Anandamide in the anterior hypothalamus diminishes defensive responses elicited in mice threatened by *Epicrates cenchria* constrictor serpents

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The purpose of this study was to investigate whether the panicolytic-like effect of different doses of anandamide microinjected into the anterior hypothalamus (AH) follows the same pattern of a bell-shaped dose-response curve observed with the same dose treatment in dorsomedial and ventromedial hypothalami. We investigated this assumption by administering the cannabinoid and vanilloid receptor agonist anandamide into the anterior hypothalamus of mice and exposing them to the real threatening situation by using our experimental model based on confrontations between rodents and wild snakes. Our findings showed a gradual decay of response, with a significant attenuation of the panic attack-like responses with anandamide at the highest dose but no effect was found after anandamide at the lowest or intermediate doses. An immunohistochemical procedure showed a lower degree of TRPV₁ receptor and moderate to higher degree of CB₁ receptors in anterior hypothalamus. In conclusion, the pattern of dose-response curve of anandamide microinjected in the AH does not seem to be the same classical pattern compared with other hypothalamic nuclei.

**Key words:** anterior hypothalamus; panic attack-like defensive behaviour; anandamide; cannabinoid receptors; *Epicrates cenchria assisi* constrictor snake.

INTRODUCTION

The neurobiological substrates of panic attacks included primarily mesencephalic structures, such as the periaqueductal grey matter (PAG) (Craske and Stein, 2016; Craske et al., 2017) and corpora quadrigemina. Pioneering studies carried out by Nashold et al. (1969), reported for the first time that stimulation of the human periaqueductal grey matter induce feelings of unconditioned fear. In this sense, similar results were subsequently demonstrated by Mobbs et al. (2007; 2009). There was also evidence for the involvement of diencephalic structures like amygdaloid complex and hypothalamus in the organisation of defensive behaviour (Craske and Stein 2016; Craske et al., 2017). In fact, Inman et al. (2018) showed that the stimulation of the human basolateral amygdala (BLA) induces strong emotional reactions accompanied by increase in heart rate. In addition, Wilent et al. (2010) demonstrated that deep brain stimulation of the ventromedial hypothalamus (VMH) induces feeling of intense fear that is also followed by autonomic reactions. These studies reveal the relevance of diencephalic structures in the organisation of panic attacks and confirm the classical pre-clinical results already demonstrated specially regarding hypothalami (Bard 1928; Hess and Bruger 1943; Di Scala et al., 1984; Schmitt et al., 1985; Brandão et al., 2018).
and Cb 2 receptors, respectively) (Lutz et al., 2015). Our group have used the oxide nitric donor SIN‑1, N‑methyl‑d‑aspartic acid (NMDA) and the GABA A receptor selective antagonist bicuculline to chemically stimulate the anterior (Falconi‑Sobrinho and Coimbra, 2018), dorsomedial (Ullah et al., 2015; Biagioni et al., 2016), ventromedial (dos Anjos‑Garcia et al., 2017; Ullah et al., 2017) and posterior (Biagioni et al., 2016; Falconi‑Sobrinho et al., 2017a; 2017b) hypothalamus, in an attempt to correlate the different pattern of defensive behaviour responses (an NO‑, NMDA‑ and bicuculline‑induced panic attack‑like effects) expressed by rodents with motivational states of fear similar to those responses reported in human being psychiatric patients.

Recently, we validated an experimental model of panic attack based on confrontations between rodents and wild snakes performed in an enriched polygonal arena for snakes designed by Coimbra et al. (2017) to study the unconditioned fear‑related defensive behaviour responses evoked by rodents in an actual threatening situation. Specifically, exposure to a snake induces increase in defensive immobility (freezing) and escape responses accompanied by enhancement in the number of Fos protein‑labelled neurons in hypothalamic nuclei of threatened rodents (Paschoalín‑Maurin et al., 2018). In the same study, these behavioural responses were attenuated by the chronic treatment with intraperitoneal administration of the selective serotonin uptake inhibitor paroxetine or the gamma aminobutyric acid (GABA)/benzodiazepine receptor agonist alprazolam, showing a suitable face, construct and predictive validities of that experimental model.

Pharmacological treatment of anxiety disorder is limited to facilitating GABA and 5‑hydroxytryptamine (5‑HT; serotonin) neurotransmission (Craske et al., 2017). However, the endocannabinoid system has emerged as a potential target for new pharmacological treatments for anxiety disorder and there is evidence for consistent results obtained in human trials (Zuardi et al., 2017). Endocannabinoids are endogenous lipids, synthesised from neuronal membranes in response to elevated intracellular calcium and this term has been used to designate endogenous ligands, such as anandamide (AEA) and 2‑arachidonoylglycerol (2‑AG) that can activate cannabinoid receptors type 1 and 2 (Cb 1 and Cb 2 receptors, respectively) (Lutz et al., 2015). The effect of these neuromodulators is limited by the re‑uptake via specific transporter and/or subsequent metabolisation by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) enzymes, respectively (Luchicchi and Pistis, 2012). Interestingly, it has already been demonstrated a great amount of the main endogenous agonist AEA into hypothalamus after systemic administration of FAAH inhibitors (Kerr et al., 2012).

It is well established in experimental animals that the compounds that inhibit AEA and 2‑AG hydrolysis reduces the anxiety‑ and panic attack‑like responses similarly to those observed with the activation of Cb 1 receptors (Patel et al., 2017). Moreover, microinjections of these compounds into the specific brain structures with limbic functions like the rostral part of the frontal cerebral cortex (Rubino et al., 2008), the amygdaloïd complex (Zarrindast et al., 2008), the substantia nigra (Almada et al., 2015) and the PAG (Finn et al., 2003) reduces the anxiety‑ and panic attack‑like responses in a dose‑dependent manner. In this sense, our group was pioneer in establishing the involvement of the hypothalamic cannabinoid signaling in the neuro‑modulation of unconditioned fear‑related behavioural responses. We showed the panicolytic‑like effects of the activation of the Cb 1 receptors in the ventromedial hypothalamus (VMH) during the bicuculline‑induced panic attack‑like effects (dos Anjos‑Garcia et al., 2017). Recently, Viana et al. (2019) corroborating our findings, also showed the same effect in the dorsomedial hypothalamus (DMH) during the NMDA‑induced panic attack‑like responses.

Lately, we used our experimental model based on confrontations between rodents and wild snakes to investigate the hypothalamic cannabinoid signaling during a real threatening situation, and we found a panicolytic‑like effect in a dose‑dependent manner (U‑shaped dose‑response curve) of anandamide microinjected into the DMH (dos Anjos‑Garcia and Coimbra, 2019). In fact, anandamide can also bind to the transient receptor potential vanilloid type 1 (TRPV 1) channel, which is an ion channel permeable to calcium ions (Elokely et al., 2016). Whereas the activation of the Cb 1 receptor in VMH (dos Anjos‑Garcia et al., 2017) and DMH (dos Anjos‑Garcia and Coimbra, 2019) diminishes defensive behavioural responses induced by the blockade of GABA A receptor and by wild snakes, respectively, the activation of the TRPV 1 channel shows opposing effects.

Although the administration of the anandamide in brain tissue is different from the procedure of inhibiting FAAH enzymes, the effects of these both procedures seem to be the same, at least considering the hypothalamic nuclei. In fact, enhancement of endogenous anandamide with FAAH inhibitors lead to a more physiological situation as recently demonstrated in another ethological approaches using a moving “robo‑beetle” as a threat (Heinz et al., 2017). However, a previous study showed that mice decreased their defensive responses during confrontation with snakes after anandamide microinjection into the substantia nigra pars reticulata (Almada et al., 2015). Another ethological study showed that administration of anandamide into the dPAG diminished the duration of defensive immobility...
displayed by rats that were exposed to the *Felis silvestris catu*s (Lisboa et al., 2014). Recently, microinjections of anandamide into the DMH also caused an inhibition of panic attack-like responses in mice threatened by wild snakes (dos Anjos-Garcia and Coimbra, 2019). That previous study used microinjections of anandamide as a cannabinoid compound into the central nervous system, hence, we ought to compared the anandamide effect in all hypothalamus extension by focused on the anterior hypothalamus (AH) especially if we consider the interconnected nuclei of the hypothalamus (Gross and Canteras, 2012).

In this sense, we demonstrated a dual role of anandamide in both VMH and DMH, an effect that seems to be consistent when we analyse the anandamide effect in other brains structures involved in panic attack-like reactions. Considering this information and the lack of studies addressing the hypothalamus in all its extension especially the AH, a hypothalamic subnucleus poorly investigated, this study was designed to test the hypothesis that microinjections of anandamide into the AH would reduce the defensive reactions induced in mice by rainbow Boidea snakes using our prey-versus-predator paradigm. We also aimed to investigate the localisation of the Cb, and TRPV, receptors in the AH.

**METHODS**

**Animals and experimental apparatus**

Male C57BL/6NTac mice, weighing 30-35 g (n=5-9 per group), were bred in the animal facility at the Ribeirão Preto Medical School of the University of São Paulo (FMRP-USP) and transferred to the Animal Care Unit of the FMRP-USP Department of Pharmacology where they were kept in a group of 5 per cage with free access to water and autoclaved food under a light/dark cycle of 12 / 12 h and at a constant room temperature of 25 ± 1°C (40-70% humidity). Two days after surgical procedure, mice were housed in groups of ten animals (habitation section) in an enriched polygonal arena. The polygonal arena (140 cm in length, 62 cm in width and 54 cm in height) consists in a parallelepiped-shape transparent acrylic enclosure. The floor of the arena was constructed from a transparent acrylic platform (divided into 20 equal rectangles) and was placed on a rectangular stainless steel plaque beneath the arena. To minimise vibration, the entire apparatus was placed on a granite rock surface (150 cm in length, 85 cm in width and 2 cm in height) elevated 83 cm above the floor. A burrow with black acrylic walls (26 cm in length, 18 cm in width and 13 cm in height) inside the polygonal arena was placed in one corner of the arena and had one entrance (diameter 5 cm) allowing the rodents to enter the burrow for protection. There were also two vertical stairs for an elevated safe platform (17 cm in length, 10 cm in width and 27 cm in height) access displayed inside the polygonal arena; one in the corner beside the burrow and other one placed at the polygonal arena sidewall (Fig. 1A). We used a translucent ceiling for the burrow to prevent place preference (Prus et al., 2009). During the habituation section, the floor of the arena was covered with sawdust and the animals were kept in a group with open access to food and water on the surface of the bedding and in a 12 h light/dark cycle in an air-conditioned room (24 ± 1°C) during three days, except during the experimental procedures performed in the light cycle. On the day of the experiment, the animals were shifted from the polygonal arena to their home cages and the arena was cleaned with 5% alcohol solution before testing.

As a source of aversive stimuli, it was used South American rainbow Boidea snakes (*Epicrates cenchria assisi*) weighing 600-800 g. The snakes were collected from the Brazilian Southeast in the countryside of Ribeirão Preto city and surrounding districts and were maintained in the main ophidiarium of the animal house of the FMRP-USP (licensed by the IBAMA Committee; process 1/35/1998/000846-1). One week before the experiments, the snakes were transferred in appropriate cages to the Laboratory of Neuroanatomy and Neuropsychobiology of the Ribeirão Preto Medical School of the University of São Paulo/Behavioural Neurosciences Institute (LNN-FMRP-USP/INeC) ophidiarium, also licensed by the Brazilian government (IBAMA 3543.6986/2012-SP and 3543.6984/2012-SP processes) and by the São Paulo State government (Secretaria do Meio Ambiente (SMA)/ Departamento de Fauna (DeFau) 15.335/2012 process; Mechanisms of Defensive Behaviour and Unconditioned fear-induced antinociception in Snake-threatened Animals (MEDUSA) Project, Sistema de Autorização e Informação em Biodiversidade (SISBIO) authorisation for activities with scientific purposes 41435-1, 41435-2, and 41435-4 processes; SIGAM authorisation of installation process 39.043/2017; Sistema Integrado de Gestão Ambiental (SIGAM) authorisation for use and handling of wild snakes process 39.044/2017). The snake enclosure of the LNN-FMRP-USP/INeC ophidiarium is illuminated by natural sunlight (and by fluorescent ultraviolet irradiation (ReptiSun; 20 W; 5UVB; Zoo Med Laboratories Inc., San Luis Obispo, California, USA) on rainy days, and has artificial waterfalls and lagoons, natural rocks, and both tropical and artificial plants. The snakes were fed at two specific times: 15 days before (with C57BL/6NTac mouse freshly killed by carbon dioxide exposure) and immediately before the start of each ex-
periment with an awake C57BL/6NTac mouse. The reason for that procedure is to minimise, in the first case, the suffering of prey during feeding process when the snakes are maintained in the main ophidiarium. In the second feeding, to normalise the influence of murine odor and pheromones of threatened prey during the feeding process inside the polygonal arena on the next mouse submitted to the experiment.

Although the snakes were fed immediately before the start of each experiment, in the case of an offensive attack triggered by the snake, we stopped the experiment and removed the guide-cannula and acrylic resin, allowing the snake feeding if we could not take the experimental animal from the predator lethal constriction. The fearlessness behaviour displayed by some mice pretreated with anandamide in a dose of 5 and 50 pmol allowed then to get closer to the snake and in some cases, inevitably preyed up. Only 17 animals were included in the statistical analysis instead of 24 originals, being seven mice discarded because of the predatory attack and feeding behaviour of constrictor snakes.

After the experiment, each snake was transferred to the LNN-FMRP-USP/IneC ophidiarium, held for a 40-day quarantine period and returned to the main FMRP-USP ophidiarium.

The experimental procedures were performed in accordance with the recommendations of the Commission of Ethics in Animal Experimentation (CONCEA) of the Ribeirão Preto Medical School of the University of São Paulo - FMRP-USP (process 227/2014). These recommendations are in agreement with the ethical principles of animal research adopted by the Brazilian College of Animal Experimentation (COBEA) and by the National Council for Animal Experimentation Control (CONCEA).

Drug

We used the cannabinoid and vanilloid receptor agonist (2-hydroxyethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (Anandamide; Tocris Bioscience, Bristol, UK), at 0.5, 5 and 50 pmol diluted in Tocrisolve TM100, a solvent containing a 1:4 ratio of soybean oil/water, emulsified with the block co-polymer Pluronic F68. The anandamide diluent was used as a vehicle control and the doses are based on previously study in other regions of hypothalamus (dos Anjos-Garcia et al., 2017; dos Anjos-Garcia and Coimbra, 2019).

Surgical procedure

Mice were anaesthetised with 100 mg/kg ketamine (Ketamine Agener, União Química Farmacêutica Nacio-

nal, Brazil) and 10 mg/kg xylazine (Dopaser, Hertape/ Calier, Juatuba, Minas Gerais, Brazil) and secured in a stereotaxic frame (David Kopf, Tujunga, California, USA). A stainless steel guide-cannula (0.6 mm outer diameter and 0.4 mm inner diameter) was implanted in the diencephalon to target the anterior hypothalamus (AH). The upper incisor bar was set 3 mm below the interaural line, such that the skull was horizontal between the bregma and lambda. The guide-cannula was vertically introduced using the coordinates AP=−0.94 mm, ML=0.5 mm, DV=−3.5 mm, with the bregma as a reference, according to the stereotaxic atlas (Paxinos and Franklin, 2001). The guide-cannula was fixed to the skull using acrylic resin. Each guide-cannula was sealed with a stainless steel wire for protection from obstruction and remained 1.5 mm above the site of drug injection to avoid lesions at the structures of interest. After surgery, each rodent was treated with an intramuscular injection of penicillin G-benzatine (120,000 UI / 0.2 mL) followed by an intramuscular injection of the analgesic and anti-inflammatory flunixin meglumine (2.5 mg/kg). The animals were maintained in post-operative recovery for five days.

Experimental protocol

The experiment was performed using independent groups of animals after five days of stereotaxic surgery and three days of habituation. Habituation was conducted to generate a non-aversive and safe environment for the mice (Uribe-Mariño et al., 2012; Twardowschy et al., 2013). On the day of the experiment, the animals were gently shifted from the polygonal arena to the home cages and were randomly assigned to one of the intra-hypothalamic microinjections. The drugs were injected through polyethylene tube (PE-10) for 15 seconds using a 5 µL syringe (Hamilton, Reno, Nevada, USA) connected to an infusion pump (Stoelting, Kiel, Wisconsin, USA). To ensure the microinjection of the drug, a bubble was created between the distilled water column and the drug. In all cases, the respective diluents were used as controls in the same volume used for drugs.

The animals initially received an intra-AH microinjection of anandamide (AEA 0.5, 5 or 50 pmol) or vehicle. Five minutes after, the rodents were placed inside the polygonal arena where they exposure to the snake for 5 min. For an additional control, other experimental group (no threat group) was treated with intra-AH microinjection of vehicle and exposed to the polygonal arena without snake. In this experiment, a volume of 100 nL was injected for each drug and respective diluents into the hypothalamic nuclei comprising 100 nL per animal.
Behavioural recordings

The behavioural responses of the mice were recorded for 5 min using a videocamera (Sony Handcam HDR-CX350, Konan, Minato-ku, Tokyo, Japan). Subsequently, the behavioural responses were analysed using the free software X-Plo-Rat version 1.1.0, developed at the Laboratory of Exploratory Behaviour of School of Philosophy, Sciences and Literature of the University of São Paulo. This software does not perform the automatic measurement of behaviours, the experimenter evaluates the behaviour and uses the software to help quantify the number and duration of each behavioural response. Scorer was unaware of the experimental design and treatment for each animal.

The behavioural analysis included defensive immobility (freezing), defined as immobility during at least 6 seconds followed by autonomic reactions, such as defecation, exophthalmia and/or micturition and orientated escape, defined as running or shifting the directions of the running toward the burrow, the elevated platforms for escape or climbing the border of the burrow (Uribe-Mariño et al., 2012; Twardowschy et al., 2013; Almada et al., 2015; Almada and Coimbra, 2015; dos Anjos-Garcia and Coimbra, 2019). According to those authors, escape and defensive immobility behaviour are typically considered panic-like responses. Additionally, we evaluated risk assessment behaviour characterised by defensive attention (alertness behaviour) and flat back approaching (elongation of the body with frontward movement toward snake). The number of crossing (stepping with four legs within a delimited rectangle in the arena after crossing the border of each section line) was recorded for quantitatively measurement of locomotor behaviour expressed by rodents. In addition, we measured the time spent inside the burrow and time spent on or above the stairs (i.e., escape platforms) as safe places.

Histology

Upon completion of the experiments, the animals were anaesthetised with 100 mg/kg ketamine and 10 mg/kg xylazine and perfused through the left cardiac ventricle using a perfusion pump. The brain was immediately removed and soaked for 4 h in fresh 4% paraformaldehyde at 4°C. After fixation, the brain was sectioned, and the diencephalon was immersed in 10% and 20% sucrose dissolved in 0.1 M sodium phosphate buffer (pH 7.3) at 4°C for at least 12 h in each solution. The tissue pieces were frozen in isopentane (Sigma-Aldrich, St. Louis, Missouri, USA), stored on dry ice, embedded in Tissue Tek and cut using a cryostat (CM 1950 Leica, Wetzlar, Germany). The slices were subsequently mounted on glass slides (coated with chrome alum gelatine to prevent detachment) and stained with haematoxylin-eosin using an autostainer (CV 5030 Autostainer XL, Leica, Wetzlar, Germany). The positions of the guide-cannula tips were defined under a motorised photomicroscope (AxioImager Z1; Zeiss, Oberkochen, Germany).

Immunohistochemical procedure

The brain was removed, fixed in PFA for 4 h, transferred to 30% sucrose for 2 days, immersed in 2-methylbutane (Sigma-Aldrich), frozen on dry ice, embedded in Tissue Tek O.C.T. and cut into 20 µm sections with a cryostat. The sections were mounted on silanised slides to prevent detachment of the sections during the incubation procedure. Independent AH sections were incubated in 0.1 M sodium phosphate buffer (LabSynth, Diadema, São Paulo, Brazil; pH 7.2) overnight. The next day, antigen retrieval with sodium citrate 10 M (pH 6.0) was performed for 30 min in a water bath at 40°C. The sections were washed three times with 0.1 M sodium phosphate buffer for 5 min each and after 0.3 M glycine (Sigma-Aldrich) for 60 min. Independent AH sections were incubated with primary antibodies (anti-Cb1 goat polyclonal IgG 1:50, Santa Cruz Biotechnology, Texas, USA, sc10066; or anti-VR1 rabbit polyclonal IgG 1:100, Abcam Plc, Cambridge, UK, ab6166) diluted in 0.1 M sodium phosphate buffer and 1% bovine serum for 24 h. The sections were washed four times with 0.1 M sodium phosphate buffer for 10 min each, simultaneously incubated with secondary antibodies (Alexa Fluor 647 donkey anti-goat IgG, 1:200; or Alexa Fluor 488 chicken anti-rabbit IgG, 1:200; both Invitrogen, Carlsbad, CA, USA) for 120 min in the dark, and washed again three more times with 0.1 M sodium phosphate buffer for 10 min each. Finally, the slides were coveredslipped with Prolong (Life Technologies), and histological sections were analysed by a motorised photomicroscopy.

Statistical analysis

The behavioural responses were recorded as the proportion of time spent in a safe place. These data are presented as a behavioural index (BI) and were calculated as previously described (dos Anjos-Garcia and Coimbra, 2019) using the following formula: \( BI = \frac{100 \times \text{number of behavioural responses}}{\text{time in seconds spent outside the safe place}} \). The behaviour duration was expressed as the time in seconds spent in a given behavioural response evoked outside of a safe place. Data from independent groups were submitted to the Shapiro-Wilk test of normality and were analysed by a parametric
test since the data fit in Gaussian distributions and the variances between groups were homogeneous for more than 80% of data. We have chosen a 5% chance of the extreme values will be identified as an outlier (Grubbs’s test with alpha set at 0.05). The number of animals were reported without occlusion of outliers and expressed as a mean ± standard error of the mean (SEM). The results were analysed using one-way analysis of variance (one-way ANOVA) followed by Tukey’s post hoc test when appropriated using treatment as main factor. The total incidence of panic attack-like responses were calculated by adding the incidence of defensive immobility and oriented escape responses. Dose-effect curves for anandamide were generated by nonlinear regression analysis to compare the findings related to AH treatment with different doses of anandamide. Results with $P<0.05$ were considered statistically significant.

**RESULTS**

**Polygonal arena for snakes, histologic confirmation of the microinjection sites and locomotor behaviour**

The experimental apparatus described in the materials and method section is illustrated in Fig. 1A. After intra hypothalamic treatment, the mice were exposed to an experimental polygonal arena without (non-threatened animal for an additional control group) or with snake (threatened animal) for recording behavioural responses.

All mice received microinjections in the anterior hypothalamic nucleus as shown in modified diagrams from stereotaxic atlas (Paxinos and Franklin, 2001) and illustrated in the Fig. 1B.

Since the behaviour frequencies were proportionally recorded in relation to the time spent outside the safe place, we measured the time spent inside the burrow and time spent on or above the stairs as shown in Fig. 1C. Note that there is no difference in time spent in a safe place ($F_{1,29}=1.44; P>0.05$), showing that all animals exposed to the snake remained equally exposed to the threatening stimulus, indicating that all threatened animals spent the same time in contact with the snake. In addition, the same occurred with the control animals (no threat-group). In the last case, we can speculate that these animals were familiarised with the whole experimental apparatus and do not necessarily stayed out of the burrow or stairs, because they were habituated for three consecutive days in the enriched polygonal arena. Additionally, the absence of sawdust during the prey versus predator exposure may be evoked an avoidance behaviour in prey, consequently spending more time in a safe place; however, they expressed neither defensive immobility nor escape responses during the test. Besides, the number of crossing was also analysed (Fig. 1D). They reflected a quantitative measurement of locomotor behaviour displayed by mice submitted to the threatening situation after treatments. The pharmacological treatments did not change the number of crossing ($F_{1,29}=2.58; P>0.05$) showing that the defensive behavioural responses observed after treatments are due to the effect of the drug but not change in locomotor activity.

**Effect of increasing doses of anandamide injected into the AH**

According to one-way ANOVA, there were significant effects of treatment on the BI ($F_{4,29}=3.98; P=0.05$) and duration ($F_{4,29}=5.85; P=0.01$) of defensive immobility. Exposure of the vehicle-treated group to the snake induced significant increases in BI and duration ($P<0.01$ in both cases) of defensive immobility compared with animals exposed to the experimental polygonal arena without the snake (non-threatened group). Only treatment with AEA at 50 pmol significantly attenuated the BI ($P<0.05$) and duration ($P<0.01$) of defensive immobility ($F_{4,29}=10.76; P<0.001$) and duration ($F_{4,29}=8.36; P<0.001$). Mice exposed to the snake also induced significant increases in BI ($P<0.001$) and duration ($P<0.01$) of oriented escape in comparison to the non-threatened group. Only intra-AH treatment with AEA at 50 pmol decreased the BI ($P<0.01$) and duration ($P<0.05$) of oriented escape. In addition, the oriented escape displayed by the 50 pmol AEA-treated group was significantly different from that displayed by the 0.5 pmol AEA-treated group both in BI and duration ($P<0.01$ in both cases; Fig. 2C-D).

Microinjections of anandamide (0.5, 5 and 50 pmol/100 nL) into the AH of mice caused a significant inhibition of panic attack-like responses in a dose-dependent manner, showing a significant correlation between dose and attenuation of the BI ($r^2=0.99; F_{1,2}=126.1; P<0.01$) and duration ($r^2=0.95; F_{1,2}=37.35; P<0.05$) of the panic attack-like responses (Fig. 2E-F).

Regarding the risk assessment behaviour, according to the one-way ANOVA, there were significant effects of treatment on the BI ($F_{4,29}=60.8; P<0.001$) and duration ($F_{4,29}=70.57; P<0.01$) of that anticipatory anxiety-like behaviour. Exposure of the vehicle-treated group to the snake induced significant increases in BI and duration ($P<0.001$ in both cases) of risk assessment compared with animals submitted to the experimental polygonal arena without the snake (non-threatened group). The
Gradual inhibition of panic responses by anandamide

**Table I.** Unconditioned fear-induced risk assessment behaviour elicited by C57BL/6NTac mice threatened by Epicrates cenchria assisi constrictor serpents.

<table>
<thead>
<tr>
<th>Risk assessment behaviour</th>
<th>BI</th>
<th>Duration (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle non threatened</td>
<td>0.58±0.20</td>
<td>1.29±0.44</td>
</tr>
<tr>
<td>Vehicle threatened</td>
<td>6.25±0.58***</td>
<td>22.66±2.14***</td>
</tr>
<tr>
<td>AEA 0.5 pmol</td>
<td>6.15±0.33</td>
<td>17.59±1.36*</td>
</tr>
<tr>
<td>AEA 5 pmol</td>
<td>1.78±0.35***</td>
<td>4.04±0.84***</td>
</tr>
<tr>
<td>AEA 50 pmol</td>
<td>0.78±0.19***</td>
<td>1.57±0.47***</td>
</tr>
</tbody>
</table>

The values are expressed by mean ± SEM. +++ P<0.001 compared with the vehicle non-threatened group. * P<0.05 and *** P<0.001 compared with the vehicle threatened group (one-way ANOVA followed by Tukey's post hoc test). BI (behavioural index). AEA (anandamide).

Fig. 1. (A) Experimental apparatus described in material and method section showing the burrow in one corner and two vertical stairs. Mice exposed to the polygonal arena without (non threatened animal) or with snake (threatened animal). (B) Schematic representation of coronal sections of the mouse brain (Paxinos and Franklin 2001) showing the histologically confirmed drug microinjection sites (filled circles) inside the anterior hypothalamus (AH). The photomicrograph shows a representative drug microinjection site (black arrow) situated in the AH. No effect was observed in time spent in a safe place (C) nor in the number of crossing (D) showing the consistency of data normalisation and the absence of locomotor changes.
hypothalamic treatment with AEA at 0.5 pmol significantly attenuated the duration ($P<0.05$) of risk assessment; however, both treatments with AEA at 5 and 50 pmol decreased the BI ($P<0.001$) and duration ($P<0.001$) of that anticipatory anxiety-like behaviour (Table I).

Fig. 2. Effect of the intra-anterior hypothalamus (AH) microinjection of anandamide (AEA; 0.5, 5 or 50 pmol/100 nl) or vehicle on the behavioural index (A and C) and duration (B and D) of defensive immobility (A and B) or oriented escape (C and D) displayed by mice during a confrontation with a snake (threatened group) or in the absence of a snake (non-threatened group). Data are presented as the means ± S.E.M.; ** $P<0.01$ and *** $P<0.001$ compared with the non-threatened group; # $P<0.05$ and ## $P<0.01$ compared with the vehicle-treated group; ++ $P<0.01$ compared with the AEA/0.5-treated group (one-way ANOVA followed by Tukey's post hoc test). Dose effect curves (E and F) were generated by nonlinear regression analysis and represent changes in the inhibition of responses to different doses of anandamide on the behavioural index (E) and duration (F).
Localisation of cannabinoid and vanilloid receptors in the AH

The immunohistochemical expression of cannabinoid and vanilloid receptors in the anterior hypothalamus of mice was determined by immunofluorescence (Fig. 3). We observed only a lower degree of TRPV1 channel immunoreactivity in the AH, which were almost exclusively in the perikarya (closed white arrow in Fig. 3D). In contrast, we found a moderate to higher expression for CB1 receptors in axonal fibres and in puncta surrounding perikarya (closed white arrow heads in Fig. 3D), suggesting that these receptors are located on terminal boutons in the AH. Consequently, we found only a limited coexpression for CB1 receptor- and TRPV1 receptor-labelling in AH cellular bodies (open arrow heads in Fig. 3D). In that diencephalic area, CB1 receptors seem to be more abundant than TRPV1 receptors, differently from those recently reported in DMH of mice (dos Anjos-Garcia and Coimbra, 2019). No labelled receptors were observed when the primary antibody was omitted (data not shown) and the specificity of the antibodies was checked in the literature where Marinelli et al. (2007) used anti-CB1 goat polyclonal IgG and anti-VR1 rabbit polyclonal IgG in order to analyse the anatomical distribution of these receptors in slices of substantia nigra.

DISCUSSION

In this work we used a polygonal arena to investigate the behavioural responses displayed by mice during the confrontation with constrictor snakes. The
polygonal arena for snakes consists in a complex experimental environment with an artificial burrow situated opposite to the arena floor occupied by snake (which remained mostly quiet along the experiments), allowing the rodents exhibit rich behavioural responses elicited by the presence of the wild snake. As expected, the exposure to a snake induced in mice an instinctive defensive response; however, intra-AH administration of anandamide caused a gradual decay in the events of defensive behaviour with significant inhibition of unconditioned defensive responses in a dose-dependent manner without affecting the motor behaviour.

It is well established the relationship between risk assessment behaviour expressed by rodents with apprehensive expectation and vigilance/scanning states, the two major behavioural manifestation present in generalized anxiety disorder, as well as the translational view between escape responses in several prey-predator paradigms model with behavioural symptoms in panic disorder, usually reported by panic syndrome patients as an urgent desire to avoid or escape from wherever threat is occurring (Blanchard et al., 2001). During the 5-minute of exposure to a snake in the enriched polygonal arena, vehicle-treated group evoked robust responses characterised primarily by defensive immobility (freezing) and oriented escape behaviours interspersed with defensive attention (alertness behaviour) and flat back approaching characterised by elongation of the body with frontward movement toward snake indicating a risk assessment state. In this sense, risk assessment can be considered a key defensive response once facilitates the acquisition of information leading to more vigorous defensive behaviours if the threatening stimulus is found, or to inhibitory responses if the threatening stimulus is far enough away or even not found.

In our experimental model, mice displayed three-fold risk assessment than escape responses. We can speculate, in an evolutionary perspective, that rodents assess risk assessment-based information to determine the choice of one defensive response or another that in this case of escape behaviour towards a safe place (oriented escape behaviour). An additional important role for risk assessment behaviour is the reduction of unnecessary vigorous/explosive defensive responses when threat is so far enough. That defensive strategy was widely recorded in the present work, since mice, in most case, assess the risk and have chosen to escape more than freeze, since they were previously habituated in de enriched polygonal arena and knowing all routes of escape towards safe places. Considering the AH, our study showed that anandamide was able to reduce the risk assessment even with at lower doses without affecting the panic attack-like responses such as defensive immobility and escape behaviours, showing that anandamide at lower doses did not impair the make decision to escape, impairment that occurred only with anandamide at a higher dose. In fact, risk assessment behaviour responds appropriately to an anxiolytic drug in several prey versus predator paradigms (Blanchard et al., 1997), including rodent versus snakes confrontation procedures (Paschoalin-Maurin et al., 2018).

Some studies describe defensive immobility (freezing) and escape/flight behaviour as passive and active responses, respectively (Heinz et al., 2017). In that study, authors showed that mice C57BL/6N confronted with “moving robo-beetle” displayed little active behaviours while shown higher incidence of passive responses. Indeed, robo-beetle does not represent a real challenger to mouse, and does not requires robust escape and/or flight responses. However, the activation of the inhibitory nigro-tectal pathways increased the passive responses and decreased the active behaviour, showing an attenuation of the threat recognition and evoked a shift from escape to risk assessment behaviour (Almada et al., 2018). Interestingly, the hyper-anxious phenotype described for some strains (e.g., HAB) showed opposite effect that is high active responses (escape and flight) and less passive responses (tolerance and freezing) and, in this case, the enhancement of anandamide signalling via intraperitoneal administration of the FAAH inhibitor URB597 in hyper-anxious phenotype (HAB) increases passive responses and reduces the flight responses without change in escape behaviour, suggesting a panicolytic-like effect (Heinz et al., 2017).

It is known that chemical stimulation of the diencephalic structures like hypothalamus are responsible for integrating more elaborate behavioural responses compared to more explosive/non-oriented responses organised by dorsal midbrain structures (Di Scala et al., 1984; Milani and Graeff 1987; Schmitt et al., 1985; Ullah et al., 2015). In this sense, our group has already reported the same pattern of responses during the confrontations between rodents and snakes (Coimbra et al., 2017). Almada and Coimbra (2015), for example, showed that the exposure to a snake induced defensive immobility (freezing) and both oriented and non-oriented escape behaviour in prey; however, the blockade of the activity of nigro-tectal GABAergic pathways with a microinjection of bicuculline in the mesencephalic tectum increased defensive immobility (freezing) and non-oriented escape reactions while decreased the oriented escape, corroborating the idea that mesencephalic structures are responsible for the organisation of more explosive/non-oriented unconditioned fear-induced response compared with diencephalic structures like hypothalamus.
cephalic structures, also during more ethological psychobiological approaches.

Although there is a great amount of behavioural response induced by snakes and based on pharmacological predictability of this experimental model (Paschoalin-Maurin et al., 2018) intra-AH treatment with anandamide decreased the unconditioned fear-induced defensive responses, suggesting a clear antiaversive/panicoletic-like effect. Our observations that the treatment of AH attenuates the responses induced by snakes corroborate our previous studies using chemical activation of the brain (dos Anjos-Garcia et al., 2017) and predator-related (dos Anjos-Garcia and Coimbra, 2019) stimuli, recruiting VMH and DMH neural networks, respectively. In first case, the treatment of the VMH with anandamide in a dose of 5 pmol attenuated bicuculline-induced panic attack-like effects, whereas the local administration of anandamide in a dose of 5 pmol decreased the unconditioned fear-induced responses, suggesting a biphasic effect of the administration of anandamide at the highest dose (50 pmol) (dos Anjos-Garcia et al., 2017).

Using snakes as a potential predator-related stimulus, the same pattern of response was shown when anandamide was administered in an intermediate dose (5 pmol), but performed in the DMH, interestingly having no effect at higher dose (50 pmol) (dos Anjos-Garcia and Coimbra, 2019). Another ethological study also showed a biphasic effect of the administration of anandamide into the PAG, attenuating unconditioned fear-related behaviour induced by cats at the same intermediate dose (Lisboa et al., 2014). In both cases, the authors showed a bell-shaped dose-response curve for all behaviours analysed in their works, without effect neither the lowest, nor the highest dose of anandamide, although, a significant inhibition of panic attack-like responses was reported with anandamide at an intermediate dose.

This pattern of modulatory effect exerted by anandamide on panic attack-like response induced by chemical or electrical stimulation of limbic and paralimbic structures, as well as caused by the exposure of rodents to unprotected spaces or to real threatening situations appears to be consistent over the cerebral cortex (Rubino et al., 2008), amygdaloid complex (Zarrindast et al., 2008), DMH (dos Anjos-Garcia and Coimbra, 2019; Viana et al., 2019), VMH (dos Anjos-Garcia et al., 2017), substantia nigra (Almada et al., 2015) and PAG (Finn et al., 2003; Casarotto et al., 2012; Batista et al., 2015). However, when we consider the treatment of the hypothalamic medial zone (Gross and Canteras, 2012) with anandamide at the same doses cited above, this effect does not seem to be the same. We showed a gradual decay of response, with a significant correlation between anandamide doses and attenuation of the panic attack-like responses. Interestingly, only the highest dose of anandamide used in the current work (50 pmol) was able to attenuate the defensive reactions of prey to the aversive effect caused by snakes. These results demonstrate that, although anandamide at 5 pmol seems to exert an antiaversive effect in several hypothalamic nuclei, like DMH and VMH, in AH this effect is achieved only with the local administration of anandamide at the highest dose. Thus, the specific role of anandamide on panic attack-like responses may vary depending on the nature of the stimulus and the brain region investigated.

Using an ethological approach (snakes as a natural wild source of aversive stimuli), our team has already demonstrated that anandamide can activate both Cb1 and TRPV1 receptors in DMH (dos Anjos-Garcia and Coimbra, 2019), resulting in panicoletic- and panicogetic-like effects, respectively. Specifically, high doses of anandamide did not decrease the mice defensive responses induced by snakes; however, the panicoletic-like effect of anandamide was revealed by the selective inhibition of TRPV1 receptors with 6-I-CPS and achieved the same effect as the intermediate dose of anandamide, which was blocked by the selective inhibition of Cb1 receptors with AM251. Previous studies, focused on dorsal midbrain electrical stimulation, had already shown the dual role of anandamide through the Cb1 receptor and TRPV1 channel, indicating the recruitment of cannabinoid and vanilloid mechanisms to modulate the defensive behaviour. In fact, pretreatment of the dPAG with the Cb1 receptor antagonist AM251 was able to prevent the panicoletic-like effect of the Cb1 receptor agonist ACEA, whereas the pretreatment with a non-selective (capsazepine) or a selective (SB366791) TRPV1 vanilloid receptor antagonist unmasked the panicoletic-like effect of ACEA in a higher dose. In addition, the binding of endogenous anandamide to Cb1 receptors reached a panicoletic-like effect, since pretreatment with Cb1 antagonist causes an inhibition of the anti-aversive effect of TRPV1 antagonists (Casarotto et al., 2012).

At the cellular level, the authors reported a great amount of the Cb1 and TRPV1 receptors into DMH with a moderate to intense coexpression for both receptors, explaining the U-shaped dose-response curve and, consequently, the differences between the lowest, intermediate and the highest dose of anandamide when administered in the dorsomedial hypothalamus (dos Anjos-Garcia and Coimbra, 2019). In contrast, we observed only a low degree of TRPV1 immunoreactivity in the AH, compared to the Cb1, immunoreactivity that seem to be more abundant in that hypothalamic nucleus. Consistently, the behavioural responses were attenuated only with anandamide at the highest dose. One
possible explanation for that intriguing finding is the pattern of TRPV₁ immunoreactivity in the AH, which explains a gradual decay of response, in a different manner from the bell-shaped dose-response curve of anandamide effect observed when centrally microinjected in the DMH and VMH.

CONCLUSION

In conclusion, this study suggests that the administration of anandamide in the AH also modulates panic attack-like behavioural responses induced by the exposure of prey to wild snakes in a different pattern from those reported when the dorsomedial and ventromedial hypothalamus were treated with that cannabinoid and vanilloid receptors agonist. These results support the significance of hypothalamic medial zone in unconditioned fear-related defensive reactions during threatening situations and the relevance of endocannabinoid signalling in the AH modulating predator-related aversive stimulus-induced defensive responses.

ACKNOWLEDGEMENTS

This work was supported by the Conselho Nacional de Pesquisa e Desenvolvimento Tecnológico (CNPq) (processes 470119/2004-7, 483763/2010-1, 474853/2013-6), Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (processes 2007/01174-1, 2012/03798-0, 2017/11855-8), and a Pro-Rectory of the University of São Paulo (USP) Research grant (NAP-USP-NuPNE; process IaPq2012-156-UsP-12.1.25440.01.6). T. dos Anjos-Garcia was financially supported by CNPq (M.Sc. fellowship, process 130124/2012-5; Sc. D. fellowship, process 141124/2014-8) and is a postdoctoral researcher supported by FAPESP (grant 2017/22647-7). Each organisation had no further role in the study design; the collection, analysis, and interpretation of the data; the writing of the report; or the decision to submit the paper for publication. N.C. Coimbra is a researcher (level 1A) from CNPq (processes 301905/2010-0 and 301341/2015-0). The authors would like to thank Daoud Hibrahim Elias-Filho for expert technical assistance with the immunofluorescence assay. D.H. Elias-Filho was supported by CNPq (grant 372877/2010-9).

REFERENCES

Falconi-Sobrinho LL, dos Anjos-Garcia T, de Oliveira R, Coimbra NC (2017a) Decrease in NMDA receptor-signaling activity in the anterior cingulate cortex diminishes defensive behaviour and unconditioned fear-induced
Gradual inhibition of panic responses by anandamide


