The Endocannabinoid System and Anxiety

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Abstract

The medical properties of *Cannabis sativa* is known for centuries. Since the discovery and characterization of the endogenous cannabinoid system, several studies have evaluated how cannabinoid compounds and, particularly, how the modulation of the endocannabinoid (eCB) system influences a wide range of functions, from metabolic to mental disorders. Cannabinoids and eCB system often exert opposite effects on several functions, such as anxiety. Although the mechanisms are not completely understood, evidence points to different factors influencing those effects. In this chapter, the recent advances in research about the relationship between eCB system and anxiety disorders in humans, as well as in animal models, will be discussed. The recent data addressing modulation of the eCBs in specific brain areas, such as the medial prefrontal cortex, amygdaloid complex, bed nucleus of *stria terminalis*, hippocampus, and dorsal periaqueductal gray, will be summarized. Finally, data from animal models addressing the mechanisms through which the eCB system modulates anxiety-related behavior dependent on stressful situations, such as the involvement of different receptors, distinct eCBs, modulation of neurotransmitters release, HPA axis and immune system activation, and plastic mechanisms, will also be discussed.

1. INTRODUCTION

1.1 Neurobiology of Anxiety, Defense Levels, and Animal Models

Fear and anxiety are adaptive defensive responses that have evolved to protect animals from dangerous stimuli (Blanchard, Blanchard, Griebel, & Nutt, 2008; Gross & Canteras, 2012; McNaughton & Corr, 2004; Tovote, Fadok, & Luthi, 2015). The distinction between these two emotions is not always clear. They are often associated with the actual (fear) vs potential (anxiety) presence of threat (Blanchard et al., 2008; Tovote et al., 2015). Gray and McNaughton (2000), however, have argued that the critical difference between anxiety and fear rests on what they called "defensive direction." Whereas fear would lead the animal to leave a dangerous situation, anxiety occurs when the animal has to enter (cautions risk assessment) or prevent entrance (passive avoidance) to such situation (McNaughton & Corr, 2004; McNaughton & Zangrossi, 2008). From these different perspectives, it is clear that the behavioral defensive responses to danger will vary considerably depending on the characteristics of the threatening stimuli, such as potential vs real, innate vs learned, proximal vs distal, and escape availability (McNaughton & Corr, 2004).

Although fear and anxiety are normal emotions, they can be considered pathological when triggered by inappropriate stimuli or are too extreme in terms of intensity and/or duration (McNaughton & Zangrossi, 2008). These defensive responses are organized by partially distinct brain systems (Canteras, Resstel, Bertoglio, Carobrez, & Guimaraes, 2010; McNaughton & Corr, 2004). A defensive circuitry, responsive to innate threat such as predators, includes the amygdala, the medial hypothalamus, and the dorsal periaqueductal gray (dPAG). Another important circuitry is related to aversive conditioning. Aversive Pavlovian conditioning allows the learning and extinction of associations between neutral and unpleasant stimuli. The neural substrate of this process is relatively well known in rodents. Acquisition and retention of fear conditioning are likely to occur in the lateral nucleus of the amygdala (Canteras et al., 2010), which projects to the central nucleus of the amygdala (CeA) by direct or indirect pathways (via the basolateral nucleus of the amygdala, BLA). The CeA, the primary output of the system, sends direct projection to the ventrolateral PAG and other structures that led to the behavioral (freezing) and autonomic changes associated to fear conditioning (LeDoux, 2000). These systems are regulated by the prefrontal cortex (PFC) and the hippocampal formation. The orbitofrontal PFC, particularly its medial part, coordinates defensive behavioral responses in a complex way, as will be discussed later (Bishop, 2007; Canteras et al., 2010). The hippocampal formation provides context information during threatening situations (Canteras et al., 2010). It also plays a significant role on fear extinction, providing the contextual information needed to acquisition and retrieval of this process (Tovote et al., 2015). In addition, a septum-hippocampus system, according to Gray's proposal, would generate anxiety in response to conflict situations. As a consequence, it increases arousal and selective attention and interrupts ongoing behaviors (Gray & McNaughton, 2000; Oehrn et al., 2015). Another brain structure that has been associated with defensive responses is the bed nucleus of the *stria terminalis* (BNST). It receives major inputs from the central (CeA) and basolateral (BLA) nucleus of the amygdala and plays an important role in anxiety responses (Tovote et al., 2015). Within these structures, moreover, different subsets of neurons can play even distinct roles. For example, independent circuitries in the medial hypothalamus are involved in social vs predator fear (Silva et al., 2013) and stimulation of distinct BNST subnuclei can result in either anxiolytic or anxiogenic responses (Tovote et al., 2015).

As will be discussed later, cannabinoid type 1 (CB_1) receptors and the enzymes responsible for the synthesis and metabolism of endocannabinoids

(eCBs) are highly expressed in all these brain regions (Hu & Mackie, 2015; Tsou, Brown, Sanudo-Pena, Mackie, & Walker, 1998). The eCB system, therefore, is in a critical position to influence defensive responses.

1.2 Cannabinoids and the eCB System

The herb *Cannabis sativa* (marijuana) has been used for centuries as a drug of abuse or as a medicine. Therefore, it is surprising that the scientific knowledge on its chemical constituents only emerged by the second half of the 20th century. This herb produces more than a hundred compounds termed cannabinoids, from which the main responsible for the typical effects of marijuana, including its abuse potential, "high," amnesia, sedation, and analgesia, is Δ^9-tetrahydrocannabinol (Δ^9-THC). Apart from Δ^9-THC, other phytocannabinois are of major medical interest, particularly cannabidiol (CBD) (Mechoulam, 1970). After the identification of Δ^9-THC as the main responsible for marijuana effects, several synthetic analogs have been obtained, which have contributed to the understanding of the pharmacological effects of marijuana. Δ^9-THC and its synthetic analogs induce typical pharmacological effects in rodents, termed the cannabinoid tetrad, consisting of hypolocomotion, catalepsy, analgesia, and hypothermia in mice (Martin et al., 1991).

The characterization of a pharmacological assay for cannabinoids enabled a better understanding of the cannabinoid pharmacology, culminating with the discovery of a receptor specific for cannabinoid compounds (Devane, Dysarz, Johnson, Melvin, & Howlett, 1988; Matsuda, Lolait, Brownstein, Young, & Bonner, 1990). This, in turn, raised the hypothesis that the body might produce its own cannabinoid compounds. Accordingly, a brain cannabinoid was isolated and characterized as an arachidonic acid derivative, arachidonoyl ethanolamide, also termed anandamide (AEA) (Devane et al., 1992). Another endogenous cannabinoid agonist, 2-arachidonoyl glycerol (2-AG) was later described (Mechoulam et al., 1995), as well as a second cannabinoid receptor (Munro, Thomas, & Abu-Shaar, 1993). These endogenous cannabinoid agonists have been termed eCBs, and the receptors have been named by their order of discovery as CB_1 and CB_2 (Howlett et al., 2002).

AEA and 2-AG are thought to be synthesized on-demand from the membrane of postsynaptic neurons after an increase in neuronal activity and to act mainly through CB_1 receptors located in presynaptic terminals. Their actions are terminated by the enzymes fatty acid amide hydrolase (FAAH) and monoacylglycerol lypase (MAGL), respectively. Altogether,

the eCBs, their receptors, and the enzymes responsible for their metabolism constitute a chemical messenger system termed the eCB system (Howlett et al., 2002; Pertwee et al., 2010). In addition, other endogenous compounds have been proposed as eCBs. Moreover, other receptors have also been included in this system, such as the transient receptor potential vanilloid type-1 (TRPV1) channel, a cation-permeable ion channel that is also activated by AEA (Di Marzo & De Petrocellis, 2012).

2. eCBs AND ANXIETY IN HUMANS

2.1 Evidence From Behavioral and Brain Image Studies

2.1.1 Pharmacological Manipulation of the Cannabinoid System

Cannabis use is generally associated with stress relief and mood elevation. However, in some cases, it produces dysphoric effects that include panic or heightened anxiety (Hall & Solowij, 1998). These bidirectional effects of cannabinoids observed in humans can be mimicked in laboratory animals, with low doses being anxiolytic whereas high doses are anxiogenic (Viveros, Marco, & File, 2005).

Additionally, some aversive effects that may result from cannabis smoking, such as anxiety and panic (Hall & Solowij, 1998), are rarely observed after oral administration of Δ^9-THC. For instance, functional magnetic resonance imaging revealed that oral Δ^9-THC reduces amygdala reactivity in healthy volunteers exposed to social signals of threat (Gorka, Fitzgerald, de Wit, & Phan, 2015; Phan et al., 2008), an effect similar to anxiolytic drugs such as benzodiazepines, although contradictory results can be also found (Bhattacharyya et al., 2010). Furthermore, an early placebo-controlled study showed that nabilone, a synthetic derivative of Δ^9-THC, reduces anxiety in patients (Fabre & McLendon, 1981). Dronabinol, another synthetic derivative of Δ^9-THC, facilitates extinction of learned fear in humans and increases activation in the ventromedial PFC and hippocampus upon presentation of conditioned stimulus (CS) (Rabinak et al., 2014, 2013). Recent evidence indicates that nabilone and Δ^9-THC relieve insomnia, nightmares, and other symptoms in individuals with posttraumatic stress disorder (PTSD) (Cameron, Watson, & Robinson, 2014; Fraser, 2009; Jetly, Heber, Fraser, & Boisvert, 2015; Roitman, Mechoulam, Cooper-Kazaz, & Shalev, 2014; Table 1). Large and well-designed controlled trials, however, are needed to better delineate the potential role of cannabinoids as an adjunct or alternative treatment to conventional approaches to PTSD management.

Table 1 Effect of Drugs Interfering With Endocannabinoid System in Humans

Drug (Dose)	Subject Condition	Model	Behavioral Effect	Physiological Alteration (If Evaluated)	References
Dronabinol (7.5 mg; oral; single administration)	Healthy volunteers	Fear extinction	↓ Recovery of fear	↓ AMY and ↑ HIP and PFC reactivity to a CS	Rabinak et al. (2014, 2013)
Nabilone (from 2 to 8 mg a day; oral; 4 weeks)	Patients suffering from anxiety		Anxiolytic		Fabre and McLendonn (1981)
Nabilone (from 0.5 to 3 mg; oral; 7 weeks)	Military personal with PTSD		↓ Nightmares		Jetly et al. (2015)
Nabilone (from 0.5 to 6 mg; oral; range, 1 day to 36 weeks)	PTSD patients		↓ Insomnia, nightmares, and PTSD symptoms		Cameron et al. (2014)
Nabilone (from 0.5 to 6 mg; oral; range, 4–12 months)	PTSD patients		↓ Nightmares, flashbacks		Fraser (2009)
CBD (300 mg; oral; single administration)	Healthy volunteers	Simulated public-speaking	Anxiolytic		Zuardi et al. (1993)
CBD (400 mg; oral; single administration)	Healthy volunteers	SPECT	Anxiolytic	Effects mediated by action on limbic and paralimbic areas	Crippa et al. (2004)

CBD (600 mg; oral; single administration)	SAD patients	Simulated public-speaking	Anxiolytic		Bergamaschi et al. (2011)
CBD (400 mg; oral; single administration)	GAD patients	SPECT	Anxiolytic	Effects mediated by action on limbic and paralimbic areas	Crippa et al. (2011)
CBD (32 mg; oral; single administration)	Healthy volunteers	Fear extinction	↑ Extinction learning		Das et al. (2013)
Rimonabant (90 mg; oral; single administration)	Healthy volunteers	Simulated public-speaking	Anxiogenic		Bergamaschi et al. (2011)

AMY, amygdala; *CS*, conditioned stimulus; *GAD*, generalized anxiety disorder; *HIP*, hippocampus; *PFC*, prefrontal cortex; *SAD*, social anxiety disorder; *SPECT*, single photon emission computed tomography; ↓, decrease/impair; ↑, increase/facilitate.

The eCB system may be involved in the pathophysiology of PTSD. An in vivo imaging study demonstrated higher CB_1 expression in specific brain areas of PTSD patients. Interestingly, increased CB_1 receptor binding was associated with a significant reduction in the peripheral concentrations of AEA and cortisol (Neumeister et al., 2013) and with increased attention bias to threat (Pietrzak et al., 2014). In line with this, PTSD patients with lower peripheral AEA levels exhibited more intrusive symptoms (Hill et al., 2013). It has also been shown that baseline anxiety inversely correlates with peripheral AEA content in healthy individuals (Dlugos, Childs, Stuhr, Hillard, & de Wit, 2012). The involvement of the eCB system in anxiety also emerged from clinical trials of the CB_1 receptor antagonist SR141716A (or Rimonabant). This drug, previously approved for the treatment of obesity and related metabolic disorders, was soon removed from the market due to its psychiatric side effects, mainly anxiety and depression (Moreira & Crippa, 2009). Recently, it was observed that Rimonabant increased public speaking anxiety in healthy humans (Bergamaschi et al., 2014).

Clinical studies also indicated that CBD has anxiolytic properties (Campos, Moreira, Gomes, Del Bel, & Guimaraes, 2012). Following the initial report that it diminishes the anxiogenic effects of high doses of Δ^9-THC (Zuardi, Shirakawa, Finkelfarb, & Karniol, 1982), it was demonstrated that CBD reduces anxiety in healthy volunteers during a neuroimaging study or after a simulated public-speaking procedure (Crippa et al., 2004; Zuardi, Cosme, Graeff, & Guimaraes, 1993). More recently, using the latter procedure, Bergamaschi et al. showed that CBD decreases anxiety in treatment-naive social phobia patients (Bergamaschi et al., 2011). CBD also enhanced consolidation of fear extinction in humans (Das et al., 2013). Neuroimaging studies show that CBD changes the activity of brain regions related to the control of emotional process. It attenuates blood oxygenation in the amygdala and the anterior and posterior cingulate cortex in subjects exposed to fearful faces, impairs the connectivity between the prefrontal and subcortical regions (Fusar-Poli et al., 2010), and decreases the activation of left amygdala–hippocampal complex and left posterior cingulate gyrus (Crippa et al., 2004).

2.1.2 Polymorphisms in the Cannabinoid Receptors/Enzymes Genes

Several studies have investigated associations between single-nucleotide polymorphisms (SNPs) in cannabinoid-related genes and fear states, anxiety and stress-related disorders. For example, in healthy, drug-free, individuals carrying the homozygous allele AA, but not the GG carriers or heterozygotes,

of the particular SNP rs2180619, located in the regulatory region of CB_1 gene (6q14-q15), presented failure to extinguish the fear-potentiated eye-blink startle reflex. In addition to that, the SNP rs1049353, located in the coding region of CB_1 gene (CNR1), was associated with normal extinction (Heitland et al., 2012). Failure to extinguish fear can lead to a state of persistent fear and anxiety, potentially resulting in PTSD (Amstadter, Nugent, & Koenen, 2009; Jovanovic et al., 2010). Also supporting possible involvement of CNR1 polymorphisms in the genetic causes of PTSD, a study evaluating the association between SNPs in the CNR1 gene and the presence of attention deficit hyperactivity disorder and PTSD in children and their parents found that SNP haplotypes in the CNR1 gene (CA and CG) were significantly associated with PTSD in some individuals (Lu et al., 2008). Moreover, carriers of the C allele of another polymorphism in the CNR1 gene (rs2023239) present increased CB_1 receptor density in the brain (Schacht, Hutchison, & Filbey, 2012), in addition to increased hippocampal volume. Considering that CB_1 receptors are expressed in the hippocampus, as will be discussed later, and that reduced hippocampal volume has been associated with the pathophysiology of PTSD, this polymorphism in the CNR1 gene could be an important factor in protecting individuals from development of PTSD after traumatic experiences.

In addition to the CB_1 receptors, studies evaluating the relationship between a common SNP in the FAAH enzyme gene (385AA), which converts a conserved proline residue to threonine (rs324420 or Pro129Thr) resulting in reduced FAAH expression in lymphocytes and increased circulating levels of AEA (Chiang, Gerber, Sipe, & Cravatt, 2004; Sipe et al., 2010), found decreased amygdala activation in response to fearful faces and trait anxiety levels (Hariri et al., 2009). Moreover, carriers of this low-expressing FAAH variant also present faster habituation of amygdala reactivity to repeated "threatening faces" (Gunduz-Cinar et al., 2013).

Genetic changes in the cannabinoid system could also interact with other neurotransmitters such as serotonin (5-HT). Although conflicting results do exist, the presence of the short allele 5-HTTLPR, a functional polymorphism in the serotonin transporter (5-HTT) gene (SLCA4) promoter, is associated with reduced 5-HTT expression (Lesch et al., 1996) and the presence of psychiatric disorders, including anxiety (Lesch et al., 1996; Ming et al., 2015; Osher, Hamer, & Benjamin, 2000; Schneck et al., 2016). Considering that CB_1 receptors can inhibit serotonin release and increase their metabolites in the rodent brain (Nakazi, Bauer, Nickel, Kathmann, & Schlicker, 2000; Tzavara et al., 2003), a study evaluated functional polymorphisms in the

promoter regions of both SLC6A and CNR1 genes in anxious phenotypes (Lazary et al., 2009). The results showed that SLC6A polymorphism was associated with higher anxiety scores only when a SNP in the promoter region of the CNR1 (rs2180619) was also present. Specifically, individuals with the genotype GG and haplotypes "GTGC" (related to lower expression of CB_1) or "AATT" (related to higher expression of CB_1) of the rs2180619, and also carrying the SS genotype of the 5-HTTLPR gene, presented higher risk for anxiety than the other variants. The authors suggest that these combinations result in higher anxiety as a consequence of deficient inhibition of serotonin release by CB_1 receptors, which in turn leads to extremely high or extremely low levels of serotonin (depending on the CB_1 haplotype), probably by a compensation mechanism, which are both implicated in anxiety disorders. Interestingly, mice-lacking CB_1 receptors particularly in serotonergic neurons have increased anxiety-like behavior independent on previous stress experience (Dubreucq et al., 2012).

In summary, these studies suggest that genetic alterations in the eCB system could predict the occurrence of fear and anxiety disorders. More studies, however, aimed particularly at unveiling the functional impact of these polymorphisms are still needed.

3. eCBs IN ANIMAL MODELS OF FEAR AND ANXIETY

Corroborating the clinical findings, studies with laboratory animals have also indicated the involvement of eCBs in anxiety and fear. Two main categories of tests have been used in these studies: (1) innate anxiety-related tests (File, Lippa, Beer, & Lippa, 2004) and (2) conditioned or learned fear-related tests (LeDoux, 2014). The first takes into consideration immediate reaction to novelty—i.e., novel environment, individual, or object—whereas the second requires learned experience about a harmful stimulus, usually paired with a previous neutral condition—i.e., footshock paired with auditory cue or context.

Considering the scope of this review, we will focus on studies investigating the impact of eCBs modulation in anxiety- and fear-like responses. Table 2, however, will also show results of studies testing the effects of synthetic cannabinoid agonists.

3.1 Effects in Innate Fear Models

The elevated plus-maze (EPM), open field, the light/dark box, and social interaction tests are the most commonly used tests to assess innate fear

Table 2 Effect of Drugs Interfering With Endocannabinoid System in Animal Models

Drug (Dose)	Possible Mechanism	Specie	Paradigm	Effect	References
SR141716A (0.25–10 mg/kg)	CB_1 antagonist/ inverse agonist	Mice, rats	EPM/ETM/ LDB/SI	Anxiogenic	Akinshola, Chakrabarti, and Onaivi (1999), Arevalo, de Miguel, and Hernandez-Tristan (2001), Balerio, Aso, and Maldonado (2006), Navarro et al. (1997), Patel and Hillardd (2006), Terzian, Micale, and Wotjak (2014), and Thiemann, Watt, Ledent, Molleman, and Hasenohrl (2009)
SR141716A (0.3–3 mg/kg)		Mice, rats	EPM/LDB	Anxiolytic	Akinshola et al. (1999), Griebel, Stemmelin, and Scatton (2005), and Rodgers, Haller, Halasz, and Mikics (2003)
SR141716A (1–10 mg/kg)		Mice, rats	EPM/EZM	No effect	Bortolato et al. (2006), Dubreucq et al. (2012), Kathuria et al. (2003), and Rodgers et al. (2003)
SR141716A (0.3–20 mg/kg)		Rat	FC—context	↓ Acquisition	Varga, Kassai, and Gyertyan (2012)
SR141716A (1–10 mg/kg)		Mice, rats	FC—cued and context	↓ Fear extinction	Finn, Beckett, et al. (2004), Llorente-Berzal et al. (2015), Marsicano et al. (2002), Pamplona, Prediger, Pandolfo, and Takahashi (2006), Plendl and Wotjakk (2010), and Suzuki et al. (2004)

Continued

Table 2 Effect of Drugs Interfering With Endocannabinoid System in Animal Models—cont'd

Drug (Dose)	Possible Mechanism	Specie	Paradigm	Effect	References
SR141716A (1–3 mg/kg)		Mice, rats	FC—cued and context	No effect	Dubreucq et al. (2012), Mazzola, Micale, and Drago (2003), and Pamplona et al. (2006)
SR141716A (1.5–5 mg/kg)		Rat	FS	↓ Fear extinction	Chhatwal, Davis, Maguschak, and Ressler (2005)
AM251 (1–10 mg/kg)		Mice	EPM	Anxiogenic	Patel and Hillardd (2006), Rodgers, Evans, and Murphy (2005), and Thiemann et al. (2009)
AM251 (0.1–5 mg/kg)		Rat	EPM	No effect	Haller et al. (2007)
AM251 (3–5 mg/kg)		Mice, rats	FC—discrete cue	↑ Fear expression No effect on fear extinction	Arenos, Musty, and Bucci (2006) and Reich, Mohammadi, and Alger (2008)
AM251 (0.3–3 mg/kg)		Mice, rats	FC—context	↓ Fear expression	Arenos et al. (2006) and Mikics et al. (2006)
AM281 (1–4 mg/kg)		Mice	LDB/OF	No effect	Rutkowska, Jamontt, and Gliniak (2006)
AM281 (1–4 mg/kg)		Mice	FC—context	↑ Fear expression	Lisboa, Gomes, et al. (2015)

Delta9-THC (0.015–5 mg/kg)	Nonselective CB_1/CB_2 agonist	Mice, rats	LDB/EPM	Anxiolytic	Berrendero and Maldonadoo (2002), Rubino et al. (2007), and Valjent, Mitchell, Besson, Caboche, and Maldonado (2002)
Delta9-THC (0.25–20 mg/kg)		Mice, rats	LDB/EPM	Anxiogenic	Long et al. (2010), O'Brien et al. (2013), Onaivi, Green, and Martin (1990), Patel and Hillardd (2006), and Schramm-Sapyta et al. (2007)
WIN55,212-2 (0.16, 0.32, 0.64, 1.25, 2.5, 5 mg/kg, IP)		Rats	Predator exposure	Anxiolytic	Lisboa, Camargo, da Silva, Resstel, and Guimaraes (2014)
WIN55,212-2 (2.5–5.0 mg/kg)		Mice, rats	FC—context	↑ Fear	Mikics et al. (2006) and Pamplona and Takahashii (2006)
CP-55940 (1–50 μg/kg)	CB_1 agonist	Mice	EPM	Low dose—anxiolytic; high dose—anxiogenic	Genn, Tucci, Marco, Viveros, and File (2004), Marco et al. (2004), and Rey, Purrio, Viveros, and Lutz (2012)
CP-55940 (75–125 μg/kg)		Rats	EPM	Anxiogenic	Arevalo et al. (2001) and Marin et al. (2003)
HU210 (5, 20 or 80 mg/kg, IP)		Rats	Ultrasound-induced aversive response	Antiaversive effects and ↑ plasma corticosterone	Finn, Beckett, et al. (2004)
HU-210 (10–50 μg/kg)		Rats	EPM	Biphasic effect	Hill and Gorzalkaa (2004)
HU-210 (0.1 mg/kg)		Rats	FC	↓ Fear expression	Mackowiak, Chocyk, Dudys, and Wedzony (2009)

Continued

Table 2 Effect of Drugs Interfering With Endocannabinoid System in Animal Models—cont'd

Drug (Dose)	Possible Mechanism	Specie	Paradigm	Effect	References
AM360 (1–3 mg/kg; acute)	CB_2 antagonist	Mice	LDB	Anxiogenic	Garcia-Gutierrez, Garcia-Bueno, Zoppi, Leza, and Manzanares (2012)
AM360 (1–3 mg/kg; chronic)		Mice	LDB/EPM	Anxiolytic	Garcia-Gutierrez et al. (2012)
AM360 (3 mg/kg)		Mice	FC—cued	↑ Fear extinction	Llorente-Berzal et al. (2015)
JWH133 (3–10 mg/kg; acute)	CB_2 agonist	Mice	LDB	No effect	Garcia-Gutierrez et al. (2012)
JWH133 (0.5–2 mg/kg)		Mice	EPM	Anxiolytic	Busquets-Garcia et al. (2011)
JWH133 (0.5–2 mg/kg; chronic)		Mice	EPM/LDB	Anxiogenic	Garcia-Gutierrez et al. (2012)
JWH015 (1–20 mg/kg)		Mice	LDB	Anxiogenic	Onaivi et al. (2008)
SB366791 (0.1–1 mg/kg)	TRPV1 antagonist	Mice	EPM	Anxiolytic	Micale et al. (2009b)
Capsazepine (1.5–10 μg/kg)		Rat	EPM	Anxiolytic	Kasckow, Mulchahey, and Geracioti (2004)
SB366791 (1–3 mg/kg)		Mice	FC—cued	No effect	Llorente-Berzal et al. (2015)
Olvanil (0.1–5 mg/kg)	TRPV1 agonist	Rats	EPM	Anxiogenic	Kasckow et al. (2004)

AM404 (1–4 mg/kg)	Reuptake inhibitor (?)	Mice	LDB	No effect	Rutkowska et al. (2006)
AM404 (0.3–10 mg/kg)		Mice, rat	EPM/MB	Anxiolytic	Bortolato et al. (2006), Braida, Limonta, Malabarba, Zani, and Sala (2007), Gomes, Casarotto, Resstel, and Guimaraes (2011), and Patel and Hillardd (2006)
AM404 (3 mg/kg)		Mice	FC—Cued	↑ Fear extinction	Llorente-Berzal et al. (2015)
AM404 (10 mg/kg)		Rat	FS	↓ Fear reinstatement	Chhatwal et al. (2005)
VDM11 (3 mg/kg)		Mice	FC—cued	No effect	Llorente-Berzal et al. (2015)
PF-3845 (1–10 mg/kg)	FAAH inhibitor	Mice—C57Bl6	MB	Anxiolytic	Kinsey, O'Neal, Long, Cravatt, and Lichtman (2011)
URB597 (0.1–10 mg/kg)		Mice, rats, Syrian hamster	EPM/EZM/LDB/	Anxiolytic	Busquets-Garcia et al. (2011), Hill, Karacabeyli, and Gorzalka (2007), Kathuria et al. (2003), Moise, Eisenstein, Astarita, Piomelli, and Hohmann (2008) Moreira, Kaiser, Monory, and Lutz (2008), and Patel and Hillardd (2006)
URB597 (0.03–10 mg/kg)		Mice, rats, Syrian hamsters	EPM/SD	No effect	Haller et al. (2009), Moise et al. (2008), Naderi et al. (2008), and Naidu et al. (2007)
URB597 (1 mg/kg)		Mice	FC—context	↓ Fear expression	Busquets-Garcia et al. (2011)

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Table 2 Effect of Drugs Interfering With Endocannabinoid System in Animal Models—cont'd

Drug (Dose)	Possible Mechanism	Specie	Paradigm	Effect	References
URB597 (0.3–3 mg/kg)		Mice	FC—context	↑ Fear extinction	Lisboa, Gomes, et al. (2015)
URB597 (0.3 mg/Kg)		Mice	FC—contex 7 days after IPE	Prevented stress-induced fear extinction impairment	Lisboa, Camargo, da Silva, et al. (2014)
AA-5-HT (0.1–5 mg/kg)	FAAH inhibitor/ TRPV1 antagonist	Mice	EPM	Anxiolytic	Micale et al. (2009b)
OMDM198 (1–5 mg/kg)		Mice	FC—cued	No effect	Llorente-Berzal et al. (2015)
JZL184 (1–408 mg/kg)	MAGL inhibitor	Mice, rat	EPM/EZM/ MB	Anxiolytic; ↑ corticosterone	Aliczki et al. (2013), Busquets-Garcia et al. (2011), Kinsey et al. (2011), and Sciolino, Zhou, and Hohmann (2011)
JZL184 (4–8 mg/kg)		Mice	FC—cued	↑ Fear expression	Llorente-Berzal et al. (2015)

EPM, elevated plus-maze; *ETM*, elevated T maze; *EZM*, elevated zero maze; *FC*, fear conditioning; *FS*, fear startle; *HB*, hole board; *IPE*, inescapable predator (rat) exposure; *LDB*, light dark box; *MB*, marble burrying; *NGT*, novelty grooming test; *NOI*, novel object investigation; *OF*, open field; *PA*, passive avoidance; *SD*, social defeat; *SI*, social interaction; ↓, decrease/impair; ↑, increase/facilitate.

and anxiety (Cryan & Holmes, 2005). They are based on the innate conflict between the potentially aversive situation (such as bright open spaces or unknown individuals) and animal's natural drive to explore unknown areas or to contact unknown/known individuals (Cryan & Holmes, 2005).

Since the development of pharmacological tools to investigate the participation of the eCB system in anxiety-like responses, conflicting data have been observed, especially regarding the use of CB_1 antagonists (Griebel et al., 2005; Haller, Bakos, Szirmay, Ledent, & Freund, 2002; Lafenetre, Chaouloff, & Marsicano, 2007; Lan et al., 1999; Patel & Hillard, 2006; Rinaldi-Carmona et al., 1994; Table 2). These contradictory results could be due to a number of factors, such as experimental condition and protocol, treatment dosage, animal strain, or even the antagonist of choice (Lafenetre et al., 2007; Parolaro, Realini, Vigano, Guidali, & Rubino, 2010). For example, although blockade of CB_1 receptor moderately increases excitatory neurotransmission (Slanina & Schweitzer, 2005), indicating a tonic eCB activity (Ruehle, Rey, Remmers, & Lutz, 2012), the two main antagonists used, SR141716A (Rimonabant) and AM251, are in fact inverse agonists (Pertwee, 2005; Sink et al., 2010). Therefore, under certain circumstances they can produce effects per se (Roberto et al., 2010). In addition, pharmacological effects of these drugs have been reported in CB_1-knockout (CB_1 KO) mice, indicating the presence of non-CB_1 action (Haller et al., 2002).

Even if discrepant results exist, possibly due to different background strain and particular experimental conditions (Haller, Varga, Ledent, Barna, & Freund, 2004; Haller, Varga, Ledent, & Freund, 2004; Ledent et al., 1999), CB_1 KO mice are more anxious when tested in anxiety models (Haller, Varga, Ledent, Barna, et al., 2004; Haller, Varga, Ledent, & Freund, 2004; Martin, Ledent, Parmentier, Maldonado, & Valverde, 2002; Table 3). Furthermore, benzodiazepine drugs do not induce anxiolytic-like effects in CB_1 KO mice (Uriguen, Perez-Rial, Ledent, Palomo, & Manzanares, 2004).

Pharmacological (Table 2) and genetic (Table 3) manipulations of the eCBs reuptake/degradation enzymatic processes also indicate their involvement in anxiety modulation. The most common tool used to inhibit eCBs reuptake is AM404 (Beltramo & Piomelli, 2000), which increases the levels of both AEA and 2-AG (Beltramo et al., 1997; Bisogno et al., 2001; Hajos, Kathuria, Dinh, Piomelli, & Freund, 2004). In the EPM, whereas low doses of AM404 showed anxiolytic-like, CB_1-dependent effect (Bortolato et al., 2006; Patel & Hillard, 2006), higher doses tend to increase anxiety (Patel & Hillard, 2006), which is probably related to AM404 targeting other molecules, such as TRPV1 channel (Zygmunt et al., 1999). The use of the FAAH

Table 3 Effect of Genetic Deletion of Endocannabinoid System Components in Animal Models

Genetic Model	Deletion Location	Specie	Paradigm	Effect	References
CB_1 KO	Ubiquitous deletion	Mice	EPM	No alteration	Dubreucq et al. (2012) and Ledent et al. (1999)
			EPM/LDB/ OF/SI/NOI	Anxiogenic	Haller, Varga, Ledent, Barna, et al. (2004), Jacob et al. (2009), Maccarrone et al. (2002), and Terzian, Micale, et al. (2014)
			FC—cued and context	↓ Fear extinction	Dubreucq et al. (2012), Jacob, Marsch, Marsicano, Lutz, and Wotjak (2012), Kamprath et al. (2009), and Marsicano et al. (2002)
			FC—context	↓ Fear expression	Mikics et al. (2006)
Glu-CB_1 KO	Deletion on glutamatergic cortical neurons	Mice	EPM/HB	No alteration	Dubreucq et al. (2012) and Rey et al. (2012)
			OF/SI/NOI	Anxiogenic	Haring, Kaiser, Monory, and Lutz (2011), Jacob et al. (2009), and Terzian, Micale, et al. (2014)
			FC—cued	↓ Fear extinction	Dubreucq et al. (2012), Kamprath et al. (2009), and Llorente-Berzal et al. (2015)
GABA-CB_1 KO	Deletion on GABAergic neurons	Mice	EMP/HB	No alteration	Rey et al. (2012)
			EPM/SI/NOI	Anxiolytic	Haring et al. (2011), Dubreucq et al. (2012), Terzian, dos Reis, Guimaraes, Correa, and Resstel (2014), and Terzian, Micale, et al. (2014)
			FC—cued	No alteration	Dubreucq et al. (2012)
			FC—cued	↓ Fear extinction	Llorente-Berzal et al. (2015)

D1-CB_1	Deletion on dopaminergic D1 receptor expression neurons	Mice	SI/NOI	No alteration	Haring et al. (2011)
			EPM/LDB/SI/NOI/NGT	Mild anxiogenic	Terzian, Drago, Wotjak, and Micale (2011)
			FC—cued and context	↓ Fear extiction	Terzian et al. (2011)
5HT-CB_1	Deletion on 5HT neurons	Mice	EPM/LDB/SI	Anxiogenic	Dubreucq et al. (2012) and Haring et al. (2015)
			FC—cued	No alteration	Dubreucq et al. (2012)
CB_2 KO	Ubiquitous deletion	Mice	EPM/LDB	Anxiogenic	Ortega-Alvaro, Aracil-Fernandez, Garcia-Gutierrez, Navarrete, and Manzanares (2011)
FAAH KO	Deletion of FAAH enzyme	Mice	EPM/LDB	Anxiolytic	Moreira et al. (2008)
DGL-alfa	Deletion of DGL-alfa enzyme	Mice—C57Bl6	LDB/EZM/OF	Anxiogenic	Jenniches et al. (2016) and Shonesy et al. (2014)
			FC—cued	↑ Fear expression/Impaired fear extinction	Jenniches et al. (2016)
MAGL	Deletion of MAGL enzyme	Mice—C57Bl6	LDB/MB	Anxiogenic	Imperatore et al. (2015)

EPM, elevated plus-maze; *ETM*, elevated T maze; *EZM*, elevated zero maze; *FC*, fear conditioning; *FS*, fear startle; *HB*, hole board; *LDB*, light dark box; *MB*, marble burrying; *NGT*, novelty grooming test; *NOI*, novel object investigation; *OF*, open field; *PA*, passive avoidance; *SD*, social defeat; *SI*, social interaction; ↓, decrease/impair; ↑, increase/facilitate.

inhibitor URB597 allows us to evaluate the role of AEA, while avoiding 2-AG effects. This drug selectively increases AEA, but not 2-AG, levels in the mouse brain after systemic injection. URB597 also induces anxiolytic-like effects in several tests, such as the EPM and zero maze, suggesting that AEA signaling induces anxiolytic-like activity (Kathuria et al., 2003). These effects are mediated via CB_1 receptors (Busquets-Garcia et al., 2011). Corroborating the pharmacological evidence, genetic deletion of FAAH in mice induced reduction in anxiety levels in different tests (Moreira et al., 2008), suggesting that FAAH inhibition is an important mechanism for the alleviation of anxiety, particularly under high aversive situations (Haller et al., 2009).

More recently, the role of 2-AG in the control of anxiety was also assessed. Inhibition of 2-AG metabolizing enzyme MAGL by JZL184 produced anxiolytic-like effects in the EPM, but also only on highly aversive conditions (Sciolino et al., 2011). Contrasting with URB597 results, however, these effects were CB_2 dependent. Additionally, animals lacking the 2-AG synthesis enzyme, diacylglycerol lipase (DAGLα-KO mice), which exhibits 80% reduction of central, but not circulating, 2-AG levels, presents greater anxiety levels (Shonesy et al., 2014), which are reversed after treatment with JZL184. Nevertheless, genetic deletion of MAGL is also reported to promote anxiogenic-like effects in mice through CB_1 receptors by reducing glutamate levels and altering BLA-medial prefrontal cortex (MPFC) connectivity (Imperatore et al., 2015).

Overall, these studies point out to an important role of eCB system in the modulation of anxiety behavior in models involving innate fear.

3.2 Effects on Induced/Learned Fear

As described for innate fear, the eCB system also plays an important role in learned fear responses. It modulates different phases of fear, from acquisition to expression of fear responses, and to reinstatement of extinct memories. As described earlier, however, contradictory results are also found, depending on the factors, such as experimental conditions, animal strains, and drug selectivity.

The classical fear-conditioning protocol is based on an association between a neutral stimulus (CS) and an aversive stimulus (unconditioned stimulus, US). After one or more CS + US pairings, the presentation of the CS alone elicits fear responses. The usual behavioral parameter evaluated

is freezing behavior, characterized by the absence of any movements except those necessary for breathing (LeDoux, 2000, 2014). However, after the CS+US association, the exposure to the CS alone can induce two (opposite) processes: reconsolidation or extinction of the aversive memory, promoting a strengthening or decrease in fear responses, respectively.

Few studies have addressed the participation of the eCB system in *fear acquisition* (Guindon & Hohmann, 2009). Rodents present increased freezing during the conditioning session after administration of the CB_1 antagonist AM251 (Reich et al., 2008), specifically for the paired CS+US presentations. Others studies did not find any impairment in memory acquisition/initial consolidation, as the pretraining administration of another CB_1 antagonist, SR141716A did not affect contextual fear expression (Suzuki et al., 2004). Interestingly, URB597 (FAAH inhibitor) enhanced aversive memory acquisition (Mazzola et al., 2009), although this result was not always observed (Laricchiuta, Centonze, & Petrosini, 2013). Moreover, the enhancing effects of URB597 on memory acquisition seem to be mediated by a bioactive lipid other than AEA, and do not involve cannabinoid receptors (Mazzola et al., 2009). Accordingly, CB_1 KO mice were able to learn a tone-foot shock association (Marsicano et al., 2002). In addition, FAAH inhibition does not affect the aversive memory consolidation phase (Busquets-Garcia et al., 2011).

Fear expression is the response to the first CS exposure after the conditioning session, resulting from acquisition, consolidation and retrieval of the fear memory. There seems that the eCB system plays a different role in cued and contextual learning. One study observed reduced freezing to context and increased freezing to tone after treatment with the CB_1 antagonist AM251 (Arenos et al., 2006), whereas a later work obtained the opposite effect with similar doses of this antagonist (Sink et al., 2010). In agreement with this later study, we recently demonstrated that administration of the CB_1 antagonist AM281 to mice increased fear expression in the contextual fear conditioning, whereas URB597 induced opposite effect (Lisboa, Gomes, et al., 2015). URB597 and the eCBs reuptake inhibitor AM404 also reduced the freezing response in another study (Llorente-Berzal et al., 2015), whereas the MAGL inhibitor JZL184 induced opposite effect depending on CB_1 signaling in GABA neurons (Llorente-Berzal et al., 2015), indicating a differential role for the 2-AG. Therefore, these data contrast to results obtained from innate fear responses, where both AEA and 2-AG attenuate anxiety-like behaviors.

The eCB system is highly involved in *extinction* of fear memory, while not being essential for appetitive memories extinction (Manwell et al., 2009). This reinforces the "on-demand" participation of the eCB system on aversive situations. CB_1 KO mice present impaired short-term (within-session) as well as long-term (between-session) extinction after cued conditioning (Marsicano et al., 2002). Also, treatment with SR141716A before extinction training produced similar results in wild-type mice. In the same study, it was suggested that CB_1 receptors are not involved in consolidation of the extinction memory, as no effect of pharmacological blockade immediately after extinction training was found (Marsicano et al., 2002). The lack of habituation to stimuli suggests that CB_1 receptor signaling critically participates in nonassociative learning processes (Kamprath et al., 2006), which is also observed for the habituation to homotypic stressors (Patel, Roelke, Rademacher, & Hillard, 2005). Furthermore, fear generalization can be observed (Reich et al., 2008), probably related to protocol intensity and due to the fact that the fear-reducing effect of the eCB system greatly depends on the strength of the aversive stimulus presented (Kamprath et al., 2009).

Additionally, several studies have shown that inhibition of eCBs uptake or AEA degradation facilitates extinction (Bitencourt, Pamplona, & Takahashi, 2008; Lisboa, Gomes, et al., 2015; Manwell et al., 2009; Pamplona, Bitencourt, & Takahashi, 2008) that is more resistant to reinstatement (Chhatwal et al., 2005). The effects of FAAH inhibition on extinction depend on CB_1 receptors (Gunduz-Cinar et al., 2013; Laricchiuta et al., 2013). In a genetic mouse model of increased nitric oxide signaling, URB597 was also able to facilitate fear extinction (Lisboa, Gomes, et al., 2015). Furthermore, URB597 treatment counteracted stress-related effects on extinction of aversive memories (Laricchiuta et al., 2013). We recently demonstrated that deficits in contextual fear extinction in mice observed a week after an inescapable predator exposure (a live rat) was attenuated by repeated treatment with URB597 (Lisboa, Camargo, da Silva, et al., 2014). Finally, genetically modified mice presenting 80% reduction of brain 2-AG showed fear extinction deficit (Jenniches et al., 2016).

In summary, these results suggest that eCBs can modify the balance between (re)consolidation and extinction of the original aversive memory. In this way, eCBs may protect the organism from excessive interference induced by aversive stimuli. Consequently, the eCB system can function as a regulatory buffer system for emotional responses, as previously suggested (Ruehle et al., 2012).

4. BRAIN SITES MODULATING CANNABINOID EFFECTS IN ANIMAL MODELS

4.1 Medial Prefrontal Cortex

The PFC is the center for executive functioning, responsible for mediating a range of cognitive, behavioral, and neuroendocrine processes (Rose & Woolsey, 1948; Uylings & van Eden, 1990). The PFC is a structural and functionally heterogeneous brain region. The MPFC in rodents is subdivided into dorsomedial and ventromedial regions (Heidbreder & Groenewegen, 2003). The ventromedial portion of the PFC (vMPFC) comprises the prelimbic (PL), infralimbic (IL), and medial orbital cortices (Heidbreder & Groenewegen, 2003; Uylings, Groenewegen, & Kolb, 2003), which have constantly been implicated in the regulation of mood, emotion, and stress. These subregions present differential modulation and connections to other brain areas (Holmes & Wellman, 2009).

The eCB system is present throughout the PFC of rodents (Marsicano & Lutz, 1999; Tsou, Brown, et al., 1998). CB_1 receptors are highly expressed in GABAergic interneurons, and in less extent in glutamatergic pyramidal neurons (Lafourcade et al., 2007). In addition to CB_1 and CB_2 receptors, eCB synthesis/degradation enzymes are also abundantly expressed in the MPFC (Basavarajappa, 2007; Marsicano & Lutz, 1999; Onaivi et al., 2006; Ramikie & Patel, 2012). Changes in CB_1 receptor expression during a stressful situation or following genetic manipulations can promote morphological changes in the corticoamygdalar pathway, along with marked dendritic atrophy in MPFC pyramidal neurons (Hill, Hillard, & McEwen, 2011). Likewise, signaling alterations in CB_1 receptor might be due to alterations in structural morphology within limbic areas after chronic stress exposure, eventually influencing amygdala-dependent information processing that shifts the emotional balance toward proaversive responses, subsequently developing pathological mood states (McLaughlin, Hill, & Gorzalka, 2014).

Under stressful situations, plastic changes in the eCB system are observed in the vMPFC. For example, AEA signaling is reduced, contributing to anxiety-like outcomes. In addition, overexpression of FAAH by lentivirus injection into the PFC elicits anxiogenic-like response in a stressful environment, whereas local URB597 administration was anxiolytic (Rubino, Realini, et al., 2008; Table 4). Such effects might involve CB_1 receptor-mediated suppression of GABAergic inhibition of PFC projection neurons, increasing PFC-amygdala pathway (McLaughlin et al., 2014). However,

Table 4 Local Effect of Drugs Interfering With Endocannabinoid System in Animal Models

Brain Region	Drug (Dose; Injection Site)	Possible Mechanism	Species	Animal Model	Main Effects	References
MPFC	AM251 (100 nmol); intra-PL + IL	CB_1 antagonist	Rats	CFC	↑ Fear expression (lower shock intensity)	Lisboa, Reis, et al. (2010)
	AM251 (100 nmol); intra-PL + IL	CB_1 antagonist	Rats	CFC	No effect (higher shock intensity)	Lisboa, Reis, et al. (2010)
	NIDA41020 (25–100 pmol); intra-PL + IL	CB_1 antagonist	Rats	CFC	↑ Fear expression	Uliana, Hott, Lisboa, and Resstel (2016)
	mAEA (0.1–10 μg); intra-MPFC	CB_1/TRPV1 agonists	Rats	EPM	Biphasic effect—low dose: anxiolytic; high dose: anxiogenic	Rubino, Realini, et al. (2008)
	AEA (5 pmol); intra-PL + IL	Partial agonist	Rat	CFC	↓ CER via CB_1 receptors	Lisboa, Reis, et al. (2010)
	2-AG (0.01–10 μg); intra-MPFC	CB_1 agonist	Rats	EPM	No effect	Rubino, Realini, et al. (2008)
	THC (2.5–25 μg); intra-CG + PL	CB_1 agonist	Rats	EPM	Anxiolytic (bell-shaped)	Rubino, Guidali, et al. (2008)
	ACEA (0.5–5 pmol); intra-PL	CB_1 agonist	Rats	EPM	Anxiolytic	Fogaca, Aguiar, et al. (2012) and Fogaca, Lisboa, et al. (2012)
	AM251	CB_1 antagonist			No effect; blocked ACEA effect	

	THC (32 nmol); intra-MPFC	CB_1 agonist	Rat	EPM	Biphasic effect—low dose: anxiolytic; high dose: anxiogenic	Rubino, Guidali, et al. (2008)
	Capsazepine (5 μg); not specified	TRPV1 antagonist	Rats	EPM	No effect; blocked methanandamide effect	Rubino, Realini, et al. (2008)
	Capsazepine (1–60 nmol); intra-PL + IL	TRPV1 antagonist	Rats	EPM/VCT	Anxiolytic	Aguiar, Terzian, Guimaraes, and Moreira (2009) and Uliana et al. (2016)
	Capsazepine (1–60 nmol); intra-PL + IL	TRPV1 antagonist	Rats	CFC	↓ Fear expression	Terzian, Micale, et al. (2014)
	6-Iodonordihydrocapsaicin (3 nmol); intra-PL	TRPV1 antagonist	Rats	EPM/VT	Anxiolytic	Fogaca, Aguiar, et al. (2012) and Fogaca, Lisboa, et al. (2012)
	6-Iodonordihydrocapsaicin (3 nmol); intra-PL + IL	TRPV1 antagonist	Rats	CFC	↓ Fear expression	Terzian, Micale, et al. (2014)

Continued

Table 4 Local Effect of Drugs Interfering With Endocannabinoid System in Animal Models—cont'd

Brain Region	Drug (Dose; Injection Site)	Possible Mechanism	Species	Animal Model	Main Effects	References
	Capsaicin (1–5 μg); intra-MPFC	TRPV1 agonist	Rats	EPM	Anxiogenic	Rubino, Realini, et al. (2008)
	Capsaicin (1 nmol); intra-PL + IL		Rats	CFC	↑ Fear expression	Terzian, Micale, et al. (2014)
	AM404 (50 pmol); intra-PL AM251 (100 pmol)	AEA uptake/ metabolism inhibitor CB_1 antagonist	Rats	EPM/VCT	Anxiolytic via CB_1 No effect; blocked AM404 effect	Lisboa, Borges, et al. (2015)
	AM404 (50 pmol); intra-PL + IL AM251 (100 pmol)	AEA uptake/ metabolism inhibitor CB_1 antagonist	Rats	CFC	↓ Fear expression via CB_1 No effect; blocked AM404 effect	Lisboa, Reis, et al. (2010)
	URB597 (0.01–0.1 nmol); intra-PL AM251 (100 pmol)	FAAH inhibitor CB_1 antagonist	Rats	EPM/VCT	Anxiolytic via CB_1 No effect; blocked URB effect	Lisboa, Borges, et al. (2015)
	URB597 (0.01–1 μg); intra-MPFC	FAAH inhibitor	Rats	EPM	Anxiolytic	Rubino, Realini, et al. (2008)
	CBD (30 nmol); intra-PL or IL	?	Rat	CFC	↓ (PL) or ↑ (IL) CER	Lemos, Resstel, and Guimaraes (2010)

dHIP	WIN55, 212-2 (1.9–5.6 nmol)	CB_1, CB_2, TRPV1 receptor agonist	Rat	EPM	Anxiogenic via CB_1	Roohbakhsh, Moghaddam, Massoudi, and Zarrindast (2007)
	WIN55, 212-2 (0.48 nmol)		Mice	Hole board	Prevented anxiogenic effect of histamine	Zarrindast, Nasehi, Piri, and Bina (2010)
	AEA (0.17 ng per side)	Partial agonist	Rat	CFC	↑ and ↓, respectively, extinction and reconsolidation	de Oliveira Alvares, Pasqualini Genro, Diehl, Molina, and Quillfeldt (2008)
	AM404 (50 pmol) bilateral	AEA uptake/ metabolism inhibitor	Rat	VCT	Anxiolytic; via CB_1	Lisboa, Borges, et al. (2015)
	URB 597 (0.01 nmol) bilateral	FAAH inhibitor	Rat	VCT/EPM	Anxiolytic; via CB_1	Lisboa, Borges, et al. (2015)
	URB 597 (0.1 and 1 μg) unilateral		Rat	EPM	Anxiolytic	Hakimizadeh, Oryan, Hajizadeh Moghaddam, Shamsizadeh, and Roohbakhsh (2012)

Continued

Table 4 Local Effect of Drugs Interfering With Endocannabinoid System in Animal Models—cont'd

Brain Region	Drug (Dose; Injection Site)	Possible Mechanism	Species	Animal Model	Main Effects	References
	AMG 9810 (unilateral, 0.01 and 0.1 nmol)	TRPV1 antagonist	Rat	EPM	Anxiolytic	Hakimizadeh et al. (2012)
	Capsaicin (1 and 10 pmol unilateral)	TRPV1 agonist	Rat	EPM	Anxiogenic	Hakimizadeh et al. (2012)
	AM251 (0.3 nmol) bilateral	CB_1 receptor antagonist/partial agonist	Rat	CFC	↑ Fear expression, via NMDA-NO pathway	Spiacci, Antero, Reis, Lisboa, and Resstel (2016)
	AM251 (0.6 pmol) bilateral	CB_1 receptor antagonist/partial agonist	Rat	CFC	Prevented the ↓ of CFC retrieval induced by corticosterone	Atsak et al. (2012)
	AM251 (5.5 or 0.27 ng per side)	CB_1 antagonist/ partial agonist	Rat	CFC	↓ and ↑, respectively, extinction and reconsolidation	de Oliveira Alvares et al. (2008)
vHIP	Capsazepine (2 nmol)	TRPV1 antagonist	Rat	EPM	Anxiolytic	Santos, Stern, and Bertoglio (2008)
	THC (16–32 nmol)	CB_1 agonist	Rat	EPM	Anxiolytic (bell-shaped)	Rubino, Guidali, et al. (2008)

	URB597 (0.03–3 nmol) AM251 (1.8–180 pmol)	AEA metabolism inhibitor CB_1 antagonist	Rat	EPM	Anxiogenic No effect	Roohbakhsh, Keshavarz, Hasanein, Rezvani, and Moghaddam (2009)
	AM404 (5–50 pmol) AM251 (0.01–1000 pmol)	AEA uptake/and metabolism inhibitor CB_1 antagonist	Rat	EPM, VCT	Anxiogenic (EPM) anxiolytic (EPM postrestraint, VCT) No effect	Campos, Ferreira, Guimaraes, and Lemos (2010)
	WIN55212-2 (11 nmol)	CB_1/CB_2/ TRPV1 agonist	Rat	CFC	Prevented SPS-induced ↓ fear extinction (injection immediately after SPS)	Ganon-Elazar and Akiravv (2013)
	2-AG (0.52 nmol)	CB_1/CB_2 agonist	Rat	CFC	↓ Fear expression. Effect disappear under chronic pain	Rea et al. (2014)
dPAG	HU210 (0.1, 1, 5 μg/rat)	CB_1 agonist	Rat	Chemical stimulation DPAG	Antiaversive effect	Finn et al. (2003)

Continued

Table 4 Local Effect of Drugs Interfering With Endocannabinoid System in Animal Models—cont'd

Brain Region	Drug (Dose; Injection Site)	Possible Mechanism	Species	Animal Model	Main Effects	References
	HU210 (5 μg/rat)	CB_1 agonist	Rat	Ultrasound-induced aversive response	Antiaversive effect	Finn, Beckett, et al. (2004)
	URB 602 (0.1 nmol)	MAGL inhibitor; CB_1 activation	Rat	Stress-induced analgesia	Analgesic	Hohmann et al. (2005)
	URB 597 (0.1 nmol)	FAAH inhibitor; via CB_1	Rat	Stress-induced analgesia	Analgesic	Hohmann et al. (2005)
	AEA (5–50 pmol)	Partial agonist; via CB_1	Rat	EPM, VCT, CFC, predator exposure	Anxiolytic	Moreira, Aguiar, and Guimaraes (2007), Lisboa, Resstel, Aguiar, and Guimaraes (2008), Resstel, Lisboa, Aguiar, Correa, and Guimaraes (2008), Lisboa, Camargo, da Silva, et al. (2014), and Lisboa, Camargo, Magesto, Resstel, and Guimaraes (2014)

	AM404 (50 pmol)	AEA uptake/ metabolism inhibitor; via CB_1	Rat	EPM, VCT, CFC	Anxiolytic	Moreira et al. (2007), Lisboa, Resstel, Aguiar, and Guimaraes (2008), and Resstel, Tavares, et al. (2008)
	URB597 (0.01–0.1 nmol)	FAAH inhibitor; via CB_1	Rat	VCT, CFC, chemical stimulation dlPAG	Anxiolytic	Lisboa et al. (2008), Resstel et al. (2008), and Viana, Hott, Resstel, Aguiar, and Moreira (2015)
	ACEA (0.05–5 pmol)	CB_1 activation	Rat	EPM, electrical stimulation dPAG	Anxiolytic	Moreira et al. (2007) and Casarotto et al. (2012)
	2AG (5–500 pmol)	Full agonist; via CB_1 and CB_2	Rat	EPM	Anxiolytic	Almeida-Santos, Gobira et al. (2013)
	URB 602 (30–300 pmol)	MAGL inhibitor; via CB_1 and CB_2	Rat	EPM	Anxiolytic	Almeida-Santos, Gobira, et al. (2013)
BLA	WIN55, 212-2 (9.6 nmol)	CB_1/CB_2/ TRPV1 agonist	Rat	Elevated platform + inhibitory avoidance	↓ Stress effect	Ganon-Elazar and Akirav (2009)
	AEA (5 pmol) bilateral	Partial agonist	Rat	EPM	No effect	Lisboa and Guimarães (2007)

Continued

Table 4 Local Effect of Drugs Interfering With Endocannabinoid System in Animal Models—cont'd

Brain Region	Drug (Dose; Injection Site)	Possible Mechanism	Species	Animal Model	Main Effects	References
	THC (3.2 nmol) unilateral	CB_1 agonist	Rat	EPM	Anxiogenic	Rubino, Guidali, et al. (2008)
	AM251 (1.8 nmol) unilateral	CB_1 antagonist	Rat	EPM	No effect	Rubino, Guidali, et al. (2008)
	AM251 (11 pmol)	CB_1 antagonist		Elevated platform + inhibitory avoidance	↑ Stress effect	Ganon-Elazar and Akirav (2009)
	CBD (7.5–30 nmol) bilateral		Rat	EPM	No effect	Lisboa and Guimarães (2007)
	AM251 (2.5–500 ng/0.5 μL; Sigma)	CB_1 antagonist	Rat	Olfactory fear-conditioning	↓ Acquisition of fear memory	Tan et al. (2011)
	WIN55,212-2 (5–500 ng/0.5 μL)	CB_1/CB_2 antagonist	Rat	Olfactory fear-conditioning	↑ Subthreshold fear-conditioning stimuli	Tan et al. (2011)
	AM404 (5–50 ng/0.5 μL)		Rat	Olfactory fear-conditioning	↑ Subthreshold fear-conditioning stimuli	Tan et al. (2011)

	URB597 (10 ng)	FAAH inhibitor	Rat	EPM	↓ Anxiogenic-like effect induced by CRH injection	Gray et al. (2015)
	AM3506 (0 or 0.1 μg/μL) preextinction	FAAH inhibitor	29S1/Sv1mJ (S1) mice	Cue-fear conditioning	↓ Freezing on retrieval	Gunduz-Cinar et al. (2013)
	Rimonabant (2 μg/μL)	CB_1 antagonist	29S1/Sv1mJ (S1) mice	Cue-fear conditioning	Prevented the ↓ freezing on retrieval induced by AM3506 (1 mg/kg i.p.).	Gunduz-Cinar et al. (2013)
	ACPA (0.4–14.5 pmol)	CB_1 agonist	Rat	EPM	Anxiolytic	Zarrindast et al. (2008)
	THC (320–470 nmol)	CB_1 agonist	Mice	EPM	Anxiogenic	Onaivi, Chakrabarti, Gwebu, and Chaudhuri (1995)
	Rimonabant (0.5, 1.5 μg/side)	CB_1 antagonist	Rat (female)	Abstinence from palatable diet cycling EPM	Anxiogenic	Blasio et al. (2013)
CeA	ACPA (0.4–14.5 pmol)	CB_1 agonist	Rat	EPM	Anxiolytic	Zarrindast et al. (2008)
	THC (320–470 nmol)	CB_1 agonist	Mice	EPM	Anxiogenic	Onaivi et al. (1995)

Continued

Table 4 Local Effect of Drugs Interfering With Endocannabinoid System in Animal Models—cont'd

Brain Region	Drug (Dose; Injection Site)	Possible Mechanism	Species	Animal Model	Main Effects	References
BNST	CBD (30 and 60 nmol) bilateral	5HT1A agonist	Rat	CFC, VCT, EPM	Anxiolytic	Gomes, Resstel, and Guimaraes (2011)
Nucleus accumbens shell	ACEA (0.05 nmol)	CB_1 receptor agonist	Rat	EPM	Anxiogenic	Kochenborger et al. (2014)
VMH	AEA (5 pmol)	CB_1 receptor activation	Rat	Panic-like responses evoked by bicuculline infusion into the VMH	Panicolytic	Dos Anjos-Garcia, Ullah, Falconi-Sobrinho, and Coimbra (in press)

2-AG, 2-arachnoylglycerol; *(m)AEA*, (metha)anandamide; *ACEA*, arachidonyl-2-chloroethylamide; *ACPA*, arachidonyl cyclopropylamide; *CFC*, contextual fear conditioning; *EPM*, elevated plus-maze; *SPS*, single-prolonged stress; *VCT*, Vogel conflict test; *THC*, delta-9-tetrahydrocannabinol; *MPFC*, medial prefrontal cortex; *PL*, prelimbic; *IL*, infralimbic; *dHIP*, dorsal hippocampus; *vHIP*, ventral hippocampus; *dlPAG*, dorsolateral periaqueductal gray; *BLA*, basolateral amygdala; *CeA*, central amygdlaa; *MeA*, medial amygdala; *BNST*, bed nucleus of stria terminalis; *VMH*, ventromedial hypothalamus; ↓, decrease/impair; ↑, increase/facilitate.

adeno-associated virus-mediated overexpression of CB_1 receptors in the MPFC did not change anxiety responses (Klugmann, Goepfrich, Friemel, & Schneider, 2011), possibly as a consequence of overexpression in regions other than PFC, such as the BNST or striatum. Also, CB_1 antagonism in the PFC does not seem to be sufficient to alter anxiety levels under mild aversive conditions (Lisboa, Reis, et al., 2010; Rubino, Realini, et al., 2008; Table 4). Pharmacological blockade of MPFC TRPV1 channels, in contrast, reduced anxiety levels in animals (Aguiar et al., 2009; Fogaca, Aguiar, Moreira, & Guimaraes, 2012; Table 4). Therefore, modulation of anxiety by AEA within the MPFC might be due to a balance between CB_1 and TRPV1 receptors (Moreira, Aguiar, Terzian, Guimaraes, & Wotjak, 2012).

The vMPFC eCB system also plays a role in the expression and extinction of conditioned fear responses. Increase in the eCB tone in this region (Table 4) attenuates fear retrieval (but not acquisition) and facilitates extinction in different paradigms (Kuhnert, Meyer, & Koch, 2013; Lin, Mao, Su, & Gean, 2009; Lisboa, Reis, et al., 2010), whereas CB_1 receptor antagonism impairs fear consolidation and extinction (Kuhnert et al., 2013; Lin et al., 2009). Moreover, CB_1 mRNA increased in the MPFC 24 h after fear conditioning (Lisboa, Reis, et al., 2010). Besides, CB_1, but not CB_2, mRNA levels correlated positively with freezing behavior in a mice strain selectively bred for low and high fear, when submitted to classical fear-conditioning protocols (Choi et al., 2012). Therefore, CB_1 receptors in the MPFC participate in emotional learning of conditioned fear memories, most likely via reciprocal interaction with the basolateral amygdala (BLA) (Laviolette & Grace, 2006). Despite evidence of CB_2 receptor expression in the MPFC (Onaivi et al., 2006), no study addressed its direct involvement in this region on the control of anxiety and fear responses.

Using a proposed PTSD model, the single-prolonged stress (SPS) test, a recent study showed that deficits in contextual fear extinction and increased glucocorticoid receptor (GR) expression in the IL portion of vMPFC observed 1 week after SPS exposure were not attenuated by local or systemic administration of the CB_1/CB_2 agonist WIN55,212-2 after stress (Ganon-Elazar & Akirav, 2013). These data together with the fact that systemic corticosterone administration increases AEA levels in amygdala and hippocampus, but not in the PFC, 10 min after injection (Hill, Karatsoreos, Hillard, & McEwen, 2010), suggest that activation of CB_1 receptors in the IL are not immediately necessary after traumatic experience to modify fear extinction a week later (Ganon-Elazar & Akirav, 2013).

Therefore, eCB signaling in the PFC represents an attractive target for the treatment of neuropsychiatric disorders characterized by broad dysfunction of emotional processing (McLaughlin et al., 2014).

4.1.1 Mechanisms

During aversive situations there was a reduction in AEA (Hill et al., 2008) and an increase in 2-AG levels in the MPFC (Patel, Roelke, et al., 2005). In addition, CB_1 receptor binding and mRNA expression also increase (Hill et al., 2008; Lisboa, Reis, et al., 2010). CB_1 receptor in cortical areas is mainly present on terminals of GABAergic interneurons and, in less extent, on glutamatergic neurons (Monory et al., 2006). Nevertheless, the relative density of CB_1 receptors on GABAergic and glutamatergic neurons is not the only parameter to consider in evaluating the role of these receptors on neurotransmitter release regulation. In fact, at least in hippocampus CB_1 receptors located on glutamatergic terminals show greater capacity to recruit G protein than when they are present on GABAergic terminals (Steindel et al., 2013). It has been suggested that anxiogenic-like responses induced by cannabinoids are mediated by CB_1 receptors located on GABAergic terminal, whereas activation of CB_1 in glutamatergic terminals produces anxiolytic-like effects (Haring et al., 2011; Lafenetre, Chaouloff, & Marsicano, 2009; Metna-Laurent et al., 2012; Terzian, dos Reis, et al., 2014).

Electrophysiological results also suggest that the eCB tone is altered in the MPFC after stress exposure (Rossi et al., 2008). Chronic stress blocks the normal reduction of inhibitory postsynaptic potentials (IPSPs) produced by CB_1 agonist administered directly into mice corticostriatal slices (Rossi et al., 2008). On the other hand, after application of CB_1 agonists, excitatory postsynaptic currents (EPSCs) were strongly depressed, whereas SR141716A reversed these responses. CB_1 antagonists per se increased glutamate release (Auclair, Otani, Soubrie, & Crepel, 2000). Also, WIN55,212-2 (CB_1/CB_2 agonist) strongly favored LTD at the apparent expense of LTP, whereas SR141716A presented the opposite profile (Auclair et al., 2000).

The hypothalamus-pituitary-adrenal (HPA) axis is also controlled by MPFC activity. Corticosterone administration rapidly induced eCB synthesis in the MPFC (Hill et al., 2011). This region, along with other limbic areas (Ulrich-Lai & Herman, 2009), inhibits the HPA axis activity by activating inhibitory projections to PVN neurons (Jankord & Herman, 2008). Furthermore, the MPFC is vastly connected to a great variety of brain areas. For instance, MPFC sends glutamatergic projections to several areas (Del Arco & Mora, 2009; Vazquez-Borsetti, Celada, Cortes, & Artigas,

2011), whereas it receives serotoninergic and noradrenergic afferents from the raphe nuclei (Charnay & Leger, 2010; Fitzgerald, 2011) or *locus coeruleus* (Fitzgerald, 2011), respectively.

Local and systemic administration of the CB1/CB_2 agonists WIN55,212-2 causes an increase in extracellular norepinephrine in the MPFC and anxiogenic-like behavior (Page et al., 2007; Page, Oropeza, & Van Bockstaele, 2008), while acute and chronic treatment with the same agonist blocked the increase in cortical pyramidal cell excitability and input resistance evoked by an alpha2-adrenergic receptor agonist (Cathel et al., 2014), suggesting that the increased cortical norepinephrine efflux may be mediated by cannabinoid receptors-mediated desensitization of α2-adrenergic receptors. Also, activation of CB_1 and CB_2 receptors, or increased eCB tone, upregulates the expression of serotoninergic 5HT2A receptors and elevates serotoninergic tone in the MPFC (Cassano et al., 2011; Franklin & Carrasco, 2012). Therefore, it is suggested that norepinephrine enhancement by eCBs is associated with anxiogenic responses, whereas cannabinoid-mediated stimulation of serotonergic signaling is associated with anxiolytic responses (Ruehle et al., 2012).

Another system involved in the MPFC CB_1 control of emotional responses is the NMDA-nitric oxide (NO) pathway. Glutamate NMDA receptors and the neuronal nitric oxide synthase (nNOS) enzyme are expressed in the MPFC (Brené, Messer, & Nestler, 1998; Vincent & Kimura, 1992). Local inhibition of NMDA receptors or NO signaling prevents the increase in aversive responses induced by CB_1 receptor blockade (Uliana et al., 2016).

4.2 Hippocampus

The septum-hippocampal system has direct and indirect connections to the amygdala and PFC, critical structures involved in defensive responses (Dejean et al., 2015). It provides contextual information associated both with conditioning and innate fear (Canteras et al., 2010; Dejean et al., 2015). In addition, the hippocampus also interferes with defensive responses by other mechanisms related to its mnemonic, neuroendocrine (inhibition of the HPA axis), spatial learning, and stimuli analysis roles (Canteras et al., 2010). Moreover, it was proposed to generate anxiety in response to conflict by interrupting ongoing behavior and increasing the level of arousal and attention, as already discussed (McNaughton & Corr, 2004).

The effects of cannabinoids on hippocampal function and plasticity are complex and depend on the aversiveness of the task (Akirav, 2011). CB_1 receptors, although prominently expressed in GABAergic terminals

(Hajos & Freund, 2002; Marsicano & Lutz, 1999), are also present in glutamatergic, serotonergic, and cholinergic neurons in the hippocampus (Katona & Freund, 2008). Low levels of CB_1 receptors are also present in progenitor cells of the subgranular zone of the dentate gyrus (Hu & Mackie, 2015). Similar to the receptors, the enzymes responsible for the synthesis and metabolism of eCBs are highly expressed in the hippocampus (for review, see Hu & Mackie, 2015).

In classical animal models of anxiety, systemic or direct injection of cannabinoid-related drugs into the hippocampus has produced contradictory results (Tables 2 and 4). The stressful experience of the animal has been proposed as a key factor to explain these conflicting findings (Campos et al., 2010). In less stressful animal models, such as the EPM, intrahippocampal administration of drugs that facilitate eCB signaling, such as AM404, produced anxiogenic effects (Roohbakhsh et al., 2007). However, AM404 was anxiolytic when injected directly into the ventral hippocampus (vHPC) of rats submitted to the Vogel conflict test (VCT), a model that involves pain and a previous stressor experience (water deprivation for 24 h). Moreover, the anxiogenic-like effect observed in the EPM after intra-vHPC AM404 injection turned into anxiolytic in rats previously submitted to an acute restraint stress (Campos et al., 2010). Corroborating this latter finding, Δ^9-THC was also anxiolytic in this brain area only when the animals have been kept isolated in their home cage (Rubino, Guidali, et al., 2008), a stressful situation that increases anxiety in the EPM (Maisonnette, Morato, & Brandao, 1993). Although the vHPC is particularly associated with anxiety (Bannerman et al., 2004; Bertoglio, Joca, & Guimaraes, 2006), contradictory results have been reported for the dorsal hippocampus (dHPC; Table 4). In the EPM, the CB_1/CB_2 agonist WIN55,212-2 induced anxiogenic effect when injected into this brain area. In contrast, enhanced eCB signaling (by AM404 or URB597) was anxiolytic in both the EPM and VCT (Lisboa, Borges, et al., 2015) (Table 4). These results suggest a regional dissociation between the dHPC and vHPC regarding stress interference on eCB modulation of anxiety responses. This possibility, however, needs to be further investigated.

Learning/memory processes involving the hippocampus are modulated by cannabinoids (Clarke et al., 2008). These effects, however, may vary depending on the route of administration, the nature of the task (aversive or not), and whether it involves emotional memory (e.g., conditioned fear and extinction learning) or nonemotional memory formation (e.g., spatial learning) (Akirav, 2011). CB_1 receptor activation, in addition to impairing

acquisition (Pamplona & Takahashi, 2006) and facilitating extinction of contextual fear memory (Akirav, 2011), also affects consolidation of this memory. This later effect is associated with a decreased expression of polysalic acid-neural cell adhesion molecules in the hippocampus, glycoproteins that have been proposed to be important for memory consolidation (Mackowiak et al., 2009). Reinforcing the involvement of the hippocampus in fear-conditioning processes, synthetic Δ^9-THC increases activation of this structure in humans during extinction of an aversive conditioned cue (Rabinak et al., 2014).

Direct drug injections into the hippocampus have also helped to unveil the role of this structure in cannabinoid interference in fear learning. Bilateral infusion of AM251 into the dHPC increases fear expression (Spiacci et al., 2016). Likewise, WIN55,212-2 injected into the ventral subiculum (in the vHIP) impaired retrieval of a contextual fear-conditioning task (Akirav, 2011). Similar effects were reported for 2-AG (Rea et al., 2014). Moreover, while 2-AG blocks fear extinction, AEA facilitates it. On the other hand, AEA blocked fear reconsolidation, whereas AM251 facilitated it. The authors suggest that the opposite effects of AEA in these two processes indicates that the hippocampal eCB system acts as a switching mechanism, deciding which process will take place by either maintaining the original memory (reconsolidation) or promoting a new learning (extinction) (de Oliveira Alvares et al., 2008).

Exposure to a model of PTSD (SPS, mentioned in Section 4.1) impaired fear extinction and increased GR expression in the vHIP a week later. Administration of WIN55,212-2 in this region or systemically attenuates these alterations (Ganon-Elazar & Akirav, 2013). Moreover, the local effect of WIN55,212 was prevented by the GR antagonist RU-486, pointing to an involvement of the HPA axis on cannabinoid effects.

4.2.1 Mechanisms

The mechanisms involved in cannabinoid regulation of emotional response by hippocampus are not clear. Hippocampal CB_1 receptors are widely expressed in cholecystokinin-expressing GABA terminals, but are also found in glutamatergic, serotonergic, and cholinergic presynaptic terminals (for review, see Akirav, 2011; Katona & Freund, 2008). Because CB_1 receptors are present at higher concentration in GABAergic compared to glutamatergic terminals, interference in the former neurotransmission has been related to cannabinoid influence in hippocampal-dependent processes (Akirav, 2011; Hajos & Freund, 2002), such as depolarization-induced

suppression of inhibition (DSI); this process facilitates LTP formation by eCB-mediated disinhibition (Wilson, Kunos, & Nicoll, 2001). However, eCBs interference with GABAergic and glutamatergic hippocampal neurotransmission may depend on the rodent species (Haller et al., 2007), brain region, and stress influence (Morena, Patel, Bains, & Hill, 2016).

CB_1 deletion in glutamatergic, but not in GABAergic, neurons impaired the behavior and neuroendocrine responses caused by forced swimming stress (Steiner et al., 2008). Recently, the contribution of these two hippocampal populations to cannabinoid effects was elegantly demonstrated by showing that reconstituting CB_1 receptor function in the dorsal telencephalic glutamatergic neurons partially restored the anxiety-like behavior phenotype of global CB_1 receptor deletion (Ruehle et al., 2013). This effect was associated with CB_1-dependent normalization of hippocampal depolarization-induced suppression of excitation, a CB_1-glutamate associated form of neural plasticity. Together, these results suggest that hippocampal CB_1 receptors located on glutamatergic terminals could be a target for the anxiolytic effects of small doses of systemically injected cannabinoids (Viveros et al., 2005).

Contrasting to CB_1, little is known about the role of hippocampal CB_2 receptors in anxiety modulation. CB_2 overexpression decreases anxiety in the EPM and light–dark tests (Garcia-Gutierrez & Manzanares, 2010). A recent study found that, different from CB_1, short-term CB_2 activation in hippocampal slices does not significantly modify synaptic activity. Long-term exposure to CB_2 agonists, on the other hand, increases synaptic activity and glutamate release. Similar effects were found in vivo after repeated administration (Kim & Li, 2015). Other mechanisms that have been associated with an anxiolytic-like or antistress effect of repeated CB_2 receptor activation are facilitation of hippocampal neurogenesis (Campos et al., 2013; Fogaca, Galve-Roperh, Guimaraes, & Campos, 2013) and antiinflammatory action, which will be further discussed.

4.3 Periaqueductal Gray Matter

The periaqueductal gray matter (PAG) has been initially investigated as an important brain site for the analgesic effects of cannabinoids (Walker, Huang, Strangman, Tsou, & Sanudo-Pena, 1999). Later on, it was proposed as a target for the antiaversive effects of these compounds, with important implications for the modulation of anxiety disorders and panic attacks. Local injection of the synthetic CB_1/CB_2 agonist HU210 in the rat dorsal portion

of the PAG (dPAG) inhibits the effects of local stimulation with an excitatory aminoacid, D,L-homocysteic acid (DLH). This compound induces a robust escape response, characterized by running in an arena, which has been proposed as a model of panic attack. HU210 prevents this response, in addition to promoting analgesia (Finn et al., 2003). Moreover, intra-DPAG injection of cannabinoids also inhibited escape responses induced by ultrasound stimulus, further suggesting that cannabinoid receptors modulate aversive responses in this structure (Finn, Jhaveri, et al., 2004). Therefore, cannabinoid mechanisms operate in the PAG to modulate pain and aversive behavior. eCBs are also locally recruited to mediated stress-induced analgesia (Hohmann et al., 2005).

Based on this literature, we have been performing extensive studies to further characterize the role of the eCB system in modulating aversive responses in the PAG, particularly in its dorsolateral portion (dlPAG). The injection of cannabinoids into the dlPAG (Table 4) induces consistent dose-dependent anxiolytic-like effect in rats tested in the EPM. This effect was observed after local AEA injection and mimicked by the selective CB_1 receptor agonist ACEA. AEA effect was prevented by the selective CB_1 receptor agonist, AM251, indicating a role for this receptor in mediating the cannabinoid effects on anxiety in the dlPAG (Moreira et al., 2007). Furthermore, local injection of AM404 and URB597 also induced anxiolytic-like effect in the Vogel test, a model based on the conflict between approaching and avoiding a stimulus, rather than on exploratory activity, supporting the role of the dlPAG in modulating the effects of cannabinoids against different types of aversive responses (Lisboa et al., 2008).

Moreover, dlPAG CB_1 activation in rats by AEA or AM404 also inhibited fear behavior in the contextual fear-conditioning model (Olango, Roche, Ford, Harhen, & Finn, 2012; Resstel et al., 2008), whereas blockade of that receptors increased behavioral and autonomic responses in the same model (Uliana et al., 2016). Similar responses have been reproduced in a more natural setting, in which AEA inhibited fear responses in rats exposed to a live predator (Lisboa, Camargo, da Silva, et al., 2014). Apart from the effects of cannabinoids acting on the CB_1 receptor, the dlPAG also mediate the antiaversive effects of CBD through non-CB_1-mediated mechanisms (Campos & Guimaraes, 2008). Finally, local injection of URB597 inhibits escape response induced by the excitatory aminoacids NMDA or DLH (Batista, Bastos, & Moreira, 2015; Batista, Fogaca, & Guimaraes, 2015; Viana et al., 2015). Thus, the eCB system might be recruited as an endogenous mechanisms protecting against aversive stimuli in the PAG.

2-AG also seems to modulate anxiety effects in the dlPAG. Local injection of an MAGL inhibitor into the dlPAG induced anxiolytic-like effects in the EPM (Almeida-Santos, Gobira, et al., 2013), indicating that both AEA and 2-AG participate in the modulation of this behavior.

4.3.1 Mechanisms

Although several studies have investigated the possible mechanisms involved in the eCB effects modulated by the dlPAG, this is still a matter of debate. Studies showing anxiolytic-like effects of eCB-related compounds administered into the dlPAG have suggested that activation of CB_1 receptors could result in decreased glutamate release, attenuating the aversiveness of the experimental situation (Fogaca, Aguiar, et al., 2012; Fogaca, Lisboa, et al., 2012; Lisboa & Guimaraes, 2012; Lisboa, Magesto, Aguiar, Resstel, & Guimaraes, 2013; Lisboa et al., 2008; Resstel et al., 2008). Accordingly, a higher and ineffective dose of AEA in the dlPAG turned into anxiolytic after local NMDA blockade (Fogaca, Gomes, Moreira, Guimaraes, & Aguiar, 2013). In addition, we previously showed that the panic-like reaction induced by administration of a NO donor into the dlPAG was attenuated by low doses of AEA and URB597 (Lisboa & Guimaraes, 2012). Moreover, a high dose of AEA attenuated the fear behavior evoked by exposing rats to a live cat only after local inhibition of NO synthesis (Lisboa, Camargo, da Silva, et al., 2014; Lisboa, Camargo, Magesto, et al., 2014). In the EPM, combination of ineffective low doses of AEA with NO signaling pathway inhibitors induced anxiolytic-like effect (Lisboa et al., 2013). Corroborating the NMDA-NO involvement in the antiaversive effect of cannabinoids, we recently demonstrated that the increase in contextual conditioned emotional response induced by blocking CB_1 receptors in the dlPAG was prevented by local NMDA antagonist or drugs inhibiting NO signaling (Uliana et al., 2016), suggesting that blockade of CB_1 receptors disinhibits glutamatergic neurotransmission and NO signaling pathway. Overall, CB_1 receptors in the dlPAG seem to decrease mainly glutamatergic/NO neurotransmission to induce anxiolytic and antifear effects, although the involvement of GABAergic mechanisms has also been reported, specially with higher doses of cannabinoids, when unbalanced glutamatergic and GABAergic mechanisms can take place (Lisboa et al., 2013; Uliana et al., 2016).

4.4 Amygdaloid Complex

The amygdala is a heterogeneous complex that modulates neuroendocrine function and is involved in the modulation of complex behaviors such as

fear, anxiety, defense, aggression, learning, and memory, including conditioning mechanisms (Adolphs, Tranel, Damasio, & Damasio, 1994; LeDoux, 1994; McNaughton & Corr, 2004; Morris et al., 1996; Phillips & LeDoux, 1992; Schafe et al., 2005; Scott et al., 1997; Sullivan et al., 2004).

The amygdaloid complex has been divided into basolateral (BLA) complex and the centromedial nuclei (central-CeA and medial-MeA amygdala nuclei), identified by cytoarchitectonic, histochemical, and immunocytochemical features (Ramikie & Patel, 2012; Sah, Faber, Lopez De Armentia, & Power, 2003). The different connections of these nuclei can be involved in the different effects promoted by them in a variety of functions, including anxiety. For instance, the amygdala hyperexcitability observed in mood and anxiety disorders has been related to impairments of the inhibitory influence of the hippocampus and PFC (Etkin, Prater, Schatzberg, Menon, & Greicius, 2009; Kim et al., 2011). Moreover, proper synchronization between amygdala, MPFC, and hippocampus is important for fear mechanisms, such as fear retrieval and extinction, and learning and memory (Lesting et al., 2013; Rei et al., 2015; Seidenbecher, Laxmi, Stork, & Pape, 2003). For instance, electrical stimulation of the BLA disrupts hippocampal CA1 LTP (Vouimba & Richter-Levin, 2005), whereas BLA lesion blocks impaired LTP and spatial memory induced by repeated stress (Kim, Koo, Lee, & Han, 2005). Moreover, optogenetic stimulation of BLA projections to the dHIP is sufficient to reproduce the molecular changes and learning and memory deficits induced by repeated stress in mice (Rei et al., 2015). The PL and IL portions of the MPFC differentially modulate CeA neurons, increasing and decreasing, respectively, CeA neurons activity and fear response, through projections to BLA and the intercalated cells nucleus (Sotres-Bayon & Quirk, 2010). Additionally, it was recently demonstrated that optogenetic stimulation and inhibition of IL axons in the BLA facilitates and impairs, respectively, fear extinction acquisition, but not retrieval, demonstrating a causal linking between MPFC and amygdala on modulation of fear memories (Bukalo et al., 2015).

CB_1 receptors are expressed in the amygdala, although at considerably higher densities in the BLA compared to the CeA or MeA (Herkenham et al., 1991; Katona et al., 2001). In addition to the receptors, eCBs are also produced in the amygdala (Bisogno et al., 1999; Herkenham et al., 1991) given that FAAH and MAGL enzymes are present in this brain area (Hu & Mackie, 2015; Tsou, Nogueron, et al., 1998). The levels of the eCBs AEA and 2-AG in different amygdala nuclei are modulated by several stressors (Blasio et al., 2013; Hill et al., 2008, 2009; Patel, Cravatt, & Hillard, 2005;

Patel, Kingsley, Mackie, Marnett, & Winder, 2009; Qin et al., 2015; Rademacher et al., 2008; and others). Local AEA levels can decrease after acute restraint stress, probably due to increased local activity of FAAH (Hill et al., 2009), whereas 2-AG is not altered after a similar procedure (Patel et al., 2009). 2-AG levels, however, increased in the BLA after chronic restraint stress (Patel et al., 2009). In contrast, exposure to a tone previous paired with footshocks increases both AEA and 2-AG release in the BLA (Marsicano et al., 2002). These data indicate that different stressors and their duration, in addition to different animal species, can differently modulate eCB levels in amygdala.

Systemic or central administration of CB_1 receptor agonists induces neuronal activation in the CeA of rats (Arnold, Topple, Mallet, Hunt, & McGregor, 2001; McGregor, Arnold, Weber, Topple, & Hunt, 1998; Patel, Cravatt, et al., 2005; Patel, Moldow, Patel, Wu, & Chang, 1998; Valjent et al., 2002; and others). In contrast, there is also a report that CB_1 agonists only induce neuronal activation in that region when combined with stress exposure (Patel, Cravatt, et al., 2005). Activation of the CeA, MeA or BLA was also observed after systemic administration of CB_1 antagonists (Newsom et al., 2012; Patel, Cravatt, et al., 2005; Patel, Roelke, et al., 2005; Sink et al., 2010).

The anxiolytic-like effects of low doses of systemic Δ^9-THC are associated with decreased amygdala activation and are prevented by the CB_1 antagonist AM251 (Rubino et al., 2007). An anxiogenic dose of AM251, in contrast, increased neuronal activation (Sink et al., 2010). In humans, anxiolytic doses of Δ^9-THC significantly reduce amygdala reactivity to social signals of threat (Phan et al., 2008). However, higher Δ^9-THC doses increased amygdala activation and anxiety feeling in similar situation (Bhattacharyya et al., 2010).

Contradictory results are also observed after local administration of cannabinoid agonists into different amygdala nuclei (Table 4). Administration of low doses of Δ^9-THC into the CeA or BLA induced anxiety-like behavior in rodents (Onaivi et al., 1995; Rubino, Guidali, et al., 2008), whereas administration of arachidonylcyclopropylamide (ACPA), a CB_1 agonist, into the CeA produced an anxiolytic-like effect (Zarrindast et al., 2008). Corroborating this latter effect, the CB_1 antagonist SR141716A in the CeA precipitated anxiety-like behavior in female rats during abstinence from palatable diet cycling (Blasio et al., 2013). In the BLA, administration of the nonselective agonist WIN55,212-2 decreased the enhancing effect of stress on inhibitory avoidance conditioning (Ganon-Elazar & Akirav, 2009),

whereas the antagonism of CB_1 receptors impaired extinction of fear conditioning (Roche, O'Connor, Diskin, & Finn, 2007) and acquisition of fear memory (Tan et al., 2011), respectively. Still in the BLA, administration of the FAAH inhibitor AM3506 facilitates fear extinction (Gunduz-Cinar et al., 2013). These later data suggest that reestablishment of an efficient AEA signaling by FAAH inhibition and/or activating cannabinoid receptors signaling could be a potential target to treat disorders associated with impaired fear extinction, such as PTSD. In fact, the fear extinction deficits and anxiety-like behavior observed a week later in a rat model of PTSD (SPS model) were attenuated by acute systemic or intra-BLA administration of WIN55,212-2, 2 min or 24 h after stress, in a CB_1-dependent way (Ganon-Elazar & Akirav, 2012). Overall, the results reported in the literature suggest that the eCB system exerts an inhibitory role in amygdala activity related to threatening situations, which is similar to what was observed with individuals carrying FAAH mutations that confer decreased FAAH, as discussed earlier in this chapter. In contrast, at high doses CB_1 agonists could facilitate anxiety-like behaviors. Studies evaluating the involvement of amygdala CB_2 receptors on modulation of anxiety-like behavior, however, are still missing.

Although administration of CBD (7.5–30 nmol) into the BLA of rats did not induce any behavioral alteration in the EPM (Lisboa & Guimarães, 2007), evidence from brain image studies in healthy humans showed that this drug did not attenuate left amygdala activity and decrease anxiety, which implicates this brain region in the effects of CBD (Crippa et al., 2004; Fusar-Poli et al., 2009). In addition, a recent study showed that although CBD in a single dose was not able to attenuate anxiety-like behavior in mice by itself, it decreased neuronal activation of the CeA and attenuated the anxiogenic-like effect induced by Δ^9-THC (Todd & Arnold, 2016).

4.4.1 Mechanisms

The mechanisms involved in the opposite effects of eCB system in amygdala, as in other brain regions, seem to involve modulation of glutamatergic and GABAergic neurotransmissions by CB_1 receptors. For instance, activation of these receptors within the BLA decreases both GABAergic and glutamatergic neurotransmissions, interfering with the plastic mechanisms of LTD and LTP, respectively (Azad et al., 2003, 2008; Katona et al., 2001). Moreover, superfusion of WIN55,212-2 onto CeA neurons decreased evoked $GABA_A$ receptor-mediated IPSPs in a CB_1-dependent way (Roberto et al., 2010). Both SR141716A and AM251 (CB_1 antagonists)

increased inhibitory responses, suggesting the presence of a tonic eCB mechanism that decreases inhibitory transmission in CeA (Roberto et al., 2010).

As commented earlier, plastic alterations within limbic regions after chronic stress exposure can result in signaling alterations in CB_1 receptors, shifting the emotional balance toward proaversive responses (McLaughlin et al., 2014). Considering that spontaneous activity of pyramidal BLA neurons is maintained at low levels by high local GABAergic inhibitory tone, stress-induced impairments in GABAergic transmission within the BLA may contribute to anxiety behavior (Quirk & Gehlert, 2003), an effect that could be modulate by eCBs. For instance, repeated stress increases 2-AG-mediated short-term synaptic suppression of GABAergic transmission in BLA slices, suggesting that 2-AG signaling is involved with adaptations induced by repeated stress (Patel et al., 2009). This effect could depend on eCB release driven, in a Ca^{+2}-independent way, by postsynaptic metabotropic glutamate receptor type 5 (mGluR5), as has already been suggested for DSI in the BLA (Zhu & Lovinger, 2005).

A recent work investigating the mechanisms by which chronic stress in the amygdala affects mGluR5 signaling and eCB synthesis resulting in molecular and behavioral alterations revealed a crucial role for the tyrosine phosphatase PTP1B (Qin et al., 2015). Stress-induced PTP1B activity in the amygdala or the genetic deletion of an endogenous inhibitor of this enzyme (LMO4) was associated with decreased mGluR5 phosphorylation, along with decreased AEA and 2-AG levels in the amygdala. These changes were associated with the presence of anxiety-like behavior and impaired fear extinction, and depended on GRs (Qin et al., 2015). Therefore, proper phosphorylation of mGluR5 is crucial for normal eCB signaling in the amygdala. Increased levels of glucocorticoids during stressful situations can disrupt this mechanism, contributing to the development of anxiety.

As will be discussed later, several studies have shown a close relationship between glucocorticoids and eCBs. CB_1 receptors in the BLA, but not in the CeA or MeA, are important modulators of the HPA axis. Their inhibition enhances HPA axis activity, increasing corticosterone levels (Ganon-Elazar & Akirav, 2009; Hill et al., 2009). Stress-induced corticosterone elevation is attenuated by intra-BLA administration of CB_1 agonists or URB597 (Ganon-Elazar & Akirav, 2009; Hill et al., 2009). These data suggest that increased FAAH activity could contribute to the decrease in BLA eCB levels, driven HPA axis activation. Corroborating this hypothesis, stress exposure mobilizes FAAH in the BLA, depleting AEA levels and increasing

neuronal excitability. In contrast, pharmacological inhibition of FAAH by AM3506 prevents the reduction of AEA levels in the BLA, in addition to preventing stress-induced dendritic hypertrophy and anxiety-like behavior (Gunduz-Cinar et al., 2013).

eCBs could also modulate anxiety behavior, at least in the BLA, due to an interaction with the corticotrophin release factor (CRF). BLA CRF1 receptors are involved in stress- and anxiety-like behavior (Jochman, Newman, Kalin, & Bakshi, 2005; Roozendaal, Brunson, Holloway, McGaugh, & Baram, 2002; Sztainberg, Kuperman, Tsoory, Lebow, & Chen, 2010). Acute restraint stress in rats induces a quick increase in FAAH activity in the amygdala pyramidal neurons (mainly BLA), an effect prevented by systemic treatment with a CRF1 antagonist. Moreover, the anxiogenic-like behavior induced by intra-BLA administration of CRF, as well as increased circulating levels of corticosterone, was attenuated by previous local administration of URB597 (Gray et al., 2015). Considering that glucocorticoids increase AEA levels in BLA (Hill et al., 2010), it was suggested that the rapid initial AEA signaling disruption mediated by CRF interfering with FAAH activity would be later counteracted by production and release of glucocorticoids as part of a negative feedback mechanism.

Overall, these results suggest that the eCB system in the amygdala, particularly in the BLA, plays an inhibitory role during threatening situations, decreasing the magnitude of HPA axis and the behavioral consequences of stress, such as anxiety and fear extinction.

4.5 Bed Nucleus of Stria Terminalis

The BNST, which is part of what is called the extended amygdala, is a highly heterogeneous and complex limbic structure associated with autonomic, neuroendocrine and behavioral functions (Crestani et al., 2013). It has reciprocal connections with the MeA and CeA (Dong, Petrovich, & Swanson, 2001; Shammah-Lagnado et al., 2000) and receives projections from the hippocampus, BLA, and MPFC (Dong, Petrovich, & Swanson, 2001; Dong, Petrovich, Watts, & Swanson, 2001; Vertes, 2006). Several studies have shown that the BNST plays a key role in expression of anxiety-like responses (Davis, Walker, Miles, & Grillon, 2010; Walker, Toufexis, & Davis, 2003).

CB_1 receptors are present in glutamatergic and GABAergic terminals in the BNST (Matsuda, Bonner, & Lolait, 1993; Puente et al., 2010; Tsou, Brown, et al., 1998). In vitro electrophysiology studies demonstrated that

CB_1 receptor activation inhibits both excitatory and inhibitory synaptic transmission in this region, which could be important for the regulation of aversive responses (Massi et al., 2008; Puente et al., 2010). Additionally, the activation of CB_1 receptors within the BNST alters the excitability of VTA dopamine neurons evoked by the stimulation of the IL portion of the MPFC and, based on those findings, it was suggested that disturbances in this circuitry might underlie abnormal emotional processing observed in disorders such as addiction and pathological stress (Massi et al., 2008). Despite these pieces of evidence, no study so far has investigated the effects of direct injections of CB_1 receptor agonists or antagonists into the BNST of animals submitted to aversive situations. However, recent studies from our group provide evidence that the BNST would be involved in the antiaversive effects of CBD.

Systemic CBD treatment attenuated the increase in c-Fos protein expression, a marker of neuronal activation, in the BNST induced by the reexposure to an aversive context previously paired with footshock (Lemos et al., 2010), suggesting a possible involvement of this structure in the effects of this drug. Afterward, we showed that intra-BNST administration of CBD attenuated freezing behavioral and the increase in heart rate and arterial pressure induced by contextual fear conditioning (Gomes et al., 2012). It should be noted that CBD did not induce any significant change in baseline values of these cardiovascular parameters. Therefore, it is unlikely that the attenuation of the cardiovascular responses to the aversive context depends on direct cardiovascular effects, but rather on an attenuation of the emotional response. In fact, intra-BNST CBD administration also induced anxiolytic-like effects in animals submitted to the EPM and VCT (Gomes, Resstel, et al., 2011). However, a direct influence of CBD in cardiovascular function could not be ruled out. Accordingly, intra-BNST administration of CBD facilitates bradycardiac baroreflex response (Alves et al., 2010). In addition, an enhanced tachycardiac response evoked by acute exposure to restraint stress, without affecting blood pressure changes, was also observed after BNST treatment with CBD (Gomes et al., 2013). These data are in contrast with those obtained after systemic or intracisternal administration of CBD, showing an attenuation of cardiovascular changes induced by restraint stress (Granjeiro, Gomes, Guimaraes, Correa, & Resstel, 2011; Resstel et al., 2009). Additionally, although the facilitation of restraint-evoked cardiovascular responses observed after intra-BNST CBD administration seems in contrast to its antiaversive effects, it suggests that CBD effects in behavioral and autonomic responses to aversive situations could

involve different neurobiological mechanisms. We cannot also exclude the possibility that neurobiological actions of CBD within BNST may depend on the type of stress.

Curiously, all the effects induced by CBD administration into the BNST were blocked by the pretreatment with the 5-HT1A receptor antagonist WAY100635, indicating that CBD acts through these receptors (Alves et al., 2010; Gomes et al., 2013, 2012; Gomes, Resstel, et al., 2011).

4.6 Other Brain Sites

The effects of cannabinoids in other brain areas that express CB_1 receptors and have been linked to anxiety, such as nucleus accumbens and hypothalamic nuclei, are less well known (Moreira, Aguiar, Resstel, et al., 2012). Onaivi and colleagues observed that administration of Δ^9-THC into the nucleus accumbens did not induce anxiety-like behavior (Onaivi et al., 1995). However, it was recently found that administration of ACEA into the shell region of the nucleus accumbens induced an anxiogenic-like effect in the EPM (Kochenborger et al., 2014). Regarding hypothalamic nuclei, several lines of evidence suggest a role for the eCBs as a negative modulator of the HPA axis. Therefore, changes in the eCB tone might be linked to stress-related diseases (Morena et al., 2016). The dorsomedial and ventromedial hypothalamic nuclei also play a role in the modulation of anxiety-like behaviors (McNaughton & Corr, 2004). Infusion of AEA into the ventromedial hypothalamus (VMH) induced a panicolytic-like effect through CB_1 receptors activation (Dos Anjos-Garcia et al., in press). Likewise, preliminary data from our group have indicated that intra-dorsomedial hypothalamus administration of drugs targeting the eCBs induces antiaversive effects, but this remains under investigation. Recently, it was showed that CB_1 receptor presynaptically expressed on medial habenula control the expression of aversive memories by selectively modulating cholinergic transmission at synapses in the interpeduncular nucleus (Soria-Gomez et al., 2015).

5. ADDITIONAL MECHANISMS INVOLVED IN THE eCB EFFECTS ON ANXIETY

5.1 Role of Specific eCBs

An important topic that remains to be further explored is the role of specific eCBs in modulating anxiety and aversive responses. AEA and 2-AG have different molecular targets in the brain and might interfere with emotional responses in particular ways. Both eCBs seem to be involved in

anxiety, as revealed by studies with compounds that selectively inhibit the enzymes reponsible for terminating their actions. As previously mentioned, systemic treatment with FAAH inhibitors, which increase mainly the levels of AEA, induces CB_1-dependent anxiolytic-like effects in experimental animals (Kathuria et al., 2003), whereas the effects of MAGL inhibition, preventing 2-AG hydrolysis, may depend on CB_2 receptor (Busquets-Garcia et al., 2011). Local injection of an MAGL inhibitor into the dlPAG also demonstrates that the anxiolytic-like effects of this compound may depend on both receptors (Almeida-Santos, Gobira, et al., 2013). Other enzymes responsible for eCB metabolism, such as the cyclooxygenase-2 (COX-2), have also been implicated in anxiety responses. For instance, site-especific inhibition of COX-2 induces anxiolytic-like effects in rats (Hermanson et al., 2013).

Other substances that have been proposed as part of the eCB system might also represent interesting targets. Hemopressin is an endogenous peptide that also binds to the CB_1 receptor, although as an inverse agonist, rather than an agonist (Heimann et al., 2007). In line with this mechanism, hemopressin administration into the dlPAG induces anxiogenic-like effects in rats (Fogaca et al., 2015).

5.2 Different Receptors and Modulation of Different Neurotransmitters

The CB_1 receptor is largely expressed throughout several brain regions related to anxiety and aversive responses (Herkenham et al., 1991), in different neuronal subpopulations (Marsicano & Lutz, 1999) and also in glia cells (Han et al., 2012; Navarrete & Araque, 2010), as already discussed. Its expression in presynaptic terminals of a variety of neuronal populations allows the modulation of several neurotransmitters released in a dose-dependent manner (Castillo, Younts, Chavez, & Hashimotodani, 2012). CB_1 are localized in GABAergic, cholinergic, glutamatergic, noradrenergic, serotonergic, among others, neuron terminals and are differentially involved in the responses to acute and chronic stress in mice (Haring, Guggenhuber, & Lutz, 2012; Lutz, Marsicano, Maldonado, & Hillard, 2015; Marsicano & Lutz, 1999; Monory et al., 2006).

CB_1 receptor activation modulates both glutamatergic and GABAergic neural subpopulations (Hoffman, Laaris, Kawamura, Masino, & Lupica, 2010; Laaris, Good, & Lupica, 2010; Monory et al., 2007) and it is broadly accepted that CB_1 localization on cortical glutamatergic and GABAergic neurons play opposing roles on the control of anxiety and fear-related

behaviors, as discussed earlier. It also has been proposed that CB_2 receptor is expressed in glia and neurons in various regions (Onaivi et al., 2006). CB_2 might modulate behavior relevant to neuropsychiatric disorders, including mood and reward (Onaivi et al., 2008), although its role in modulating anxiety-related responses has remained uncertain. This topic, therefore, warrants further investigation. Since its effects have been mostly related to the immune system, this will be addressed further.

The effects of low doses of cannabinoids are accepted to depend on the presence of CB_1 receptor on cortical glutamatergic neurons, whereas the anxiogenic effect of higher doses is mediated by CB_1 receptors on forebrain GABAergic neurons (Lutz et al., 2015). Animals with specific CB_1 deletion on glutamatergic neurons showed increased anxiety-like responses in highly aversive or arousing situations (Haring et al., 2011; Jacob et al., 2009; Lafenetre et al., 2009; Rey et al., 2012), in addition to deficits in fear learning and active-passive copying strategies (Metna-Laurent et al., 2012). Specific rescue of CB_1 receptors gene on cortical glutamatergic neurons restored anxiety-like, but not fear-related behavior (Ruehle et al., 2013). In contrast, deletion of CB_1 receptors in forebrain GABAergic neurons reduced anxiety-like or neophobic behavior in mild aversive or arousing conditions (Haring et al., 2011; Lafenetre et al., 2009; Rey et al., 2012). Also, although CB_1 receptors in GABAergic neurons do not seem to have an essential role in fear alleviation (Dubreucq et al., 2012), other studies reported decreased freezing in conditional mutant mice (Llorente-Berzal et al., 2015), as well as a more efficient switch from passive to active avoidance strategies (Metna-Laurent et al., 2012).

Another possible step in the eCB action mechanism involves NO signaling, as already commented. NO is involved in several stress-related disorders (Guimaraes, Beijamini, Moreira, Aguiar, & de Lucca, 2005) and interacts with the eCB system in the dlPAG (Batista, Bastos, et al., 2015; Batista, Fogaca, et al., 2015; Lisboa & Guimaraes, 2012; Lisboa et al., 2013; Uliana et al., 2016) and in the dHIP (Spiacci et al., 2016) on the control of emotions. Moreover, pharmacological and genetic inhibition of nitric oxide synthase (neuronal, nNOS; or inducible, iNOS) induce changes in anxiety- and fear-like behaviors (Buskila et al., 2007; Kelley, Anderson, & Itzhak, 2010; Kelley, Balda, Anderson, & Itzhak, 2009; Lisboa, Gomes, et al., 2015; Wultsch et al., 2007). Recently, it was showed that the fear extinction disruption observed in mice-lacking iNOS was attenuated by the FAAH inhibition (Lisboa, Gomes, et al., 2015), suggesting that increased eCB tone could balance NO signaling toward fear and anxiety relief.

Several studies demonstrated that the eCB system could also modulate monoaminergic systems (Haj-Dahmane & Shen, 2011; Laviolette & Grace, 2006; Micale et al., 2009a; Nakazi et al., 2000; Terzian et al., 2011). CB_1 agonists, for example, stimulate the release of norepinephrine and the expression of different adrenergic receptors in the rodent PFC, as previously mentioned (Carvalho, Mackie, & Van Bockstaele, 2010; Oropeza, Mackie, & Van Bockstaele, 2007; Oropeza, Page, & Van Bockstaele, 2005). Under acute stress exposure, an ineffective dose of URB597 increased norepinephrine levels in PFC and BLA of rats in a CB_1-dependent way (Bedse et al., 2015), suggesting a possible noradrenergic mechanism by which eCBs modulate stress responses. These effects could be due to increased firing of noradrenergic neurons, since URB597 enhances this mechanism, in addition to inducing antidepressant-like effect in mice (Gobbi et al., 2005). In relation to serotonin, mice-lacking CB_1 receptors in serotonergic neurons are more anxious and less sociable than control littermates, but only under stressful situations (Haring et al., 2015). Added to that, increases of eCB tone produces anxiolytic-like responses via serotoninergic 5-HT1A and 5-HT2A receptors (Bambico et al., 2010), also enhancing serotonin neurons firing activity (Gobbi et al., 2005). In addition, eCBs could downregulate serotonin release (Haring, Marsicano, Lutz, & Monory, 2007). There is also evidence for the serotonin involvement in the biphasic effects of cannabinoid drugs, since the anxiogenic-like effect of a higher dose of the CB_1 agonist CP55,940, but not the anxiolytic effect of a lower dose, was prevented by a 5-HT1A antagonist (Marco et al., 2004). CB_1 and eCBs also interact with the dopaminergic system, since deletion of dopamine D3 receptor, for example, promotes anxiolytic-like behaviors accompanied by changes in AEA and 2-AG levels in several brain areas (Micale et al., 2009a). Moreover, CB_1 deletion on dopaminergic D1- expressing neurons induces anxiety- and fear-like behaviors in mice (Terzian et al., 2011).

Apart from the cannabinoid receptors, other molecular targets for AEA expressed in brain regions related to anxiety might be involved in eCB effects on anxiety. The expression of the TRPV1 channel, which is activated by AEA, as already discussed, has been reported in various brain regions (Toth et al., 2005). This topic, however, has been controversial. Recently, its expression was detected at significant levels only in specific brain regions, including the PAG (Cavanaugh et al., 2011). In contrast, other studies have not only detected TRPV1 expression in the brain but also observed a significant colocalization with CB_1 in various brain regions (Cristino et al., 2006), including the dlPAG (Casarotto et al., 2012). These results are in line

with pharmacological evidence for a reciprocal interaction between CB_1 and TRPV1 in mediating the effects of AEA. At low doses, this eCB may bind predominantly to CB_1 receptor and exert anxiolytic effects. At higher levels, however, it may activate TRPV1 at the same synapse and promote anxiogenic and proaversive responses (Almeida-Santos, Moreira, Guimaraes, & Aguiar, 2013; Casarotto et al., 2012; Fogaca, Aguiar, et al., 2012; Fogaca, Lisboa, et al., 2012; Lisboa & Guimaraes, 2012; Terzian, Aguiar, Guimaraes, & Moreira, 2009).

Therefore, considering eCBs role in emotional processing, the fine-tune control of neurotransmitters release and different receptors involved assures the necessary balance in neuronal signaling, especially under stressful situations, as suggested elsewhere (Kano, Ohno-Shosaku, Hashimotodani, Uchigashima, & Watanabe, 2009; Fogaca, Lisboa, et al., 2012). Together, these results could help to explain why the resultant effect on anxiety behavior of drugs interfering with eCB signaling depend on several factors, such as the location and type of cannabinoid receptors and the stressors characteristic, including their duration.

5.3 Modulation of the HPA Axis and the Immune System

Exposure to acute and/or repeated stressors increases anxiety in rodents and is associated with the onset of several psychiatric disorders in humans. Cannabinoid effects in animal models of anxiety are also influenced by stress, being usually more marked in highly stressful situations (Morena et al., 2016). Several plastic mechanisms seem to be responsible for this influence. Activation of CB_1 receptors modulates several mechanisms of synaptic plasticity such as LTP, LTD, and depolarization-induced suppression of inhibition/excitation (DSI/E) that are influenced by stress (for review, see Morena et al., 2016). Stress, on the other hand, changes eCB signaling (Morena et al., 2016). For example, chronic unpredictable stress (CUS) decreases CB_1 receptors and reduces AEA levels, and eCB-mediated synaptic plasticity in brain areas associated with anxiety such as MPFC, amygdala, and hippocampus (Gorzalka & Hill, 2011; Hill et al., 2008), as discussed previously in this chapter. In adult rats, CUS decreases CB_1 receptors in the hippocampus of male rats by still unknown mechanisms. This reduction seems to involve GABAergic terminals, with the less inhibition resulting in enhanced local excitatory transmission (Reich, Mihalik, Iskander, Seckler, & Weiss, 2013). In this way, considering that stressful situations decrease AEA levels and drive AEA to act preferentially in CB receptors located in GABAergic terminals in the hippocampus, we could argue that

administration of an AEA metabolism/uptake inhibitor into the ventral hippocampus turns the anxiogenic effect of this drug in nonstressed rats into anxiolytic effect after restraint stress since it is normalizing the local neurotransmission (Campos et al., 2010).

Since the effects of chronic stressors on eCBs are similar to those produced by repeated corticosterone administration, activation of the HPA axis has been proposed as the causal factor relating stress and changes in eCB signaling (Gorzalka & Hill, 2011). Glucocorticoid exogenous administration, similar to stress, preferentially modulates eCB signaling in glutamatergic or GABAergic terminals, depending on the region (Morena et al., 2016). The eCB system also seems to be very sensitive to early stress in a gender-dependent manner. Maternal deprivation, for example, increases expression of most eCB-related genes (Marco et al., 2014).

Although there are some inconsistent data, stress exposure or corticosterone administration treatment increases 2-AG, but decreases AEA levels, in the hippocampus and other brain regions (Morena et al., 2016). The decline in AEA levels induced by stress seems to depend on increased expression/activity of FAAH caused by a rise in CRF signaling. This initial AEA decrease has been proposed to mediate the initiation of the stress response. The subsequent corticosterone-dependent increase in 2-AG levels would facilitate the termination of this response (Morena et al., 2016). Furthermore, CB_1 KO mice exhibit increased CRF mRNA levels in the PVN, suggesting impaired HPA fast feedback mechanism, showed by sustained HPA axis activity (Steiner et al., 2008).

These results suggest that the hippocampus, together with other brain regions such as the MPFC and BLA, could be one of the structures where the HPA and eCB systems interact to modulate stress-induced changes in anxiety behavior (Lee & Gorzalka, 2012; Morena et al., 2016). For example, blockade of dorsal hippocampal CB_1 receptors with AM251 prevented the impairment of contextual fear retrieval induced by systemic injection of corticosterone in rats (Atsak et al., 2012). Moreover, WIN55,212-2 administered before each daily restraint stress episode for 2 weeks prevented the long-lasting anxiogenic effects, impaired LTP in the *vHIP-accumbens* pathway and hippocampal-dependent learning performance, and reduced GR expression in the hippocampus (Abush & Akirav, 2013). In addition, exposure to stressors such as SPS upregulates GR in the hippocampus, in the BLA and frontal cortex, an effect prevented by WIN55,212-2 systemic administration (Ganon-Elazar & Akirav, 2013).

The attenuation of stress consequences by cannabinoids seems to depend on GR activation, since they can be prevented by the GR antagonist RU-486. In the BLA, however, the stress-induced increase in glutamate currents depends on mineralocorticoid receptors (Karst, Berger, Erdmann, Schutz, & Joels, 2010). Beta-adrenergic receptors could also be involved, since propranolol injected into the hippocampus blocked the memory retrieval impairment induced by WIN55,212-2. These results suggest that stressful-arousing situations increase GCs levels in the hippocampus that would interact, by nongenomic mechanisms, with the noradrenergic system to impair emotional memory retrieval (Hill et al., 2010).

Glucocorticoids, similar to noradrenergic signaling, are well known to modulate immune response induced by stressors (Carrillo-de Sauvage et al., 2013), including microglia activation (Frank, Thompson, Watkins, & Maier, 2012). They can also suppress hippocampal and amygdala eCB signaling (Bowles et al., 2012). These alterations have been implicated in anxiety-like behavior induced by stressful situations involving the immune system activation (Reader et al., 2015). Microglia, the resident immune cells in the brain, peripheral macrophages and other immune cells express cannabinoid receptors (Atwood, Huffman, Straiker, & Mackie, 2010; Ehrhart et al., 2005; Maresz, Carrier, Ponomarev, Hillard, & Dittel, 2005; Martin-Moreno et al., 2011; Nunez et al., 2004; and others) and functional eCB degradation enzymes (Tham, Whitaker, Luo, & Webb, 2007). Under pathological conditions, expression of CB_2 and production of eCBs are increased (Cabral & Marciano-Cabral, 2005; Stella, 2009). It has been suggested that such increase may contribute to defense mechanisms through accumulation of antiinflammatory microglia phenotype (Stella, 2009).

Cannabinoids and eCBs can interfere on microglia activation and the production of inflammatory mediators (Henry, Kerr, Finn, & Roche, 2016; Lisboa, Gomes, Guimaraes, & Campos, 2016; Mecha, Carrillo-Salinas, Feliú, Mestres, & Guaza, 2016). Over the past years, increasing amount of evidence points to an important modulatory role of immune system in anxiety- and stress-related disorders (Baker, Nievergelt, & O'Connor, 2012; Capuron & Miller, 2011; Haroon, Raison, & Miller, 2012; Maes, Leonard, Myint, Kubera, & Verkerk, 2011; O'Donovan et al., 2015; Yirmiya, Rimmerman, & Reshef, 2015; and others). In contrast, microglia cells also contribute to the CNS homeostasis and plasticity, removing redundant synapses and eliminating dying neurons, in addition to modulating neurotransmitter release and neurogenesis (Beumer et al., 2012; Jones & Lynch, 2015; Kettenmann, Kirchhoff, & Verkhratsky, 2013; Ziv et al., 2006).

There is a substantial body of evidence suggesting that cannabinoid receptors, mostly CB_2 on microglia, may be potential targets for cannabinoid drugs that modulate immune system associated disorders, including psychiatry disorders (Lisboa, Gomes, et al., 2016). In vitro and in vivo studies have revealed that activation of those receptors suppresses the release of proinflammatory cytokines, enhances the release of antiinflammatory cytokines and reduces microglia activation and proliferation (Ehrhart et al., 2005; Fernandez-Ruiz, Pazos, Garcia-Arencibia, Sagredo, & Ramos, 2008; Romero-Sandoval, Horvath, Landry, & DeLeo, 2009; Walter et al., 2003; and others). Additionally, an extensive literature shows that cannabinoid drugs modulate stress-induced immune system alterations along with behavioral effects. For instance, the increased proinflammatory profile in the frontal cortex induced by subchronic stress in mice was attenuated by pharmacological CB_2 activation or by genetic overexpression of this receptor, whereas CB_2 deletion induced effects similar to stress (Zoppi et al., 2014). Similar results were reported with CB_1 activation and CB_1 KO mice (Zoppi et al., 2011), suggesting that both receptors exert an inhibitory role in stress-induced neuroinflammation and its behavioral consequences. We recently provided the first evidence linking microglia cells, cannabinoid receptors, and anxiety by showing that administration of the nonselective cannabinoid receptors agonist WIN55,212-2 daily before social defeat stress in mice attenuates stress-induced immune system activation, including brain microglia activation and anxiety-like behavior (Lisboa, Niraula, et al., 2016).

In addition to stress exposure, other evidence for interaction between cannabinoid and immune systems on anxiety modulation came from studies with IL-1β signaling. Central administration of IL-1β, a proinflammatory cytokine that has been related to learning and memory, HPA axis modulation, and neurogenesis, induces anxiety-like behavior similar to a social defeat stress paradigm in mice. The stress effect is dependent on the IL-1β receptor signaling (Rossi, Sacchetti, et al., 2012). Moreover, both IL-1β and stress (Rossi et al., 2008) alter the sensitivity of $CB_{1/GABA}$ receptors in striatum in a cholesterol- and TRPV1 receptors-dependent fashion (Maccarrone et al., 2008; Rossi et al., 2008; Rossi, Sacchetti, et al., 2012). Meanwhile, it was also showed that IL-1β increases the frequency of glutamatergic sEPSCs in the striatum via TRPV1 channels (Rossi, Furlan, et al., 2012). Different eCBs (2-AG and AEA, respectively) and intracellular mechanisms, however, are related to modulation of $CB_{1/GABA}$ and $CB_{1/Glutamate}$ (De Chiara et al., 2013; Rossi, Sacchetti, et al., 2012). These data suggest that inflammatory mediators, such as IL-1β, induce anxiety by interfering with eCB signaling and

alteration of GABA and glutamate neurotransmissions. In contrast, activation of CB_1 and CB_2 receptors could attenuate anxiety disorders by inducing IL-1ra release, an endogenous antagonist of IL-1β, by glial cells (Molina-Holgado et al., 2003). In addition to the effects on immune cells and directly modulation of the HPA axis, as aforementioned, considering the relationship between cannabinoids and IL-1β signaling and that this signaling pathway modulates the HPA axis activity and, consequently, glucocorticoid release (Goshen, Yirmiya, Iverfeldt, & Weidenfeld, 2003), cannabinoids could also indirectly interfere with the HPA axis through IL-1β signaling. Overall, the studies published so far indicate that eCB signaling could modulate anxiety behavior by interfering with the HPA axis and immune system activation, dampening the overactivation of the immune system.

5.4 Plastic Mechanisms

Two cellular mechanisms have been particularly associated with the behavioral consequences of stress exposure: decreased hippocampal neurogenesis and dendritic remodeling (Castren & Hen, 2013). In adult mice, repeated treatment with drugs that facilitate eCB signaling share similarities with antidepressants. Both activate intracellular pathways that regulate cell proliferation and neural cell survival such as mitogen-activated protein kinase and phosphoinositide 3-kinase/Akt or PKB signaling (Fogaca, Galve-Roperh, et al., 2013). Nonselective CB_1 and CB_2 agonists, or drugs that facilitate eCB signaling, could enhance adult hippocampal neurogenesis (Fogaca, Galve-Roperh, et al., 2013). This effect is similar to that observed with antidepressants and has been associated with the anxiolytic/antistress effects of repeated treatment with these drugs (Campos et al., 2013; Jiang et al., 2005). The effects of cannabinoid agonists on neurogenesis, however, could also be deleterious. For example, adolescent exposure to CB_1 agonists could interfere with hippocampal function by decreasing neurogenesis (Abboussi, Tazi, Paizanis, & El Ganouni, 2014) and Δ^9-THC can reduce hippocampal cell proliferation and impair spatial memory (Wolf et al., 2010).

CB_1 receptor maintenance of functional hippocampal homeostasis is proposed to depend on the structural synaptic plasticity of pyramidal neurons (Monory, Polack, Remus, Lutz, & Korte, 2015). However, this role depends on their synaptic localization. Deletion of CB_1 receptors located in glutamatergic or GABAergic terminals in the hippocampus increase and decrease, respectively, the density of dendrite spines in the dentate gyrus. These effects were associated with similar changes in long-term potentiation (Monory et al., 2015).

Exposure to cannabinoid agonists can decrease dendritic spine density in the dentate gyrus, a finding that was associated with memory impairment (Candelaria-Cook & Hamilton, 2014). A direct interference by cannabinoids on stress-induced synaptic remodeling in the hippocampus, however, has not been investigated.

6. CLOSING REMARKS AND FUTURE DIRECTIONS

It has become increasingly clear by both preclinical and clinical studies that cannabinoids interfere with anxiety responses. The final outcome, however, depends on several factors such as the drugs and doses used, the aversiveness of the task, and the animal species. These cannabinoid drugs exert these multifaceted effects by interfering with components of the eCB system. Numerous pieces of evidence now suggest that this system regulates and buffers exaggerated responses to several insults, including threatening and aversive stimuli. Several studies have also suggested that eCB dysfunctions play a role in the pathophysiology of anxiety- and stress-related disorders. Consequently, their components are potential targets for the development of new therapeutic approaches for these disorders. Along this review, several of these targets have been discussed. However, paralleling the increase in the number of studies investigating the modulatory effects of cannabinoids on emotions, our knowledge about the complexity of the eCB system and the neurobiology of anxiety is also exponentially growing. The challenge for future studies and the development of new therapeutic targets is to integrate these recent findings in an intelligible picture.

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