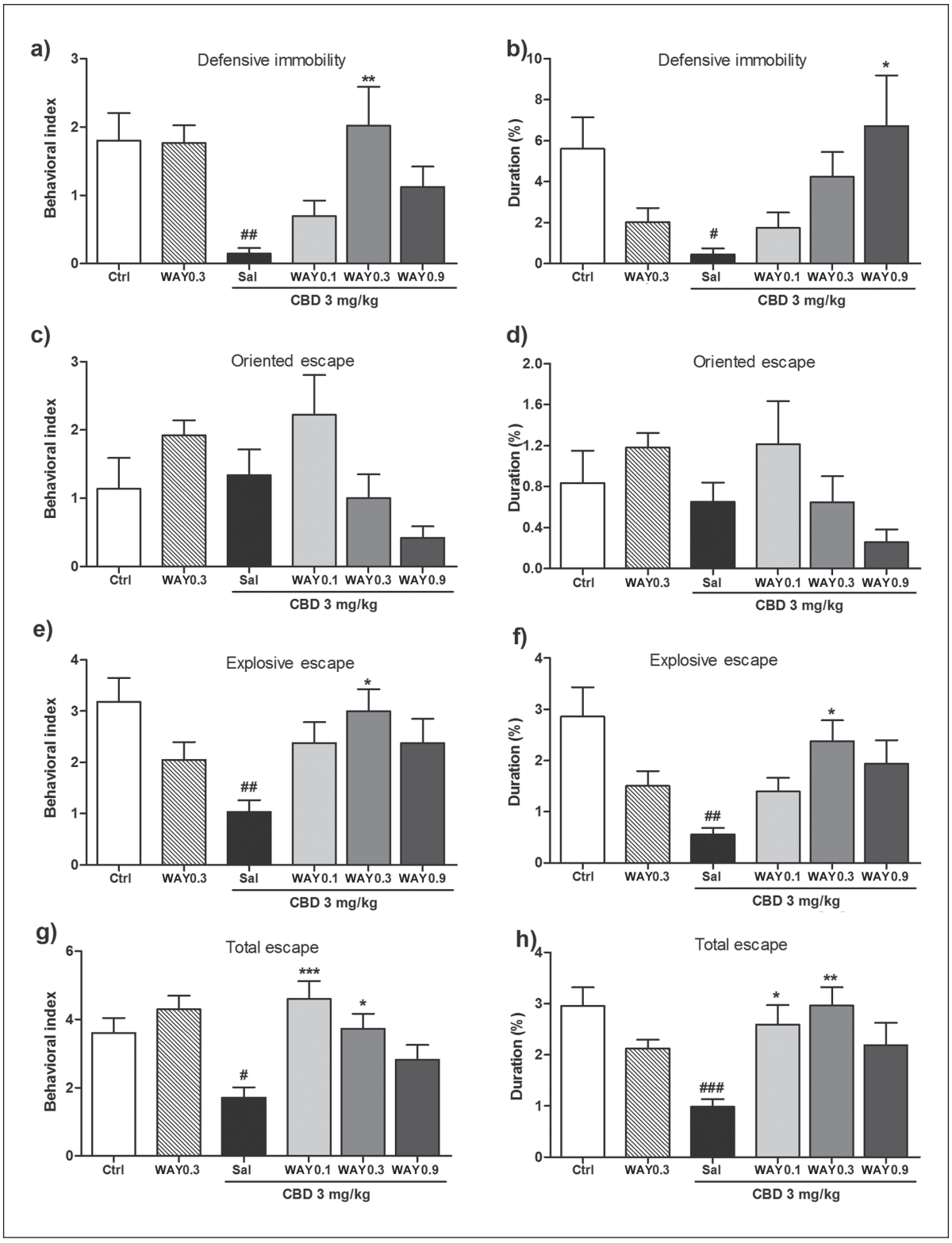

Figure 2. The outside the burrow panic-like responses displayed by the mice. The effect of intraperitoneal administration of the vehicle or cannabidiol (CBD) at 3 mg/kg and saline or WAY-100635 at 0.1 mg/kg, 0.3 mg/kg and 0.9 mg/kg on the behavioral index (a) and duration of defensive immobility (b), behavioral index (c) and duration of oriented escapes (d), behavioral index (e) and duration of explosive escapes (f) and behavioral index (g) and duration of total escape responses (h) during the confrontation with the snake after a three-day habituation period in the arena. The columns represent the means, and the bars represent the SEM with n=9–15 per group.

$^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ when compared with the saline+vehicle-treated group (Ctrl), and $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ when compared with the saline+CBD-treated group.

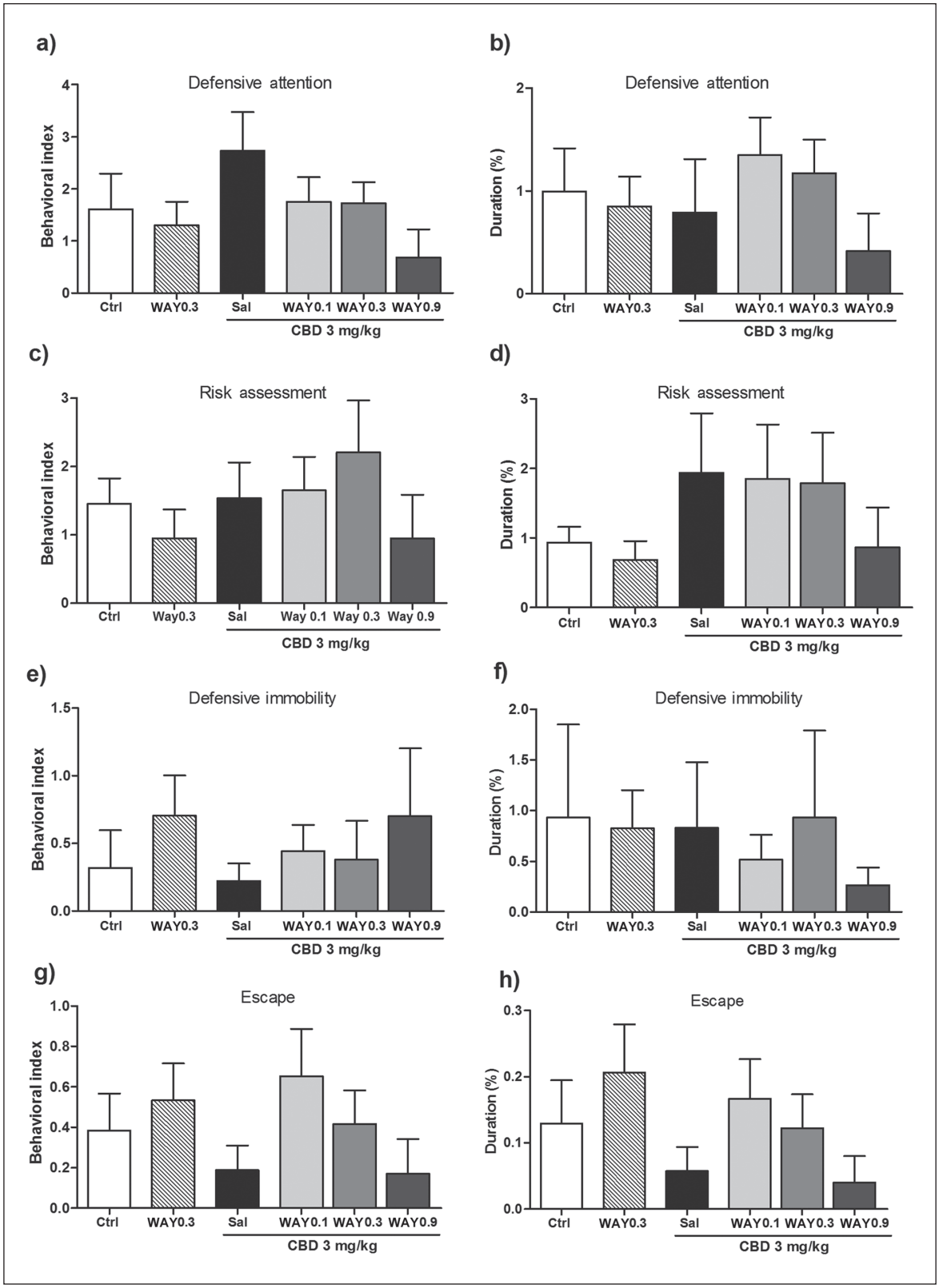

Figure 3. The inside the burrow behavioral responses displayed by the mice. The effect of intraperitoneal administration of vehicle or cannabidiol (CBD) at 3 mg/kg and saline or WAY-100635 at 0.1 mg/kg, 0.3 mg/kg and 0.9 mg/kg on the behavioral index (a) and duration of defensive attention (b), behavioral index (c) and duration of risk assessment (d), behavioral index (e) and duration of defensive immobility (f) and behavioral index (g) and duration of escape responses (h) during the confrontation with the snake after a three-day habituation period in the arena. The columns represent the means, and the bars represent the SEM with n=9–15 per group.

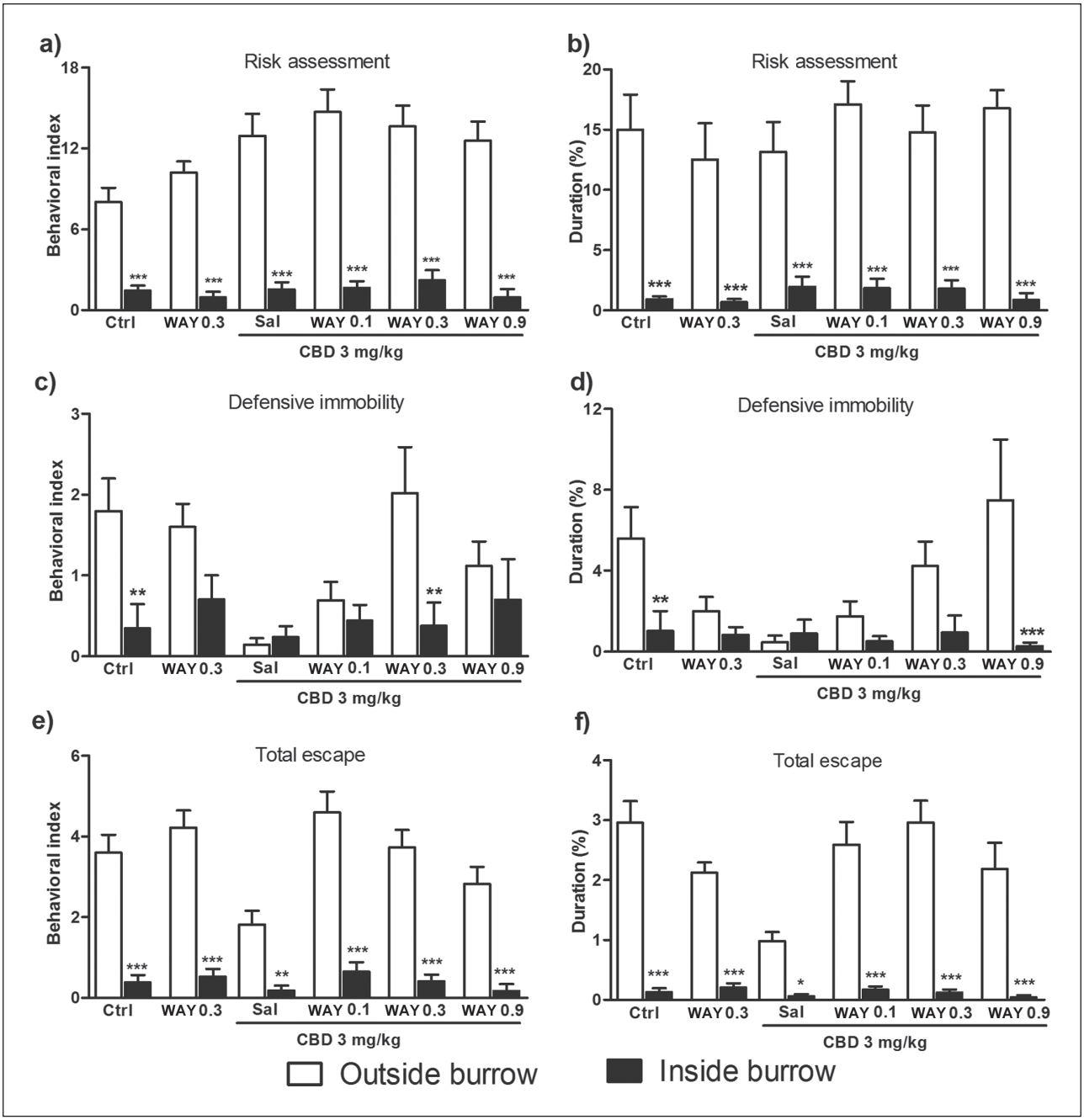

Figure 4. The outside versus inside the burrow behaviors displayed by the mice. The effect of intraperitoneal administration of the vehicle or CBD at 3 mg/kg and saline or WAY-100635 at 0.1 mg/kg, 0.3 mg/kg and 0.9 mg/kg on the behavioral index (a) and duration of risk assessment (b), behavioral index (c) and duration of defensive immobility (d) and behavioral index (e) and duration of escape responses (f). The columns represent the means, and the bars represent the SEM with n=9–15 per group.
*p<0.05, **p<0.01 and ***p<0.001 when the identical behaviors displayed inside and outside the burrow were compared.

panic-like reactions than non-specific anxiety-related behavioral responses.

Anxiety and panic are evoked by different scenarios, although unconditioned and conditioned fear-related responses can be elicited in both cases. Defensive behaviors, such as risk assessment, are elicited in the organism in situations of potential danger and are commonly related to anxiety. However, responses such as oriented and explosive escapes and defensive immobility are exhibited in circumstances in which the aversive stimulus is not only present but also within a proximal distance and represents an obvious threat to the survival of the organism (Blanchard et al., 2003; McNaughton and Corr, 2004; Motta et al., 2009). Both passive and active defensive responses were widely evoked by the mice in the constrictor snake presence.

The CBD decreased the defensive behavioral responses related to the imminent danger in the present experimental model of panic attack-like reactions in which the aversive connotation of several visual and odoriferous stimuli from the snakes have been fully recognized as potentially threatening.

In an attempt to verify the underlying mechanism of panicolytic-like effects of CBD, the present work showed that the decrease of defensive behaviors caused by the peripheral treatment with CBD was impaired by a previous injection of WAY-100635. This result indicates that the activation of 5-HT_{1A} receptors is critical for at least a portion of the CBD panicolytic effects. These findings are in accordance with previous reports that suggest that the CBD-induced inhibition of escape responses that are generated by the electrical stimulation of the dorsal columns of the periaqueductal gray matter (Soares et al., 2010) are blocked by previous microinjections of WAY-100635. Additional studies have shown that a CBD-induced anxiolytic-like effect is dependent on the involvement of 5-HT_{1A} receptors (Campos and Guimarães, 2008; Resstel et al., 2009; Soares et al., 2010). In fact, several studies have shown that a microinjection of serotonin in the dPAG induces anti-aversive effects by interacting with the 5-HT_{1A} and 5-HT_{2A} receptors (Graeff, 1990, 1994; Nogueira and Graeff, 1995). Notably, there is also evidence that the anxiolytic-like effects of CBD microinjected into the mesencephalon were not blocked by the previous administration of AM251 (a CB1 partial antagonist) or capsazepine (a vanilloid-receptors antagonist) (Campos and Guimarães, 2008), which supports the relevance of the recruitment of a serotonin-mediated system in the anti-aversive effect of CBD.

It was also observed in the present work that threatened animals run to the burrow during confrontations with a predator. The peripheral treatment with CBD caused a reduction in the time spent outside the burrow, which suggests that these animals had a preference to stay inside the burrow, a safe place, to avoid a possible confrontation with the predator, an inhibitory avoidance that can be considered an anxiety-related response. However, this place preference was reversed by the pretreatment with WAY-100635.

In addition, considering the possibility of an invasion of the burrow by the predator, some aversive stimulus-induced reactions were also elicited even in the safety of the burrow immediately after the prey versus predator encounter. However, the present findings show that when defensive behaviors evoked outside the burrow were compared with those behaviors evoked inside the burrow, significant decreases were observed in panic attack- and anxiety-related responses, such as defensive immobility, explosive escape behavior and risk assessment. This evidence suggests that the burrow could reduce the expression of some fear-related behaviors, which was expected. Therefore, the burrow could represent a safe place for the mice with its low illumination and narrow halls, thus bringing more security. Consequently, the burrow could also have anxiolytic- and panicolytic-like effects per se.

In summary, the present results suggest that peripheral treatment with CBD decreases the expression of defensive responses associated with panic in the model of panic reactions induced by prey versus predator encounter. This antipanic-like effect appears to be mediated by an activation of the 5-HT_{1A} receptors, which suggests that the complex action of CBD involving the serotonin-mediated system could play a pivotal role in the regulation of emotional states.

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Conflict of interest

The authors declare no conflict of interest.

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References

Biagioni AF, de Freitas RL, da Silva LA, et al. (2013) Serotonergic neural links from the dorsal raphe nucleus modulate defensive behaviours organised by the dorsomedial hypothalamus and the elaboration of fear-induced antinociception via locus coeruleus pathways. *Neuropharmacology* 67: 379–394.

Biagioni AF, Silva JA and Coimbra NC (2012) Panic-like defensive behavior but not fear-induced antinociception is differently organized by dorsomedial and posterior hypothalamic nuclei of *Rattus norvegicus* (Rodentia, Muridae). *Braz J Med Biol Res* 45: 328–336.

Bisogno T, Hanus L, de Petrocellis L, et al. (2001) Molecular targets for cannabidiol and its synthetic analogues: Effect on vanilloid VR1 receptors and on the cellular uptake and enzymatic hydrolysis of anandamide. *Br J Pharmacol* 134: 845–852.

Blanchard DC and Blanchard RJ (1984) Affect and aggression: An animal model applied to human behavior. In: Blanchard RJ and Blanchard DC (eds) *Advances in the Study of Aggression*, Vol. I. New York: Academic Press, pp.2–63.

Blanchard DC and Blanchard RJ (1988) Ethoexperimental approaches to the biology of emotion. *Annu Rev Psychol* 39: 43–68.

Blanchard DC, Griebel G and Blanchard RJ (2001) Mouse defensive behaviors: Pharmacological and behavioral assays for anxiety and panic. *Neurosci Biobehav Rev* 25: 205–218.

Blanchard DC, Griebel G and Blanchard RJ (2003) Conditioning and residual emotionality effects of predator stimuli: Some reflections on stress and emotion. *Prog Neuropsychopharmacol Biol Psychiatry* 27: 1177–1185.

Blanchard RJ, Griebel G, Henrie JA, et al. (1997) Differentiation of anxiolytic and panicolytic drugs by effects on rat and mouse defense test batteries. *Neurosci Biobehav Rev* 21:783–789.

Blanchard DC, Griegel G, Rodgers RJ, et al. (1998) Benzodiazepine and serotonergic modulation of antipredator and conspecific defense. *Neurosci Biobehav Rev* 22: 597–612.

Campos AC and Guimarães FS (2008) Involvement of 5HT_{1A} receptors in the anxiolytic-like effects of cannabidiol injected into the dorsolateral periaqueductal gray of rats. *Psychopharmacology* 199: 223–230.

Carlini EA, Leite JR, Tannhauser M, et al. (1973) Letter: Cannabidiol and Cannabis sativa extract protect mice and rats against convulsive agents. *J Pharm Pharmacol* 25: 664–665.

Carrier EJ, Auchampach JA and Hillard CJ (2006) Inhibition of an equilibrative nucleoside transporter by cannabidiol: A mechanism of cannabinoid immunosuppression. *Proc Natl Acad Sci USA* 103: 7895–7900.

Crippa JA, Zuardi AW and Hallak JE (2010) Therapeutical use of the cannabinoids in psychiatry. *Rev Bras Psiquiatr* 32: 56–66.

Darwin C (1872) *The Expression of the Emotions in Man and Animals*. London: John Murray.

Da Silva LF, Coimbra NC and Menescal-de-Oliveira L (2012) Rostral ventromedial medulla modulates nociception and tonic immobility behavior through connections with the A7 catecholaminergic region. *Behav Brain Res* 233: 422–427.

Da Silva LF, Coimbra NC and Menescal-de-Oliveira L (2013a) Rostral ventromedial medulla connections in Cavia porcellus and their relation with tonic immobility defensive behavior: A biotinylated dextran amine neurotracing study. *Neurosci Lett* 535: 116–121.

Da Silva LA, de Freitas RL, Eichenberger GCD, et al. (2013b) Chemical neuroanatomical and psychopharmacological evidence that κ receptor-mediated endogenous opioid peptide neurotransmission in the dorsal and ventral mesencephalon modulates panic-like behaviour. *Eur J Pharmacol* 698: 235–245.

Davis M, Hitchcock JM and Rosen JB (1987) Anxiety and the amygdala: Pharmacological and anatomical analysis of the fear-potentiated startle paradigm. In: Bower GH (ed.) *The Psychology of Learning and Motivation: Advances in Research and Theory*. New York: Academic Press, pp.263–305.

De Freitas RL, Salgado-Rohner CJ, Biagioni AF, et al. (2013a) NMDA and AMPA/Kainate glutamatergic receptors in the prelimbic medial prefrontal cortex modulate the elaborated defensive behavior and innate fear-induced antinociception elicited by GABAA receptor blockade in the medial hypothalamus. *Cereb Cortex*. Epub ahead of print 2013. DOI: 10.1093/cercor/bh001.

De Freitas RL, Salgado-Rohner CJ, Hallak JEC, et al. (2013b) Involvement of medial prefrontal cortex on panic-like elaborated defensive behavior and innate fear-induced antinociception elicited by $GABA_A$ receptor blockade in the medial hypothalamus: Role of the endocannabinoid CB_1 receptor. *Int J Neuropsychopharmacol* 16: 1781–1798.

Di Marzo V, Piscitelli F and Mechoulam R (2011) Cannabinoids and endocannabinoids in metabolic disorders with focus on diabetes. *Handb Exp Pharmacol* 203: 75–104.

Eichenberger GCD, Ribeiro SJ, Osaki MY, et al. (2002) Neuroanatomical and psychopharmacological evidence for interaction between opioid and GABAergic neural pathways in the modulation of fear and defense elicited by electrical and chemical stimulation of the deep layers of the superior colliculus and dorsal periaqueductal gray matter. *Neuropharmacology* 42: 48–59.

Fusar-Poli P, Crippa JA, Bhattacharyya S, et al. (2009) Distinct effects of {delta}9-tetrahydrocannabinol and cannabidiol on neural activation during emotional processing. *Arch Gen Psychiatry* 66: 95–105.

Gorman JM, Kent JM, Sullivan GM, et al. (2000) Neuroanatomical hypothesis of panic disorder, revised. *Am J Psychiatry* 157: 493–505.

Graeff FG (1990) Brain defense systems and anxiety. In: Burrows GD, Roth M, Noyes R Jr (eds) *Handbook of Anxiety: The Neurobiology of Anxiety*. Vol. 3. Amsterdam: Elsevier Science Publishers, pp. 307–354.

Graeff FG (1994) Neuroanatomy and neurotransmitter regulation of defensive behaviors and related emotions in mammals. *Braz J Med Biol Res* 27: 811–829.

Gray JA and McNaughton N (2000) *Précis of the Neuropsychology of Anxiety: An Enquiry into the Functions of the Septo-Hippocampal System*, 2nd ed. New York: Oxford University Press.

Griebel G, Blanchard DC and Blanchard RJ (1996) Predator-elicited flight responses in Swiss-Webster mice: An experimental model of panic attacks. *Prog Neuro-Psychopharmacol Biol Psychiatry* 20: 185–205.

Guimarães FS, Chiaretti TM, Graeff F, et al. (1990) Antianxiety effect of cannabidiol in the elevated plus-maze. *Psychopharmcology (Berl)* 100: 558–559.

Guimarães-Costa R, Guimarães-Costa MB, Pippa-Gadioli L, et al. (2007) Innate defensive behavior and panic-like reactions evoked by rodents during aggressive encounters with Brazilian constrictor snakes in a complex labyrinth: Behavioural validation of a new model to study affective and agonistic reactions in a prey versus predator paradigm. *J Neurosci Meth* 165: 25–37.

Hubbard DT, Blanchard DC, Yang M, et al. (2004) Development of defensive behavior and conditioning to cat odor in the rat. *Physiol Behav* 80: 525–530.

LeDoux JE (1986) Neurobiology of emotion. In: LeDoux JE and Hirst W (eds) *Mind and Brain*. New York: Cambridge University Press, pp.301–354.

Lobão-Soares B, Walz R, Prediger RDS, et al. (2008) Cellular prion protein modulates defensive attention and innate fear-induced behaviour evoked in transgenic mice submitted to an agonistic encounter with the tropical coral snake *Oxyrhopus guibei. Behav Brain Res* 194: 129–137.

Lopes LT, Patrone LG, Bícego KC, et al. (2012) Periaqueductal gray matter modulates the hypercapnic ventilatory response. *Pflügers Arch-Eur J Physiol* 464: 155–166.

Mackowiak M, Chocyk A, Dudys D, et al. (2009) Activation of CB1 cannabinoid receptors impairs memory consolidation and hippocampal polysialylated neural cell adhesion molecule expression in contextual fear conditioning. *Neuroscience* 158: 1708–1716.

Malfait AM, Gallily R, Sumariwalla PF, et al. (2000) The nonpsychoactive cannabis constituent cannabidiol is an oral anti-arthritic therapeutic in murine collagen-induced arthritis. *Proc Natl Acad Sci USA* 97: 9561–9566.

Marco EM and Viveros MP (2009) Functional role of the endocannabinoid system in emotional homeostasis. *Rev Neurol* 48: 20–26.

McNaughton N and Corr PJ (2004) A two-dimensional neuropsychology of defense: Fear/anxiety and defensive distance. *Neurosci Biobehav Rev* 28: 285–305.

Mechoulam R, Parker LA and Gallily R (2002) Cannabidiol: An overview of some pharmacological aspects. *Chem Phys Lipids* 121: 35–43.

Mishima K, Hayakawa K, Abe K, et al. (2005) Cannabidiol prevents cerebral infarction via a serotonergic 5-hydroxytryptamine1A receptor-dependent mechanism. *Stroke* 36: 1077–1082.

Mobbs D, Petrovic P, Marchant JL, et al. (2007) When fear is near: Threat imminence elicits prefrontal-periaqueductal gray shifts in humans. *Science* 314: 1079–1083.

Moreira FA, Grieb M and Lutz B (2009) Central side-effects of therapies based on CB1 cannabinoid receptor agonists and antagonists: Focus on anxiety and depression. *Best Pract Res Clin Endocrinol Metab* 23: 133–144.

Motta SC, Goto M, Gouveia FV, et al. (2009) Dissecting the brain's fear system reveals the hypothalamus is critical for responding in subordinate conspecific intruders. *Proc Natl Acad Sci USA* 106: 4870–4875.

Nogueira RL and Graeff FG (1995) Role of 5-HT receptor subtypes in the modulation of dorsal periaqueductal gray generated aversion. *Pharmacol Biochem Behav* 52: 1–6.

Resstel LB, Tavares RF, Lisboa SF, et al. (2009) $5\text{-}HT_{1A}$ receptors are involved in the cannabidiol-induced attenuation of behavioural and cardiovascular responses to acute restraint stress in rats. *Br J Pharmacol* 156: 181–188.

Roser P, Vollenweider FX and Kawohl W (2008) Potential antipsychotic properties of central cannabinoid (CB(1)) receptor antagonists. *World J Biol Psychiatry* 11: 208–219.

Russo EB, Burnett A, Hall B, et al. (2005) Agonistic properties of cannabidiol at 5-HT1A receptors. *Neurochem Res* 30: 1037–1043.

Silva RC and Brandão ML (2000) Acute and chronic effects of gepirone and fluoxetine in rats tested in the elevated plus-maze: An ethological analysis. *Pharmacol Biochem Behav* 65: 209–216.

Soares VP, Campos AC, Bortoli VC, et al. (2010) Intra-dorsal periaqueductal gray administration of cannabidiol blocks panic-like response by activating 5-HT_{1A} receptors. *Behav Brain Res* 213: 225–229.

Spiacci A Jr, Coimbra NC and Zangrossi A Jr (2012) Differential involvement of dorsal raphe subnuclei in the regulation of anxiety- and panic-related defensive behaviors. *Neuroscience* 227: 350–360.

Thiemann G, Watt CA, Ledent C, et al. (2009) Modulation of anxiety by acute blockade and genetic deletion of the CB1 cannabinoid receptor in mice together with biogenic amine changes in the forebrain. *Behav Brain Res* 200: 60–67.

Thomas A, Baillie GL, Phillips AM, et al. (2007) Cannabidiol displays unexpectedly high potency as an antagonist of CB1 and CB2 receptor agonists in vitro. *Br J Pharmacol* 150: 613–623.

Thomas BF, Gilliam AF, Burch DF, et al. (1998) Comparative receptor binding analyses of cannabinoid agonists and antagonists. *J Pharmacol Exp Ther* 285: 285–292.

Uribe-Mariño A, Francisco A, Castiblanco-Urbina MA, et al. (2012) Anti-aversive effects of cannabidiol on innate fear-induced behaviors evoked by an ethological model of panic attacks based on a prey vs the wild snake *Epicrates cenchria crassus* confrontation paradigm. *Neuropsychopharmacology* 37: 412–421.

Weltson A, Pippa-Gadioli L, Koji-Narazaki F, et al. (2002) Neuromorphologic evidence for c-Fos-immunoreactive neurons in forebrain, diencephalon and brainstem structures involved in the elaboration of panic and fear in golden hamster after aggressive encounter with South American coral snake. *Int J Psychophysiol* 45: 156–157.

Zuardi AW, Antunes Rodrigues J and Cunha JM (1991) Effects of cannabidiol in animal models predictive of antipsychotic activity. *Psychopharmacology* 104: 260–264.