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FACULTY OF MEDICINE OF RIBEIRÃO PRETO
Laboratory of Neuroanatomy and Neuropsychobiology
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The innate defensive behaviour and unconditioned fear-induced antinociception evoked by NMDA receptor activation in the medial hypothalamus are modulated by the intradiencephalic treatment with cannabidiol: the role of CB1 cannabinoid receptor

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Thesis presented to the Department of Neurosciences and Behavioural Sciences of Ribeirão Preto Medical School of the University of São Paulo for obtaining the title of ***Scientiae Doctor***.

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Supervisor: Norberto Cysne Coimbra, **M.D., M.Sc., Sc. D.**

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CATÁLOGO

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I DEDICATE THIS THESIS

TO

PROFESSOR DR. NORBERTO CYSNE COIMBRA
M.D., M.Sc., Sc.D., Postdoct (Oxford)

SUPERVISOR

OF

MY DOCTORAL RESEARCH PROJECT

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RESUMO

O comportamento de defesa inato e a antinocicepção induzida pelo medo incondicionado induzidos pela ativação de receptores NMDA no hipotálamo medial são modulados pelo tratamento intradiencefálico com cannabidiol: papel do receptor canabinoide CB1

O papel dos canabinoides exógenos nas regiões do cérebro com um número modesto de receptores cannabinoides, por exemplo, o hipotálamo ventromedial, ainda não está plenamente esclarecido. Algumas pesquisas de nosso grupo, não obstante, mostraram o hipotálamo ventromedial (HVM) exerce modulação de reações comportamentais provocadas pelo medo inato em animais submetidos a um modelo de ataques de pânico. Crises de pânico foram induzidas em animais de laboratório por N-metil-D-aspartato (NMDA), um aminoácido excitatório que, ao ser microinjetado em estruturas do sistema encefálico de aversão, estimula reações comportamentais defensivas no sistema nervoso central que mimetizam as respostas defensivas eliciadas por roedores confrontados com serpentes. Apesar do mecanismo de sinalização endocanabinoide mediado pelos receptores CB1 desempenhar um papel na modulação da neurotransmissão excitadora e inibitória no SNC, ainda há escassez de evidências morfológicas que embasem a distribuição dos receptores CB1 no HVM. Por conseguinte, este estudo foi idealizado para explorar a forma específica de distribuição dos receptores CB1 no HVM e, posteriormente, estudar a implicação desses receptores na modulação de respostas comportamentais defensivas, seguidas por antinocicepção induzida pelo medo, moduladas por endocanabinoides e evocadas por microinjetação de NMDA no HVM. Uma cânula-guia feita de aço inoxidável foi implantada no cérebro do roedor, e direcionada para o HVM por meio de cirurgia estareotóxica. Três diferentes doses de cannabidiol (CBD) foram microinjetadas no HVM. A dosagem mais eficaz foi utilizada após o pré-tratamento do hipotálamo medial com um antagonista do receptor CB1, o AM251, seguido da microinjeção NMDA no HVM. Os resultados demonstraram que as respostas

comportamentais defensivas evocadas em resposta à administração intra-HVM de NMDA (6 nmol) foram diminuídas por microinjeções intra-hipotalâmicas de CBD na dose mais alta (100 nmol). Estes efeitos, no entanto, foram atenuados pela administração do antagonista do receptor CB1, AM251, na dose de 100 pmol no HVM. Além disso, a antinocicepção induzida pelo medo foi atenuada pela administração intra-diencefálica de CBA, o que foi revertido pelo pré-tratamenot do HVM com AM251. Esses dados sugerem que o CBD causa efeitos panicolíticos, quando administrado no HVM, envolvendo o mecanismo de sinalização do receptor CB1-endocannabinoide.

Palavras-chave: Comportamento de defesa, ataques de pânico, antinocicepção induzida pelo medo, cannabidiol, receptores canabinoides de tipo 1, hipotálamo Ventromedial, NMDA

ABSTRACT

The innate defensive behaviour and unconditioned fear-induced antinociception evoked by NMDA receptor activation in the medial hypothalamus are modulated by the intradiencephalic treatment with cannabidiol: the role of CB1 cannabinoid receptor

The impacts of exogenous cannabinoids, such as the chemical constituents of *Cannabis sativa* like cannabidiol (CBD), on brain regions having a modest number of cannabinoid receptors, for example, the ventromedial hypothalamus, are not yet surely knew. A few researches have shown evidence that ventromedial hypothalamus (VMH) neurons play a role in modulating innate fear-induced behavioural reactions in rodents submitted to experimental models of panic attack, for example those based on prey versus wild snake confrontation paradigm. The panic attack-like state was also potentially induced in laboratory animals by N-Methyl-D-aspartate (NMDA), an excitatory amino acid, which stimulates neurons that organize defensive behavioural reactions in the central nervous system. Despite the fact that CB1 receptor-mediated endocannabinoid signaling mechanism underlies the antiaversive effect of exogenous anandamide in medial hypothalamus, there is still a lack of morphological evidence to support the distribution of CB1 receptors in the VMH. Henceforth, this study was designed to explore the specific pattern of distribution of the CB1 receptors in the VMH and, subsequently, the implication of these receptors in the endocannabinoid-modulated defensive behavioural responses followed by fear-induced antinociception evoked by NMDA microinjected in the VMH. A stainless steel guide-cannula was embedded in the rodent's brain coordinated towards VMH by means of stareotaxic surgery. Three different doses of cannabidiol (CBD) were microinjected in the VMH. The most effective dose was used after the pretreatment with the CB1 receptor-antagonist AM251, followed by NMDA microinjection in the VMH. The outcomes demonstrated that the defensive behavioural responses evoked in response to intra-VMH administration of NMDA (6 nmol) were decreased by intra-hypothalamic microinjections of CBD at the highest dose (100 nmol).

These effects, however, were blocked by the administration of the CB1 receptor-antagonist AM251 (100 pmol) in the VMH. In addition, the fear-induced antinociception elicited by VMH chemical stimulation diminished after the VMH treatment with CBD, an effect reversed by the intra-diencephalic pretreatment with AM251. These findings suggested that CBD causes panicolytic-like effects when administered in the VMH, and that antiaversive effect recruits the CB1 receptor-endocannabinoid signaling mechanism in VMH.

Keywords: Defensive behaviour, Panic attack-like responses, innate fear-induced antinociception, cannabinoid receptor type-1, ventromedial hypothalamus, NMDA

LIST OF ABBREVIATIONS AND ACRONYMS

AEA	anandamide or N-arachidonoylethanolamine
AH	anterior hypothalamus
AM251	N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide
ACEA	arachidonyl-2-chloroethylamide
ABS	acrilonitrile butadiene styrene
ACC	anterior cingulate cortex
ATP	adenosine triphosphate
2-AG	2-arachidonoyldopamine
BLA	basolateral amygdala
BSA	bovine serum albumin solution
BNST	brainstem
CB1	cannabinoid receptor type-1
CBD	cannabidiol
CNS	central nervous system
CONCEA	national council for control of animal experimentation
CEUA	commision of ethics in animal research
cAMP	cyclic adinocine monophosphate
cVMH	central part of the ventromedial hypothalamus
DSM-5	diagnostic and statistical menual of mental disorders 5th edition
DV	dorso-ventral
DNA	deoxyribunucleic acid
DAB	3,3-diaminobenzidine
DMSO	dimethyl sulfoxide
dPAG	dorsal periaqueductal grey matter
dMH	dorsomedial hypothalamus
dhSC	dorsal horn of the spinal cord
dhSM	dorsal horn of the spinal medulla
ECS	endocannabinoid system
EPM	elevated plus maze
ETM	elevated T maze
enCBs	endocannabinoids

exCBs	exogenous cannbinoids
GABA	gamma-aminobutyric acid
$GABA_A$	gamma-aminobutyric acid receptor type-A
Gi/o	a heterogenous G protein subunit
g	gram
HHA	hypothalamus-hypophysial-adrenal-axis
HE	hematoxylin and eosin
$5\text{-}HT_{1A}$	5-Hydroxytryptamine type-1A or serotonin
IASP	international association for the study of pain
K+	potassium
LH	lateral hypothalamus
mRNA	messenger ribonucleic acid
MH	medial hypothalamus
mM	millimole
mL	milliliter
min	minute
NMDA	N-methyle-D-aspartate
NaCl	sodium chloride
Ni/Cr	nickle crome alloy
nmol	nano-mole
O-AEA	o-arachidonoyl ethanolamine
OD	outer diameter
PNS	peripheral nervous system
PCR	polymerase chain reaction
PFC	prefrontal cortex
PH	posterior hypothalamus
PAG	periaqueductal grey matter
dPM	dorsal premammilary nucleus
PBS	phosphate buffered saline
RNA	ribonucleic acid
SEM	standard error of mean
SR141716	rimonabant
SAL	saline

THC	delta-9-tetrahydrocannabinol
TRPV1	transient receptor potential vanilloid type-1
TFLs	tail flick latencies
μL	microliter
VMH	ventromedial hypothalamus
dmVMH	dorsomedial division of the ventromedial hypothalamus
VCT	vogel conflict test
veh	vehicle

Summary

1 INTRODUCTION

Fear is considered to be a factor of the acute stress response to dangerous stimuli, which may unfavorable to the integrity and stability of the individual. However, it establishes a maladaptive reaction of anxiety-like states such as generalized anxiety, phobia, and panic-like responses (Mobbs and Kim, 2015). Several neurological mechanisms are suggested to be involved in the organisation of these anxiety or panic attack-like states displayed by laboratory animals, which include glutamatergic, serotonergic, GABAergic, and noradrenergic systems (Schenberg, 2010). Recently, researchers have shown an increasing interest in evaluating the effects of endocannabinoid system in the modulation of defensive behaviour, suggesting that this system has a vital role in the regulation of anxiety-like states, mood disorders and other emotional responses. CB1 receptors in the brain are thought to be involved in the modulation of these endocannabinoid signaling system in anxiety and emotional states. There are also shreds of evidence, showing that alkaloids from *Cannabis sativa* have a broad range of effects on the brain neural systems involved in the regulation of emotions (Viveros et al., 2007). Cannabidiol, the major non-psychoactive component of the *Cannabis sativa* plant, have shown to attenuate the anxiety and panic attack-like states and other behaviours related to the innate fear in experimental animals (Uribe-Mariño et al., 2012), as well as in human beings (Tambaro and Bortolato, 2012). The functional neuroanatomy involved in the fear, anxiety, and panic-like responses investigated so far includes thalamus, hypothalamus, amygdaloid complex, hippocampus, periaqueductal grey matter, and locus coeruleus (Gorman, Liebowitz, Fyer, & Stein, 1989; Gorman, Kent, Sullivan, & Coplan, 2000; Sobanski & Wagner, 2017). In recent years, the researchers have given attention to evaluate the role of ventromedial hypothalamus in the organisation of fear and anxiety, and panic attack-like defensive behaviours (Freitas et al., 2009; Ullah et al., 2015; dos Anjos-Garcia et al., 2017).

1.1 Panic syndrome

Panic disorder is a state of anxiety, which is currently viewed as a psychiatric disorder. Several names have been given to this issue previously. The first run through, in an American civil war battled in 1871, Jacob Mendes da Costa found an anxiety disorder, which he named "Da Costa's disorder" or "soldier's heart" or "shore disorder" described by dyspnea, fatigue, and difficulty in breath, sweating, and palpitation (Da Costa, 1951; Wooley, 1982; Pichot, 1996). Some other names were likewise given, for example, "neurosis anxiety" by Sigmund Freud (1894); neurocirculatory asthenia", in 1918 by Oppenheimer (Oppenheimer, 1942) and "stress disorder" (Nixon, 1993), all these were ascribed by the individual exhibiting "anxiety attacks" (Mendel and Klein, 1969). The Diagnostic and Statistical Manual of Mental Disorders (5th ed.; DSM-5) of the American Psychiatric Association, defines the panic syndrome as an anxiety disorder characterised by panic attacks, defined as recurrent and unexpected episodes of intense fear, terror, discomfort, or apprehension associated with a sense of death or imminent danger with an immediate need to escape from that situation (American Psychiatric Association, 2013). During these attacks, the individual may experience symptoms such as shortness of breath, palpitations, tremors, sweating, chills or hot flashes and paresthesias, chest pain or discomfort, suffocation, and fear of death or of going mad or lose self-control. Commonly, the individual with panic syndrome exhibits agoraphobia, which is the fear of places or situations in which they may feel unprotected or from which they cannot easily evade (Katon, 2006; Taylor, 2006).

Fear, present in panic syndrome, can be considered as a trigger for defensive behaviour that represents an innate part of the survival instinct of the species, where the individual protects himself against aversive, dangerous and threatening situations such as: unknown environments, the silhouette of predators, emotional expressions that indicate rage

and threat of attack, odour or noise of a predator, threatening vocalisations of animals that have acquired warning significance by consistently precede the occurrence of noxious and painful stimuli (Steimer, 2002). In this sense, the emotional state of fear is manifested through adaptive responses to the risk of imminent or potential danger. Although panic-stricken fears of fear in the panic syndrome are uniquely human, there is evidence of correlation with defensive behavioural responses of animals in perilous situations have been used for a better understanding of the neural circuits involved in the organisation and elaboration of the defensive behaviour evoked by aversive conditions (Brandão et al., 1994; Biagioni et al., 2012; Almada and Coimbra, 2015). By analysing the behaviour of the animals, once exposed to aversive stimuli, it becomes possible to reproduce aspects related to anxiety disorder, for example, in the panic that occurs in humans, such as psychopathological symptoms and pharmacotherapeutic effects (Steimer, 2011).

1.2 Endocannabinoid system

The endocannabinoid system (ECS) is an endogenous neuromodulatory system, to which the alkaloids from *Cannabis sativa*, for example, delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD), interacted to apply its neuromodulatory impacts on behavioural and emotional states in the central nervous system (CNS) (Lu and Mackie, 2016). The endocannabinoids (enCBs) are synthesised ‘on demand’ and act to discharge the neurotransmitter at the synaptic level bringing about excitation or inhibition of the post-synaptic neuron (Hashimotodani et al., 2013). The discovery of the receptors to cannabinoids begins in the year 1988 when the research group of Howlett reported the characterisation of a biologically active cannabinoid type-1 (CB1) receptor in the rat brain (Devane et al., 1988), which was then cloned by the expression of its complementary DNA and also its mRNA was found in those brain regions where the CB1 receptors were reported in much amounts,

suggesting that they have attenuating effects on the CNS through cannabinoids (Matsuda et al., 1990). Another group of researchers from Cambridge, UK, cloned the second cannabinoid receptor called Cannabinoid type-2 (CB2) receptor in the peripheral spleen tissue by using the PCR techniques designed for studying of G protein-coupled receptors (Munro et al., 1993). The cannabinoid receptors have been portrayed in numerous species, including human beings, monkey, pig, puppy, rodent, and mouse; however, not in bugs. It was demonstrated that mammals synthesise endogenous agonists or ligands called enCBs to these receptors (Di Marzo et al., 1998; Mechoulam et al., 1998). The main endocannabinoid identified was the N-arachidonoylethanolamide (anandamide; AEA); however, other compounds have been included in the endocannabinoid group, such as the 2-arachidonoylglicerol (2-AG), the O-arachidonoylethanolamine (virodhamine; O-AEA) and the N-arachidonoyldopamine (Devane et al., 1988; Sugiura et al., 1995; Porter et al., 2002; Walker et al., 2002). For this reason, the specific receptors to enCBs, and each endocannabinoid itself constitutes the so-called endocannabinoid system (De Petrocellis et al., 2004).

The enCBs for example, AEA and 2-AG, have been well evaluated by researchers and showed their possible mechanisms of action. They, when bind to the cannabinoid receptors, exhibit their functions by coupling with $G_{i/o}$ protein (Reggio, 2010). In this way, the levels of cyclic AMP inside the cell are decreased and it activates mitogen-activated protein kinases. Moreover, the cannabinoid receptors after activation act to modulate the ion channels also through the $G_{i/o}$ protein coupling mechanism, which leads to the activation and inhibition of potassium and calcium channels, respectively (Howlett et al., 2010). CB1 receptors through coupling with Gs protein, can modulate the synthesis of cAMP, under certain circumstances (Eldeeb et al., 2016). There is evidence showing that these enCBs are released from the post-synaptic membranes when required. They move to act upon the CB1 receptors located on the pre-synaptic membrane resulting in the decrease of neurotransmitter release in a

heterosynaptic manner. This is considered to work as a critical feedback mechanism to regulate and balance both the inhibitory and excitatory neurotransmissions and, henceforth, is a leading system which intervenes adaptation at the level of the synapse (Alger and Kim, 2011; Castillo, 2012; Kano, 2014).

Since the revelation of Δ9-tetrahydrocannabinol (THC) as the fundamental psychoactive compound of *Cannabis sativa*, and the cloning of cannabinoid receptors and the recognition of their endogenous ligands (endocannabinoids; enCBs), our comprehension of the endocannabinoid signaling and their molecular mechanism has increased significantly. They are suggested to have a specific role at the synaptic clefts of both the inhibitory and excitatory synapses by their retrograde signaling mechanism leading to decrease or inhibit the neurotransmitters discharge (Pertwee, 2006). It has also suggested that by adjusting synaptic quality, enCBs can manage an extensive variety of neuronal functions, such as motor control, cognition, pain and defensive behaviours (Castillo et al., 2012). The retrograde signaling is the main mechanism by which enCBs regulate the functions of the synapse. In this, during the signal transmission at the nerve terminals, the endocannabinoid is produced at the post-synaptic membrane. This enCB then passes through the synaptic cleft towards the presynaptic membrane where it binds to the CB1 receptors leading to the decreased release of neurotransmitter from the synaptic vesicles (Kano et al., 2009). However, it is also reported that the enCBs can follow the non-retrograde signaling mechanism, in which they act either on the transient receptor potential vanilloid type 1 (TRPV1) or on the CB1 receptors present in the postsynaptic cells and tissues modulating the synaptic transmission. In a recent research, it was suggested that enCBs can modulate the presynaptic and postsynaptic neuronal functions indirectly through the astrocytes signaling (Zhu and Lovinger, 2005; Ohno-Shosaku et al., 2012). CB1 receptors are the class of G protein-coupled receptors, which attach to G_i/G_o proteins leading to inhibit the activity of adenyl cyclase which is an energy regulatory

enzyme in cells, shaping action potential by activating the potassium channels, and cause excitation of neuronal cells by inhibiting the voltage-gated calcium channels (Howlett et al., 2002).

CB1 receptors are present abundantly in the central nervous system while in small amounts in peripheral organs. CB1 receptors are expressed abundantly in major structures of the limbic system, including the hippocampus and basolateral complex of the amygdala (BLA), as well as in the rostral areas of the frontal lobe "prefrontal" cortex (PFC), which is closely linked with limbic structures (McPartland et al., 2009). The CB1 receptors are expressed on the terminals of serotonergic, dopaminergic and noradrenergic neurons (Morena and Campolongo, 2014; Häring et al., 2007; Hermann et al., 2002; Oropeza et al., 2007), GABAergic neurons (Marsicano and Lutz, 1999; Azad et al., 2008; Morozov et al., 2009), and glutamatergic neuronal terminals (Kawamura et al., 2006; Monory et al., 2006).

1.2.1 The role of endocannabinoid system in defensive behaviour

There are evidence, which suggest that ECS plays a vital role in the manipulation of behavioural and emotional reactions such as anxiety and panic attack-like defensive behaviours, which enable a person to expel itself from dangerous circumstances (Uribe-Mariño et al., 2012; Fernandes et al., 2013). Higher vertebrate animals show complex behaviours and they can adopt the exhibition of defensive reactions to specific ecological cues (Sih et al., 2011). The rats and mice show various bahaviours related to anxiety and panic attack-like states such as risk assessment, defensive attention, defensive immobility, escape, expresses by running and jumping, and time spent inside the burrow/inhibitory avoidance, in a threatening environment (Coimbra et al., 2017). The role of ECS in the regulation of stress, emotions, fear and anxiety responses reported various times which is later on confirmed when

the utilisation of *Cannabis sativa* extract resulted in euphoria (Akirav, 2011; Temple et al., 2014).

Numerous evidence show the involvement of ECS in the organizsation of behavioural responses (Chhatwal and Ressler, 2007). Chaperon and Thiébot (1999) showed a decrease in the locomotor activity and a profound dose-related catalepsy after cannabinoid injections in rats. Corroborating this finding, Compton et al. (1996) and Ledent et al. (1999) suggested that the injection of CB1-receptor antagonists, such the SR141716A, and mice knockout for CB1-receptor respectively, caused an increase of locomotor activity, and also potentiated the stimulant effect of apomorphine (Masserano et al., 1999). In addition, the cannabinoid CB1 receptor is involved in the moduclation of haloperidol-induced catalepsy (Medeiros et al., 2016).

It is known that the CB1 receptor is widely distributed in the limbic system and in cortical areas, suggesting that this receptor is involved in the control of emotions (Breivogel and Childers, 1998; Tsou et al., 1998; Martin et al., 2002; dos Anjos-Garcia et al., 2017; Lange et al., 2017). Some studies have been shown that CB1 knockout animals display anxiogenic response in different behavioural models, including the open-field test, the dark-light box test, and in the elevated plus maze test (Haller et al., 2002; Urigüen et al., 2004). In addition, the cannabinoids can also modulate the hypothalamus-hypophysis-adrenal (HHA), changing the release of several neurotransmitters involved in the emotional behaviour (Akirav, 2011). According to this, Urigüen et al. (2004) showed that anxiogenic responses observed in CB1 knockout mice were followed by alterations in HHA axis action, showing hypersensitivity to the stress and a reduction in the action of anxiolytic drugs, such as buspirone, in the dark-light test.

Our team has also recently demonstrated the role played by CB1-cannabinoid receptor in anandamide modulatory effecs on panic-like behaviours elicited by GABAergic

disinhibition in the medial hypothalamus (dos Anjos-Garcia et al., 2017). Indeed, the hypothalamus, especially its ventromedial division and the anterior nucleus, was found with evenly distributed CB1 receptors (Wittmann et al., 2007). Amongst the different hypothalamic nuclei, the higher amounts of CB1 receptors were expressed in lateral, medial and magnocellular preoptic nuclei, while low levels in the premammillary nucleus of the hypothalamus. However, the mRNA for CB1 receptors was expressed in lesser amounts in various hypothalamic nuclei, but the hybridisation was strongest detected in the anterior nucleus and in the ventromedial division of hypothalamus (Marsicano and Lutz, 1999). According to Schlicker and Kathmann (2001) and Alger (2002), many axonic terminals in the central nervous system express CB1 receptors, with the function of inhibiting the release of both inhibitory and excitatory neurotransmitters. Freund et al. (2003) showed that the CB1 receptors are present in glutamatergic and GABAergic projections in the above structures, modulating inputs to these nuclei. Marsicano and Lutz (1999) showed immunoreactivity to CB1 receptors in paraventricular, and ventromedial hypothalamic nuclei, in the infundibular region and in the lateral hypothalamic area, situated on glutamatergic neurons. It is also known that these endocannabinoid receptors are involved with the function of monoaminergic autoreceptors such as α_2-noradrenergic and 5-HT_{1A} serotonergic receptors (Demuth and Molleman, 2006).

1.3 Phytocannabinoids and psychiatric disorders

Amongst the phytocannabinoids that naturally exist in the chemical composition of *Cannabis sativa* plant, Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD) are the most popular (Mechoulam and Gaoni, 1965). CBD is considered as the major non-psychoactive alkaloid of the *Cannabis sativa* plant, constituting up to 40% of its extract. In the last decade, numerous studies demonstrated that CBD plays a role in the treatment of different psychiatric

disorders (Mechoulam et al., 2007; Zuardi, 2008; Izzo et al., 2009). Preclinical and clinical studies showed that the CBD exert different pharmacological and neuropsychological actions such as antidepressant, anxiolytic, antipsychotic, and neuroprotective (Guimarães et al., 1990; Zanelati et al., 2010; Bergamaschi et al., 2011; Esposito et al., 2011; Campos et al., 2012; Uribe-Mariño et al., 2012). However, the exact mechanism of action regarding these effects is not yet fully understerstood (Izzo et al., 2009). The CBD mechanism of action is thought to have an impact to diminish anandamide hydrolysis or its re-uptake (Bisogno et al., 2001; Campos et al., 2013), which can enhance endocannabinoid signaling, and its consequences for behavioural reactions are seemed to be due to the CB1 receptor-mediated endocannabinoid signaling mechanism (Bitencourt et al., 2008; dos Anjos-Garcia et al., 2017). In addition to interaction with endocannabinoid system, the antidepressant and anxiolytic effects of CBD could rely upon the neurotransmission of GABAergic and glutamatergic neuronal systems (Campos and Guimarães, 2008; Fogaça et al., 2014)

So, we can suggest that the interaction between different neurotransmitters, specially endocannabinoids, endogenous opioid peptides and serotonin in different structures involved in emotional and physiological disorders, like the PAG, the hypothalamus, and the amygdaloid complex, can integrate threatening cues coming from sensory organs and starting behavioural responses critical for the survival of the animal.

1.4 Defensive behaviour and hypothalamus

Several animal species have the abilities to cope with dangerous environments and to adopt strategies for their survival (Sih et al., 2011). They show defensive behaviour to the aversive stimuli (Guimarães-Costa et al., 2007; Biagioni et al., 2012; Coimbra et al., 2017). The neural substrates for these defensive behaviours and their mechanism of action are hardwired inside the brain and are considered an innate response (Brandão et al., 1994; Labar

and Ledoux, 2011; da Silva et al., 2013). Different regions of the hypothalamus are now known to play a significant role in the modulation of defense behaviour (Motta et al., 2009; Biagioni et al., 2012; Ullah et al., 2015, 2017). The confrontation of animal species to its natural predators elicits defensive behavioural responses such as defensive attention, risk assessment, defensive immobility, and escape, expressed by jumping and running (Almada and Coimbra, 2015; Guimarães-Costa et al., 2007). Different regions of the hypothalamus (Biagioni et al., 2012; Ullah et al., 2015; dos Anjos-Garcia et al., 2017), and hypothalamic efferent connexions (Ullah et al., 2017) have been significantly related to innate fear (Biagioni et al., 2013, 2016), antipredatory behavioural responses (Paschoalin-Maurin et al., 2018), and instinctive fear-induced antinociception (Biagioni et al., 2013, 2016; Falconi-Sobrinho et al., 2017; Falconi-Sobrinho and Coimbra, 2018). The PMd, VMHdm, dMH stimulation exhibit defensive behavioural responses with the expression of Fos protein (Dielenberg and McGregor, 2001). The medial hypothalamic regions, such as dMH, stimulation elicit defensive behaviour-induced neurovegetative activation, such as tachycardia, hyperventilation, and hypertension (Nascimento et al., 2010). The electrical and chemical stimulation of the medial hypothalamus has been demonstrated in many studies to modulate the defensive behaviour involving the somatomotor and autonomic responses during natural threats (Fernandez De Molina and Hunsperger, 1962; Lipp and Hunsperger, 1978; Schmitt et al., 1985; Lammers et al., 1988; Silveira and Graeff, 1988; Wilent et al., 2010). Regarding this, it is suggested that there is a complex neuronal network (Canteras and Swanson, 1992) participating in elaboaration of these defensive behavioural responses, involving the anterior hypothalamus (Falconi-Sobrinho and Coimbra, 2018), the premammillary nucleus of the hypothalamus, and the ventromedial hypothalamic nuclei (dos Anjos-Garcia et al., 2017), and the activation of these regions (Ullah et al., 2015, 2017) and the exposure to natural predators (Paschoalin-Maurin et al., 2018) elicit strong defensive reactions, followed by nuclear Fos

protein increase in the neuronal cells. Recently, several researches have been focusing on anterior (Falconi-Sobrinho et al., 2018), posterior (Biagioni et al., 2012; Falconi-Sobrinho et al., 2017), dorsomedial (Biagioni et al., 2013, 2016; Freitas et al., 2009; de Freitas et al., 2013, 2014), and ventromedial (de Freitas et al., 2013, 2014), divisions of the hypothalamus, in addition to the dorsomedial division of the ventromedial hypothalamic nucleus (Ullah et al., 2015, 2017; dos Anjos-Garcia et al., 2017) to produce panic attack-like defensive responses (Guimarães-Costa et al., 2007; Freitas et al., 2009; Biagioni et al., 2012; Uribe-Mariño et al., 2012; de Freitas et al., 2013; da Silva et al., 2013, 2015; Twardowschy and Coimbra, 2015; Viana et al., 2015; Ullah et al., 2015, 2017; dos Anjos-Garcia et al., 2017; Falconi-Sobrinho et al., 2017; Roncon et al., 2017; Coimbra et al., 2017; Falconi-Sobrinho and Coimbra, 2018). It was also morphologically demonstrated the neuronal activation of the anterior, posterior, dorsomedial, ventromedial, lateral, posterior periventricular, and dorsal premammillary hypothalamic nuclei in prey confronted to a natural predator, reversed by chronic treatment with panicolytic medicines (Canteras et al., 1997; Guimarães-Costa et al., 2007; Uribe-Mariño et al., 2012; Coimbra et al., 2017; Paschoalin-Maurin et al., 2018). Confrontations of prey with wild venomnous snakes elicit panic attack-related defensive responses, such as defensive immobility and non-oriented escape (Coimbra et al., 2017; Paschoalin-Maurin et al., 2018), and the immobility was noticed on the optogenetic activation of VMH (Lin et al., 2011; Falkner et al., 2014). Therefore, it can be suggested that VMH plays an important role in the modulation of panic attack-related defensive behavioural responses, although, the exact neuronal mechanism of the modulation of that defensive behaviour elicited by the VMH activation remained elusive.

1.5 Pain

Pain is an upsetting sensation regularly caused by strong or harming stimuli (Lumley et al., 2011). The International Association for the Study of Pain broadly utilised definition characterises pain as "a repulsive and unpleasant sensory and emotional experience related with real or potential tissue injury or described in a condition of such injury (Eccleston and Crombez, 1999; Lascaratou, 2007).

Nociceptive pain occurs when the special pain receptors called nociceptors are stimulated (Zaki et al., 2016). The noxious stimuli sensitise the nociceptive sensory fibres; releasing chemical mediators, which can directly act upon the nociceptive receptors and stimulate them (Julius and Basbaum, 2001; Woolf and Ma, 2007). The stimuli for the release of these chemical mediators might be chemical, thermal, or mechanical, which bind and/or mediate their specific receptors to release different types of mediators, such as histamine, bradykinin, serotonin, ATP, arachidonic acid metabolites, cytokines, adenosine, substance P, excitatory amino acids, nitric oxide, endogenous opioid peptides, neurotrophins, acetylcholine, somatostatin, among others. These chemical mediators transmit the nociceptive signals by changing the permeability of the neuronal membrane to generate an action potential (Basbaum and Fields, 1984).

A neuropathic pain, classified according to the IASP as a pathologic painful clinical feeling, is usually due to a dysfunction or primary injury of the central or peripheral nervous system structures. There also exists another type of pain called psychogenic pain, which occurs due to a change in the psychological state of the body and is mainly observed during anxiety (Fürst, 1999; Colloca et al., 2017).

The nociception is a perception of pain in the CNS sensed due to the activation of nociceptive receptors in a specific stimulated site (Tracey, 2017). When the action potential, generated because of the nociceptive stimuli, reaches the spinal and supraspinal structures, the multidimensional complexes, and neuronal mechanisms involve in this perception and are

called cognitive-emotional, affective-motivational, and sensorial-discriminative (Bromm and Lorenz, 1998; Marchand, 2008). Actually, we can say that pain is a complex experience not only involved in the transmission of noxious stimuli but also processed by a series of social, emotional, environmental, cultural, and cognitive behavioural experiences (Hansen and Streltzer, 2005).

1.6 Fear-induced antinociception

It is suggested to use the terms nociception and antinociception instead of pain and analgesia, respectively, in experimental animal studies (Yarnitsky et al., 2014). The antinociceptive process may be defined as a reduction in the capability to perceive pain afer the limbic and paralimbic system structures activation (Coimbra et al., 1992; Coimbra and Brandão, 1997), as well as during exposure to a threatening situation (Coimbra et al., 2017). It has been considered an essential part of fear-induced defensive behaviour (Coimbra et al., 2006, 2017). It is appropriate and crucial in aversive situations, for example, exposure of an animal to a predator, to a dangerous or an unreceptive environment (Zylberberg and Deweese, 2011; Coimbra et al., 2017). This process is indispensable to evoke innate or conditioned fear-induced defense behaviour (Coimbra et al., 2017), instead of pain or sickness-induced recuperative behaviour (Bassi et al., 2012, 2018). In this condition, if the defensive behavioural response against aversive situations results in the decrease of nociceptive impulses, and ultimately in a decrease in the perception of pain, it is appropriate that the pain inhibition system is positively recruited (Coimbra et al., 2006). On the other hand, if the pain inhibitory system not recruited positively, the animal could acquire a recovery behavior, due to suffering, rather than displaying a defensive posture, to preserve its physical integrity.

The relationship between fear and defensive behaviour has been shown and this theory was named as the perceptual-recovery model (Bolles and Fanselow, 1980; Bouton et al.,

2001; Dunsmoor and Paz, 2015). That pain-induced recovery model would be responsible for restoring the normal state of the individual who had suffered an injury, whereas, in the defensive phase, the activation of the descending pain inhibitory system causes the reaction of the aversive stimulus followed by antinociception (Bassi et al., 2018).

The antinociception is a process of inhibition of the traveling of action potential alongside the neuronal axon generated by peripheral pain stimuli, for example, in the dorsal horn of the spinal cord (dhSC). Supraspinal structures, such as the periaqueductal gray matter (PAG), the major raphe nucleus and the ventromedial rostral bulb have been suggested as important regions involved in pain modulation, due to their direct projections to the dorsal horn of the spinal cord (Basbaum and Fields, 1984; Tracey and Mantyh, 2007; Ossipov et al., 2010). The periaqueductal grey matter was one of the first regions to be explored in this sense (Reynolds, 1969; Ossipov et al., 2014). Evidence shows that antinociceptive processes can be generated in all PAG regions (Rhodes, 1979; Coimbra et al., 1992, 2006; Yarnitsky et al., 2014). Some researchers point to the ventro-lateral region of the PAG, as being the most effective in producing antinociception (Wang, 1976; Gebhart and Toleikis, 1978), alongside sites located in the dorsomedial and dorsolateral columns (Coimbra and Brandão, 1997; Castellan-Baldan et al., 2006). In this sense, some studies have shown that the electrical stimulation of PAG promoted antinociception preceded by behavioural reactions of explosive escape (Fardin et al., 1984). Similar responses and innate fear-induced antinociception can be elicited by dorsal mibbrain GABAergic disinhibition (Coimba et al., 2006).

However, no less important structures located in the brainstem, such as the locus coeruleus (LC) and the dorsal raphe nucleus, have been suggested as participatory regions in the downward inhibitory control of pain (Basbaum and Fields, 1984; Bassi e al., 2018). Neuroanatomical studies, performed through retrograde neurotracking in the dorsoal horn of the spinal cord, showed noradrenergic projections from the locus coeruleus (Kwiat and

Basbaum, 1992), which modulate the input of the nociceptive impulse into the neuraxis, through its downward connections with the endogenous opioid interneurons present in the close to the first ascending pathway synapse (Millan, 2002).

Other studies have shown that nociception, after stimulation of the dorsal raphe nucleus, has decreased. That mesencephalic region is one of the major nuclei of the raphe, whereby serotonergic fibres descend to the gelatinous substance of the dorsalhorn of the spinal cord. These pathways can be projected onto brain interneurons, which in turn hyperpolarise the neurons responsible for transmitting the nociceptive message (Melzack et al., 1982). The study of the participation of other supraspinal structures, such as the cerebral cortex, thalamus, and hypothalamus in the pain modulatory process has also been the target of several researchers. Although these more rosy structures are often more stimulated, there is evidence that the antinociception produced in these regions is transmitted via PAG (Hagbarth and Kerr, 1954; Rhodes, 1979). Hagbarth and Kerr (1954), after electrically stimulating the reticular formation, cerebellum and the cerebral cortex, observed that the activation of these structures resulted in a blockage of nociceptive neurotransmission at the spinal cord level.

In the year 1988, Aimone et al. suggested that the lateral hypothalamus (LH) would be involved with the descending system of pain inhibition. After electrically stimulating this structure, the animals responded with an increase in tail-withdrawal latency (antinociception), as measured by the tail-flick test. In another approach in the same structure, researchers observed that electrical stimulation of LH caused antinociception equitably, preceded by jumping reactions (Mayer and Liebeskind, 1974), which suggests an antinociception that follows the defensive behaviour of escape.

Another study proposed that the dorsal-medial hypothalamus (dmH), as well as the posterior hypothalamus (PH), appear to recruit the dorsal columns of the periaqueductal grey matter for the organisation of at least part of the antinociception induced by panic attack-like

defensive responses evoked by the chemical stimulation of such hypothalamic nuclei of *Rattus norvegicus* (Biagioni et al., 2012). In fact, there is evidence that hypothalamic descending projections, in focus on the posterior hypothalamus, particularly those that target PAG, are involved in the modulation of complex behavioural responses (Vertes and Crane, 1996; Ullah et al, 2017). Thus, neuroimaging approaches have shown that traumatic nociceptive pain, for example, activates neurons of both the periaqueductal grey matter and the hypothalamus (Hsieh et al., 1996).

Finally, our team has demonstrated that the activation of medial hypothalamic nuclei (Freitas et al., 2009; de Freitas et al., 2013, 2014; Biagioni et al., 2013, 2016), the anterior hypothalamus (Falconi-Sobrinho and Coimbra, 2018) and the posterior hypothalamus (Falconi-Sobrinho et al., 2017) also elicit unconditioned fear-induced antinociception, with similar intensity and duration to that displayed by prey threatened by rattle snakes in a dangerous environment (Coimbra et al., 2017).

1.7 Hypothesis

Considering the neuronal activation of medial hypothalamic nuclei of prey confronted with venomous snakes (Paschoalin-Maurin et al., 2018), the panicolytic-like effect of cannabidiol in prey threatened by constrictor snakes (Uribe-Mariño et al., 2012), the panicolytic-like effect of CB1-mediated anandamide signaling mechanisms in the medial hypothalamus (dos Anjos-Garcia et al., 2017), the hypothesis of the present work is that cannabidiol will decrease both VMH-stimulation produced defensive behaviour and unconditioned fear-induced antinociception via the recruitment of cannabinoid type 1 receptor.

2 OBJECTIVES

2.1 General objective

To study the role played by CB1 cannabinoid receptor in the effect of VMH treatment with cannabidiol during VMH chemical stimulation.

2.2 Specific objectives

- Investigation of CB1 receptor distributions in the ventromedial hypothalamus different subnuclei;
- Investigation of the effect of VMH treatment with cannabidiol on defensive behavior elicited by chemical stimulation of the medial hypothalamus;
- Investigation of the role played by CB1 cannabinoid receptor in the effect of cannabidiol on defensive behaviour elicited by chemical stimulation of the medial hypothalamus;

3 MATERIAL AND METHODS

3.1 Ethical approval

All experimental trials were led in strict consistency with the Ethical Commission in Animal Experimentation of the FMRP-USP, which fulfills the principles of ethics for animal research adopted by the National Council for Control of Animal Experimentation (CONCEA), and were approved by the Commission of Ethics in Animal Research (CEUA-FMRP-USP) (process 107/2012).

3.2 Animals

Male *Wistar* rats (*Rattus norvegicus,* Rodentia, Muridae), weighing in between 240 and 260 g were purchased from the animal breeding colony located at Ribeirão Preto Medical School of the University of São Paulo (FMRP-USP). They were rested for 2 days in the animal house of the Department of Pharmacology (FMRP-USP) in order to calm down rodents, due to induction of stress during transportation. They were given free access to water and food *ad libitium* in a centrally air-conditioned environment with a temperature between 22 to 24ºC with the daily light/dark cycle (lights turned on at 7:00 a.m.).

3.3 Immunohistochemical staining technique

Rodents under deep urethane anesthesia (1.25 g/kg, IP) were undergone intra-cardial perfusion using 20 ml saline trailed by 20 ml 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.3). Brains were quickly removed and were placed overnight in 4% paraformaldehyde solution at a controlled temperature of 4°C, shifted to 10% sucrose solution for overnight at 4°C, and again in 20% sucrose solution for overnight at 4°C. The samplings were fixed in OCT paraffin-gel and were coronally sectioned (6 µm) on a cryostat machine (Leica CM 1950, Wetzlar, Germany), and were mounted on the silanised glass slides. Immunohistochemical staining for CB1 receptors was performed on paraffin-fixed brain

sections using a polyclonal anti-CB1 IgG antibody (N-15, sc-10066, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The sectioned brain tissues were brought to de-paraffinisation in xylene solvent and were graded washed by ethanol solutions. The process of antigen retrieval was made by treating the slides with citrate buffer (10 mM) and incubated for 10 min at 100°C on high pressure. The slides were treated with 3% hydrogen peroxide and were immediately washed with Tris buffer after 15 min. The blockage solution (BSA 3%) was performed for 1 h. The anti-CB1 antibody (Santa Cruz Biotechnology) diluted in phosphate buffer (PBS) with a ratio of 1:400 was applied to the tissue sections and were incubated for 1 h, at room temperature. These rat brain slices were then incubated with immuno-peroxidase polymer for 1h and 30 min, and the lights were turned off for adding the DAB solution in a controlled environment from 3 to 10 min. Brain slices were dipped in the Harris-hematoxylin and rested for 1 min to stain. The slides were graded washed with alcohol, ABS, and xilol solutions to dehydrate them and covered with coverslips using mounting medium. The slides were analysed under a motorised photomicroscope (AxioImager Z1, Carls Zeiss Straβe, Oberkochen, Germany).

3.4 Experimental apparatus

An acrylic semi-transparent parallelepiped-shaped polygonal arena (Coimbra et al., 2017) having dimensions 154 × 72 × 64 cm, and the inner surfaces of the walls were provided with a thin film having capacity of 80% light reflection in order to avoid the visual contact of animal with the experimenter (Ullah et al., 2015; Biagioni et al., 2016). The arena's floor was drawn with red lines to divide it into 20 small but equal rectangles for experimental purposes and was placed on a stainless steel sheet. This whole apparatus was then placed on a table elevated 83 cm from the ground, and its surface was made up of granite rock (170 × 85 × 2 cm). A semi-simulated dark-hued acrylic burrow (36 × 26 × 12.5 cm), having two entries at

opposite sides, was set on a corner of the field imitating as a sheltered place for animals to escape in. To avoid place preference, a translucent ceiling was used for the burrow (Prus et al., 2009). The animals were habituated for three days inside this apparatus with easy access to food *ad libitium* and water, and the room temperature (24 ± 1 ºC) was maintained through an air-conditioning during the whole 12h light/dark cycle except at the time of experiment carried out in the daytime (Almada and Coimbra, 2015).

3.5 Drugs

The polyclonal anti-CB1 IgG antibody (N-15, sc-10066, Santa Cruz Biotechnology, Santa Cruz, CA, USA), N-methyl-D-aspartate (NMDA; 6nmol Sigma®) dissolved in physiological saline (NaCl; 0.9%), Cannabidiol (CBD; ~99,9% pure; in a dose of 25, 50, and 100 nmol; STI-Pharmaceuticals, UK), the CB1 receptor antagonist N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251; Tocris Bioscience, 147 Bristol, UK) at 100 pmol diluted in 10% DMSO (Almeida-Santos et al., 2013) were used in the present work.

3.6 Surgical procedure

The surgical procedure of animals was carried out by settling their heads in a stereotaxic machine's frame through the iron rods embedded in the ears (David Kopf, Tujunga, California, USA) after profoundly anaesthetising them with the solution of ketamine (92 mg/kg, Ketamine Agener, União Química Farmacêutica Nacional, Brazil) mixed with xylazine (9.2 mg/kg, Dopaser®, Hertape/Calier, Juatuba, Minas Gerais, Brazil). A stainless steel-guided cannula (external and internal diameters of 0.6 and 0.4 mm, respectively) was inserted in the diencephalon directed to the ventromedial hypothalamus (VMH). The upper incisor bar was set 3 mm underneath the interaural line, and the skull was flat between the

bregma and lambda. Taking bregma as a point of reference the guide cannula was vertically implanted in the aimed structure utilising the stereotaxic coordinates (AP=-2.64 mm, ML=-0.6 mm, DV=-8.4 mm) provided in the rat brain in stereotaxic coordinates atlas by Paxinos and Watson (2007). The guided-cannula was firmly settled at the position by a stainless steel screw screwed into the skull followed by pouring an acrylic gum. A thin stainless steel wire was inserted to seal the cannula in order to avoid its obstruction. Each animal was then treated with penicillin G benzathine (120.000UI/0.2mL; i.m) and flunixin meglumine (2.5 mg/kg; i.m) so that to avoid inflammation and pain. The animals were put for postoperative recovery for 5 days.

3.7 Experimental procedure

The animals (n=6-8 per group) were put for experiment after five days of stareotaxic surgical procedure and three days of habituation in the polygonal arena. The safe and non-invasive environment was created and was adopted by the rodents while putting them for habituation (Uribe-Mariño et al., 2012; Almada and Coimbra, 2015; Ullah et al., 2015; Biagioni et al., 2016). 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-diencephalic treatments (intra-VMH microinjections). The drugs were microinjected in either at right or left-brain hemisphere, using a 5µL Hamilton syringe (Merck, Darmstadt, Germany) connected to a polyethylene tube having at its one end a dental needle (OD: 0.3mm), with a length of 1 mm longer than the guide-cannula. The syringe was fitted in an automated infusion pump (Master Flex L/S TM; Sydney, Australia) and the drugs were injected through specific rates. A bubble was produced between the column of the drug and the column of distilled water both taken in the same tube. The position of the bubble was marked from time to time in order to ensure the movement of the drug. In all cases, the same volumes of the

respective diluents were used as controls. In the initial set of experiments, the rodents at first got an intra-VMH microinjection of cannabidiol (CBD 25, 50 or 100 nmol; 0.2 μL) or vehicle (veh), by one more microinjection of NMDA in a concentration of 6 nmol/0.2 μL or vehicle (veh) after 5 minutes. In the second set of experiments, the rodents at first got an intra-VMH microinjection of (AM251 in a concentration of 100 pmol/0.1 μL or vehicle (veh), trailed by another microinjection of NMDA (6 nmol/0.1 μL) or vehicle (veh). Each animal from all the sets of experiments, after receiving the last microinjection, was put inside the polygonal arena to record the behavioural reactions for 10 minutes. In this case, we utilised a volume of 0.1 μL for each drug and its separate diluents (veh) microinjected into the VMH, with a total volume of 0.3 μL received per rodent. Taking into account the past shreds of evidence recommending that the spreading of substances microinjected in the CNS into the tissue encompassing the site of infusion is directly proportional to the volume infused (Myers, 1966; Routtenberg, 1972). Accordingly to Myers (1966), a volume of 0.5 μL causes a normal spread of 1.04 mm, and for this reason, volumes no bigger than 0.4 μL were microinjected into the VMH in the present work. Drug treatments and doses were based on the literature.

3.8 Behavioural recordings

The rodents were recorded for their behavioural responses during their free movements inside the polygonal arena each for 10 minutes using a video camera (Sony Handycam HDR-CX350, Tokyo, Japan) settled on the stand. These recorded behavioural reactions, consequently, were analysed utilising a programming X-Plo-Rat software version 1.1.0, developed at the Laboratory of Exploratory Behaviour at the Ribeirão Preto School of Philosophy, Sciences and Literature of the University of São Paulo. This product does not play out the automatic estimation of behavioural reactions, but the experimenter assesses and, run the software to help evaluate the number and duration of each behavioural reaction. The

behavioural psychoanalysis including the alertness (defensive attention), characterised as interruption of ongoing behaviours for up to six seconds, followed by an attentive posture, as well as behaviours characterised by small head movements, rearing and smelling. Freezing (defensive immobility) was measured when the rodent demonstrated absence of movement for at least six seconds, except for respiration, followed by autonomic reactions, such as defecation, exophthalmia and/or micturition. The number of crossings (stepping with four legs within a delimited rectangle on the arena floor after crossing the border of each section line) was measured for estimation of the number of escape reactions characterised by running. The escape defensive behaviour was classified as either oriented or non-oriented escape behaviour. Oriented escape was more organised, depicted by racing to the burrow or to the elevated platforms, while non-oriented escape was considered as a vigorous running in directions of the arena opposite to that in which was situated the entrance of the burrow. In addition, the climbing to the roof of the burrow and to the side borders of the arena, vertical jumps were too counted as oriented escape behaviour. The time spent inside after escape to the burrow was also recorded. The freezing and escape defensive behavioural responses are considered as panic attack-like defensive reactions whereas alertness is included in the anxiety-like defensive responses (Shekhar et al., 1994; Blanchard et al., 2001; Borelli et al., 2004; Coimbra et al., 2017).

3.9 Nociceptive testing

Nociceptive thresholds were measured using the tail-flick test and were compared in independent groups of rats. Each animal was placed in a restraining apparatus (Insight, Ribeirão Preto, SP, Brazil) with acrylic walls, and its tail was placed on a heating sensor (Tail-Flick Analgesia Instrument; Insight, Brazil). The progressive heat elevation was automatically interrupted when the animal removed its tail from the apparatus. The current

raised the temperature of the coil (Ni/Cr alloy; 26.04 cm in length x 0.02 cm in diameter) at a rate of 9°C/s, starting at room temperature (approximately 20°C), and small current intensity adjustments were performed, if necessary, at the beginning of the experiment (baseline records) to obtain three consecutive tail-flick latencies (TFLs) between 2.5 s and 3.5 s. If the animal did not remove its tail from the heater within 6 s, the apparatus was turned off to prevent skin damage. Three baseline measurements of control TFLs were recorded at 5-min intervals.Tail-flick latencies were, likewise, estimated for 60 min instantly after the elaboration of defensive behavioural responses.

3.10 Histological procedure

Upon completion of the experiments, the animals were anaesthetised with 1 mL/100 g of 5% chloral hydrate (Vetec Química Fina®, Duque de Caxias, Rio de Janeiro, Brazil) and perfused through the left ventricle using a perfusion pump (Master Flex® L/STM peristaltic tubing pump, East Bunker Court Vernon Hills, Illinois, USA). The blood was washed out using 40 mL of cold, oxygenated, Ca++ -free Tyrod's buffer followed by 200 of ice-cold 4% (w/v) paraformaldehyde (LabSynth, Brazil) in 0.1 M PBS (LabSynth, Brazil), pH 7.3, over 15 min, at a pressure of 50 mmHg. The brain was immediately removed and soaked for 4 h in fresh fixative (4% paraformaldehyde) at 4ºC. 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 4ºC 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

according to the Paxinos and Watson atlas (2007) under a motorised photomicroscope (AxioImager Z1; Zeiss, Oberkochen, Germany). The data from rats with guide-cannula tips located outside the VMH were not included in the statistical analyses.

3.11 Statistical analysis

One-way analysis of variance (ANOVA) followed by Newman Keuls *post hoc* tests was used to analyse the behavioural studies data. Data from the nociceptive threshold experiments collected immediately after the end of the defensive behaviour were submitted to a repeated measures two-way ANOVA (RM-ANOVA) followed by Tukey's *post hoc* tests. Behavioural data are expressed as mean and tail-flick latencies are expressed as mean ± S.E.M for n=6 rats per group. $P < 0.05$ was considered statistically significant. The software used for statistical analysis and graph plotting was GraphPad Prism version 7.0.

4 RESULTS

4.1 Immunohistochemistry

The morphological procedure demonstrated a profuse distribution of CB1-cannabinoid receptor-labelled perikarya and in neuronal fibres in dorsomedial division, central, and ventrolateral subnuclei of the ventromedial hypothalamic nucleus, as shown in figure 1.

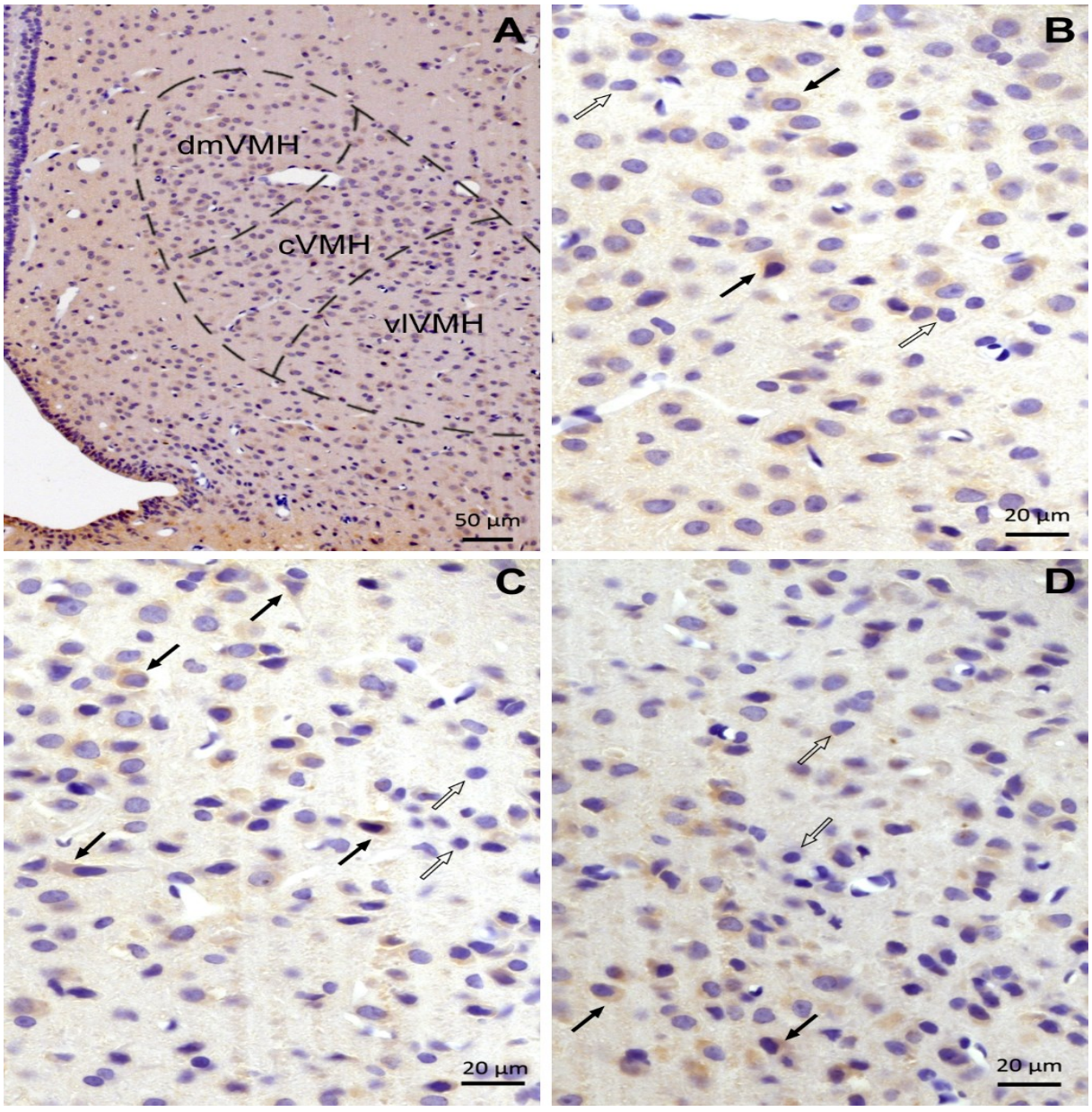

Figure 1 – Photomicrographs of coronal sections of Wistar *Rattus norvegicus* (Rodentia, Muridae) at the level of medial hypothalamus (A), showing cannabinoid type 1 (CB1) receptor-like immunohistochemical staining in the ventromedial nucleus of the hypothalamus (VMH). Black arrows indicate CB1 receptor-labelled perikarya surrounded by CB1-negative neuronal bodies (open arrows) situated in the dorsomedial (B), central (C), and ventrolateral (D) divisions of the VMH. Counter-staining: Harris haematoxylin.

4.2 Histologically confirmed sites of the microinjections

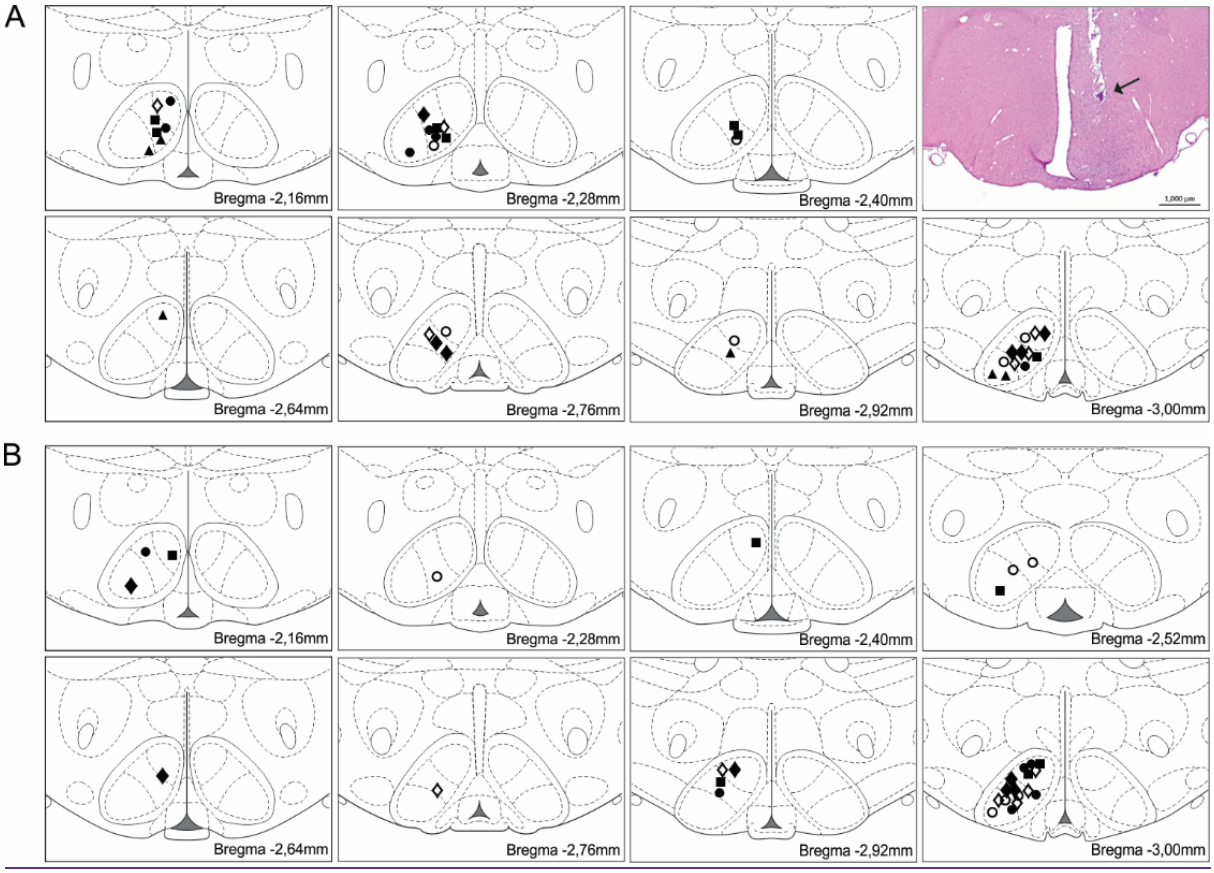

Figure 2 – Schematic coronal sections of the *Rattus norvegicus*' brain depicted in modified diagrams of the Paxinos and Watson's rat brain in stereotaxic coordinates atlas (2007), showing (A) sites of intra-VMH microinjections of vehicle + vehicle (○), vehicle + NMDA 6 nmol (●), CBD at 25 (▲), 50 (■) and 100 (♦) nmol + NMDA, 6 nmol + vehicle (◊) and photomicrograph of a coronal section of the Wistar rat's brain, showing (black arrow), a representative microinjection of drugs in the VMH. Counter-staining: Hematoxylin-eosin. (B) vehicle + vehicle + vehicle (○), vehicle + vehicle + NMDA 6 nmol (●), vehicle + CBD 100 + NMDA (■), AM251 100 pmol + CBD 100 nmol + NMDA (♦), AM251 100 pmol + CBD 100 nmol + NMDA (◊) corresponding to pharmacological experiment 2.

4.3 *Experiment 1:* Effects of the VMH pretreatment with CBD on innate fear-related behavioural reactions and unconditioned fear-induced antinociception

4.3.1 *Behavioural reactions*

According to the one-way ANOVA followed by a Newman-Keuls *post hoc* test, there was a significant effect of treatment on the number ($F_{5,31}$= 8.011, $P < 0.001$) and duration ($F_{5,31} = 6.995$, $P < 0.001$) of running, number ($F_{5,31} = 3.668$, $P < 0.001$) of jumps and number ($F_{5,31} = 5.112$, $P < 0.001$) of crossings. Intra-VMH microinjections of NMDA at 6 nmol elicited panic attack-like escape behaviour, expressed by running and jumping ($P < 0.01$ and $P < 0.05$, respectively) and increased the duration of running ($P < 0.01$) and number of crossing ($P < 0.05$) when compared to (10% DMSO + physiological saline)-treated group, as shown in Figure 3*A-D*. In addition, there was a significant effect of treatment on the number of escape to the burrow ($F_{5,31} = 27.52$, $P < 0.001$) and time spent inside after escape to the burrow ($F_{5,31} = 4.318$, $P < 0.001$). Intra-VMH treatment with NMDA at 6 nmol increased the number of escape to the burrow and time spent inside after escape to the burrow (Newman-Keuls' *post hoc* test; $P <0.001$ and $P < 0.05$, respectively) than the vehicle + vehicle-treated control group, as shown in Figure 3*E-F*.

Interestingly, only the highest dose of CBD (100 nmol) microinjected into the VMH was able to impair the innate fear-related defensive behaviours. According to Newman-Keuls*post hoc* test, the intra-VMH pretreatment with CBD (100 nmol) attenuated the number ($P < 0.05$) and duration ($P < 0.05$) of running, and inhibited the jumping responses ($P < 0.05$), the number of escape to the burrow ($P < 0.001$) and, consequently, time spent inside after escape to the burrow ($P < 0.05$), as shown in Figure 3*A-F*.

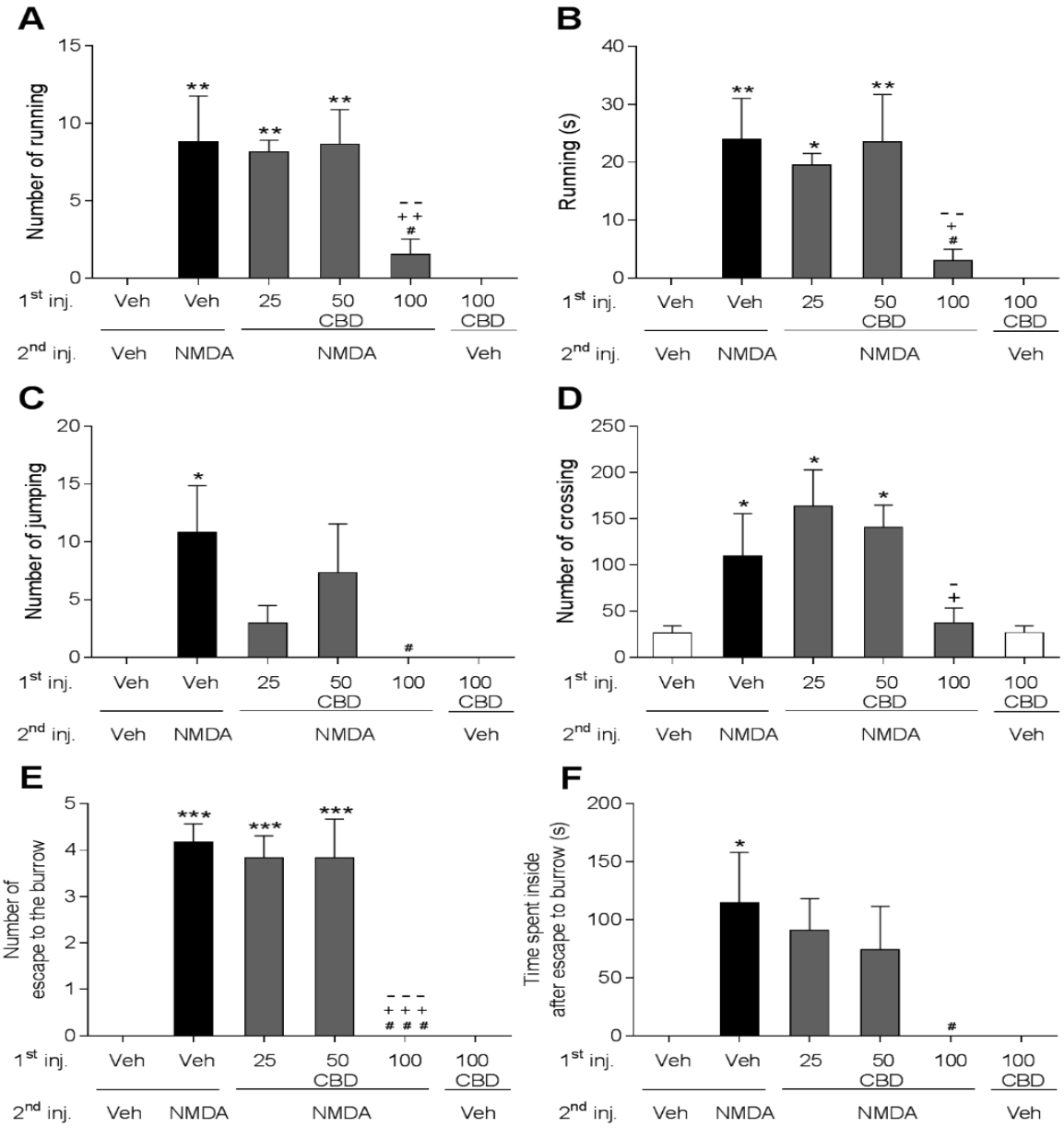

Figure 3 – Effect of central microinjections of cannabidiol (25, 50 and 100 nmol/0.1 µL) or vehicle (NaCl 0.9%/0.1 µL) into the ventromedial hypothalamus (VMH) on the number and duration of running (A and B), number of jumping (C), number of crossing (D), number of escape to the burrow (E) and time spent inside after escape to the burrow (inhibitory avoidance) (F) induced by microinjection of 6 nmol NMDA or vehicle in the ventromedial hypothalamus. Columns represent mean, and bars the standard error of the mean (S.E.M.); n = 6 per group. *P < 0.05, ** P < 0.01, *** P < 0.001, as compared to veh + veh-treated group; $^{\#}$ P < 0.05, $^{\#\#\#}$ P < 0.001, compared to veh + NMDA-treated group; $^{+}$ P < 0.05, $^{++}$ P < 0.01; $^{+++}$ P < 0.001, compared to CBD 25 nmol + NMDA-treated group ; $^{-}$ P < 0.05, $^{--}$ P < 0.01; $^{---}$ P < 0.001, compared to CBD 50 nmol + NMDA-treated group, according to two-way ANOVA followed by Newman-Keuls' *post hoc* test.

4.3.2 *Fear-induced antinociception*

The panic attack-like defensive behaviours that were evoked by the NMDA microinjected into the VMH were followed by a significant antinociceptive response. According to two-way ANOVA, there were significant effects of treatment ($F_{5.31}$ = 34.55; P< 0.001) and of time ($F_{9.279}$ = 126.6; P< 0.001), as well as a significant interaction between treatment and time ($F_{45.279}$ = 26.91; P< 0.001). According to Tukey's *post hoc* test, the intra-VMH microinjections of NMDA increased antinociception compared with VMH-vehicle + vehicle treatment at the time 0 to 50 min after the defensive behaviours (P< 0.05). In comparison to the VMH-vehicle + NMDA treatment, the intra-VMH microinjections of CBD at the higher doses (50 and 100 nmol) decreased the unconditioned fear-induced antinociception at the same times (P< 0.05), while the CBD microinjected into the VMH at the lower dose (25 nmol) reduced the antinociception at the time 30 to 50 min after panic attack-like defensive behaviour (Tukey's *post hoc* test; P< 0.05). Regarding the responses between the doses of CBD on fear-induced antinociception, the attenuator effect caused by the CBD at higher doses (50 and 100 nmol) was different from that caused by the CBD at the lower dose (25 nmol) at the times 0, 10, 20 and 40 after defensive behaviour, according to Tukey's *post hoc* test (P< 0.05 in all cases). In addition, the attenuating response of CBD (100 nmol) microinjected into the VHM on the fear-induced antinociceptive effect was different from that caused by CBD injected at the intermediate dose (50 nmol) at 0 to 30 min after panic attack-like defensive behaviour evoked by intra-VMH microinjections of NMDA (Tukey's *post hoc* test; P< 0.05), as shown in Figure 4*G*.

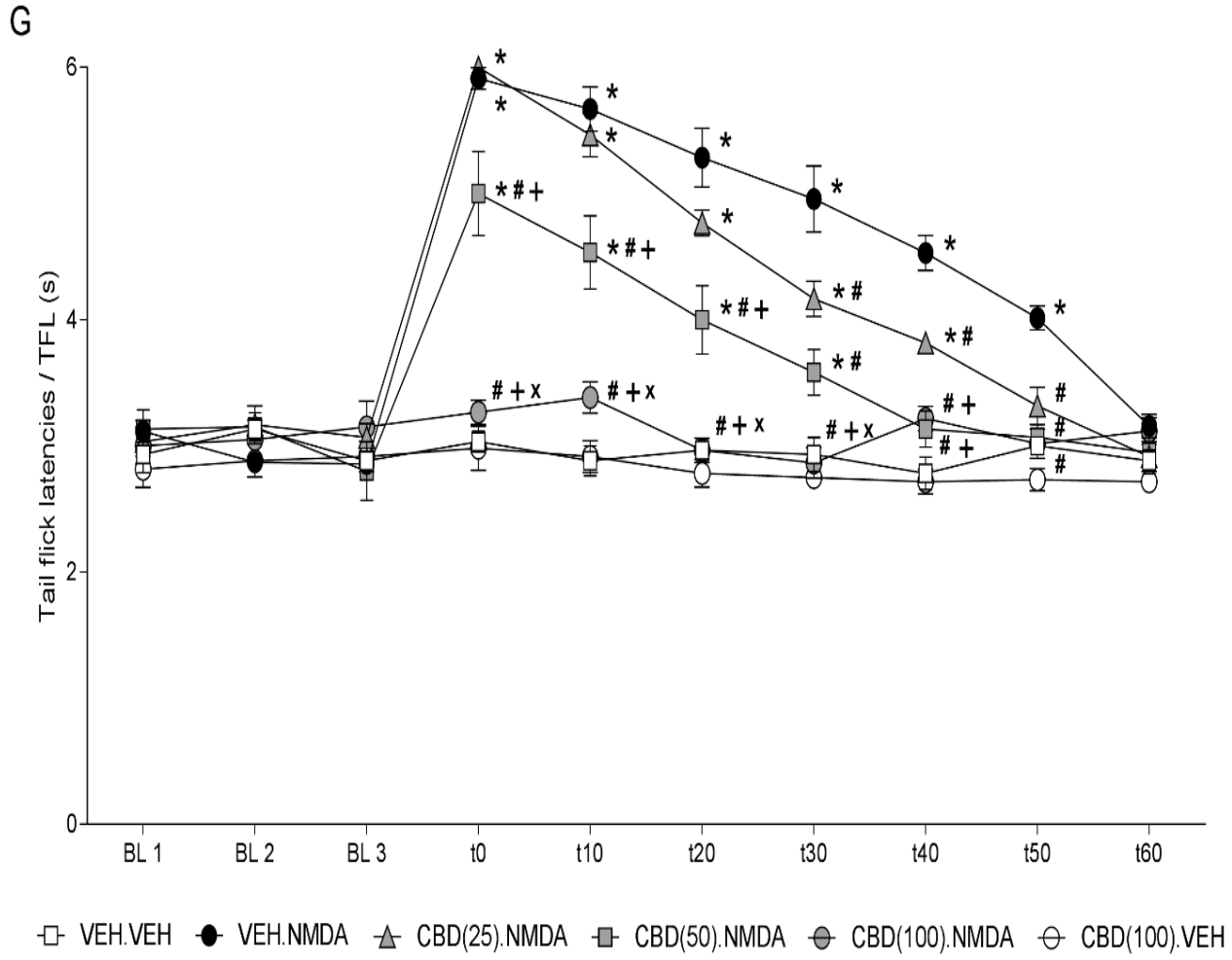

Figure 4 – Effect of central microinjections of cannabidiol (25, 50 and 100 nmol/0.1 µL) or vehicle (NaCl 0.9%/0.1 µL) into the ventromedial hypothalamus (VMH) on unconditioned fear-induced antinociception elicited by chemical stimulation of VMH with N-mehyl-D-aspartate (NMDA) at 6 nmol. Columns represent mean, and bars the standard error of the mean (S.E.M.); n = 6 per group. *P < 0.05, as compared to veh + veh-treated group, $^{\#}$ P < 0.05, $^{+}$ P < 0.05, compared to CBD 25 nmol + NMDA-treated group, $^{-}$ P < 0.05, compared to CBD 50 nmol + NMDA-treated group, according to repeated measure two-way ANOVA followed by Tukey's *post hoc* test. TFL: tail-flick latencies.

4.4 *Experiment 2:* Effects of the VMH pretreatment with AM251 on innate fear-related behavioural reactions and unconditioned fear-induced antinociception

4.4.1 *Behavioural reactions*

Similarly to the results obtained in the first experiment, the intra-VMH microinjections of NMDA (6 nmol) induced panic attack-like defensive behaviour, expressed by escape reactions. In addition, these fear-related behaviours were attenuated by pre-treatment of VMH with CBD (100 nmol). Interestingly, local microinjections of AM251 inhibited the antiaversive effects of VMH pretreatment with CBD on panic attack-like defensive behaviours evoked by intra-VMH microinjections of NMDA.

According to the one-way ANOVA followed by a Newman-Keul's *post hoc* test, there was a significant effect of treatment on the number ($F_{4.25}$= 10.29, $P < 0.001$) and duration ($F_{4.25}$ = 11.1, $P < 0.001$) of running, number ($F_{4.25}$ = 3.43, $P < 0.001$) of jumps, number ($F_{4.25}$ = 7.117, $P < 0.001$) of crossing and number of escape to the burrow and time spent inside after oriented escape to the burrow ($F_{4.25}$, = 10.24,$P < 0.001$ and $F_{4.25}$, = 4.514, $P < 0.001$, respectively). According to Tukey's *post hoc* test, intra-VMH microinjections of NMDA at 6 nmol preceded by vehicle + vehicle increased the number ($P < 0.01$) and duration ($P < 0.01$) of running, the number ($P < 0.05$) of jumping, the number ($P < 0.05$) of crossing ($P < 0.05$), and number of escape to the burrow and time spent inside the safe place after the oriented escape to the burrow ($P < 0.01$, in both), when compared to vehicle + vehicle + vehicle-treated group, as shown in Figure 5*A-F*. The intra-VMH treatment with vehicle + CBD+ NMDA decreased the number ($P < 0.01$) and duration ($P < 0.01$) of running, the number of jumping and crossings ($P < 0.05$ in both cases), and number of escape to the burrow and time spent inside the safe place after the oriented escape to the burrow ($P < 0.01$ in both cases), when compared to vehicle + vehicle + NMDA-treated group, as shown in Figure 5*A-F*. In addition, both AM251 + CBD + NMDA- and AM251 + vehicle + NMDA-

treated groups were able to increase the number ($P < 0.001$ and $P < 0.01$, respectively) and duration ($P < 0.001$ and $P < 0.01$, respectively) of running, the number ($P < 0.01$) of crossing and the number ($P < 0.01$ and $P < 0.001$, respectively) of escape to the burrow when compared to vehicle + CBD + NMDA-treated group, as shown in Figure 5*A*-*F*.

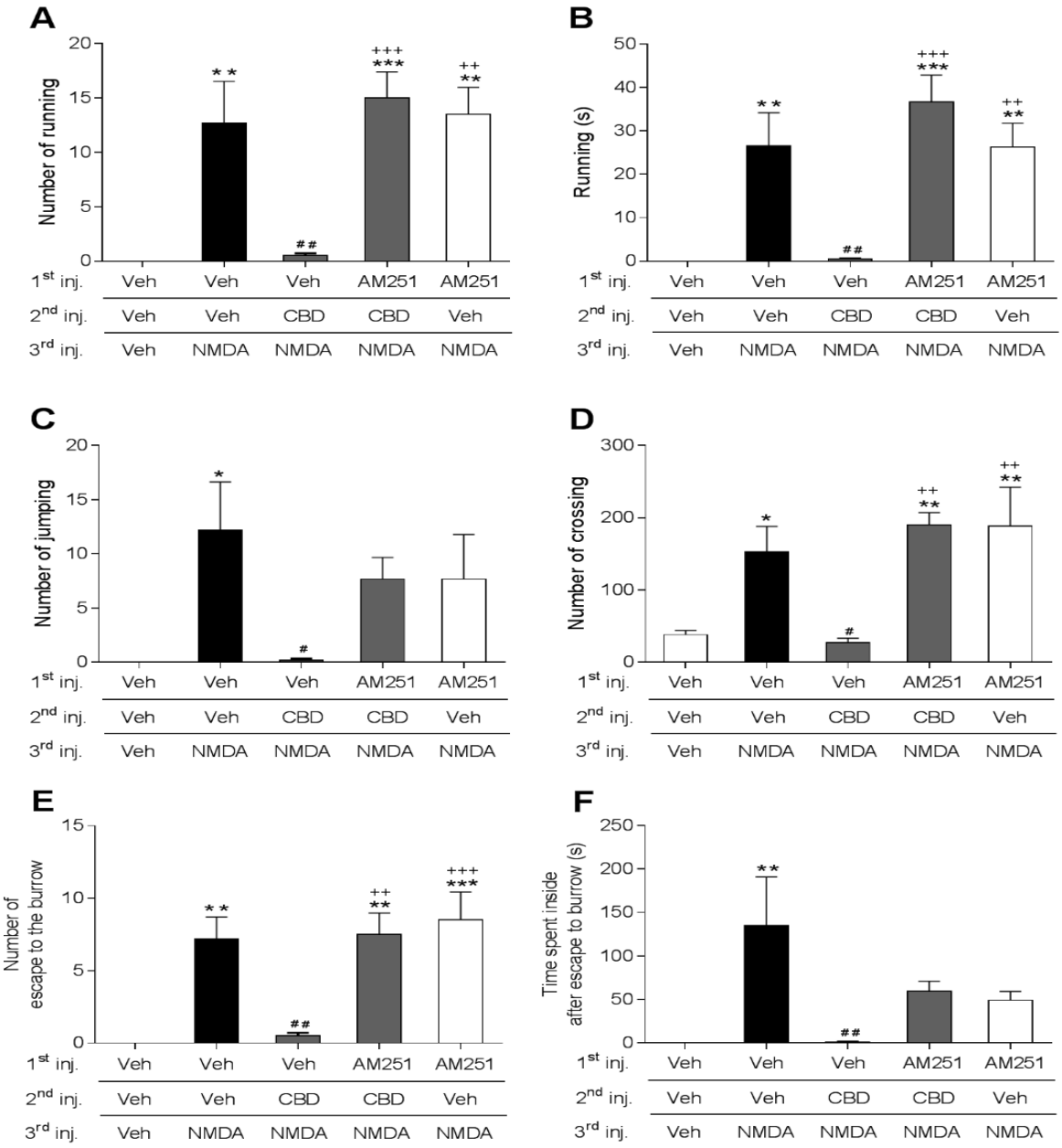

Figure 5 – Effect of central microinjections of cannabidiol (100 nmol/0.1 µL), AM251 (100 pmol) or vehicle (NaCl 0.9%/0.1 µL) into the ventromedial hypothalamus (VMH) on the number and duration of running (A and B), number of jumping (C), number of crossing (D), number of escape to the burrow (E) and time spent inside after escape to the burrow (inhibitory avoidance) (F) induced by microinjection of 6 nmol NMDA or vehicle in the ventromedial hypothalamus. Columns represent mean, and bars the standard error of the mean (S.E.M.); n = 6 per group. *P < 0.05, ** P < 0.01, *** P < 0.001, as compared to veh + veh-treated group; $^{\#}$ P < 0.05, $^{\#\#\#}$ P < 0.001, compared to veh + NMDA-treated group; $^{+}$ P < 0.05, $^{++}$ P < 0.01; $^{+++}$ P < 0.001, compared to CBD 25 nmol + NMDA-treated group ; $^{-}$ P < 0.05, $^{--}$ P < 0.01; $^{---}$ P < 0.001, compared to CBD 50 nmol + NMDA-treated group, according to two-way ANOVA followed by Newman-Keuls' *post hoc* test.

4.4.2 *Fear-induced antinociception*

The induction of panic attack-like defensive reactions in the VMH by local microinjections of NMDA was followed by significant antinociception compared to the nociceptive responses of the vehicle + vehicle + vehicle control group.

According to two-way RM-ANOVA, there were significant effects of treatment ($F_{4.25}$ = 29.99; P< 0.001) and of time ($F_{9.225}$ = 186.1; P< 0.001), as well as a significant interaction between treatment and time ($F_{26.225}$ = 34.9; P< 0.001). According toTukey's *post hoc* test, the activation of NMDA receptors in the VHM significantly increased unconditioned fear-induced antinociceptive response compared to the VMH vehicle + vehicle + vehicle-treatment at the time 0 to 50 min after defensive behaviour (P< 0.05). Compared to VHM vehicle + vehicle + NMDA-treatment, the local microinjections of CBD reduced unconditioned fear-induced antinociceptionin the same times (0-50 min) after defensive behaviours, according to Tukey's *post hoc* test; P< 0.05. Both VMH AM251 + vehicle + NMDA and AM251 + CBD + NMDA treatments increased antinociceptive response when compared to vehicle + vehicle + vehicle- and vehicle + CBD + NMDA-treatment at the time 0 to 30 min after panic attack-like defensive behaviour (P< 0.05). In addition, both VMH AM251 + vehicle + NMDA- and AM251 + CBD + NMDA-treatment were different from the vehicle + vehicle + NMDA-treatment only at time 50 after panic attack-defensive behaviours (according to Tukey's *post hoc* test; P< 0.05), as shown in Figure *6*.

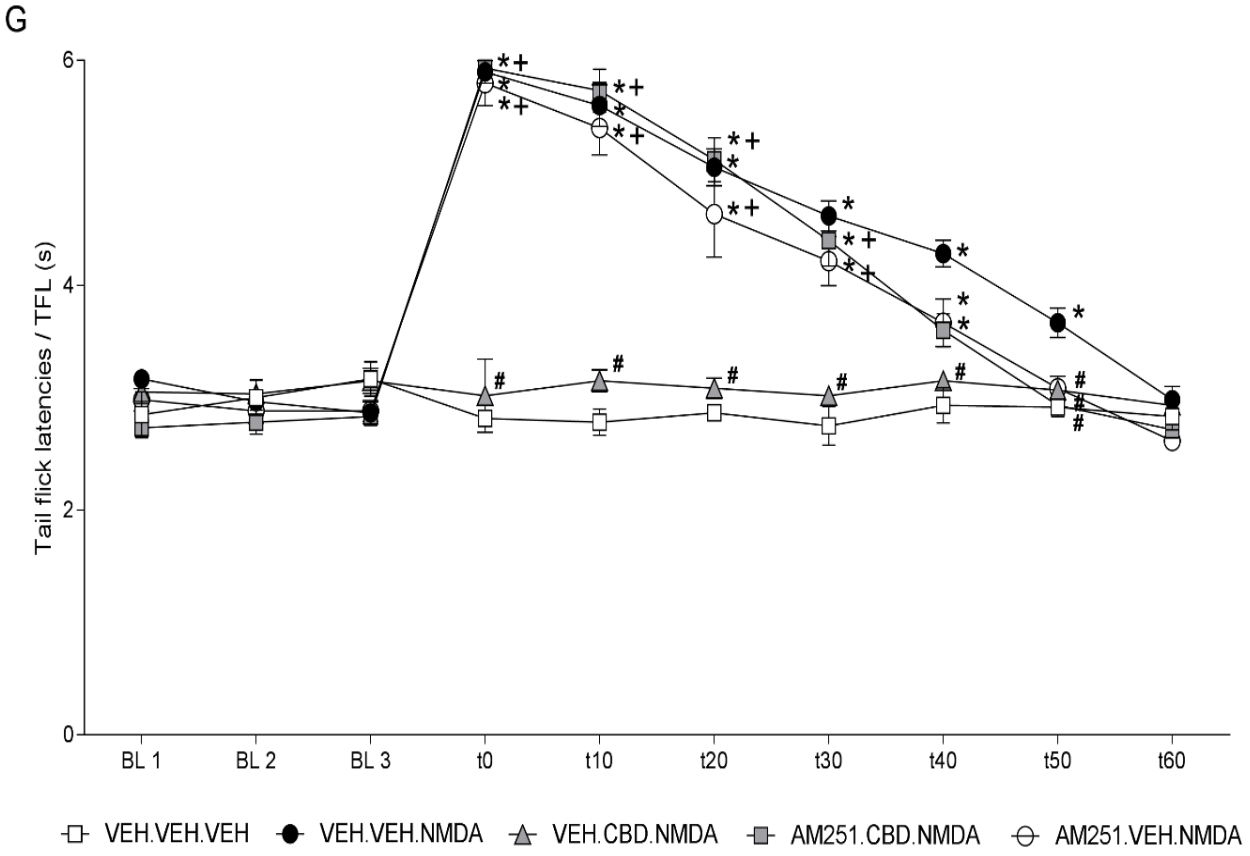

Figure 6 – Effect of central microinjections of cannabidiol (100 nmol/0.1 µL), AM251 (100 pmol) or vehicle (NaCl 0.9%/0.1 µL) into the ventromedial hypothalamus (VMH) on unconditioned fear-induced antinociception elicited by chemical stimulation of VMH with N-mehyl-D-aspartate (NMDA) at 6 nmol. Columns represent mean, and bars the standard error of the mean (S.E.M.); n =6 per group. *P < 0.05, ** P < 0.01, *** P < 0.001, as compared to veh + veh + veh-treated group; $^{\#}$ P < 0.05, $^{\#\#\#}$ P < 0.001, compared to veh + Veh + NMDA-treated group; $^{++}$ P < 0.01; $^{+++}$ P < 0.001, compared to Veh + CBD (100 nmol) + NMDA-treated group, according to repeated measure two-way ANOVA followed by Tukey's *post hoc* test.

5 DISCUSSION

The current investigation provides evidence that the treatment of VMH with cannabidiol microinjection plays a crucial role in the modulation of panicolytic-like defensive behaviours and unconditioned fear-induced antinociception involving the endocannabinoid-mediated signaling system, in which the CB1 cannabinoid receptor is recruited. The semi-natural polygonal experimental arena involves a complex exploratory environment with an artificial burrow for the examination and segregation between the oriented and non-oriented escape defensive behaviours. The panic attack-like aversive state and, subsequently, the unconditioned fear-induced antinociception displayed by rodents were induced by chemical diencephalic stimulation with excitatory amino acids (NMDA) microinjections in the VMH. The local activation of CB1 receptors by intra-VMH microinjections of CBD prevented the panic attack-like aversive reactions as well as antinociception; however, this state was inverted by the pretreatment of AM251, the selective CB1 receptor antagonist. We, likewise, provided the morphological confirmation of the existence of CB1 receptors all the way through VMH by immunohistochemical labelling techniques using a specific anti-CB1-polyclonal antibody.

The chemical stimulation of VMH neurons with NMDA was employed to provoke panic-like state in rats, expressed by running, jumping, increased duration of running and increased number of crossings, behavioural events of escape to the burrow and time spent inside that safe place, after the oriented escape to the burrow, a behavioural evidence for inhibitory avoidance, one of the most common anxiety-related symptoms of panic syndrome patients. Several studies show that the stimulation of medial hypothalamus elicits defensive behavioural responses (Freitas et al., 2009; Wilent et al., 2010; Biagioni et al., 2012; de Freitas et al., 2014), with an oriented behavioural pattern (Ullah et al., 2015, 2017) in comparison to the explosive/non-oriented escape behaviour elicited by electrical and chemical stimulation of dorsal midbrain structures (Nashold et al., 1969; Ribeiro et al., 2005; Castellan-

Baldan et al., 2006). Amusingly, mainly superior colliculus strata control the horizontal jumps (da Silva et al., 2015), while the escape responses in the form of vertical jumps are organised by medial hypothalamic nuclei (de Freitas et al., 2014; Ullah etal., 2017) and, the latter case has shown in this study. Oriented escape responses, like running towards the burrow, vertical jumps, climbing the top of the burrow and climbing the walls of the field are reactions keeping in mind the goal to locate a sheltered place (Biogioni et al., 2012; Ullah et al., 2015, 2017). It was noticed that these reactions were also displayed by rodents threatened by a natural predator (Uribe-Mariño et al., 2012; Twardowschy et al., 2013; Almada et al., 2015; Almada and Coimbra, 2015; Coimbra et al., 2017). Interestingly, both anxiety-related responses and panic attack-like reactions displayed by rodents' confrontated with snakes in the polygonal arenas diminish after the chronic treatments with either anxiolytic drugs or with panicolytic medicines (Paschoali-Marurin e al., 2018). Indeed, defensive behavioural responses like escape to the sheltered place are ethologically acquired and are responsive to panicolytic pharmacological treatment (Coimbra et al., 2017). The outcomes appeared in this examination are likewise comparative, when VMH was treated with CBD, brought about panicolytic-like responses, contemplating that the CB1 receptors through endocannabinoid-mediated signaling system diminished the escape defensive reaction. The CB1 receptors are contemplated possessing a modulator influence on the control of panic-like behaviour, and it was noticed when a selective CB1 receptor agonist and later than NMDA was microinjected in the dlPAG demonstrated a lessening in the vigorous locomotor responses employed by aversive state (Viana et al., 2015). The endocannabinoid-mediated signaling may regulate neuronal activity engaged with various kinds of defensive responses and, furthermore, may have an impact over conditioned fear (Lutz et al., 2015).

Despite the involvement of CB1-cannabinoid receptor in the panicolytic-like effect of intrahypothalamic treatment with CBD, we cannot rule out the potential recruitment of other

sites of action. In fact, peripheral treatments with CBD showed to be mainly panicolytic in rodents threatened by constrictor snakes in the same experimental environment used in the present work, decreasing non-oriented escape behaviour and defensive immobility. Interestingly, that CBD produced panicolytic-like effect decreased significantly after the peripheral pretreatment with the 5-H_{1A} selective antagonist WAY100635 (Twardowschy et al., 2013).

In addition, we established the reponses to antinociceptive stimuli subsequent to the recruitment of defensive behavioural reactions through the administration of NMDA in the VMH. Fear-induced antinociception is a reaction of pain inhibition estimated as an imperative part of the innate defensive behaviour (Bolles and Fanselow, 1980; Coimbra et al., 2006). It endorses the inhibitory pain descending connexions, organising the fear-induced antinociception like that provoked in rodents harmed by predators in a dangerous environment (Coimbra et al., 2017). These descending pain modulatory pathways cause antinociception at the level of the dorsal-horn of the spinal cord by blocking the transmission of the ascending nociceptive stimuli (Ossipov et al., 2014). In contemplates, it has been proposed that the descending pain inhibitory pathways recruited by hypothalamic neuronal cells can be controlled by the limbic cortex area (Bushnell et al., 2013), as well as by anterior cingulate gyrus (Falconi-Sobrinho et al., 2017) connected to hypthalamic nuclei. Actually, the significance of the pain inhibitory system is estimated by the defensive behaviours elaborated by rodents confronted to with a given predator is that the activation of that descending modulatory system would underly unconditioned or conditioned fear-induced defensive behaviours instead of recovery behaviour, increasing the chances of survival of the animals in a dangerous situation. On the contrary, without the existence of that system, which enhances the risk resistance of a threatened organism, the wounded animal will elict a pain-induced behaviour being subject of additional lesions and death?. Unconditioned fear-induced

antinociception is also exhibited by laboratory animals submitted to the chemical and electrical stimulation of the medial hypothalamus (Freitas et al., 2009; de Freitas et al., 2013, 2014), as well as after the activation of the deeplayers of the superior colliculus and the periaqueductal grey matter (Coimbra et al., 1992; Coimbra and Brandão, 1997; da Silva et al., 2013). Even so, comparable antinociceptive reactions after the tonic immobility defensive behavioural response with an impediment of motility can be evoked (Ferreira and Menescal-de-Oliveira, 2012), and the involvement of GABAergic, serotonergic and endogeneous opioid peptidergic systems was also reported elsewhere (Favaroni Mendes and Menescal-de-Oliveira, 2008). Besides, the endogenous pain modulatory pathways, which are thought to have a key role in the organisation of these fear-induced antinociceptive reactions, receives inputs from the the dorsal midbrain structures (Coimbra et al., 2006) that in turn are target of VMH efferent neurons (Ullah et al., 2017), and send projections to the dorsal horn of the spinal cord (da Silva et al., 2013).

This work demonstrates that the indirectly activation of CB1 receptor through a non-psychoactive exogenous cannabinoid (CBD) microinjected in the ventromedial hypothalamus could modulate the defensive behavioural responses by involving of endocannabinoid system. Anxiolytic effects of CBD in models of generalised anxiety have been linked to specific receptor mechanisms in several brain regions activated during the exposure to different neuropsychobiological models. Treatment of dPAG with CBD showed anxiolytic effects in the EPM, VCT, and ETM tests. Microinjection of CBD into the bed nucleus of the stria terminalis (BNST) using EPM and VCT tests and, also in the central nucleus of the amygdaloid complex caused anxiolytic-like effects. Finally, it has shown that the anxiolytic effects of systemic CBD partially depend on the activation of $GABA_A$ receptor in the EPM but not in the VCT model (Blessing et al., 2015). Actually, the anxiolytic impacts initiated by anandamide through CB1 receptor were blocked by the pretreatment with the selective CB1

receptor antagonist AM251, microinjected in the mesencephalic structure. Here, we microinjected the CBD rather than anandamide and, the structure focused was VMH rather than mesencephalic structure, an innovative procedure. In another investigation, using the electrical stimulation of dPAG procedure, the pre-treatment of the dorsal midbrain with AM251 prevented the panicolytic-like effects of the CB1 receptor (Casarotto et al., 2012). Recently, it was demonstrated that the nitric oxide donor SIN-1 induces flight reactions expressed displayed by laboratory animals in a circular arena (Kalouda and Pitsikas, 2015).

Our findings were obtained in the present study by central administration of CBD; however, they were reversed by the pre-treatment of AM251, in an experimental model clarifying the difference between the oriented and non-oriented escape reactions, as a result of the the chemical activation the ventromedial hypothalamic neurons. The panicolytic-like effects obtained pharmacologically in the present study are relevant to the mechanism of actions of CBD explained in some studies. The mechanism of the activation of CB1-receptor is targeted in many studies to test the anxiolytic effects of drugs such as inhibition of fatty acid amide hydrolase enzyme (FAAH), use of psychoactive and non-psychoactive alkaloids of *Cannabis sativa*, the THC and CBD, respectively. Interestingly, CBD is thought to activate the CB1 receptors indirectly, either through enhancing its constitutional activity or increasing the level of AEA while inhibiting the FAAH (Blessing et al., 2015). CBD also acts as an agonist for transient receptor potential cation channel subfamily V member 1 (TRPV1) channels. It has been recommended that TPRV1 channels may be recruited for some of the CBD effects (Iannotti et al., 2014). In addition, the microinjection of capsazepine, a TPRV1 antagonist, into the dPAG antagonised the anxiolytic-like effects evoked by central administration of CBD in the dorsal midbrain (Soares et al., 2010). This data suggest that CBD can modulate defensive behavioural responses acting through complex pharmacological systems. Moreover, CBD exerts some actions on GABAergic receptors regulating anxiety-

like effects, sedation or ataxia, and, another evidence of the complex pharmacological actions of CBD and enhancing its psychotherapeutic functions (Bakas et al., 2017). Indeed, CBD acts as an allosteric modulator of $GABA_A$ receptors, changing the shape of that receptor to reduce the anxiety by enhancing the soothing effects of GABA neurotransmitter (Thomet et al., 2000). It was extensively demonstrated that CBD has antianxiety and sedative activities. Even though CBD has low affinity for cannabinoid receptors, however, it enhances the activities of the endocannabinoids by inhibiting the reuptake or hydrolysis of the their degrading enzyme (Bisogno et al., 2001). Moreover, as stated above, CBD can also act upon $5\text{-}HT_{1A}$ receptors enhancing its activities, increasing the level of serotonin to coup with anxiety-like behaviour (Resstel et al., 2009). Likewise, correlation amongst endocannabinoids and endogenous opioid peptides has been accounted (Manzanares et al., 1999). It was reported that at least part of the CBD panicolytic-like effects seems to be due to its action on neostriato-nigral disinhibitory pathways (da Silva et al., 2015), similar sites on which anandamide acts for decreasing unconditioned fear-induced behaviours displayed by mice threatened by *urutu-cruzeiro* pit vipers (Almada et al., 2015). These structures of the midbrain connected by neostriato-nigral disinhibitory/nigrotectal inhibitory GABAergic neural pathways are additionally modulated by endogenous opioid peptides system (Castellan-Baldan et al., 2006; Twardowschy and Coimbra, 2015).

It is suggested that the CB1 receptor-mediated signaling via non-traditional transmitters, for example, endovanilloids and endocannabinoids, can likewise control defensive behavioural responses evoked in the dlPAG by modulating either the discharge or the effects of the classical neurotransmitters like glutamate, GABA, serotonin, and so forth (Antonio López-Moreno et al., 2008). A few confirmations appeared that these modulatory functions involve glutamatergic neurotransmission. The CB1 receptors recruitment after activation with low *quanta* of AEA, decreases the release of glutamate, which attenuate

defensive behavioural responses (Azad et al., 2003, 2008). It is additionally announced that CB1 receptor is in charge of the suppression of cannabinoid-induced synaptic transmission at the synapse, diminishing the glutamate release in hippocampus (Takahashi and Castillo, 2006). By repressing glutamate discharge, the CB1 receptor may produce the long lasting activities of hippocampal neurons (Misner and Sullivan, 1999), thereby potentially resulting in the decrease of panic-like defensive behaviour, considering the connections between the dorsal hipoccampus and the rostral parts of the frontal lobe (Almada et al., 2015) .

Morphologically, it is observed that the CB1 receptors are expressed for the most part on the axon terminals (Mackie, 2005; Domenici et al., 2006; Kendall and Yudowski, 2016), including the substantia nigra pars reticulata (Almada et al., 2015), a key structure involved in the control of emotions (Coimbra and Brandão, 1993; Ribeiro et al., 2005; Castellan-Baldan et al., 2006), controlling the synaptic transmission of the excitatory and inhibitory neurons (Kano, 2014; Monory et al., 2015; Busquets-Garcia et al., 2017;). In fact, CB1 receptors are expressed on both GABAergic and glutamatergic neurons (Hill et al., 2007; Albayram et al., 2011). Different research groups have demonstrated and distinguished these receptors in the hypothalamus on glutamatergic neurons, and, some has shown the high proportion of CB1 receptors on the axonal terminals of GABAergic neurons (Reguero et al., 2011; Hrabovszky et al., 2012). Studies have also shown the immunoreactivity of CB1 receptors observed in the ventromedial and paraventricular parts of the lateral and infundibular hypothalamic regions (Cottone et al., 2005; Wittmann et al., 2007).

Summarizing this study, the morphological evidence of CB1 cannabinoid receptor immunolabelling on VMH neurons, together with the present pharmacological evidence that demonstrates a clear CBD panicolytic-like effect on defensive responses elicited by chemical stimulation of VMH, highlight the CB1 cannabinoid receptor-mediated signaling in the antiaversive effects of intra-hypothalamic treatment with CBD. The modulatory action of

CBD via endocannabinoid system in panic attack-like emotional responses elicited by the VMH chemical stimulation provides a novel information about the brain sites on which CBD can exert its panicolytic effect, enhancing the perspective of the treatment of mental disorders with *Cannabis sativa* chemical components.

6 CONCLUSION

- The chemical activation of NMDA receptors in the ventromedial hypothalamus elicits panicogenic defensive behavioural responses;
- The cannabinoid type-1 receptor are widely distributed in dorsomedial, central and ventrolateral subnuclei of the ventromedial hypothalamus;
- Cannabidiol, when administered in the ventromedial hypothalamus causes a clear panicolytic-like effect;
- At least part of the panicolytic-like effects of intra-hypothalamically administered cannabidiol are due to the recruitment of CB1 cannabinoid receptor;
- The panicolytic-like effect of cannabidiol microinjected into the medial hypothalamus can be mainly due to its action on central subnuclei of the ventromedial hypothalamus, in which the majority of the microinjections were made.

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