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The Rodent-versus-wild Snake Paradigm as a Model for Studying Anxiety- and Panic-like Behaviors: Face, Construct and Predictive Validities

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Abstract—Using an innovative approach to study the neural bases of psychiatric disorders, this study investigated the behavioral, morphological and pharmacological bases of panic attack-induced responses in a prey-versus-coral snake paradigm. *Mesocricetus auratus* was chronically treated with intraperitoneal administration of the selective serotonin uptake inhibitor paroxetine or the gamma aminobutyric acid (GABA)/benzodiazepine receptor agonist alprazolam at three different doses and were then confronted with a venomous coral snake (*Micrurus frontalis, Reptilia, Elapidae*). The threatened rodents exhibited defensive attention, flat back approaches, defensive immobility, and escape defensive responses in the presence of the venomous snake, followed by increases in Fos protein in limbic structure neurons. Chronic administration of both paroxetine and alprazolam decreased these responses with morphological correlates between the panicolytic effect of both drugs administered at the highest dose and decreases in Fos protein-immunolabeled perikarya found in the amygdaloid complex, hypothalamus and periaqueductal gray matter columns, which are structures that make up the encephalic aversion system. These findings provide face, construct and predictive validities of this new experimental model of anxiety- and panic attack-like behavioral responses displayed by threatened prey confronted with venomous coral snakes.

Key words: paroxetine, alprazolam, rodent-versus-snake paradigm, defensive behavior, venomous coral snakes, *Micrurus frontalis*.

INTRODUCTION

Several attempts to correlate experimental models in rodents with some pathological anxiety state, such as generalized anxiety disorder (GAD) and panic disorder syndrome (PD), have been undertaken (Bard, 1928; Hess and Brüger, 1943; Griebel et al., 1996; Spiacci Jr. et al., 2012; da Silva et al., 2017). There is evidence that instinctive stimuli signaling danger induce defensive reactions and activate encephalic circuits that are responsible in animal tests for generating and elaborating aversive states (Adams, 1979; Blanchard and Blanchard, 1988), which have been interpreted as a motivational state of fear in human beings (Nashold et al., 1969; Wilent et al., 2010).

Predator silhouettes, emotional expressions indicating rage and imminent attack, odors or sounds, threatening postures of a potential predator, and any other factor that might indicate the occurrence of noxious or painful

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Abbreviations: ANOVA, analysis of variance; DAB, 3,3′-diaminobenzidine; GAD, generalized anxiety disorder; PD, panic disorder; PMd, dorsal pre-mammillary; PVNp, posterior periventricular.

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stimuli have been frequently found in rodent-versus-snake paradigms (Guimarães-Costa et al., 2007; Almada and Coimbra, 2015; Coimbra et al., 2017a,b). In threatening situations, rodents can use sonorous clues to evaluate dangerous situations in their territory (Owings et al., 2002). Ground squirrels threatened by *Crotalus viridis oreganus* distinguished between rattlesnakes close to their shelters and those situated further away, and they clearly associated rattling sounds with the predator, proving to be able of assessing determinants of snake dangerousness based on acoustic cues (Swaisgood et al., 1999a,b).

Consequently, the defensive antipredatory behavioral repertory can be displayed as inhibitory avoidance and defensive attention (Coimbra et al., 2017a,b), defensive immobility (Uribe-Mariño et al., 2012), escape behavior (Twardowschy et al., 2013) and fear-induced antinociception (Coimbra et al., 2006, 2017a; Biagioni et al., 2013, 2016a; de Oliveira et al., 2017). In cases from which it is impossible to escape, some rodents exhibit tonic immobility (Leite-Panissi et al., 2003), or there might be elicited a set of threatening postures and vocalizations that can be followed by an attack (Blanchard and Blanchard, 1988).

Consistent findings have been reported since the work of Blanchard and Blanchard (Blanchard and Blanchard, 1988), based on threatening stimulus-induced interspecific confrontations in attempts to better understand these behavioral responses (Guimarães-Costa et al., 2007; Almada and Coimbra, 2015). According to this point of view, elucidating the neural bases of innate fear could be achieved by anti-predatory responses induced by a potential natural predator (Canteras et al., 1997; Comoli et al., 2003; Coimbra et al., 2017a). Several studies using ethological approaches have shown the neural substrates and neurochemical bases of instinctive stimuli signaling danger (Blanchard et al., 1990; Griebel et al., 1996). Furthermore, our research team showed that different species of rodents, such as *Rattus norvegicus* (Coimbra et al., 2017a), *Mus musculus* (Lobão-Soares et al., 2008; Uribe-Mariño et al., 2012; Twardowschy et al., 2013; Almada and Coimbra, 2015; Almada et al., 2015) and *Meriones unguiculatus* (Guimarães-Costa et al., 2007), displayed defensive responses when threatened by wild snakes. Although there have been several reports of rodent exposure to cats (Martinez et al., 2011), cat odor (do Monte et al., 2008; Souza and Carobrez, 2016), fox odor (Vincenz et al., 2017), and even wild snakes (Swaisgood et al., 1999a,b; Uribe-Mariño et al., 2012; Almada and Coimbra, 2015), few experiments have shown the neural bases of the antipredatory responses displayed by threatened rodents, and the majority of these reports have related experiments using rats confronted with cats/cat odor or fox odor (Blanchard et al., 2005; de Lima et al., 2017; Vincenz et al., 2017). However, there has been a lack of morphological and pharmacological evidence in terms of the validity of the experimental model using wild snakes as a threatening stimulus in a controlled, dangerous environment. The advantages of using venomous coral snakes in this model, is the exploratory behavior displayed by these snakes in the polygonal arena that enhances the probability of prey-versus-snake encounters, as well as the great (100%) survival index of threatened laboratory animals (Coimbra et al., 2017a).

In this sense, GAD has been related to defensive behaviors displayed in response to potential, as well as in real, but distant, threats, which consist of approach-avoidance conflict eliciting inhibitory behaviors and defensive attention. Conversely, models of PD consider the freezing and escape responses induced by a real and proximal threat related to panic attacks (Shekhar, 1994; Blanchard et al., 2001; Borelli et al., 2004; Ribeiro et al., 2005; Castellan-Baldan et al., 2006). We used, in the current work, the venomous coral snake *M. frontalis* in an attempt to provide threatening stimuli to Syrian hamsters, aimed at the activation of as many aversion-related structures of the limbic system as possible, resulting in the organization of defensive behavioral responses, which are suggestive of innate fear and are correlated with a motivational state of fear in humans, as described above.

Thus, the purpose of the present work was to provide validation of a new model of panic attack using a combination of ethological, morphological and pharmacological methodologies. These approaches allowed for the careful investigation of the neural substrates and the role played by serotonin- and GABA/benzodiazepine-mediated systems in the modulation of instinctive fear displayed by rodents in a threatening situation.

EXPERIMENTAL PROCEDURES

Animals

Male Syrian hamsters (*Mesocricetus auratus,* Rodentia, Cricetidae) weighing 100–150 g (n = 6–8 per group) from the animal house of the School of Medicine of Ribeirão Preto of the University of São Paulo (FMRP-USP) were used in the present work as prey. The Syrian hamsters (n = 76) were kept (four in a cage) in an experimental environment (48 h prior to the experiments), with free access to food and water under a light/dark cycle of 12/12 h (lights on from 7 a.m. to 7 p.m.) at a room temperature of 23 ± 1 °C. Females were excluded from this experiment to avoid the effects of different levels of estrogen and progesterone in each estrous cycle on behavioral responses elicited in the presence of coral snakes and to keep the total number of laboratory animals confronted by venomous snakes as small as possible.

As a source of threatening stimuli, we used wild male venomous coral snakes (*Micrurus frontalis, Reptilia, Elapidae*) weighing 200–150 g. The coral snakes (n = 3) were collected from Brazilian Cerrado and Atlantic forest and were maintained in an ophidiarium. The ophidiarium is a naturally lit compartment with calcareous rocks, tropical plants, and artificial thermal caves and burrows. The venomous coral snakes used in these experiments are commonly found in Grand Cerrado of Central Brazil and in Brazil Southeastern Atlantic forest, and consist of a natural source of visual

and olfactory stimuli with aversive connotations for the snakes' habitual prey and other small animals. One week before the experiments, the South American coral snakes were maintained in a polygonal arena for snakes with appropriate shelter and water situated in the Neuroanatomy and Neuropsychobiology Laboratory of the Ribeirão Preto Medical School of the University of São Paulo (LNN-FMRP-USP)/Behavioral Neurosciences Institute (INeC) ophidiarium. This snake facility was licensed by the Brazilian Federal government (IBAMA Committee; processes 3543.6986/2012-SP and 3543.6984/2012-SP) and by the São Paulo State government (SMA/DeFau 15.335/2012; MEDUSA Project, SISBIO processes 41435-1, and 41435-2; SIGAM 034399/14; NIS 1181484; authorization for installation process 39.043/2017; authorization of use and handling process 39.044/2017). The snake enclosure was maintained under a light/dark cycle of 12/12 h (lights on from 7 a.m. to 7 p.m.) and at a constant room temperature of 27 °C ± 1 °C (50–70% humidity). Our technicians fed the venomous coral snakes with *Oxyrhopus guibei* puppies at two specific times: 24 h before and immediately before the start of each experimental session. On these occasions, the coral snakes displayed hunting behaviors and predatory attacks, followed by searching responses, capture of their natural prey and behavioral feeding responses. All of the experiments were performed according to the recommendations of the FMRP-USP Commission of Ethics in Animal Experimentation (process 015/2003), consistent with the ethical principles for animal research adopted by the National Council for Animal Experimentation control (CONCEA) and approved by the FMRP-USP Commission of Ethics in Animal Research (CETEA).

Prey-versus-snake paradigm

For rodent-versus-snake threatening encounters, a semi-transparent acrylic enclosure was used, which consisted of a polygonal arena for the snakes. The polygonal arena is a model of panic attack designed by Coimbra et al. (2017a,b) to study instinctive fear-induced behaviors displayed by small rodents in the presence of wild snakes. The polygonal arena used in the current investigation consisted of a high walled crystal acrylic parallelepiped shape polygonal environment 154 cm in length, 72 cm in width and 64 cm in height. The inner walls were coated with a reflective film aiming to provide 80–90% light reflection to minimize visual contact of the coral snake with the surrounding experimental area and to target its attention toward the golden hamster. The roof of the polygonal arena has 31 (0.9 mm diameter) holes situated on each side for air changes, and has a fluorescent lamp in the center of its inner surface, providing an illuminance of 30 footcandles (200 Lux). A fluorescent line (4.2 mm in width; Pritt Mark-it) was used to divide the arena into 20 equal rectangular compartments. The acrylic base of the arena was positioned over a rectangular, stainless steel platform, and the complete apparatus was placed on a granite rock surface (2 cm in length 85 cm in width and 170 cm in height) 83 cm from the laboratory floor to minimize any vibratory stimuli.

Procedures

After one week of habituation in the LNN-FMRP-USP/INeC Ophidiarium, each snake was carefully placed inside the polygonal arena. The rodent-versus-snake confrontation experiment was preceded by exposure of a naïve hamster (not included in the statistical analysis) to the snake. Thus, each experimental group was eventually subjected to the same residual odor stimulus provided by the coral snake or rodent. However, a different snake was used for each naïve experimental group after at least one week of resting in the ophidiarium, and the experimental polygonal arena was cleaned with 5% alcohol solution before each experimental group underwent testing, as previously published (Coimbra et al., 2017a). No hamster was habituated to the polygonal arena before testing. However, the rodents were previously habituated to the laboratory environment. In addition, the no-threat group (submitted to the presence of a cleaned toy snake) was submitted to a completely clean environment that was previously washed with tap water and cleaned with a 5% alcohol solution. The hamsters were exposed to coral snakes in a random fashion during the evening according to the experimental groups, as described below. The rodents were then placed into the apparatus in a location opposite from the snake. After 15 min of confrontation, each hamster was removed from the polygonal arena, and after cleaning the experimental environment with distilled water, the other rodents were placed one at a time into the polygonal arena to be confronted with the snake. Each rodent remained in the arena for the same amount of time. The snakes were exposed to a maximum of 8 rodents (one at a time) and were then transferred back into the snake enclosure in the LNN-FMRP-USP/INeC Ophidiarium for a quarantine period to minimize any stressful effects due to the threatening encounter. After the quarantine, the venomous snakes were then transferred to the FMRP-USP main ophidiarium. In no case did a coral snake eat an experimental Syrian hamster (100% prey survival). The experimental groups of Syrian hamsters received intraperitoneal chronic treatment with physiological saline, paroxetine (at 5, 10 and 20 mg/kg) or alprazolam (at 1, 2 and 4 mg/kg) for 21 days (n = 8 per group). Ten minutes after the last injection, the rodents were exposed to the coral snake for 15 min. For an additional control, another experimental group (no-threat group) was treated with intraperitoneal administration of physiological saline for 21 days and was exposed to the polygonal arena with a toy coral snake (n = 6).

Behavioral response recordings

The rodent behaviors were recorded as follows: (a) defensive attention (alertness behavior), operationally defined as the interruption of ongoing behavior for up to five seconds, with occasional analysis of the environment (i.e., attentive posture, with short head movements, rearing and smelling the surrounding air

with attentional behavior directed toward the snake); (b) defensive immobility (freezing behavior) when they presented immobility (operationally defined as the absence of head and body movements at least for 6 s, except that required for respiration accompanied by autonomic reactions: defecation, urination and piloerection); (c) escape responses, recorded as important features of the defensive behavior of the rodents, suggesting an instinctive fear-related response, expressed as running and jumping; and (d) flat-back approach, indicating cautious behavior expressed by the rodents as forward elongation of the body with frontward movement by slowly pulling their hind bodies.

We consider escape responses and defensive immobility behaviors as panic-like responses unlike risk assessment behavioral responses (defensive attention and flat-back approaches), which are considered anxiety-like responses. Close contacts, followed by sniffing, careful touching with the front paws and/or nose, and eventually ‘‘exploratory biting” (by some rodents) between hamsters and coral snakes, were considered interactions between the rodent and the snake. All of the behavioral reactions were captured by a video camera (Sony Handcam HDR-CX350, Tokyo, Japan) for subsequent blinding of researchers for analysis.

Drugs

The 5-hydroxytryptamine uptake inhibitor paroxetine hydrochloride (5, 10 and 20 mg/kg, Eli Lilly & Co., Indianapolis, IN, USA) and alprazolam (1, 2 and 4 mg/kg, Sigma Chemical Co., St. Louis, MO, USA), a GABA/benzodiazepine receptor agonist, were used in this study. The drugs were dissolved in 0.9% NaCl shortly before use. Physiological saline was also used in the control group.

Immunohistochemistry of c-Fos-labeled neurons

Two hours after the rodent-versus-coral snake confrontation, the group of Syrian hamsters that received paroxetine at 20 mg/kg, alprazolam at 4 mg/kg, or physiological saline and the no-threat group were deeply anesthetized with ketamine at 92 mg/kg (Ketamine Agener, União Química Farmacêutica Nacional, São Paulo, Brazil) and xylazine at 9.2 mg/kg (Calmium, União Química Farmacêutica Nacional, São Paulo, Brazil) and were perfused through the left cardiac ventricle with 0.1 M phosphate-buffered saline (Na_2HPO_4 + $NaH_2PO_4{\cdot}H_2O$ + NaCl + distillate H_2O), followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The encephalons were obtained, immersed in fixative for 2 h (4 °C) and then were kept in 10% and 20% sucrose solutions in 0.1 M PBS for cryoprotection. They were then fast frozen in isopentane (−40 °C) and sectioned with a cryostat (Leica CM 1950, Wetzlar, Germany) at −19 °C. Two adjacent series of 40-μm-thick encephalic slices were obtained according to the stereotaxic atlas of the golden hamster brain (Morin and Wood, 2001).

One encephalic coronal section series was stained with Nissl and used for neuroanatomical comparison. The other brain section series was collected in 0.1 M PBS and was subsequently free-floating processed according to the avidin–biotin procedure, using the PK 6101 Vectastain ABC Elite peroxidase rabbit IgG kit (Vector Laboratories, Burlingame, CA, USA). The reactions were performed at room temperature under constant agitation. The encephalic slices were first incubated with 1% H_2O_2 for 10 min and washed four times with 0.1 M PBS (5 min each). To avoid non-specific binding, the slides were then incubated in bovine serum albumin (2%) plus goat serum (10%) for 1 h, followed by overnight incubation with anti-Fos protein rabbit polyclonal primary antibody (Santa Cruz, CA, USA, SC-52) at 1:2000 in 0.1 M PBS, enriched with 0.2% Triton-X and 0.5% bovine serum albumin. The slices were washed again (three times for 5 min each time) with 0.1 M PBS, incubated for 1 h with biotinylated anti-rabbit goat IgG secondary antibody (Vectastain, Vector Laboratories, CA, USA) at a concentration of 1:400 in 0.1 M PBS plus 0.5% BSA, and then washed three times (5-min duration per wash) in 0.1 M PBS. After washing again (three 5-min washings) in 0.1 M PBS, the slices were then incubated with the avidin–biotin–peroxidase complex in 0.1 M PBS (A and B solution of the Vectastain ABC Vector Laboratories kit) at 1:250 in 0.1 M PBS for 1 h. The encephalic coronal sections were then washed three more times in 0.1 M PBS (5 min per wash). Fos protein immunoreactivity was revealed by the addition of 0.02% 3,3′-diaminobenzidine (DAB), to which hydrogen peroxide (0.04%) was added immediately before use. The slices were then washed three times with 0.1 M PBS.

Quantification of Fos protein-labeled perikarya

The cerebral and brainstem tissue sections were mounted on gelatin-coated silanized (siliconized) slides and dehydrated for histological analysis. The neuronal cells were then counted under a motorized photomicroscope (AxioImager Z1, Carl Zeiss Straβe, Oberkochen, Germany). A blinded researcher obtained photomicrographs from 1118 encephalic slices showing Fos protein-labeled structures, related to the no-threat group (195 sections; $n = 6$), physiological saline + threat-treated group (326 sections; $n = 8$), chronic paroxetine + threat-treated group (240 sections; $n = 8$) and alprazolam + threat-treated group (357 slices; $n = 8$). The average of 37 images selected was calculated for each golden hamster. For the counting process, the background signal intensity average was 41.83, and the nucleus intensity signal average was 82.57. All nuclei with sizes between 33.19 μm^2 and 98.5 μm^2 (55.4 μm^2 in average) were counted. Neuronal nuclei expressing DAB reaction product greater than the tissue background levels were counted as Fos protein labeled. Counting of Fos protein-labeled perikarya was performed under magnification of 10× in one field per area that encompassed the entire encephalic region, which was included in the quantification. Structures of the same shape and size per encephalic area were

used for each Syrian hamster. The same light and threshold conditions were employed for all histological encephalic sections. Fos protein-staining intensity could vary from one structure to another. To ensure the accuracy of the recordings and to avoid variations among the same nuclei in different Syrian hamsters, the background of every area was recorded and digitally subtracted from the area under investigation. All of the encephalic regions were bilaterally counted for each Syrian hamster. The encephalic structures analyzed in the present work are indicated below. The neuronal nuclei were individually counted and expressed as the number of c-Fos protein-labeled nuclei per 0.1 mm^2.

We considered lateral (LA), basolateral (BLA), basomedial (BMA), medial (MeA) and central (CeA) nuclei of the amygdaloid complex as prosencephalic key limbic structures. The diencephalic limbic structures include the anterior (AH), dorsomedial (DMH), ventromedial (VMH), posterior (PH), lateral (LH), posterior periventricular (PVNp) and dorsal premammillary (PMd) hypothalamic nuclei. In the mesencephalic division of the central nervous system, we considered the rostral, intermediate and caudal divisions of the dorsomedial (dmPAG), dorsolateral (dlPAG) and lateral (lPAG) columns of the periaqueductal gray matter, which are midbrain structures considered the main output of the encephalic aversion system.

Statistical analysis

The behavioral experiments were randomly performed. All of the data from independent groups were submitted to the Shapiro–Wilk test of normality and to Bartlett's test of homogeneity of variances using GraphPad Prism software, version 7.0. The experimental data followed the Gaussian distributions, and equal variances were found in the sample distributions for 70% of data. The behavioral data for the no-threat and saline-treated groups were analyzed using the unpaired *t* test for independent samples and a one-way analysis of variance (ANOVA) followed by the Newman−Keuls multiple comparison post hoc test to compare the effects of different doses of paroxetine and alprazolam with the saline-treated group. Morphological qualitative analyses were performed using an AxioImager Z1 photomicroscope with AxioVision software, version 4.7, and quantitative analyses were performed using ImageJ software. The morphological data were analyzed by a one-way ANOVA, followed by Tukey's multiple comparison post hoc test. The significance level was set at $p < 0.05$.

RESULTS

Neuroethological analysis

As shown in Figs. 1 and 2, hamster exposure to the coral snake evoked risk assessment, expressed by defensive attention and flat back approaches, defensive immobility, and escape behavior expressed by jumps and running, compared with the rodents' exposure to the toy coral snake. In addition, exposure to a live snake induced a significant decrease in interaction, compared with the exposure of Syrian hamster to the toy coral snake. The effects of different doses of chronic treatment with paroxetine and alprazolam on the behavioral responses displayed by rodents are also shown in these figures.

Effects of chronic treatment with paroxetine and alprazolam on the behavioral responses evoked in rodents confronted by coral snakes

Exposure to a live venomous snake (physiological saline-treated group) induced a significant increase in the frequency (number of behavioral events) ($t_{12} = 4.68$; $p < 0.001$) and duration ($t_{12} = 2.57$; $p < 0.001$) of defensive attention, compared with the animals exposed to the toy coral snake (no-threat group). According to a one-way ANOVA followed by Newman–Keuls' post hoc test, there was a significant effect of the treatment on the frequency [$F_{(6,49)} = 6.58$, $p < 0.001$] and duration [$F_{(6,49)} = 5.92$, $p < 0.001$] of defensive attention. The chronically treated group with the highest dose of paroxetine (20 mg/kg) showed decreased frequency ($p < 0.01$) of defensive attention displayed with exposure to the coral snake (saline-treated group). Hamsters in the 20-mg/kg paroxetine chronically treated group were different from the group that received paroxetine at 5 mg/kg ($p < 0.01$ for the frequency). The intermediate and highest doses of paroxetine (10 and 20 mg/kg) decreased the duration ($p < 0.05$ and $p < 0.01$, respectively) of defensive attention and were different from the group treated with paroxetine at 5 mg/kg ($p < 0.05$ and $p < 0.01$, respectively). The group chronically treated with alprazolam at 2 and 4 mg/kg showed decreased frequency ($p < 0.05$ and $p < 0.001$, respectively) of defensive attention, compared to the response displayed by physiological saline-treated hamsters exposed to the coral snake. The frequency of defensive attention displayed by Syrian hamsters chronically treated with alprazolam at 4 mg/kg was significantly different from that displayed by rodents treated with alprazolam at the lower dose of 1 mg/kg ($p < 0.05$). In addition, chronically treated groups with alprazolam at all doses (1, 2 and 4 mg/kg) showed decreased duration ($p < 0.05$, $p < 0.01$ and $p < 0.01$, respectively) of defensive attention (Fig. 1A, B).

The wild venomous coral snake (physiological saline-treated group) induced a significant increase in the frequency ($t_{12} = 3.46$; $p < 0.01$) and duration ($t_{12} = 2.99$; $p < 0.05$) of defensive attention, compared with the animals exposed to the toy coral snake (no-threat group). According to a one-way ANOVA, there was a significant effect of the treatment on the frequency [$F_{(6,49)} = 7.85$, $p < 0.001$] and duration [$F_{(6,49)} = 5.28$, $p < 0.001$] of defensive immobility. According to the Newman–Keuls post hoc test, the group chronically treated with paroxetine at doses of 10 and 20 mg/kg decreased the frequency ($p < 0.001$ in both doses) and duration ($p < 0.01$ in both doses) of defensive immobility induced by predator exposure (physiological saline-treated control group). The groups chronically treated with paroxetine at 10 and 20 mg/kg were different from the group that received paroxetine at 5

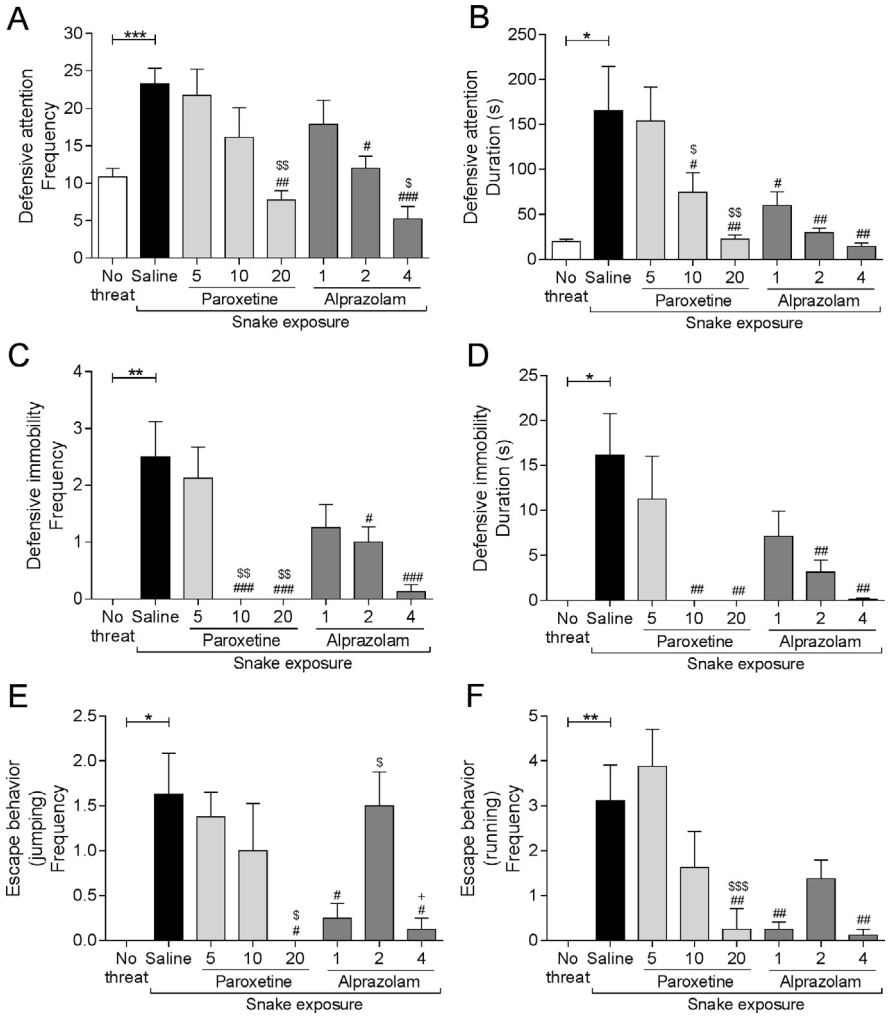

Fig. 1. Effect of intraperitoneal chronic treatment with paroxetine (5, 10, and 20 mg/kg) or alprazolam (1, 2, and 4 mg/kg) in Syrian hamsters exposed to confrontation with the venomous coral snake *Micrurus frontalis* on the frequency and duration of defensive attention (A, B) and defensive immobility (C, D) and on the frequency of escape behavior expressed by jumps (E) and running (F). The columns represent the means, and the error bars represent the S.E.M.; $n = 6–8$ hamsters per group. $^{*}p < 0.05$, $^{**}p < 0.05$ and $^{***}p < 0.001$ compared to the no-threat group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared to physiological saline-treated group; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ and $^{\$\$\$}p < 0.001$ compared to the lower dose used in the same treatment; $^{+}p < 0.05$ compared to the intermediate dose used in the same treatment.

mg/kg ($p < 0.01$) regarding the frequency of defensive immobility but not duration ($p > 0.05$). The chronically treated groups with the intermediate and highest doses of alprazolam (2 and 4 mg/kg) showed decreased frequency ($p < 0.05$ and $p < 0.001$, respectively) of defensive immobility, compared to the exposure of the physiological saline-treated group to the coral snake. The groups chronically treated with alprazolam at 2 and 4 mg/kg also showed decreased duration ($p < 0.01$ in both doses) of defensive immobility. These data are shown in Fig. 1C, D.

Threatening by a live coral snake (physiological saline-treated group) induced a significant increase in the frequency of escape behavior expressed by jumps ($t_{12} = 3.03$; $p < 0.05$) and running ($t_{12} = 3.38$; $p < 0.01$), compared with the animals exposed to the toy coral snake (no-threat group). There was a significant effect of the treatment on the frequency of jumps [$F_{(6,49)} = 4.55$, $p < 0.001$] and running [$F_{(6,49)} = 6.47$, $p < 0.001$], according to a one-way ANOVA. The chronically treated group with the highest dose of paroxetine (20 mg/kg) showed a decreased frequency of jumps ($p < 0.05$) and running ($p < 0.01$), according to the Newman–Keuls post hoc test. The group chronically treated with paroxetine at 20 mg/kg displayed jumps and running responses significantly different from those displayed by the group treated with paroxetine at 5 mg/kg ($p < 0.05$ and $p < 0.01$, respectively). The group chronically treated with alprazolam at 1 and 4 mg/kg decreased the frequency of jumps ($p < 0.05$) and running ($p < 0.01$). The frequency of jumps displayed by alprazolam at 4 mg/kg chronically-treated group was significantly different from that displayed by alprazolam at 2 mg/kg chronically-treated group ($p < 0.05$) and there was significant differences between the incidence of jumps displayed by Syrian hamsters treated with alprazolam at the lower doses (1 and 2 mg/kg). These data are shown in Fig. 1E, F).

Exposure to *Micrurus frontalis* (physiological saline-treated group) induced a significant increase in the frequency ($t_{12} = 3.52$; $p < 0.01$), but not in the duration ($t_{12} = 2.00$; $p > 0.05$), of flat back approaches, compared with the animals exposed to the toy coral snake (no-threat group). There was a significant effect of the treatment on the frequency [$F_{(6,49)} = 5.06$, $p < 0.001$] and duration [$F_{(6,49)} = 5.47$, $p < 0.001$] of flat-back approaches, according to a one-way ANOVA. Chronic treatment with paroxetine at all doses (5, 10 and 20 mg/kg) decreased the frequency ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively) and duration ($p < 0.05$, $p < 0.01$, $p < 0.001$, respectively) of flat-back approaches, according to the Newman–Keuls post hoc test. The group chronically treated with alprazolam at all doses (1, 2 and 4 mg/kg) also showed a decreased frequency ($p < 0.05$, $p < 0.05$ and $p < 0.001$, respectively) of flat-back approaches, and the group chronically treated with alprazolam at 2 and 4 mg/kg also showed decreased duration ($p < 0.01$ and $p < 0.001$ respectively) of flat-back approaches, according to the Newman-Keuls post hoc test. These data are shown in Fig. 2A, B.

Exposure to a live venomous coral snake (physiological saline-treated group) reduced the duration

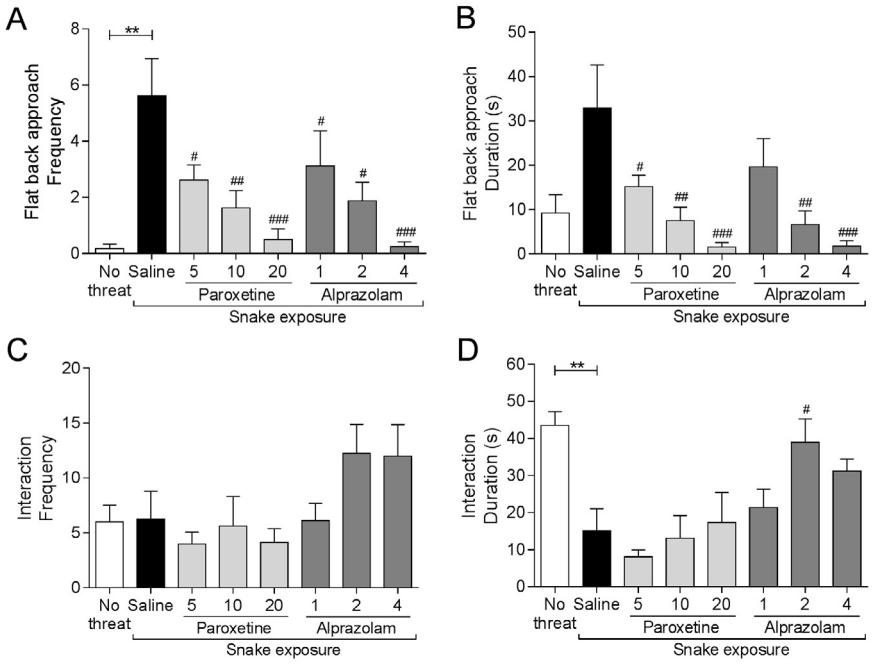

Fig. 2. Effect of intraperitoneal chronic treatment with paroxetine (5, 10, and 20 mg/kg) or alprazolam (1, 2, and 4 mg/kg) in Syrian hamsters exposed to confrontation with the venomous coral snake *Micrurus frontalis* on the frequency and duration of flat back approach (A, B) and on the interaction between the hamsters and coral snakes (C, D). The columns represent the means, and the error bars represent the S.E.M.; n = 6–8 hamsters per group. $^{**}p < 0.01$ compared to the no-threat group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared to physiological saline-treated group.

(t_{12} = 3.71; $p < 0.01$), but not the frequency (t_{12} = 0.08; $p > 0.05$), of the interaction between rodents and coral venomous snakes, compared with the animals exposed to the toy coral snake (no-threat group). According to a one-way ANOVA, there was no effect of the treatment on the frequency [$F_{(6,49)}$ = 2.51, $p > 0.05$] of interaction between rodents and snakes. However, regarding the duration of rodent-versus-snake interaction, there was a significant effect of the treatment [$F_{(6,49)}$ = 3.81, $p < 0.01$]. The Newman-Keuls post hoc test indicated that only the group chronically treated with alprazolam at 2 mg/kg increased the duration ($p < 0.05$) of the interactions with the snake, as shown in Fig. 2C, D.

Morphological analyses of the snake threatening and pharmacological treatment effects on Fos protein-immunolabeled neurons

Syrian hamsters chronically pre-treated with physiological saline and confronted with coral snakes expressed Fos protein-immunolabeled neurons in the prosencephalic, diencephalic and mesencephalic structures related to the organization of innate fear-related behavior, considering the amygdala, hypothalamic nuclei and periaqueductal gray matter columns, as shown in the representative photomicrographs provided at the top of Figs. 3–5.

The representative photomicrographs provided in the middle of Figs. 3–5 show the effects of exposure to the venomous coral snake and of chronic treatment with either paroxetine or alprazolam at the highest doses (20 and 4 mg/kg, respectively).

According to a one-way ANOVA, followed by Tukey's post hoc test, Syrian hamsters exposed to the venomous coral snake (physiological saline-treated group) displayed panic attack-related defensive responses as described above and an increase in the number of Fos protein-immunolabeled neurons in prosencephalic neurons, including the LA [$F_{(3,26)}$ = 19.66, $p < 0.001$], BLA [$F_{(3,26)}$ = 28.23, $p < 0.001$], BMA [$F_{(3,26)}$ = 16.84, $p < 0.001$], MeA [$F_{(3,26)}$ = 15.28, $p < 0.001$] and CeA [$F_{(3,26)}$ = 11.63, $p < 0.001$] nuclei of the amygdaloid complex, compared to Syrian hamsters exposed to the toy coral snake (no-threat group). Chronic pretreatment with paroxetine at the highest dose (20 mg/kg) decreased the number of Fos protein-immunolabeled neurons in the LA ($p < 0.001$), BLA ($p < 0.001$), BMA ($p < 0.01$), MeA ($p < 0.01$) and CeA ($p < 0.01$) amygdaloid complex nuclei. Chronic pretreatment with alprazolam at the highest dose (4 mg/kg) also decreased the number of Fos protein-immunolabeled neurons in the LA ($p < 0.001$), BLA ($p < 0.001$), BMA ($p < 0.001$), MeA ($p < 0.001$) and CeA ($p < 0.001$) amygdaloid nuclei. These data are shown in Fig. 3 (bottom), and the raw data are represented as the mean ± standard error of the mean (S.E.M.) in Table 1.

According to a one-way ANOVA, followed by Tukey's post hoc test, exposure to the venomous coral snake also increased the number of Fos protein-immunolabeled perikarya in the diencephalic limbic structures, including the AH [$F_{(3,26)}$ = 17.20, $p < 0.001$], DMH [$F_{(3,26)}$ = 21.04, $p < 0.001$], VMH ([$F_{(3,26)}$ = 14.45, $p < 0.001$], PH [$F_{(3,26)}$ = 9.78, $p < 0.001$], LH [$F_{(3,26)}$ = 38.15, $p < 0.001$], PVNp [$F_{(3,26)}$ = 17.48, $p < 0.001$], and PMd [$F_{(3,26)}$ = 39.08, $p < 0.001$]. Chronic pretreatment with paroxetine at the highest dose (20 mg/kg) decreased the number of Fos protein-immunolabeled perikarya in the AH ($p < 0.001$), DMH ($p < 0.01$), VMH ($p < 0.001$), LH ($p < 0.001$), PVNp ($p < 0.001$), and PMd ($p < 0.001$) but not in the PH ($p > 0.05$). Chronic pretreatment with alprazolam at the highest dose (4 mg/kg) decreased the number of Fos protein-immunolabeled perikarya in the AH ($p < 0.001$), DMH ($p < 0.01$), VMH ($p < 0.01$), PH ($p < 0.01$), LH ($p < 0.001$), PVNp ($p < 0.001$), and PMd ($p < 0.001$) hypothalamic nuclei. These data are shown in Fig. 4 (bottom), and the raw data are presented as the mean ± S.E.M. in Table 1.

Regarding the mesencephalic division of the central nervous system, exposure to a venomous coral snake

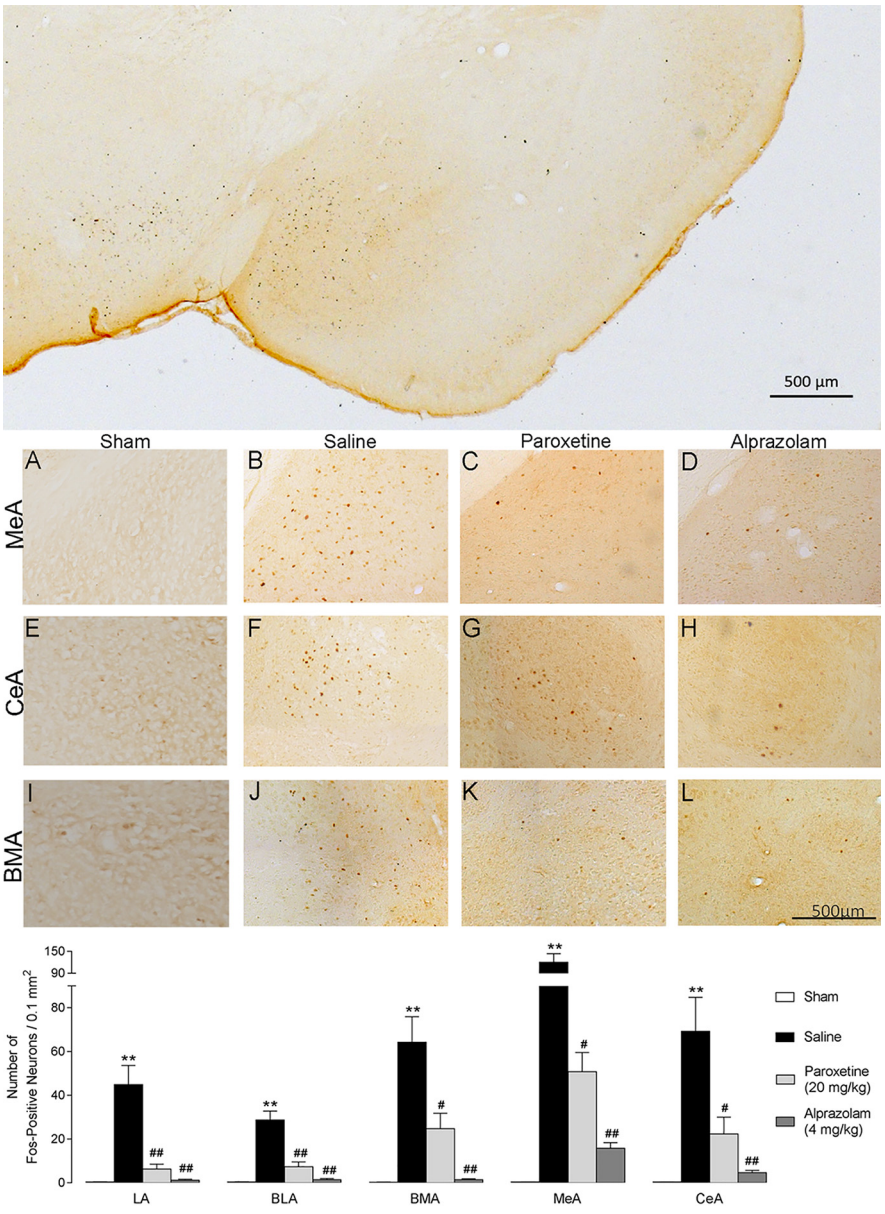

Fig. 3. Representative photomicrograph of a coronal section of the encephalon at the level of the amygdaloid nuclei (upper panel). Photomicrographs show Fos protein-immunolabeled nuclei in neuronal cells in the medial (A–D), central (E–H) and basomedial (I–L) amygdaloid nuclei of Syrian hamsters exposed to a toy snake (sham group, A, E and I) and those pre-treated with intraperitoneal chronic injections of saline (B, F and J), paroxetine at 20 mg/kg (C, G, and K) or alprazolam at 4 mg/kg (D, H, and L) and exposed to venomous coral snakes. Bottom of the panel shows the number of Fos protein-labeled neurons/0.1 mm^2 in the lateral (LA), basolateral (BLA), basomedial (BMA), medial (MeA) and central (CeA) nuclei of the amygdaloid complex. The columns represent the means, and the error bars represent the S.E.M. n = 6–8 hamsters per group. $^{**}p < 0.001$ compared to sham group; $^{\#}p < 0.01$ and $^{\#\#}p < 0.001$ compared to saline-treated group. The scale bars shown in the top and in L represent 500 µm in all photomicrographs.

increased the number of Fos protein-immunolabeled neurons in the rostral divisions of the dmPAG [$F_{(3,26)}$ = 24.84, $p < 0.001$], dlPAG [$F_{(3,26)}$ = 35.06, $p < 0.001$] and lPAG [$F_{(3,26)}$ = 18.91, $p < 0.001$], in the intermediate divisions of the dmPAG [$F_{(3,26)}$ = 51.73, $p < 0.001$], dlPAG [$F_{(3,26)}$ = 51.58, $p < 0.001$] and lPAG [$F_{(3,26)}$ = 51.56, $p < 0.001$], and in the caudal divisions of the dmPAG [$F_{(3,26)}$ = 13.53, $p < 0.001$], dlPAG [$F_{(3,26)}$ = 30.81, $p < 0.001$] and lPAG [$F_{(3,26)}$ = 40.41, $p < 0.001$], according to a one-way ANOVA, followed by Tukey's post hoc test. Chronic pretreatment with paroxetine and alprazolam at the highest doses (20 and 4 mg/kg, respectively) decreased the number of Fos protein-immunolabeled neurons in all divisions of the dmPAG ($p < 0.001$), dlPAG ($p < 0.001$) and lPAG ($p < 0.001$) columns. These data are shown in Fig. 5 (bottom), and the raw data are presented as the mean ± S.E.M. in Table 1.

DISCUSSION

Effects of snake exposure and pharmacological treatments on defensive behavior

Syrian hamsters confronted with the venomous coral snake *M. frontalis* displayed anxiety- and panic-like behaviors, and treatment with paroxetine and alprazolam significantly attenuated both anxiolytic- and panicolytic-like behavioral responses. Defensive attention and flat back approach behaviors evaluated in the present paradigm consist of a defensive strategy that comprises risk assessment defensive behaviors. These responses have already been described elsewhere (Coimbra et al., 2017a) and are considered a generalized anxiety-related response (Shekhar, 1994; Blanchard et al., 2001; Borelli et al., 2004). For this reason, the decrease in these behaviors after chronic treatment with paroxetine and alprazolam suggested some anxiolytic-like effects of these drugs. In contrast, the defensive immobility and escape behaviors evaluated in this work consisted of panic attack-related responses induced by a real and proximal threat, as recently described by Coimbra et al. (2017a,b) and elsewhere (Uribe-Mariño et al., 2012; Twardowschy et al., 2013). In addition, the interaction between rodents and snakes is also considered an unconditioned fear-related behavior.

Benzodiazepines, such as alprazolam, would be expected to reduce anxiety-like behaviors, and serotonin uptake inhibitors, such as paroxetine, would be expected to reduce panic-like behavioral responses. However, in the present rodents-versus-venomous coral snake paradigm, both drugs diminished both anxiety-related responses and panic attack-related behavioral reactions.

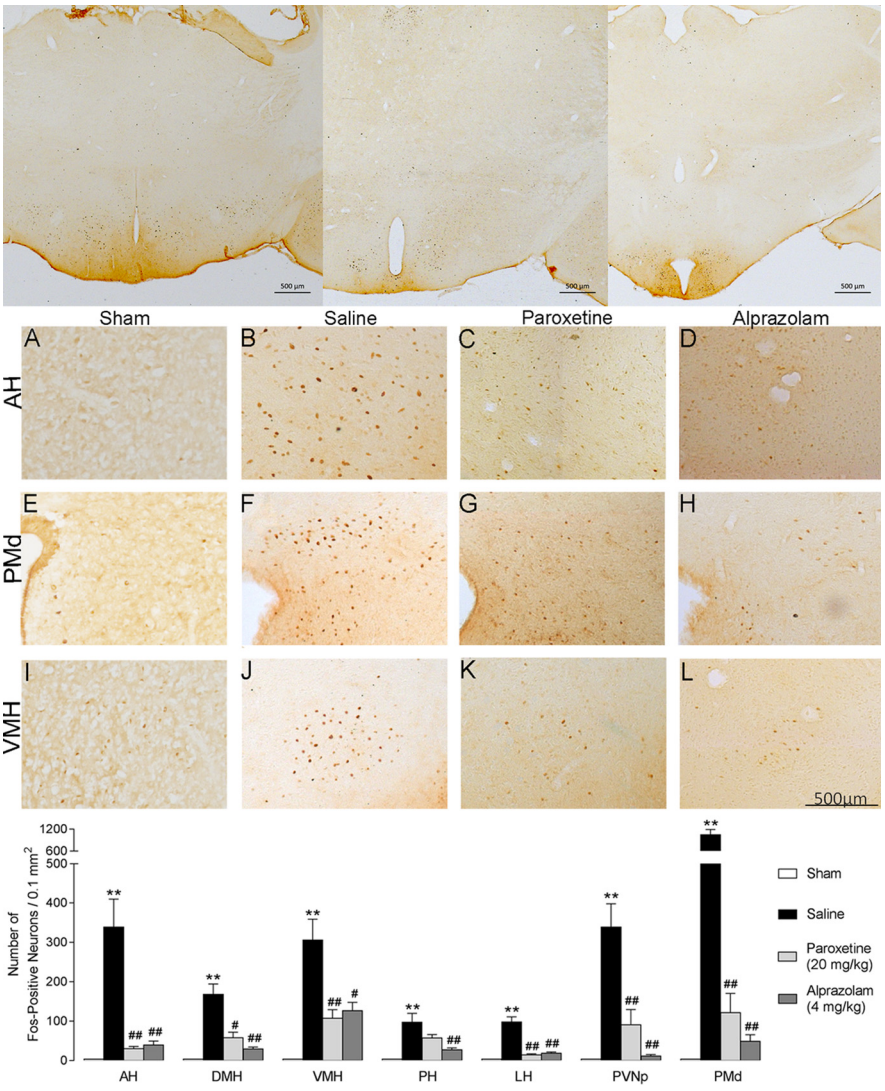

Fig. 4. Representative photomicrographs of coronal sections of the encephalon at the level of the hypothalamic nuclei (upper panel). Photomicrographs show c-Fos-immunolabeled nuclei in neuronal cells in the anterior (A–D), dorsal pre-mammillary (E–H) and ventromedial (I–L) hypothalamic nuclei of Syrian hamsters exposed to a toy snake (sham group, A, E and I) and those pre-treated with intraperitoneal chronic injections of saline (B, F and J), paroxetine at 20 mg/kg (C, G, and K) or alprazolam at 4 mg/kg (D, H, and L) and exposed to venomous coral snakes. Bottom of the panel shows the number of Fos protein-labeled neurons/0.1 mm^2 in the anterior (AH), dorsomedial (DMH), ventromedial (VMH), posterior (PH), lateral (LH), posterior periventricular (PVNp) and dorsal pre-mammillary (PMd) hypothalamic nuclei. The columns represent the means, and the error bars represent the S.E.M. $n = 6–8$ hamsters per group. $^{**}p < 0.001$ compared to sham group; $^{\#}p < 0.01$ and $^{\#\#}p < 0.001$ compared to saline-treated group. The scale bars shown in the top and in L represent 500 µm in all photomicrographs.

In clinical practice, serotonin reuptake inhibitors are generally considered the first-line drugs for short- and long-term treatments of GAD. A clinical report by Pollack et al. (2001) showed, in an 8-week double-blind placebo-controlled study, that 20 and 40 mg of paroxetine significantly improved Hamilton Anxiety Rating Scale scores and clinical symptoms of anxiety in humans, compared to placebo. In addition, clinical evidence has suggested a putative effect of benzodiazepines in GAD due to their rapid onset of effect and good tolerability (Reinhold and Rickels, 2015). Rickels et al. (2005) in 4-week double-blind placebo-controlled study showed that alprazolam at 1.5 mg caused a significant reduction in Hamilton Anxiety Rating Scale scores, compared with placebo. In fact, both alprazolam and diazepam improved the anxiety scale and seemed to be effective in the treatment of GAD (Elie and Lamontagne, 1984). Therefore, the present results reinforced the idea that GABA/benzodiazepine receptor activation with alprazolam and serotonin reuptake inhibition with paroxetine caused anxiolytic-like effects, and the rodent-versus-snake paradigm might have utility for the investigation of anxiolytic drugs, given the pharmacological predictability of our model.

Both paroxetine and alprazolam have consistent and similar effects on panic-like responses except for rodent-versus-snake interaction behavior since only alprazolam was able to increase this behavior. Supported by those authors, decreases in these behaviors after chronic treatment with paroxetine and alprazolam suggest a panicolytic-like effect caused by these drugs in the present panic attack experimental paradigm. Our data agree with previous studies performed by Griebel et al. (1995), who showed that only chronic treatment with alprazolam decreased defensive responses displayed by rodents in a mouse defense test battery, diminishing the distance between prey and a potential predator, which is a panicolytic-like effect.

In the pharmacotherapy of PD, medicines with efficacy usually include selective serotonin or norepinephrine reuptake inhibitors, tricyclic antidepressants, monoamine oxidase inhibitors, and benzodiazepines (Pull and Damsa, 2008). A twelve-week double-blind placebo-controlled study performed in humans showed that paroxetine at doses of 20, 40, and 60 mg caused substantial reduction in panic attack episodes, compared with placebo (Oehrberg et al., 1995). Another 10-week double-blind placebo controlled study, designed by Sheehan et al. (2005), indicated that controlled release of paroxetine abolished panic attack episodes, compared to placebo. Benzodiazepines, such as alprazolam, are effective treatments for PD with or without agoraphobia, alone or in association with serotonin reuptake inhibitors (Ballenger et al., 1988; Andersch et al., 1991). In fact, alprazolam is widely used in psychiatry for the treatment of PD (Pecknold et al., 1988; Shelton et al., 1993; Klein,

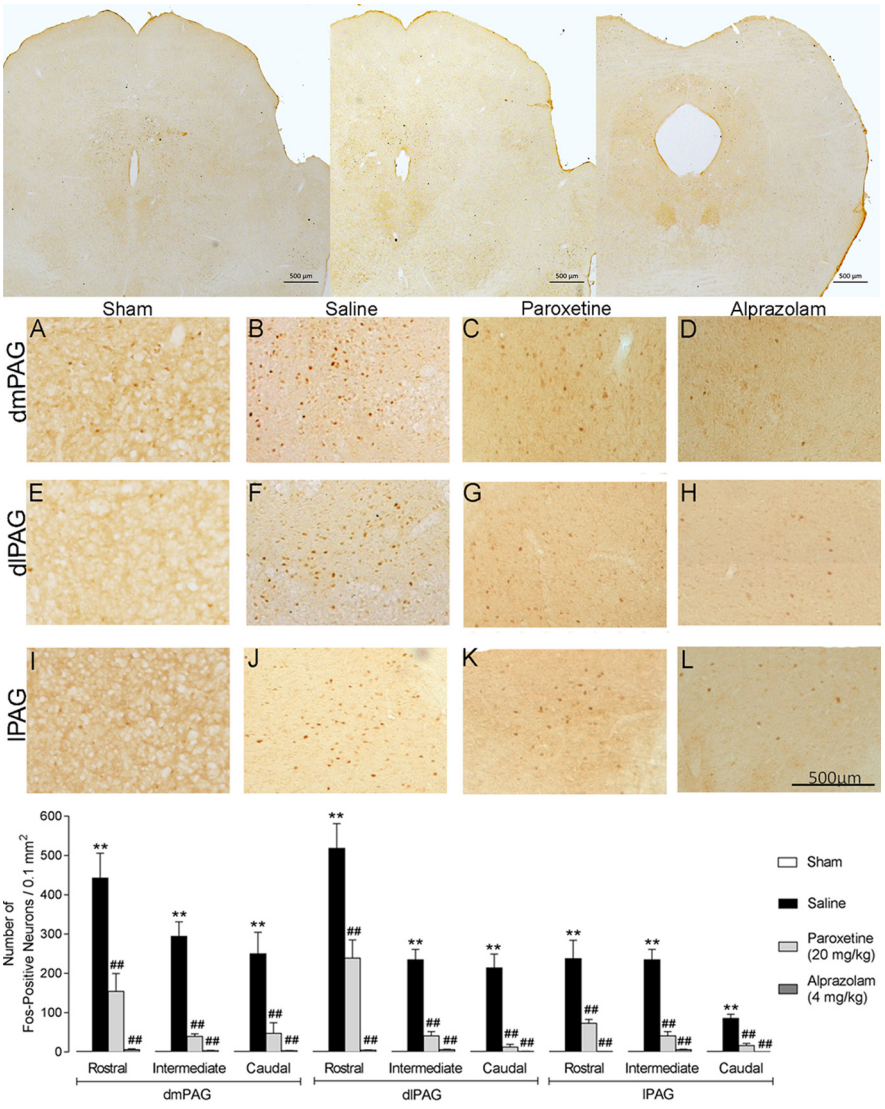

Fig. 5. Representative photomicrographs of coronal sections of the encephalon at the level of the periaqueductal gray matter columns (upper panel). Photomicrographs show c-Fos-immunolabeled nuclei in neuronal cells in the dorsomedial (A–D), dorsolateral (E–H) and lateral (I–L) columns of the periaqueductal gray matter of Syrian hamsters exposed to a toy snake (sham group, A, E and I) and those pre-treated with intraperitoneal chronic injections of saline (B, F and J), paroxetine at 20 mg/kg (C, G, and K) or alprazolam at 4 mg/kg (D, H, and L) and exposed to venomous coral snakes. Bottom of the panel shows the number of Fos protein-labeled neurons/0.1 mm^2 in the rostral, intermediate and caudal divisions of the dorsomedial (dmPAG), dorsolateral (dlPAG) and lateral (lPAG) columns of the periaqueductal gray matter. The columns represent the means, and the error bars represent the S.E.M. n = 6–8 hamsters per group. $^{**}p < 0.001$ compared to sham group; $^{\#\#}p < 0.001$ compared to saline-treated group. The scale bars shown in the top and in L represent 500 μm in all photomicrographs.

2002; Rickels, 2004). Therefore, the panicolytic-like effects found in the present study using alprazolam and paroxetine suggested that the present rodent-versus-snake paradigm could have utility for the investigation of new panicolytic drugs, providing predictive validities of our experimental model.

Effect of predator exposure and c-Fos activation neuronal patterns

The morphological approach of this study demonstrated that confrontations between rodents and wild coral snakes were able to induce increases in the number of Fos protein-labeled neurons in limbic structures of the rodents, correlated with unconditioned fear states in humans (Nashold et al., 1969; Wilent et al., 2010). In fact, the exposure of Syrian hamsters to *Micrurus frontalis* caused Fos protein increases in prosencephalic, diencephalic and mesencephalic structures involved in the elaboration of unconditioned fear-induced behavior. Supporting this evidence, according to Blanchard and Blanchard (1990a,b) and McNaughton and Corr (2004), defensive psychological distance is mapped to the neural level, with the shortest defensive distances recruiting periaqueductal gray neuronal activity and the largest defensive distances recruiting prosencephalic structures.

In other words, the structures that participate in the organization of defensive behaviors in rodents are exactly the same as those evoking fear in humans, providing a theoretical construct for our model (Belzung and Lemoine, 2011). Confrontation showed activation of amygdaloid complex neurons, in which there is augmentation of Fos protein-labeled cells in the LA, BLA, BMA, and CeA and more pronounced increases in MeA. Studies have revealed that rats exposed to cats, cat odor or non-predator-related threats resulted in Fos protein-labeled neurons in the MeA, LA and BMA. However, when exposed only to the cat odor, there was marked activation in the posteroventral region of the medial amygdaloid nucleus (Dielenberg et al., 2001). Thus, the posteroventral division of the medial amygdaloid complex nucleus is particularly responsive to the predatory odor in rodents and seems to exert a key role in the processing of pheromone-related identification of defensive cues from a potential predator (Blanchard and Blanchard, 1972; Swanson and Petrovich, 1998). Considering these findings, the activation of the MeA in Syrian hamsters confronted with venomous coral snakes could be a consequence of the odor of a snake with a limbic connotation. Although the CeA is commonly involved in the expression of conditioned fear-related responses (LeDoux, 2000), this work revealed an interesting increase in Fos protein-immunolabeled nuclei in the CeA, suggesting the involvement of this amygdaloid nucleus in innate

Table 1. Effects of intraperitoneal microinjections of physiological saline, paroxetine or alprazolam on the number of c-Fos protein-labeled neurons in encephalic regions of Syrian hamsters exposed to a toy or live venomous coral snake. Data are represented as the mean ± S.E.M. from 6 to 8 animals per group. All encephalic regions were bilaterally counted for each animal. Lateral (LA), basolateral (BLA), basomedial (BMA), medial (MeA) and central (CeA) nuclei of the amygdaloid complex. Anterior (AH), dorsomedial (DMH), ventromedial (VMH), posterior (PH), lateral (LH), posterior periventricular (PVNp) and dorsal pre-mammillary (PMd) hypothalamic nuclei. Rostral, intermediate and caudal divisions of the dorsomedial (dmPAG), dorsolateral (dlPAG) and lateral (lPAG) columns of the periaqueductal gray matter

Structure	Nucleus	No Threat	Saline	Paroxetine (20 mg/kg)	Alprazolam (4 mg/kg)
Amygdala	LA	0.42 ± 0.08	44.86 ± 8.80	6.26 ± 2.25	1.17 ± 0.45
	BLA	0.39 ± 0.07	28.70 ± 4.12	7.33 ± 2.17	1.41 ± 0.52
	BMA	0.28 ± 0.05	64.25 ± 11.70	24.69 ± 7.13	1.40 ± 0.41
	MeA	0.37 ± 0.02	120.27 ± 23.70	50.80 ± 8.80	15.72 ± 2.66
	CeA	0.36 ± 0.05	69.29 ± 15.55	22.33 ± 7.68	4.66 ± 0.99
Hypothalamus	AH	3.48 ± 0.03	338.72 ± 71.42	29.86 ± 5.85	39.50 ± 9.94
	DMH	3.39 ± 0.04	168.53 ± 26.04	58.10 ± 13.70	29.02 ± 5.12
	VMH	3.40 ± 0.01	306.04 ± 52.97	107.36 ± 21.67	126.58 ± 21.02
	PH	3.39 ± 0.03	97.49 ± 22.33	57.46 ± 8.47	27.37 ± 4.42
	LH	3.42 ± 0.03	97.91 ± 12.84	14.35 ± 2.66	18.59 ± 2.86
	PVNp	3.44 ± 0.02	338.72 ± 59.28	90.51 ± 38.95	11.44 ± 3.56
	PMd	3.37 ± 0.11	1049.55 ± 142.12	121.76 ± 48.74	48.35 ± 17.16
dmPAG	Rostral	1.46 ± 0.08	442.18 ± 63.45	154.14 ± 45.54	5.82 ± 2.34
	Intermediate	1.23 ± 0.06	294.53 ± 36.54	39.01 ± 7.25	3.11 ± 0.91
	Caudal	1.29 ± 0.06	249.86 ± 54.76	47.73 ± 26.73	3.05 ± 0.81
dlPAG	Rostral	1.31 ± 0.03	518.68 ± 62.51	238.85 ± 46.28	4.05 ± 1.21
	Intermediate	1.35 ± 0.07	234.35 ± 26.96	40.81 ± 11.12	5.52 ± 1.75
	Caudal	1.43 ± 0.08	213.93 ± 35.22	12.41 ± 6.93	1.23 ± 0.65
lPAG	Rostral	1.43 ± 0.14	237.34 ± 47.33	72.54 ± 10.47	1.30 ± 0.29
	Intermediate	1.45 ± 0.07	234.35 ± 26.96	40.81 ± 11.12	5.52 ± 1.75
	Caudal	1.47 ± 0.09	85.30 ± 10.84	16.40 ± 5.35	1.01 ± 0.01

fear-induced responses. It is well established that, in instinctive fear, the LA, BLA and BMA amygdaloid nuclei integrate other relevant cues with the perception of a potential predator, such as those from aversive stimulus perception-related structures of the brain, including the auditory cortical areas (McDonald, 1998). This information justifies the lower, but significant, labeling of Fos protein in neurons from this nucleus when compared with MeA and CeA of the hamsters confronted by *M. frontalis* coral snakes because these species do not signal with sonorous cues, like rattlesnakes do (Coimbra et al., 2017a).

Our data also showed a significant increase in Fos protein-labeled neurons in the AH, DMH, VMH, PH, LH, PVNp, with a particularly greater expressive increase in the PMd hypothalamic nucleus after confrontation with the coral snake. The AH, VMH and PMd have been described as important structures of the medial hypothalamic defense system (Gross and Canteras, 2012), and they are critically involved in the organization of instinctive defensive responses to natural predators (Canteras et al., 1997). This outcome is a very interesting finding because the same structures were activated in hamsters during their confrontations with venomous coral snakes. Interestingly, despite South American coral snakes not usually being predators of small rodents, they live underground, increasing the possibility of threatening interactions with small rodents, and they behave as potential predators, with offensive postures and regular motor behaviors displayed during the confrontations performed in the present work. Increases in Fos protein-immunolabeled neurons in the hypothalamic defense system nuclei provided additional evidence that the venomous coral snakes elicited the same pattern of neuronal activation found in other prey-versus-predator paradigms, as described above. The PVNp displayed significant Fos protein labeling in this study. Currently, this outcome is the first piece of evidence that this nucleus is involved in instinctive reactions of defense.

Some authors consider the oriented escape responses to be consequences of the activation of the hypothalamic nucleus (dos Anjos-Garcia et al., 2017; Falconi-Sobrinho et al., 2017a,b; Ullah et al., 2015), different from those elicited by activation of the periaqueductal gray matter (PAG), which elicits non-oriented or explosive escape behaviors (Biagioni et al., 2016b; Ullah et al., 2017). Periaqueductal gray matter columns are involved in distinct patterns of behavioral and physiological responses that can be critical to the survival of animals, according to distal or proximal threatening stimuli (Bandler and Depaulis, 1991; Carrive et al., 1997; Vianna and Brandão, 2003), including those recorded in human trials (Mobbs et al., 2007). Our findings demonstrated that the rostral divisions of the dmPAG and dlPAG columns were more activated compared to the intermediate and caudal divisions. The patterns of immunolabeled neurons in the periaqueductal gray matter in hamsters confronted with venomous coral snakes were similar to those presented by rats confronted with cats (Canteras and Goto, 1999), thus providing evidence that rodents of different species have a preserved innate aversion to snakes.

In conclusion, the experimental paradigm used in this work provided a face, construct and predictive validities

for studying anxiety- and panic attack-like behaviors. The face and construct validities were supported by the behavioral responses and the structures activated during these responses resembling those seen in anxiety and panic states in humans (Nashold et al., 1969; Mobbs et al., 2007; Wilent et al., 2010). The predictive validity was supported by the observation that the drugs used for the treatment of anxiety and panic syndrome in clinical practice (Pull and Damsa, 2008; Reinhold and Rickels, 2015) decreased the rodents' behavioral responses observed during confrontations with wild venomous snakes. In conclusion, the present study provided an ethological, morphological and pharmacological validation of an innovative animal model for the study of anxiety- and panic attack-correlated behaviors based on confrontations between small rodents and wild snakes. The ethological experimental model used here was shown to be a useful tool for the study of the behavioral defensive responses related to anxiety and panic states in humans, correlated with neural structures of the limbic system. The present neuropsychobiological approach, based on the rodent-versus-snake paradigm, has putative utility for the investigation of new potential anxiolytic and panicolytic agents.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest with respect to the work presented herein.

AUTHORS' CONTRIBUTIONS

T. Paschoalin-Maurin and T. dos Anjos-Garcia performed the experiments, analyzed and interpreted the data, and wrote the manuscript. L.L. Falconi-Sobrinho and R.L. de Freitas performed part of the morphological and pharmacological experiments. T. dos Anjos-Garcia and L.L. Falconi-Sobrinho also interpreted the data, wrote the manuscript and collaborated on the design of the figures. J.P.C. Coimbra performed the control experiments with toy coral snakes. N.C. Coimbra designed the enriched polygonal arena for the snakes, designed the experiments, analyzed and interpreted the data, wrote the manuscript, and approved the final manuscript. All of the authors have approved the final version of the manuscript. We are entirely responsible for the scientific content of this paper.

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