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Review

Neuroanatomical substrates involved in cannabinoid modulation of defensive responses

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Abstract

Administration of *Cannabis sativa* derivatives causes anxiolytic or anxiogenic effects in humans and laboratory animals, depending on the specific compound and dosage used. In agreement with these findings, several studies in the last decade have indicated that the endocannabinoid system modulates neuronal activity in areas involved in defensive responses. The mechanisms of these effects, however, are still not clear. The present review summarizes recent data suggesting that they involve modulation of glutamate and GABA-mediated neurotransmission in brain sites such as the medial prefrontal cortex, amygdaloid complex, bed nucleus of the stria terminalis, hippocampus and dorsal periaqueductal gray. Moreover, we also discuss results indicating that, in these regions, the endocannabinoid system could be particularly engaged by highly stressful situations.

Keywords

Amygdala, anxiety, bed nucleus of the stria terminalis, cannabidiol, endocannabinoid, hippocampus, medial prefrontal cortex, periaqueductal gray

Introduction

Fear and anxiety could be seen as emerging properties of interacting brain regions that mediate defensive responses in animals exposed to threatening stimuli (Gray and McNaughton, 2000; McNaughton and Corr, 2004; Morgane et al., 2005). These responses depend on factors such as distance (proximal versus distal), environment (escape availability) and nature (potential versus real, innate versus learned) of the stimulus (Gray and McNaughton, 2000; McNaughton and Corr, 2004; Sandford et al., 2000). They are organized by at least partially distinct and hierarchical brain systems that include a behavioural inhibition system, responsible for the suppression of behaviours that could enhance danger, and an antipredator defensive system, involved in immediate responses to threatening stimuli. The hierarchy of these systems has been confirmed by recent neuroimaging studies in humans showing that initial detection of a potential threat engages mostly forebrain areas, such as the ventromedial prefrontal cortex, anterior cingulate cortex, amygdala, hippocampus and hypothalamus. These areas seem to be involved in early threat responses, including the assignment and control of fear (Mobbs et al., 2009). When the chance of a predatory attack is high, however, midbrain structures such as the dorsolateral periaqueductal gray (PAG) would be preferentially engaged, resulting in fast, active defensive strategies (Mobbs et al., 2009). Additional networks could also be responsible for responses such as avoidance (Graeff, 1994; McNaughton and Corr, 2004).

Dysfunctions in these defensive brain areas have been related to pathological anxiety in humans. For example, several neuroimaging studies have shown abnormalities in the prefrontal cortex of anxiety disorder patients, with decreased neuronal activity in disorders characterized by intense fear, such as panic, post-traumatic stress disorder (PTSD) and phobias, and increased activity in disorders that involve worry and rumination such as generalized anxiety and obsessive-compulsive disorder (Milad and Rauch, 2007). In addition to prefrontal cortex changes, patients with PTSD also have a smaller hippocampus volume and increased activity in the amygdala (Quirk and Mueller, 2008). Panic patients, on the other hand, show, when treated with panic symptoms-inducing drugs, increased activation of the parahippocampal gyrus, the superior temporal lobe, the anterior cingulate, cerebellar vermis, insula, temporal poles, the hypothalamus, and PAG (Boshuisen et al., 2002; Javanmard et al., 1999).

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Diverse neurotransmitters have been shown to modulate these defensive responses. As will be discussed in this review, several pieces of evidence suggest that, in addition to classical neurotransmitters such as gamma-aminobutyric acid (GABA), glutamate and serotonin, endocannabinoids (eCBs) may also play an important role in the modulation of behavioural responses to threatening stimuli.

eCBs act as neurotransmitters in several brain regions. They have been characterized after studies with Δ^9-tetrahydrocannabinol (THC) and synthetic related substances. THC is the main constituent that accounts for the psychotomimetic effects of the herb *Cannabis sativa*. Despite its ancient use either as a medicine or as a drug of abuse, it was only in the middle of the last century that the chemistry of this plant started to be elucidated (Mechoulam, 1970). In addition to THC, several other compounds, such as cannabidiol (CBD) and cannabigerol, have also been identified in this plant and referred to as phytocannabinoids (Izzo et al., 2009). The characterization of these phytocannabinoids has rendered it possible to develop synthetic compounds that could mimic typical effects of THC. This, in turn, fostered pharmacological studies on this area.

For decades it remained unclear how cannabinoids would exert their effects. However, in 1988, a binding site for these compounds was finally detected in the mouse brain (Devane et al., 1988). The identification of this cannabinoid receptor, now named CB1, led to the hypothesis that an endogenous ligand should exist in mammals. Indeed, a cannabinoid receptor agonist was isolated from the porcine brain in 1992 (Devane et al., 1992). This substance, arachidonoyl-ethanolamide, was named anandamide (AEA), after *ananda*, the Sanskrit word for 'bliss'. Later on, other endogenous cannabinoid receptor agonists, or endocannabinoids, were isolated. They are all arachidonic acid derivates, and include 2-arachidonoyl glycerol (2-AG), N-arachidonoyl dopamine, noladin ether and virodhamine (Howlett et al., 2002). A few years later a CB2 receptor was also described (Munro et al., 1993). Both CB1 and CB2 are metabotropic receptors, with the former probably being the main receptor responsible for the behavioural effects of cannabinoids.

A particular feature of the CB1 receptor is its predominant location in presynaptic terminals. Indeed, contrary to classical neurotransmitters, eCBs act in retrograde fashion, as they are produced in and released from the postsynaptic neuronal membrane, acting presynaptically to decrease neurotransmitter release (Wilson and Nicoll, 2001). Anandamide actions terminate after an internalization process followed by hydrolysis by an enzyme called fatty acid amid hydrolase (FAAH) in the postsynaptic neuron (Cravatt et al., 1996). 2-AG, on the other hand, is degraded by the enzyme monoacylglycerol lipase (MAGL) (Dinh et al., 2002). Anandamide can also target the transient receptor potential vanilloid type-1 channel (TRPV1), an ion channel permeable to calcium that, contrary to CB1, could facilitate glutamate release (Xing and Li, 2007).

Natural or synthetic cannabinoids often yield complex responses in experimental models of anxiety. As extensively reviewed elsewhere (Moreira et al., 2009a, 2009b; Viveros et al., 2005), these drugs produce bell-shaped or biphasic dose–response curves in several animal models including the elevated plus-maze (EPM), elevated T-maze and the zero maze, the light–dark test, and the Vogel conflict test. Usually the anxiolytic-like effects occur at low doses, whereas anxiogenic-like or no effects are observed with high doses (Moreira and Lutz, 2008; Moreira et al., 2009a, 2009b; Viveros et al., 2005). Similar results have also been observed in humans (Zuardi et al., 2006). The reasons for these complex effects remain unknown.

CB1 receptors and other components of the eCB system are present in brain regions associated with defensive responses, including the medial prefrontal cortex (mPFC), amygdala, hippocampus, hypothalamus and PAG (Howlett et al., 2002). Cannabinoids are able to modulate the release of several neurotransmitters that mediate or modulate those responses, including glutamate (Jin et al., 2007), GABA (Szabo et al., 1998), glycine (Jennings et al., 2001), serotonin (Nakazi et al., 2000) and cholecystokinin (Schlicker and Kathmann, 2001). Among them, GABA and glutamate usually play a pivotal and opposite roles, with the former inhibiting and the latter facilitating anxiety (Moreira et al., 2009a). The contributions of these different neurotransmitters on cannabinoid effects in specific behavioural situations are poorly understood and could depend on several variables, including animal species and brain region (Haller et al., 2007; Lafenêtre et al., 2007; Lutz, 2009). Studies employing intra-cerebral injections into specific structures, therefore, could help to elucidate the role of cannabinoids on anxiety. In the last few years a number of studies have employed this approach to investigate the effects of cannabinoids in brain areas related to defensive behaviours.

The main pharmacological tools used in these studies were the non-selective (CB1, CB2, TRPV1) agonists, such as AEA; selective CB1 receptor agonists and antagonists, such as arachidonoylchloroethanolamide (ACEA) and AM251, respectively; AEA transporter inhibitor, such as AM404; FAAH inhibitor, such as URB597; TRPV1 receptor agonist and antagonist, such as capsaicin and capsazepine, respectively (Pertwee, 2008; Saito et al., 2010). The effects of CBD have also been investigated. This phytocannabinoid causes anxiolytic effects in animal models and clinical studies (Guimarães et al., 1990, 1994; Moreira et al., 2006; Resstel et al., 2006b; Zuardi et al., 1993). Although some of these effects have been associated with an agonist action at 5-HT1A receptors (Campos and Guimarães, 2008), CBD can also facilitate eCBs' signalling by inhibiting the metabolism and reuptake of AEA (Bisogno et al., 2001). This mechanism seems to be involved at least on the drug facilitatory effect of aversive conditioning extinction (Bitencourt et al., 2008) and decrease of marble burying behaviour (Casarotto et al., 2010).

The main results regarding the intra-cerebral effects of these compounds in animal models of anxiety are described below. In addition to anxiety, however, the eCB system also plays a significant role in the extinction of aversive memories (Lutz, 2009; Marsicano et al., 2002), a phenomenon that has been particularly associated with the pathophysiology of PTSD (Cannistraro and Rauch, 2003). Since this topic will be specifically addressed in another paper of this Journal issue (Gomes et al, 2011), only intra-cerebral cannabinoid effects on the expression of conditioned aversive responses will be discussed here.

The medial prefrontal cortex

The mPFC is an executive brain region that regulates several neural processes (Dalley et al., 2004). It is proposed to play a crucial role in interfacing processes involved in fear responses originated in limbic and neocortical regions (Conde et al., 1995; Sesack et al., 1989; Takagishi and Chiba, 1991). Neurons located in this region show increased activity during the expression and acquisition of Pavlovian fear associations (Baeg et al., 2001; Laviolette et al., 2005; Sotres-Bayon et al., 2006). The mPFC receives information about context and electrical shock associations through afferents from the amygdala and the hippocampus (Jay and Witter, 1991). Moreover, similar to these structures (Anagnostaras et al., 1999; Galeno et al., 1984; Gentile et al., 1986; Iwata et al., 1986; Kim and Fanselow, 1992; LeDoux et al., 1988; Maren et al., 1997; Maren and Fanselow, 1997), lesions of the mPFC decrease behavioural and cardiovascular responses associated with conditioned fear (Resstel et al., 2006a).

The ventral portion of the mPFC (vmPFC) seems to be particularly associated with emotional regulation (Frysztak and Neafsey, 1991; Resstel et al., 2006a, 2008b, 2008d) and its abnormal functioning has been related to several neuropsychiatric disorders such as schizophrenia, depression, PTSD and obsessive-compulsive disorder (Abbruzzese et al., 1995). Experiments employing c-Fos protein as a marker of neuronal activation showed that in rodents the vmPFC, particularly its infralimbic (IL) and prelimbic (PrL) subregions (Beck and Fibiger, 1995; Dias and Aggleton, 2000), is activated by exposure to dangerous and/or stressful stimuli (Duncan et al., 1996; Morrow et al., 2000; Rubino et al., 2007). It modulates neuroendocrine, autonomic and behavioural responses that include activation of the hypothalamic–pituitary–adrenal (HPA) axis and changes in breathing, heart rate and blood pressure (Jinks and McGregor, 1997; Resstel and Correa, 2006; Sullivan and Gratton, 2002).

Components of the eCB system are present in the mPFC of rats and humans (Bisogno et al., 1999; Di Marzo et al., 2000; Egertova et al., 2003; Glass et al., 1997; Hájos and Freund, 2002; Herkenham, 1992; Herkenham et al., 1990, 1991; Mailleux and Vanderhaeghen, 1992, Mato and Pazos, 2004; Thomas et al., 1997; Tsou et al., 1998a, 1998b). Also, cannabinoids can modify prefrontal cortex activity (Brett et al., 2001; Freedland et al., 2002; Lundqvist et al., 2001; Margulies and Hammer, 1991; O'Leary et al., 2000, 2002; Whitlow et al., 2002), an effect that has been related to the cognitive and emotional consequences of *Cannabis* intake.

In an initial study, Bortolato et al. showed that the anxiolytic effects observed after systemic administration of the AEA transporter inhibitor AM404 were associated with increased levels of AEA, but not 2-AG, in the prefrontal cortex. These effects were prevented by pre-treatment with the CB1 antagonist rimonabant (SR141716A) (Bortolato et al., 2006). Moreover, although opposite effects have been found with a much higher dose (5 mg/kg, Egerton et al., 2001), the anxiolytic-like effect of THC observed at the dose of 0.75 mg/kg was accompanied by decreased Fos expression in the mPFC (Rubino et al., 2007) and increased levels of phosphorylated CREB (pCREB) and ERK, all effects attenuated by pre-treatment with AM251. In a subsequent work, Rubino et al. (Rubino et al., 2008a) directly tested if the mPFC could be involved in the anxiolytic effects of THC. They observed that local administration of this drug decreased anxiety in rats submitted to the EPM. Similar effects were observed with a low dose of the AEA analogue methanandamide (mAEA) (Rubino et al., 2008b) and with FAAH inhibitors, in the Vogel conflict test (see Table 1).

Fear conditioning is another paradigm that has been extensively used to investigate the effects of cannabinoids on emotional responses. In this model an aversive unconditioned stimulus, usually an electrical footshock, is associated with the presentation of a neutral conditioned stimulus, which can be a discrete stimulus such as a light or a tone (Fendt and Fanselow, 1999; Resstel et al., 2009), or the environment context (Beck and Fibiger, 1995; Blanchard and Blanchard, 1972; Fendt and Fanselow, 1999). The conditioned emotional response (CER) evoked by re-exposure to the context or to the discrete stimulus is characterized by freezing behaviour and changes in autonomic activity (heart rate and arterial blood pressure increases) (Resstel et al., 2006a; Sullivan et al., 2004; Vianna et al., 2008). All these responses are attenuated by prototype anxiolytics such as diazepam, further suggesting its relationship to anxiety-like behaviour (Beck and Fibiger, 1995; Fanselow and Helmstetter, 1988; Malkani and Rosen, 2000; Resstel et al., 2006b).

Although there are contradictory results that could depend on the different protocols and species (rats or mice) used (for review see Resstel et al., 2009), several studies have shown that the eCB system can interfere with fear conditioning (Arenos et al., 2006; Chhatwal et al., 2005; Finn et al., 2004a; Marsicano et al., 2002; Mikics et al., 2006; Pamplona and Takahashi, 2006; Reich et al., 2008; Roche et al., 2007; Suzuki et al., 2004), usually attenuating the CERs and facilitating extinction of aversive memories. Concerning the former effect, intra-mPFC injections of AEA or AM404 were able to attenuate the freezing behaviour and cardiovascular changes induced by re-exposure to the aversive context (Lisboa et al., 2010). These effects were prevented by local AM251 pre-treatment, indicating that they were mediated by CB1 receptor activation. Although AM251 alone failed to change behaviour in animals tested in the EPM, this drug was able to increase the conditioned fear response when low electrical footshock intensity was employed as the aversive stimulus (Lisboa et al., 2010). Corroborating this finding, animals overexpressing the FAAH enzyme in the prefrontal cortex, which would cause a decrease in AEA levels, showed increased anxiety-related behaviour compared with controls (Rubino et al., 2008b). As will be discussed below, the contrasting effects of the CB1 receptor antagonist in animals submitted to the EPM and contextual fear conditioning (CFC) models suggest that the eCB system plays a significant inhibitory role on defensive responses under more stressful/aversive situations.

CB1 receptors are localized presynaptically in glutamatergic neural terminations in the mPFC (Auclair et al., 2000). Accordingly, CB1 receptor agonists such as WIN55,212-2 and CP55,940 (Devane et al., 1988) decreased excitatory postsynaptic currents (EPSCs) whereas the CB1 receptor antagonist SR141716A increased them (Auclair et al., 2000).

Table 1. Effects of intra-cerebral administration of cannabinoid-related drugs in rodents models of anxiety

Brain Region	Drug (dose)	Possible mechanism	Species	Animal model	Main effect	Reference
mPFC	mAEA (0.277–27.7 nmol) Unilateral	CB1/TRPV1 agonist	Rat	EPM	Anxiolytic/Anxiogenic (bell-shaped)	Rubino et al. (2008b)
	AEA (5 pmol) Bilateral	CB1/TRPV1 agonist	Rat	CFC	↓ CER	Lisboa et al. (2010)
	THC (32 nmol) unilateral	CB1 agonist	Rat	EPM	Anxiolytic (bell-shaped)	Rubino et al. (2008a)
	AM251 (18 nmol) Unilateral	CB1 antagonist	Rat	EPM	No effect	Rubino et al. (2008a)
	AM251 (100 pmol) Bilateral	CB1 antagonist	Rat	CFC (lower shock intensity)	↑ CER	Lisboa et al. (2010)
	AM404 (50 pmol) Bilateral	AEA uptake/metabolism inhibitor	Rat	CFC	↓ CER	Lisboa et al. (2010)
	URB597 (0.0296–2.96 nmol) Unilateral	AEA metabolism inhibitor	Rat	EPM	Anxiolytic (bell-shaped)	Rubino et al. (2008b)
	Capsaicin (16.37–32.74 nmol)	TRPV1 agonist	Rat	EPM	Anxiogenic	Rubino et al. (2008b)
	Capsazepine (13.27 nmol)	TRPV1 antagonist	Rat	EPM	No effect; blocked mAEA 10 μg effect	Rubino et al. (2008b)
	Capsazepine (1–60 nmol) Bilateral	TRPV1 antagonist	Rat	EPM, VCT	Anxiolytic	Aguiar et al. (2009)
	CBD (30 nmol)	?	Rat	CFC	↓ (PrL) or ↑ (IL) CER	Lemos et al. (2010)
BLA	WIN55, 212-2 (9.6 nmol)	CB1/TRPV1 agonist	Rat	Elevated platform + Inhibitory avoidance	↓ stress effect	Ganon-Elazar and Akirav (2009)
	THC (3.2 nmol) Unilateral	CB1 agonist	Rat	EPM	Anxiogenic	Rubino et al. (2008a)
	AM251 (1.8 nmol) Unilateral	CB1 antagonist	Rat	EPM	No effect	Rubino et al. (2008a)
	AM251 (11 pmol)	CB1 antagonist	Rat	Elevated platform + Inhibitory avoidance	↑ stress effect	Ganon-Elazar and Akirav (2009)
	Rimonabant (100 nmol) Unilateral	CB1 antagonist	Rat	CFC	↓ extinction	Roche et al. (2007)
CeA	ACPA (0.4–14.5 pmol)	CB1 agonist	Rat	EPM	Anxiolytic	Zarrindast et al. (2008, 2010a)
	THC (320–470 nmol)	CB1 agonist	Mice	EPM	Anxiogenic	Onaivi et al. (1995)
BNST	CBD (30 and 60 nmol) Bilateral	5-HT1A agonist	Rat	CFC, VCT, EPM	Anxiolytic	Gomes et al. (2010)
Dorsal hippocampus	WIN55, 212-2 (1.9–5.6 nmol)	CB1 agonist	Rat	EPM	Anxiogenic	Roohbakhsh et al. (2007)
	WIN55, 212-2 (0.48 nmol)	CB1 agonist	Mice	Hole-board	Prevented anxiogenic effect of histamine	Zarrindast et al. (2010b)
	AM404 (50 pmol) Bilateral	AEA uptake/metabolism inhibitor	Rat	VCT	Anxiolytic	Nejo et al. (2009)
	URB597 (0.01 nmol) Bilateral	AEA metabolism inhibitor	Rat	VCT	Anxiolytic	Nejo et al. (2009)
Ventral hippocampus	THC (16–32 nmol)	CB1 agonist	Rat	EPM	Anxiolytic (bell-shaped)	Rubino et al. (2008a)
	AM404 (5–50 pmol)/AM251 (0.01–1000 pmol)	AEA uptake/metabolism inhibitor/CB1 antagonist	Rat	EPM, VCT	Anxiogenic (EPM)/anxiolytic (EPM post-restraint, VCT)/ No effect	Campos et al. (2010)

(continued)

Table 1. Continued

Brain Region	Drug (dose)	Possible mechanism	Species	Animal model	Main effect	Reference
	URB597 (0.03–3 nmol)/AM251 (1.8–180 pmol)	AEA metabolism inhibitor/ CB1 antagonist	Rat	EPM	Anxiogenic/No effect	Roohbakhsh et al. (2009)
	Capsazepine (2 nmol)	TRPV1 antagonist	Rat	EPM	Anxiolytic	Santos et al. (2008)
dPAG	AEA (0.5–50 pmol)	CB1/TRPV1 agonist	Rat	EPM, VCT, CFC	Anxiolytic, ↓ CER	Moreira et al. (2007), Lisboa et al. (2008), Resstel et al. (2008c)
	ACEA (0.05–5 pmol)	CB1 agonist	Rat	EPM	Anxiolytic	Moreira et al. (2007)
	HU210 (0.25–12.9 nmol))	CB1 agonist	Rat	Escape induced by chemical stimulation, ultrasound induced hyperlocomotion	Antiaversive	Finn et al. (2003, 2004b)
	WIN55, 212-2 (3–30 pmol)	CB1/TRPV1 agonist	Rat	EPM	Anxiolytic	Campos and Guimarães (2009)
	AM251 (10–100 pmol)	CB1 antagonist	Rat	EPM, VCT	No effect	Moreira et al. (2007), Lisboa et al. (2008)
	URB597 (0.01 nmol)	AEA metabolism inhibitor	Rat	VCT	Anxiolytic	Lisboa et al. (2008)
	AM404 (50 pmol)	AEA uptake/metabolism inhibitor	Rat	VCT, CFC	Anxiolytic ↓ CER	Lisboa et al. (2008), Resstel et al. (2008c)
	Capsazepine (10–60 nmol)/ Capsaicin (0.01–1 nmol)	TRPV1 antagonist/agonist	Rat	EPM, VCT	Anxiolytic	Terzian et al. (2009)
	CBD (10–60 nmol)	5-HT1A agonist	Rat	EPM, ETM and escape induced by dPAG stimulation	Anxiolytic/Panicolytic	Campos and Guimarães (2008), Soares et al. (2010)

Drugs: ACEA, arachidonoylchloroethanolamide; ACPA, arachidonyl cyclopropylamide; AEA, anandamide; CBD, cannabidiol; mAEA, methanandamide.
Regions: BLA, basolateral amygdala; BNST, bed nucleus of the stria terminalis; CeA, central amygdala; mPFC, medial prefrontal cortex; IL, infra-limbic cortex; PrL, prelimbic cortex; dPAG, dorsal periaqueductal gray.
Animal models: CER, conditioned emotional response, CFC, contextual fear conditioning; EPM, elevated plus-maze; ETM, elevated T-maze; VCT, Vogel conflict test.

In agreement with these observations, systemic administration of a CB1 receptor antagonist increased neuronal activation in the vmPFC (Alonso et al., 1999). These results suggest that glutamatergic EPSCs evoked in vmPFC cells are tonically inhibited by endogenous cannabinoids through CB1 receptors. Taken together, these findings indicate that cannabinoids could act through a presynaptic mechanism to suppress excitatory synaptic activity in the frontal cortex (Li et al., 2010). In this way, a CB1-mediated inhibition of glutamate release in the mPFC could be a potential mechanism of fear adaptation (Kamprath et al., 2009; Lisboa et al., 2010).

Intra-mPFC administration of a higher dose of mAEA enhanced anxiety-like behaviours, an effect that was prevented by a TRPV1 receptor antagonist (Rubino et al., 2008b). High doses of some cannabinoids such as AEA and WIN55,212-2 have been shown to activate these receptors (Di Marzo et al., 2001; Pertwee, 2008). TRPV1 receptors can increase glutamate release, which would facilitate defensive responses in the mPFC (Marsch et al., 2007; Resstel et al., 2008b; Terzian et al., 2009; Toth et al., 2005). Together, these findings suggest that the biphasic dose–response curve caused by AEA in the mPFC depends on activation of TRPV1 receptors by a high drug concentration (Rubino et al., 2008b). Corroborating this possibility, anxiogenic effects were observed after local administration of capsaicin, a TRPV1 receptor agonist (Rubino et al., 2008b), whereas capsazepine, an antagonist at these receptors, induced anxiolytic-like effects in rats submitted to the EPM and in the Vogel conflict test (Aguiar et al., 2009) (see Table 1).

Systemic administration of the non-psychotomimetic phytocannabinoid CBD was also able to block CERs evoked by contextual aversive conditioning (Resstel et al., 2006b), an effect that was associated with decreased c-Fos expression in the PrL, IL and bed nucleus of the stria terminalis (BNST) regions (Lemos et al., 2010). This effect may involve a drug action in the PrL, since direct CBD injection into this region also decreased the CERs. Interestingly, opposite effects were observed when the drug was injected into the IL (Lemos et al., 2010) (see Table 1). The mechanisms of these opposite effects remain to be investigated.

The amygdaloid complex

The amygdala is a heterogeneous gray matter complex (Pitkanen et al., 1997) that modulates neuroendocrine functions and behavioural responses related to fear, anxiety, defence, aggression, memory, and learning (Adolphs et al., 1994; LeDoux, 2000; McNaughton and Corr, 2004; Morris et al., 1996; Scott et al., 1997). These functions involve specific amygdaloid nuclear groups identified by cytoarchitectonic, histochemical, and immunocytochemical features (for review see Sah et al., 2003; Swanson and Petrovich, 1998). Among them, three have been the focus of much of the research concerning defensive behaviour: the basolateral complex (BLA), central nucleus (CeA) and medial nucleus (MeA) (Sah et al., 2003). Lesions of the amygdala block the effects of unconditioned and conditioned stimulus in a variety of behavioural situations (Blanchard and Blanchard, 1972; LeDoux et al., 1988, 1990; Luiten et al., 1985; Rosen, 2004). Together with the PAG and hypothalamus, it is proposed to be responsible for the behaviour and neurovegetative responses to threatening stimuli (Graeff, 1994).

CB1 receptors are present in the BLA and, at lower levels, in the CeA and MeA (Herkenham et al., 1990; Matsuda et al., 1993; McDonald and Mascagni, 2001; Tsou et al., 1998a). Immunohistochemical studies have also demonstrated that BLA neurons contain the FAAH enzyme (Tsou et al., 1998b). Tissue content of AEA and 2-AG in the amygdala is modulated in response to 'psychological' stressors, as first demonstrated by Marsicano et al. (2002) and later by others (Patel et al., 2005b; Rademacher et al., 2008), and restraint stress increases the hydrolytic activity of FAAH and decreases AEA levels throughout the amygdala (Hill et al., 2009). Studies investigating the effects of intra-amygdaloid administration of cannabinoids on anxiety, however, have produced conflicting results. Systemic or intracerebroventricular (i.c.v.) administration of CB1 receptor agonists induced c-Fos expression in the CeA of rats (Arnold et al., 2001; McGregor et al., 1998, 2004; Patel et al., 1998, 2005a; Valjent et al., 2002). Furthermore, intra-CeA administration of THC produced anxiety-like responses in mice on the EPM (Onaivi et al., 1995). Similar results were verified when THC was injected into the BLA in rats (Rubino et al., 2008a) (see Table 1). In healthy volunteers, THC increased amygdala activation in response to fearful faces, an effect directly correlated with anxiety feelings (Bhattacharyya et al., 2010).

Contrasting with these findings, Zarrindast et al. (2008, 2010a) observed that microinjection of arachidonylcyclopropylamide (ACPA), a CB1 receptor agonist, into the CeA produces anxiolytic-like effects. Moreover, the anxiolytic-like effects of low doses of THC injected systemically were associated with decreased c-Fos expression in the amygdala, an effect prevented by AM251 (Rubino et al., 2007). Corroborating this finding, an anxiogenic systemic dose of the CB1 receptor antagonist AM251 increased c-Fos expression in the amygdala (Sink et al., 2010). Similar effects on c-Fos expression were observed with rimonabant in the CeA and BLA (Patel et al., 2005a). Moreover, local administration into the BLA of a CB1 receptor agonist and a FAAH inhibitor attenuated stress-induced corticosterone secretion, whereas microinjection of a CB1 receptor antagonist increased it (Hill et al., 2009). Similar effects were observed in animals exposed to an elevated platform stress. In this case, intra-BLA administration of WIN55,212-2 also decreased the enhancing effect of stress on inhibitory avoidance conditioning (Ganon-Elazar and Akirav, 2009) (see Table 1). In humans, anxiolytic doses of THC significantly reduced amygdala reactivity to social signals of threat (Phan et al., 2008). Together, these results suggest that the eCB system exerts an inhibitory role in amygdala activity related to threatening stimuli and decreases the magnitude of HPA axis response to stressful stimuli. At high doses, however, CB1 agonists injected into this region could facilitate anxiety-related responses. As in other brain regions, these opposite effects could be related to a CB1-mediated decrease of glutamatergic or GABAergic neurotransmission, respectively (Azad et al., 2003, 2008; Katona et al., 2001). Although Katona et al. (2001) have observed no effect of cannabinoids on GABAergic transmission in this region, a recent study by

Roberto et al. (2010) verified that CB1 receptor activation in the CeA decreased evoked $GABA_A$ receptor-mediated inhibitory postsynaptic potentials. In this study, it was also observed that superfusion of the CB1 receptor antagonists onto CeA neurons augmented inhibitory responses, suggesting that tonic eCB activity decreases inhibitory transmission in CeA (Roberto et al., 2010). Effects on glutamatergic transmission in the CeA, however, have not been studied. No data regarding the cellular effects of cannabinoids in the MeA are available.

The amygdala has also been closely associated to conditioning to both discrete and contextual cues (Beck and Fibiger, 1995; Phillips and LeDoux, 1992; Schafe et al., 2005; Sullivan et al., 2004), and several studies suggest that it is involved in the effects of cannabinoids on consolidation and extinction of aversive memories (Bucherelli et al. 2006; Cannich et al. 2004; Campolongo et al., 2009; Ganon-Elazar and Akirav, 2009; Lin et al., 2006; Marsicano et al., 2002; Roche et al., 2007). Indeed, exposure to a tone previously paired with footshock increases both AEA and 2-AG release in the BLA, but not in the PFC (Marsicano et al., 2002). Either systemic (Marsicano et al., 2002) or local (Roche et al., 2007) injection of rimonabant impairs the extinction of CER.

Although we could not detect any significant effects of CBD (7.5–30 nmol) after BLA injections in rats submitted to the EPM (Lisboa et al., unpublished data), the drug was able to attenuate activity in the left amygdala and hippocampus regions and decrease anxiety in healthy subjects, suggesting that this region could also be implicated in the effects of this drug (Crippa et al., 2004; Fusar-Poli et al., 2009).

Therefore, even though CB1 expression in some amygdaloid nuclei has been reported to be low (Katona et al., 2001; Tsou et al., 1998a), local cannabinoid injection does induce changes in defensive-related responses. This expression, however, has been a matter of debate in some brain regions or neural populations. In any case, even if modestly, different amygdaloid nuclei seem to partially account for the behavioural effects of cannabinoids.

The bed nucleus of the stria terminalis

The BNST is a highly heterogeneous and complex limbic structure associated with autonomic, neuroendocrine and behavioural functions (Casada and Dafny, 1991; Dunn, 1987; Dunn and Williams, 1995). It has reciprocal connections with the MeA and CeA (Dong et al., 2001a; Shammah-Lagnado et al., 2000) and receives projections from the hippocampus (Dong et al., 2001b), BLA (Dong et al., 2001a) and the mPFC (Vertes, 2006). Several studies have shown that the BNST is critically involved with the expression of anxiety-like responses (Davis et al., 2010; Walker et al., 2003), including CERs and cardiovascular responses to aversive situations (Crestani et al., 2009; Resstel et al., 2008a; Sullivan et al., 2004).

CB1 receptors are present in GABAergic neurons in the BNST (Matsuda et al., 1993; Tsou et al., 1998a). Similar to the CeA, CB1 receptor agonists are also able to induce c-Fos expression in this region (Arnold et al., 2001; Patel et al., 1998; Valjent et al., 2002). Activation of group I metabotropic glutamatergic receptors in the BNST induced a transient depression of excitatory synaptic transmission that is CB1 receptor dependent (Grueter et al., 2006). More recently, Puente et al. demonstrated that activation of CB1 receptors inhibits excitatory and inhibitory synaptic transmission in this region, which could be important for the regulation of aversive responses (Puente et al., 2010). Despite these pieces of evidence, no study so far has investigated the effects of direct injections into the BNST of CB1 receptor agonist or antagonist in animals submitted to animal models of anxiety. A recent study from our group showed that CBD administration into the BNST is able to attenuate CERs and produces anxiolytic-like effects in rats submitted to the EPM and Vogel conflict test by activating local 5-HT1A receptors (Gomes et al., 2010) (see Table 1).

The hippocampal formation

In addition to mnemonic processing, spatial learning and navigation, the septum–hippocampal system is also proposed to perform context analysis in threatening situations, and to generate anxiety in response to conflict by interrupting ongoing behaviour and increasing the level of arousal and attention (Gray and McNaughton, 2000; McNaughton and Corr, 2004). CB1 receptors are widely expressed in presynaptic terminals in the hippocampus. Although these receptors are prominently present in GABAergic terminals (Marsicano and Lutz, 1999), they can also be found in other hippocampal neuronal subpopulations, including glutamatergic, serotonergic and cholinergic (for review see Katona and Freund, 2008).

Direct injections of cannabinoids into the hippocampus have produced opposite results (Campos et al., 2010; Nejo et al. 2009; Roohbakhsh et al., 2007, 2009; Rubino et al., 2007) (see Table 1). Although the mechanisms responsible for these contradictory results are not yet clear, it has been argued that they are related to the stressful experiences of the animal (Campos et al., 2010). Enhancement of eCB signalling in the dorsal or ventral hippocampus induced anxiogenic effects in non-stressed rats tested in the EPM (Roohbakhsh et al., 2007). However, anxiolytic effects were found in animals submitted to the Vogel conflict test, an animal model that involves a stressful experience represented by 48 h of water deprivation. Moreover, in the EPM, the anxiogenic effect of AM404, an AEA uptake and metabolism inhibitor, turned into an anxiolytic one when the animals were previously (24 h) submitted to a 2 h restraint period (Campos et al. 2010). Contrasting with these results, Rubino et al. (2008a) showed anxiolytic effects of THC in the ventral hippocampus of rats submitted to the EPM. In this study, however, the rats were maintained isolated in their home cages, a well-known stress factor in these animals (Maisonnette et al., 1993).

Although the ventral hippocampus, a region closely connected to the prefrontal cortex and to subcortical structures associated with the HPA axis, including the BNST and amygdala (Krettek and Price, 1977; Petrovich et al., 2001; Siegel and Tassoni, 1971), has been proposed to play a preferential role in anxiety-related behaviours (Bannerman et al. 2004; Bertoglio et al., 2006), the effects of cannabinoid in anxiety

models after injection into this region seem to be similar to those observed after dorsal hippocampus administration (see Table 1).

The molecular mechanisms of these effects are not clear. Activation of CB1 receptors in the hippocampus seems to control various forms of long-term synaptic plasticity such as long-term potentiation (LTP) at excitatory synapses and depolarization-induced suppression of inhibition (DSI) that results in a facilitation of LTP formation by endocannabinoid-mediated disinhibition (Wilson et al., 2001). While the role of CB1 receptors located in GABAergic terminals seems to be well established (Hájos and Freund, 2002), the picture regarding glutamatergic synaptic transmission is not so clear. Using a genetic dissection of THC effects in mice, Monory et al. (2007) showed that several important pharmacological effects of this drug do not depend on functional expression of CB1 receptors on GABAergic interneurons, but rather on other neuronal populations, particularly glutamatergic neurons. In addition, deletion of CB1 receptors in glutamatergic, but not in GABAergic, neurons promoted a decrease in the behaviour and neuroendocrine responses induced by forced swimming stress (Steiner et al., 2008). Moreover, Jacob et al. (2009) investigated the consequences of CB1 deletion in glutamatergic neurons in animals exposed to several models of anxiety-related behaviours. These animals showed increased open-arms avoidance during a re-exposure to the EPM and increased thigmotaxis in a bright open field, though no phenotype was detected in the light–dark test and in the first exposure to the EPM. The authors suggest that the involvement of the endocannabinoid system may depend on the controllability of the stress situation (see below).

Besides interference with neurotransmitter release, plastic changes in the hippocampus, represented by increased neurogenesis, have also been associated with the anxiolytic effects of cannabinoids (Jiang et al., 2005).

The periaqueductal gray

The midbrain PAG presents a morphofunctional organization that comprises dorsomedial, dorsolateral (dlPAG), lateral and ventrolateral columns (Bandler et al., 2000; Carobrez, 2003). In addition, the dorsal portion of PAG (dPAG), which includes the dorsomedial and dlPAG columns, is responsible for elaborating active defensive strategies, such as fight and flight behaviours to proximal threats (Aguiar and Guimarães, 2009; Bandler et al., 2000; Beijamini and Guimarães, 2006; Carrive, 1993; Canteras and Goto, 1999). The ventrolateral columns, on the other hand, are more relevant for mediating analgesia and to elaborate passive stress-coping strategies, such as freezing to conditioned aversive stimuli (Bandler et al., 2000). The PAG has also been related to the modulation of subtle defensive behaviours, such as the fear-like responses observed in rats submitted to the EPM or exposed to a novel open field (Nagahara and Handa, 1997) and other stressful stimuli, including immobilization (Cullinan et al., 1995), footshocks (Campeau et al., 1997a) and audiogenic stress (Campeau et al., 1997b).

Several neurotransmitters have been shown to modulate defensive responses in the PAG including glutamate, serotonin, GABA, nitric oxide and neuropeptides (Adamec et al., 1999; Aguiar and Guimarães, 2009; Blanchard et al., 1992; Guimarães et al., 2005; McGregor et al., 2004; Moreira and Guimarães, 2008). eCBs may also function as neural mediators in this structure, as suggested by several pieces of evidence. For instance, CB1 receptors and the FAAH enzyme are expressed throughout the midbrain in a complementary fashion (Egertova et al., 2003; Herkenham, 1991). Moreover, increased expression of c-Fos protein in the PAG has been reported after systemic or intracerebroventricular administration of CB1 agonists (McGregor et al., 1998; Navarro et al., 1997). Thus, this brain region has also been proposed as a possible brain site for the anti-aversive effects of cannabinoids.

In an initial investigation, Finn et al. observed that intra-dPAG administration of the synthetic cannabinoid HU210 reduced escape reactions induced by either local injections of the excitatory amino acid, D, L-homocysteic acid, or by ultrasound exposure (Finn et al., 2003, 2004b) (see Table 1). To further investigate a possible role of eCBs in the dlPAG on anxiety modulation, our group showed that local injection of AEA causes anxiolytic-like effects in rats submitted to EPM, which are blocked by previous treatment with AM251 (Moreira et al., 2007). AEA produced a bell-shaped dose-response curve, with higher doses being ineffective. Anxiolytic-like effects of AEA were mimicked by the selective CB1 agonist, ACEA, and were potentiated by previous treatment with AM404. This drug, however, was without effect by itself in this model (Moreira et al., 2007). The anxiolytic effects of AEA in the dlPAG were confirmed in the Vogel conflict test and CFC models (Lisboa et al., 2008; Resstel et al., 2008d). In these studies AEA uptake/metabolism inhibitors also induced anxiolytic-like effects (Lisboa et al., 2008; Resstel et al., 2008d), consistent with the effects observed after systemic administration (Kathuria et al., 2003; Moreira et al., 2008).

In line with results observed in other brain structures, it seems that under more stressful situations, such as those promoted by the Vogel conflict test or CFC, the eCB system in the dlPAG is engaged to a greater extent than when animals were exposed to the EPM. Indeed, studies with knockout mice revealed that the endocannabinoids become relevant in modulating stress preferentially when the stress is inescapable or its intensity is high (Jacob et al., 2009; Kamprath et al., 2009). Interestingly, painful stimuli, such as those used in those two tests, increase AEA levels in the dPAG (Hohmann et al., 2005; Walker et al., 1999).

Further implicating the PAG eCB system in defensive behaviour regulation, we have recently observed that AEA injected into the rat dlPAG reduced the defensive responses induced by cat exposure (Lisboa and Guimarães, unpublished results). Taken together, these results strongly suggest that the eCB system plays an important role in defensive behaviours mediated by the PAG.

Studies employing slices from the PAG showed that activation of CB1 receptors inhibits GABAergic and glutamatergic synaptic transmission (Vaughan et al., 2000), which may be related to the biphasic effects on anxiety-like responses observed with CB1 receptor agonists in the PAG (Moreira et al., 2007). However, PAG neurons also express TRPV1 receptors (Toth et al., 2005) which, as discussed above, are

proposed to facilitate glutamate release and anxiety-like responses (Marsch et al., 2007; Rubino et al., 2008b; Terzian et al., 2009). Similar to the mPFC (Aguiar et al., 2009; Rubino et al., 2008b), local injection of the TRPV1 receptor antagonist capsazepine was anxiolytic by itself (Terzian et al., 2009) and prevented the decrease in open-arm exploration caused by the higher dose of WIN55,212-2 (Campos and Guimarães, 2009) (see Table 1).

Intra-dlPAG administration of CBD also induced anxiolytic effects in the EPM and Vogel conflict test models (Campos and Guimarães, 2008). In addition, this treatment inhibited escape latencies in the elevated T-maze and increased the electrical current threshold to induce flight responses after dPAG stimulation, suggesting a panicolytic effect (Soares et al. 2010). Similar to the BNST, these effects were prevented by pre-treatment with a 5-HT1A receptor antagonist (Campos and Guimarães, 2008; Soares et al., 2010) (see Table 1), suggesting that the anti-aversive effects of CBD involve serotonin- rather than eCB-related mechanisms (Izzo et al., 2009).

It is worth noting that some animal models of anxiety-related behaviour employed in these studies are based on the suppression of punished responses, such as electric shock in the Vogel conflict test (Lisboa et al., 2008; Moreira et al., 2006). Thus, nociceptive responses might be a potential confound factor in the study of anxiolytic-like drugs. This is particularly relevant in studies with drug injection into the PAG, since this structure is classically recognized as a major site for analgesic drugs, including cannabinoids (Palazzos et al., 2006). Therefore, we performed control experiments to exclude the possibility that the results described above would be solely secondary to antinociceptive effects. This was done by testing the active drug doses in the anxiety-related tests in a model of nociceptive responses, the tail-flick test. Since no treatment was active in this test, it can be concluded that the PAG may at least partially account for both the antinociceptive and anxiolytic-like effects of cannabinoids (Palazzos et al., 2006).

Finally, endocannabinoids in the dPAG have also been reported to mediate stress-induced analgesia (Hohmann et al., 2005). Rats previously submitted to footshock have an increase in endocannabinoids in this structure. In addition, local injection of rimonabant inhibits, whereas FAAH- or MAGL inhibitors enhances, stress-induced analgesia. Thus, in addition to opioid mechanisms, the endocannabinoid system in the PAG seems to also mediate this consequence of stress (Hohmann et al., 2005).

As for the ventrolateral columns, most works have focused on the mediation of the analgesic effects of cannabinoids (Palazzos et al., 2006). Whether local eCBs also contribute to defensive responses, such as freezing, remains to be investigated.

Other brain sites

The effects of cannabinoids in other brain areas that express CB1 and have been related to emotional responses, such as the medial hypothalamus and nucleus accumbens, are less well known (Moreira and Lutz, 2008). Local cannabinoid injection into the nucleus accumbens did not modify anxiety-like behaviours (Onaivi et al., 1995); instead, it induced rewarding effects (Zangen et al., 2006). We are currently investigating the effects of cannabinoids administration into the dorsomedial and ventromedial hypothalamic nuclei, two regions where antagonism of glutamate-mediated neurotransmission causes anxiolytic-like effects (Jardim and Guimarães, 2004; Jardim et al., 2005).

Conclusions and perspectives

The present review showed that intra-cerebral injections of CB1 receptor agonists into brain regions closely associated with defence behaviour clearly decrease anxiety-like behaviours in distinct animal models, suggesting that these areas could be involved in the anxiolytic effects observed after systemic administration of these compounds. However, paralleling results obtained after systemic injections, intra-cerebral cannabinoid administration also causes bell-shaped or biphasic dose–response curves.

At least two pharmacological mechanisms have been involved in these effects: (i) activation of TRPV1 receptors by high concentration of some of these compounds (AEA, WIN55,212-2); and (ii) CB1-mediated inhibition of GABAergic and glutamatergic synaptic transmissions (Vaughan et al., 2000), which play opposite roles in anxiety modulation (Moreira et al., 2007). In addition, cannabinoid effects appear to depend on environmental conditions, particularly stress experience. The results observed after intra-cerebral injections of CB1 receptor antagonists or AEA uptake/metabolism inhibitors suggest a preferential involvement of endogenous cannabinoids in situations where the animal was subjected to stressful situations (see Table 1). This agrees with the proposed role of endocannabinoids as part of a 'stress buffer system', recruited by high-demand situations (Lutz, 2009; Moreira and Lutz, 2008; Viveros et al., 2007). Corroborating this proposal, decreased open-arm exploration of the EPM in CB1 receptor knockout mice can only be detected when the intensity of light is very high (Haller et al., 2004). In addition, the anxiolytic-like effects of FAAH-blocker are more consistently seen when the behavioural tests are performed in highly aversive environments, rather than in mildly stressful circumstances (Haller et al., 2009; Moreira et al., 2008).

Stressful inescapable events can promote long-lasting behavioural consequences (for review, see de Kloet et al., 2005). Neural correlates of these stress-induced behavioural changes have been shown in the amygdala, prefrontal cortex, hippocampus and PAG (Conrad et al., 1999) and have also been described for the eCB system (Hill et al., 2005, 2009; Sutt et al., 2008). For example, hippocampal CB1 receptors are sensitive to stressful environments (Hill and Gorzalka, 2006), and eCBs levels increased in the hippocampal formation after a single dexamethasone injection (Hill et al., 2010). Moreover, exposure to chronic unpredictable mild stress produced an up-regulation of hippocampal FAAH levels and decreased CB1 receptor expression in male, although not in female, rats (Reich et al., 2009). However, the specific plastic changes induced by stress on CB1 receptors and CB1-related systems responsible for the opposite cannabinoid effects observed in brain areas such as the hippocampus are far

from clear. One possibility that needs to be investigated is that stressful experiences produce a dissimilar impact on CB1 receptors located in GABAergic and glutamatergic terminals, leading to a greater decrease in glutamate release compared to GABA in response to cannabinoid agonists. Corroborating this possibility, a recent study showed that, at least in the striatum, CB1 receptors related to GABAergic and glutamatergic neurons have distinct regulatory mechanisms (De Chiara et al., 2010). Moreover, epileptic patients showed a significant reduction in the fraction of CB1-positive glutamatergic, but not GABAergic, axon terminals, which could be related to the harmful effects of increased network excitability (Ludányi et al., 2008).

Another important point is that, notwithstanding the major role of AEA as a CB1 ligand (Katona and Freund, 2008), a crosstalk between this cannabinoid and 2-AG may exist in some brain regions, such as in the striatum (Di Marzo and Maccarrone, 2008; Maccarrone et al., 2008). This may be relevant considering that 2-AG is also a major endogenous ligand of CB1 receptors (Katona and Freund, 2008; Tanimura et al., 2010). However, as can be seen in Table 1, studies aimed at investigating the specific role of 2-AG in anxiety control employing, for example, intra-cerebral administration of MAGL inhibitors, are still sparse and need to be performed in the nearby future.

As a last point, our results with CBD suggest that cannabinoids could also modify anxiety-like behaviours acting in specific brain sites by mechanisms that do not directly involve the eCB system.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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