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The role of the brain's endocannabinoid system in pain and its modulation by stress

Louise Corcoran[1,3], Michelle Roche[2,3], David P. Finn[1,3]

[1]Pharmacology and Therapeutics, School of Medicine, National University of Ireland Galway, Ireland; Ireland.

[2]Physiology, School of Medicine, National University of Ireland Galway, Ireland;

[3]Galway Neuroscience Centre and Centre for Pain Research, NCBES, National University of Ireland Galway, Ireland.

Short Running Title: The supraspinal endocannabinoid system in pain and stress

Abstract:

Stress has a complex, bidirectional modulatory influence on pain. Stress may either reduce (stress-induced analgesia) or exacerbate (stress-induced hyperalgesia) pain depending on the nature, duration and intensity of the stressor. The endogenous cannabinoid (endocannabinoid) system is present throughout the neuroanatomical pathways that mediate and modulate responses to painful stimuli. The specific role of the endocannabinoid system in the brain in pain and the modulation of pain by stress is reviewed herein. We first provide a brief overview of the endocannabinoid system, followed by a review of the evidence that the brain's endocannabinoid system modulates pain. We provide a comprehensive evaluation of the role of the endocannabinoid system supraspinally, and particularly in the rostral ventromedial medulla, periaquaductal grey, amygdala and prefrontal cortex, in pain, stress-induced analgesia

and stress-induced hyperalgesia. Increased understanding of endocannabinoid-mediated regulation of pain and its modulation by stress will inform the development of novel therapeutic approaches for pain and its co-morbidity with stress-related disorders.

Key Words: Nociception; fear; Cannabinoid CB_1 receptor; anandamide; 2-arachidonyl glycerol; descending pain pathway

Abbreviations: eCB – Endocannabinoid; Cannabinoid – CB; PAG – periaquaductal grey; RVM – rostral ventromedial medulla; PFC – prefrontal cortex; SIA - stress-induced analgesia; SIH - stress-induced hyperalgesia; FCA - fear-conditioned analgesia; THC - Δ^9-tetrahydrocannabinol; CB_1 - cannabinoid type 1; CB_2 - cannabinoid type 2, AEA – anandamide; 2-AG - 2-arachidonoyl glycerol; CNS – central nervous system; PLD - phospholipase D; NAPE - N-acyl phosphatidylethanolamine; AC – adenylate cyclise; MAPK - mitogen-activated protein kinase; FAAH - fatty acid amide hydrolase; MAGL - monoacylglycerol lipase; FLAT - FAAH-like transporter; FABP – fatty acid binding protein; TRPV1 - transient receptor potential vanilloid 1; PPARs – peroxisome proliferator-activated receptors; PEA - *N*-palmitoylethanolamide; OEA - *N*-oleoylethanolamide; i.c.v. – intracerebroventricular; GiA - gigantocellular reticular nucleus; VPL - ventral posterolateral nucleus of the thalamus; SNL - spinal nerve ligation; ACC - anterior cingulate cortex; HMBA - 4-hydroxy-3-methoxybenzylamine; NGF – nerve growth factor; CUS - chronic unpredictable stress; CCK – cholecystokinin; GiA - gigantocellular reticular nucleus; WKY – wistar-kyoto; SD – sprague dawley; dlPAG - dorsolateral periaquaductal grey; lPAG – lateral periaquaductal grey; vlPAG – ventrolateral periaquaductal grey; dPAG - dorsal periaquaductal grey; dmPAG – dorsomedial periaquaductal grey; CCI - chronic constriction

injury; NSAIDs - non-steroidal anti-inflammatory drugs; OX – orexin; mGlu - metabotropic glutamate receptors; LA - lateral nucleus; BLA - basolateral nucleus; CeA – central nucleus of the amygdala; ABA - accessory basal nucleus; MeA - medial nucleus; mPFC – medial prefrontal cortex; IL – infralimbic cortex; PrL – prelimbic cortex; SNI - spared nerve injury.

Introduction:

Pain can be defined as 'an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage' (International Association for the Study of Pain [IASP] Task Force on Taxonomy, 1994). Recent data indicate that approximately 20% of the population suffer from chronic pain, the majority of whom also suffer from some other disability or mood disturbance (Blyth, et al., 2001; Demyttenaere, et al., 2007; Vos, et al., 2012). Chronic pain is usually defined as pain persisting for over 3 months. It may be neuropathic, inflammatory or idiopathic in nature (Aguggia, 2003). Epidemiological studies of 289 diseases and injuries concluded that chronic pain conditions were among the 10 conditions resulting in the longest number of years lived with disability (Vos, et al., 2012). Current pharmacotherapies for pain management lack efficacy in many patients, with ~40% of patients with chronic pain unsatisfied with their treatment (Breivik, Collett, Ventafridda, Cohen, & Gallacher, 2006). Furthermore, the annual economic cost of pain in the US has been estimated at a staggering $560 - $625 billion annually, including direct and indirect costs (for review see Gaskin & Richard, 2012; McCarberg & Billington, 2006; Turk, 2002). Despite the efforts of the research community and the pharmaceutical industry to invest in and develop new drugs to manage pain, chronic pain in particular continues to represent a major unmet clinical need. Thus, further research is needed to understand fully the

neurobiological mechanisms of pain, and its modulation, with a view to identifying novel targets and developing new, superior analgesics.

Increasing evidence over the past two decades has demonstrated that the endogenous cannabinoid (endocannabinoid; eCB) system has a regulatory role in pain processing and perception (Woodhams, Sagar, Burston, & Chapman, 2015). This regulatory function is facilitated by the expression of the eCB signalling machinery at neuronal synapses within all components of the pain pathway. Activation of cannabinoid receptors on presynaptic nerve terminals generally functions to reduce neurotransmission, resulting primarily in antinociception/analgesia. However, depending on physiological and pathological state, the tissue concentration of eCBs and expression levels of eCB-sensitive receptors can vary (Alexander & Kendall, 2007; Woodhams, et al., 2015), and with it the regulatory potential of this system on nocieptive processing.

The intensity and severity of perceived pain does not necessarily correlate with the degree of tissue damage, injury or inflammation occurring. The importance of context and modulation of pain by emotion is now widely recognised. Stress, fear and anxiety exert important modulatory influences on pain (Asmundson & Katz, 2009; N.N. Burke, Finn, & Roche, 2015; Butler & Finn, 2009; Fitzgibbon, Finn, & Roche, In Press; Ford & Finn, 2008; Jennings, Okine, Roche, & Finn, 2014; Okine, et al., 2014; Rhudy & Meagher, 2000, 2001; Wiech & Tracey, 2009). Regardless of arousal level, positive emotions generally act to inhibit pain, while negative emotions with low to moderate arousal tend to enhance pain, and negative emotions with high arousal inhibit pain (de Wied & Verbaten, 2001; Dougher, 1979; Meagher, Arnau, & Rhudy, 2001; Rhudy & Meagher, 2000, 2001, 2003a, 2003b). Thus, a complex relationship exists between emotion and pain processing. Cannabinoid (CB) receptors are localized in brain regions involved in the modulation of pain including the rostral ventromedial medulla (RVM), the periaqueductal grey (PAG), amygdala and prefrontal cortex (PFC) (Herkenham, et al.,

1991; Tsou, Brown, Sanudo-Pena, Mackie, & Walker, 1998) with these brain regions also key components of stress, fear and anxiety circuitry. Stress and fear have been shown to alter levels of eCBs in these brain regions (Hill, et al., 2013; Hill, et al., 2005; Hohmann, et al., 2005; Jennings, et al., 2014; Olango, Roche, Ford, Harhen, & Finn, 2012; Patel, Cravatt, & Hillard, 2005; Rademacher, et al., 2008), (for review see Carrier, Patel, & Hillard, 2005; Morena, Patel, Bains, & Hill, 2015). Thus, the eCB system is an important common denominator in pain, stress and fear and its role in the aforementioned brain regions in pain and the modulation of pain by stress is the main focus of this Chapter.

We will consider the role of the supraspinal eCB system in acute and chronic pain, as well as its role in both stress-induced analgesia (SIA) and stress-induced hyperalgesia (SIH). The role of the spinal and peripheral eCB system in pain or stress-pain interactions is beyond the scope of this review but has been reviewed previously by ourselves and others (Butler & Finn, 2009; Finn, 2010; Hohmann & Suplita, 2006; Jennings, et al., 2014; Maccarrone, et al., 2015; Olango & Finn, 2014; Walker & Hohmann, 2005).

The endocannabinoid system

The medicinal properties of the *Cannabis sativa* plant have been known for millennia but it was not until the mid to late nineteenth century that its therapeutic potential was examined scientifically. The discovery of Δ^9-tetrahydrocannabinol (THC), the major psychoactive component of the plant *Cannabis sativa* in the 1960s (Mechoulam & Gaoni, 1967) led to extensive studies that have revealed the mechanisms underlying the physiological and pharmacological effects of the eCB system.

The eCB system as we know it today consists of CB type 1 (CB_1) (Devane, Dysarz, Johnson, Melvin, & Howlett, 1988; Matsuda, Lolait, Brownstein, Young, & Bonner, 1990) and CB type

2 (CB_2) receptors (Munro, Thomas, & Abu-Shaar, 1993), their endogenous ligands *N*-arachidonylethanolamide (anandamide, AEA) and 2-arachidonoyl glycerol (2-AG) (Devane, et al., 1992; Mechoulam, et al., 1995; Sugiura, et al., 1995), and the enzymes responsible for their synthesis and degradation. AEA and 2-AG are the best characterised eCBs, however there are a number of other endogenous ligands with affinity and activity at CB_1 and CB_2 receptors including 2-AG ether (noladin ether), virodhamine, *N*-arachidonyl dopamine (NADA) and others (for review see Battista, Di Tommaso, Bari, & Maccarrone, 2012; Di Marzo, 2008; Di Marzo, Stella, & Zimmer, 2015; Henry, Kerr, Finn, & Roche, 2015; Pertwee, 1997, 2001).

The CB receptors in the adult human brain and spinal cord are distributed in a heterogeneous fashion (Glass, Dragunow, & Faull, 1997). CB_1 receptors are the most predominant CB receptor subtype in the CNS, (Glass, et al., 1997; Herkenham, et al., 1991; Pertwee, 1997), with particularly high density in brain regions that are key components of the descending inhibitory/facilitatory pain pathways and the stress/fear/anxiety circuitry. CB_2 receptors, although expressed in the CNS (Baek, Zheng, Darlington, & Smith, 2008; Concannon, Okine, Finn, & Dowd, 2015; Onaivi, et al., 2006; Van Sickle, et al., 2005; Zhang, et al., 2014) are mainly distributed in the periphery with particularly high density on cells and tissues of the immune system (Berdyshev, 2000; Munro, et al., 1993; Sugiura, et al., 1995) CB_1 and CB_2 receptors are Gi/o protein-coupled receptors negatively coupled to adenylate cyclase (AC), (Howlett, 1985; Howlett, Mukhopadhyay, Shim, & Welsh, 1999) and positively coupled to mitogen-activated protein kinase (MAPK) (Bouaboula, et al., 1995). Upon binding to CB_1 receptors, eCBs also inhibit N- and P/Q-type voltage-activated Ca^{2+} channels and induce inwardly rectifying K^+ currents, resulting in inhibition of neurotransmitter release (Demuth & Molleman, 2006).

The biosynthetic pathways for AEA are not fully characterized but the best described mechanism involves the formation of AEA from the precursor N-

arachidonoylphosphatidylethanolamine (NAPE), due to the hydrolytic activity of the phospholipase D enzyme known as NAPE-PLD (Bisogno, Ligresti, & Di Marzo, 2005). 2-AG is synthesized almost exclusively by phospholipase C (PLC) hydrolysis producing 1,2-diacylglycerol which is then converted to 2-AG by diacylglycerol lipases (DAGL) (Di Marzo, 2008; Howlett & Mukhopadhyay, 2000). For a more complete discussion of the biosynthetic routes for AEA and 2-AG please refer to Chapter 1. AEA is primarily degraded to arachidonic acid and ethanolamine by the enzyme fatty acid amide hydrolase (FAAH), located in the endoplasmic reticulum of the postsynaptic neuron (Cravatt, et al., 1996; Giang & Cravatt, 1997), (for review see Otrubova, Ezzili, & Boger, 2011). FAAH also catabolises additional *N*-acylethanolamines including *N*-palmitoylethanolamide (PEA) and *N*-oleoylethanolamide (OEA) which themselves do not have appreciable activity at CB_1 or CB_2 receptors but which can elevate levels of AEA through substrate competition at FAAH (Di Marzo, et al., 1994; Sugiura, et al., 1995). In contrast, 2-AG is primarily metabolized to arachidonic acid and glycerol by the enzyme monoacylglycerol lipase (MAGL) (Ueda, Tsuboi, Uyama, & Ohnishi, 2011), with other enzymes including FAAH, ABHD6 and ABHD12 accounting for a modest degree of 2-AG catabolism (Blankman, Simon, & Cravatt, 2007; Goparaju, Ueda, Yamaguchi, & Yamamoto, 1998). FAAH is primarily a postsynaptic enzyme, whereas MAGL is presynaptic (Egertova, Cravatt, & Elphick, 2003; Gulyas, et al., 2004; Tsou, Nogueron, et al., 1998), (for review see Blankman & Cravatt, 2013; Di Marzo, 2008; Lichtman, Blankman, & Cravatt, 2010).

The mechanisms underlying eCB biosynthesis, signalling and degradation are relatively well understood although controversy remains surrounding the mechanisms by which eCBs are transported across cell membranes. It has been proposed that due to their lipophilic nature, eCBs are readily transported via a simple diffusion mechanism (Glaser, et al., 2003; Kaczocha, Hermann, Glaser, Bojesen, & Deutsch, 2006) while others suggest the existence of a protein

facilitated transport process (Beltramo & Piomelli, 2000; Hillard, Edgemond, Jarrahian, & Campbell, 1997). Most recently, a FAAH-like transporter (FLAT) has been described as the main mediator for AEA transport (Fu, et al., 2012). Furthermore, fatty acid binding proteins (FABPs) are small cytoplasmic lipid transport proteins (Furuhashi & Hotamisligil, 2008) located both peripherally (De Leon, et al., 1996) and in the CNS (Yamamoto, et al., 2009). FABP5 and FABP7 are capable of binding eCBs and regulating their signalling and catabolism by FAAH (Cravatt, et al., 2001; Kaczocha, Glaser, & Deutsch, 2009; Kaczocha, Vivieca, Sun, Glaser, & Deutsch, 2012).

In addition to the two classical CB receptors (CB_1 and CB_2), several lines of evidence suggest that eCBs act at numerous other non-CB_1/non-CB_2 including the transient receptor potential vanilloid 1 (TRPV1), members of the nuclear receptor family of peroxisome proliferator-activated receptors (PPARs), and the G-protein coupled receptors GPR55 and GPR119 (Alexander & Kendall, 2007; Brown, 2007; O'Sullivan, 2007).

The endocannabinoid system in the brain regulates pain

Considerable effort has been invested in investigating the brain regions involved in mediating the anti-nociceptive effects of eCBs and CB receptor agonists. Later sections will discuss in more detail the role of the eCB system in individual brain regions in pain and its modulation by stress. Presented in this section is an overview of the studies that have identified a role of the eCB system, supraspinally, in the modulation of pain (and summarised in Figure 1).

Strong evidence of a role for the supraspinal eCB system in the modulation of pain was provided by (Hohmann, Tsou, & Walker, 1999). Here, systemic administration of the CB receptor agonist WIN55,212-2 resulted in an anti-nociceptive effect in the tail flick test in rats. Transection of the spinal cord and thus blockade of descending pain processes, inhibited the

CB-induced suppression of noxious heat-evoked activity in the tail flick test, thus indicating that WIN55,212-2 acted supraspinally to mediate its anti-nociceptive efficacy. This study paved the way for the investigation of the role of supraspinal sites in CB-induced anti-nociception.

Anti-nociceptive activity of CB receptor agonists had been demonstrated in the mouse and rat tail flick tests following intracerebroventricular (i.c.v.) administration. Specifically, i.c.v. injection of the cannabinoid agonist WIN55,212-2, Δ^9-THC and CP-55,940 produced antinociception in the rat tail flick test (Lichtman, Cook, & Martin, 1996; Martin, Lai, Patrick, Tsou, & Walker, 1993). In spinally transected rats, i.c.v. administration of the CB_1 receptor antagonist/inverse agonist rimonabant completely blocked the anti-nociceptive effects of Δ^9-THC and CP 55,940 in the rat tail flick test, indicating that these effects are mediated through CB_1 receptors in the brain. The antagonist failed to block the effects of morphine, indicating its selectivity for CB receptors (Lichtman & Martin, 1997). However, when administered via the same route, i.c.v. administration of Δ^9-THC enhances the anti-nociceptive potency of morphine (Welch, Thomas, & Patrick, 1995) suggesting a synergistic interaction between the opioid and CB systems. Similar to Martin et al., 1993, i.c.v. injection of WIN 55,212-2 and THC produced dose related anti-nociceptive effects in the mouse tail flick test (Raffa, Stone, & Hipp, 1999). In addition i.c.v. administration of WIN 55,212-2 induces anti-nociception in the mouse tail flick and paw withdrawal test (Fang, et al., 2012). It was also shown that rimonabant has greater efficacy in the mouse tail flick test at a supraspinal rather than spinal level when blocking the action of THC, HU210, CP55,940, and AEA (Welch, Huffman, & Lowe, 1998). Thus, taken together supraspinal CB_1 receptors are important in modulating pain processes. Although i.c.v. administration of pharmacological agents is a useful means of investigating the contribution of the brain in general, alternative approaches are required to study the role of the eCB system in specific brain regions in pain and its modulation by stress.

These approaches and the results obtained are discussed later in the review for each of the key brain regions that comprise the descending pain pathways (RVM, PAG, amygdala and prefrontal cortex). Additionally, less characterized mechanisms and targets will also be discussed towards the end of the review. Lines of evidence implicating the supraspinal eCB system in pain, stress and their interaction, will be considered briefly now.

[Figure 1 here]

The modulation of pain by stress: role for the brain's endocannabinoid system

Both painful (Alexander & Kendall, 2007; Kwilasz, et al., 2014; Walker, Huang, Strangman, Tsou, & Sanudo-Pena, 1999; Woodhams, et al., 2015) and aversive (stress/fear) (Hill, et al., 2013; Hill, et al., 2005; Hohmann, et al., 2005; Jennings, et al., 2014; Olango, et al., 2012; Patel, et al., 2005; Rademacher, et al., 2008) stimuli have been shown to alter eCB levels and expression of key components of the eCB system in supraspinal regions (for review see Morena, et al., 2015). As highlighted earlier, emotion and stress can profoundly impact on

nociceptive processing, with chronic stress paradigms shown to enhance pain perception under a variety of experimental conditions. Chronic unpredictable stress (CUS), a widely used model for inducing anxiety and depressive-like behaviour in mice, has been shown to enhance thermal (hot plate test) and mechanical (von Frey) hyperalgesia. It has also been shown to induce long lasting widespread hyperalgesia following intramuscular injection of nerve growth factor (NGF) (Lomazzo, et al., 2015). The FAAH and MAGL inhibitors URB597 and JZL184 attenuated the CUS-induced anxiety-related behaviour in the light-dark box and thermal hyperalgesia in the hot plate test. URB597 significantly reduced the widespread hyperalgesia

induced by combining CUS and NGF in this study, while JZL184 had no significant effect. Both drugs enhanced the levels of AEA and 2-AG respectively in the midbrain and cingulate cortex (Lomazzo, et al., 2015). These data highlight the strong potential for pharmacological inventions aimed at increasing eCB levels supraspinally in both anxiety- and pain-related disorders.

I.c.v. administration of rimonabant increases levels of the stress hormones, adrenocorticotropic hormone and coricosterone, in rats, suggesting a role for supraspinal CB_1 receptors in the neuroendocrine response to stress (Manzanares, Corchero, & Fuentes, 1999). CB_1(-/-) knockout mice develop normal mechanical hypersensitivity but more pronounced anxiety-related behaviour following partial sciatic nerve ligation, indicating a potential role for the EC system in chronic co-morbid pain/anxiety disorders (Racz, Nent, Erxlebe, & Zimmer, 2015). Indeed, the acquisition, expression and extinction of fear-related behaviour have all been shown to involve eCB signalling, (see Chhatwal & Ressler, 2007). Our group has shown an interaction between the eCB and opioid systems in fear-conditioned analgesia (FCA). FCA was modelled by assessing formalin-evoked nociceptive behaviour in an arena previously paired with footshock. Systemic administration of the FAAH inhibitor URB597 enhanced FCA in rats, an effect blocked by the CB_1 and CB_2 receptor antagonists rimonabant and SR144528, respectively (Butler, Rea, Lang, Gavin, & Finn, 2008). These findings corroborated and extended our earlier work demonstrating that CB_1 receptors play a key role in mediating FCA (Finn, et al., 2004). The use of transgenic mice lacking components of the eCB system further implicates a role for eCBs in stress-induced analgesia (Valverde, Ledent, Beslot, Parmentier, & Roques, 2000) and studies that have investigated the role of the EC system in specific brain regions in FCA/SIA will be discussed in detail below.

It has been shown that the neuropeptide cholecystokinin (CCK) plays a role in pain sensitivity via its regulation of opioid tone in the CNS. A recent study has demonstrated an interaction

between CCK and ECs in the regulation of SIA (Kurrikoff, Inno, Matsui, & Vasar, 2008). Intraperitoneal rimonabant prevented SIA, in the tail flick test, in response to footshock in wild-type mice. SIA was present in CCK type 2 receptor deficient mice regardless of rimonabant treatment while naloxone weakened SIA in both wild type and CCK type 2 receptor-deficient mice. The CCK_2 receptor gene, along with genes implicated in eCB-mediated neurotransmission, were upregulated in the mesolimbic area of the brain. CCK_2 receptors may therefore modulate the action of eCBs. This study demonstrates a clear involvement of the CCK_2 receptor in eCB-mediated SIA.

See Table 1 for a summary of studies (excluding those focused on the RVM, PAG, amygdala and PFC) investigating the role of the brain's eCB system in pain and its modulation by stress. Tables 2-5, Figure 2 and the sections that follow below then deal with the role of the eCB system within the RVM (Table 2), PAG (Table 3), amygdala (Table 4) and PFC (Table 5) in pain and its modulation by stress.

[Table 1 here]

The role of the endocannabinoid system in the rostral ventromedial medulla (RVM) in pain, stress-induced analgesia and stress-induced hyperalgesia

Pain:

The RVM is made up of the nucleus raphe magnus, the nucleus gigantocellularis pars alpha (GiA) and the adjacent reticular formation; and is a major component of the descending inhibitory pain pathway (Meng, Manning, Martin, & Fields, 1998). CB_1 receptors have been shown to be expressed in the RVM using receptor autoradiography and immunohistochemistry (Glass, et al., 1997; Herkenham, et al., 1991; Herzberg, Eliav, Bennett, & Kopin, 1997; Mailleux, Parmentier, & Vanderhaeghen, 1992; Thomas, Wei, & Martin, 1992; Tsou, Brown,

et al., 1998). The RVM contains ON and OFF cells which are involved in descending facilitation and inhibition of nociception, respectively (Vanegas, Barbaro, & Fields, 1984), and it projects to the dorsal horn of the spinal cord and the trigeminal nucleus to exert bi-directional control over nociception (Aicher, Hermes, Whittier, & Hegarty, 2012; Basbaum & Fields, 1984).

The RVM shares connections with the PAG, forming the PAG-RVM pathway (Basbaum & Fields, 1984). CBs activate descending analgesia via this pathway through a process of 'GABA disinhibition'. According to the GABA disinhibition hypothesis of analgesia, CB_1 receptor-mediated inhibition of GABAergic interneurons in the PAG and RVM results in disinhibition of projection neurons within the descending inhibitory pain pathway, resulting in analgesia (Basbaum & Fields, 1984; Lau & Vaughan, 2014; Szabo & Schlicker, 2005).

Microinjection of the CB receptor agonists WIN55,212-2 and HU210 into the RVM suppressed nociceptive behaviours in the tail flick test, an effect attenuated by co-administration with the CB_1 receptor antagonist rimonabant (Martin, Tsou, & Walker, 1998). Nociceptive behaviour remained unchanged upon CB receptor agonist injection outside of the RVM. CBs induce antinociception by modulating neuronal activity in the RVM and inactivation of the RVM prevents CB-induced analgesia (Meng, et al., 1998). As previously mentioned, ON-cells in the RVM increase firing in response to painful stimuli whereas OFF-cells decrease firing, facilitating and inhibiting pain respectively. Intra-RVM microinjection of WIN55,212-2 increases tail flick latencies while inhibiting ON-cell activity and increasing OFF-cell activity, thus decreasing nociception. Co-infusion with rimonabant blocked these effects, indicating a role for the eCB-CB_1 receptor system in the RVM in nociception (Meng & Johansen, 2004).

Microinjection of WIN 55,212-2 into the GiA resulted in behavioural analgesia in the rat tail flick test, an effect blocked by co-administration of rimonabant (Monhemius, Azami, Green,

& Roberts, 2001). In the same study, animals with partial sciatic nerve ligation were given intra-GiA WIN 55,212-2 and rimonabant and intraplantar formalin, contralaterally to the site of nerve ligation. Formalin-evoked nociceptive behaviour was significantly reduced in partial nerve ligated rats, an effect reversed by microinjection of rimonabant into the GiA. This study demonstrated a role for the CB_1 receptor in GiA-mediated antinociception and modulation of nociceptive transmission in both acute pain and chronic neuropathic pain (Monhemius, et al., 2001)

SIA:

To our knowledge only one study to date has investigated the role of the eCB system in the RVM in the modulation of pain by acute stress (SIA). Intra-RVM administration of rimonabant attenuated SIA in a rat model that combined footshock and a tail flick test. The FAAH inhibitor and TRPV1 agonist AA-5-HT, administered systemically or intra-RVM, enhanced SIA in rats in a CB_1 receptor-dependent manner (Suplita, Farthing, Gutierrez, & Hohmann, 2005). This study provides evidence for an important role of CB_1 receptors in the RVM in mediating and modulating SIA.

SIH:

While there is evidence for a role of the RVM in SIH (for review see Jennings, et al., 2014), few studies have specifically investigated the role of the eCB system in the RVM in SIH. Genetic background plays a key role in determining the effect of stress on pain. The Wistar-Kyoto (WKY) rat displays increased sensitivity to noxious stimuli and exhibits a depressive/anxiety-like phenotype and hyper-sensitivity to stress, compared with other rat strains including Sprague-Dawley (SD) rats (Burke, et al., 2010; O'Mahony, et al., 2010). We

have recently reported an impairment in pain-related mobilization of the eCBs AEA and 2-AG, along with their synthesising enzymes, NAPE-PLD and DAGL, respectively, in the RVM of WKY rats compared with SD rats, following intraplantar injection of formalin (Rea, et al., 2014). Systemic administration of AM251 potentiated while systemic administration of the FAAH inhibitor URB597 attenuated hyperalgesia to formalin injection in WKY rats, but not SD rats, an effect mediated by CB_1 receptors in the RVM. These data suggest eCB dysfunction in the RVM underlies the hyper-sensitivity to noxious stimuli in WKY rat model of negative affective state (Rea, et al., 2014).

See Table 2 for a summary of studies investigating the role of the endocannabinoid system in the RVM in pain and its modulation by stress.

[Table 2 here]

The role of the endocannabinoid system in the periaquaductal grey (PAG) in pain, stress-induced analgesia and stress-induced hyperalgesia

Pain:

The PAG is a midbrain/brainstem structure that can be divided into four columns along its rostro-caudal axis: dorsomedial (dmPAG), dorsolateral (dlPAG), lateral (lPAG) and ventrolateral (vlPAG) columns (Bandler & Keay, 1996). Exposure to an aversive stimulus activates the descending inhibitory pain pathway, of which the PAG is a key component. The PAG, via the RVM, modulates nociceptive transmission at the level of the spinal cord (Fields, Heinricher, & Mason, 1991). The PAG possesses a larger density of CB receptors than other brainstem structures (Herkenham, et al., 1991). CBs act in the PAG to inhibit GABAergic and glutamatergic synaptic transmission and to produce analgesia by a disinhibitory mechanism (Vaughan, Connor, Bagley, & Christie, 2000).

CB_1 receptor-mediated anti-nociception and increased levels of AEA were reported following electrical stimulation of the dorsal PAG (dPAG) and lPAG (Walker, et al., 1999). These authors also showed that subcutaneous injection of formalin elicited a pain response in rats and substantially increased AEA levels in the PAG, measured by *in vivo* microdialysis. Increased levels of the eCBs, AEA and 2-AG,were also seen in the PAG and RVM of rats 7 days post chronic constriction injury (CCI) of the sciatic nerve, when hyperalgesia and mechanical allodynia were observed to be maximal (Petrosino, et al., 2007).

Intra-vlPAG administration of morphine in rats enhanced the anti-nociceptive effect of the CB_1 receptor agonist HU-210 in the hot plate test (Wilson-Poe, Pocius, Herschbach, & Morgan, 2013). Likewise, intra-vlPAG and systemic administration of HU-210 enhanced the anti-nociceptive effect of morphine (Wilson, Maher, & Morgan, 2008). This study provides evidence for a dual role of morphine and CBs in pain and antinociception. Formalin-evoked nociceptive behaviour was reduced following microinjection of HU210 into the dorsal PAG (dPAG) of rats, an effect blocked by co-administration with the CB_1 receptor antagonist rimonabant (Finn, et al., 2003). Microinjection of CP-55,940 into the vlPAG, but not the posterior dlPAG or the anterior vlPAG, areas produces antinociception in the rat tail flick test (Lichtman, et al., 1996). WIN55,212-2 increased tail-flick latencies following microinjection into the rat dlPAG, (Martin, Patrick, Coffin, Tsou, & Walker, 1995). Microinjection of WIN55,212-2 into the PAG increased the latency of the nociceptive response in the plantar test in rats, an effect blocked by co-administration with rimonabant. MPEP, a metabotropic glutamate receptor mGlu5 antagonist, also completely blocked the anti-nociceptive effect of WIN55,212-2 (Palazzo, et al., 2001), indicating a CB_1-glutamatergic interaction in the PAG in mediating CB-induced analgesia.

Studies investigating the analgesic effect of the nonsteroidal anti-inflammatory drugs (NSAIDs) in supraspinal structures indicate a role for eCBs and CB_1 receptors in the PAG and

RVM. Inflammation-induced hyperalgesia can be attenuated by microinjection of the NSAID metazinol into the PAG (Vazquez, Escobar, Ramirez, & Vanegas, 2007). Injection of the CB_1 receptor antagonist AM251 into the PAG or RVM reverses metazinol-induced analgesia, suggesting a role for the eCB system in these brain regions in NSAID-induced analgesia (Escobar, et al., 2012).

TRPV1, a target of AEA, is expressed in the PAG (Palazzo, Rossi, & Maione, 2008) and a role for TRPV1 in pain modulation in the PAG has also been demonstrated. Intra-dlPAG injection of the TRPV1 agonist capsaicin increased the latency of nociceptive responses in the rat plantar test (Palazzo, et al., 2002). A higher dose administered to the same region produced opposite effects, decreasing the latency of nociceptive responses and inducing hyperalgesia followed by analgesia (McGaraughty, et al., 2003). Similar to Palazzo et al. (2002), intra-vlPAG administration of capsaicin also increased the latency of nociceptive responses in the hot-plate responses in rats (Liao, Lee, Ho, & Chiou, 2011), (for review see Starowicz, Nigam, & Di Marzo, 2007). Thus, TRPV1 agonism in the PAG elicits anti-nociceptive effects in several pain models. For a recent review see Madasu, Roche & Finn, In Press.

Intra-vlPAG injection of the FAAH inhibitor URB597 produced a robust hyperalgesic response at low doses, an analgesic response at high doses, and a biphasic effect on nociception at intermediate doses, in the rat plantar test (Maione, et al., 2006). AEA and 2-AG levels were increased in a dose-dependent manner following URB597 administration into the vlPAG. Co-administration of a low dose of URB597 with the CB_1 receptor antagonist AM251 converted the hyperalgesic effect to an analgesic one, while co-administration of URB597 with both the TRPV1 antagonist capsazepine and AM251 abolished all effects. In comparison, the early hyperalgesic effect of the intermediate dose of URB597 was blocked by AM251, while the later URB597-induced analgesic effect became hyperalgesic following TRPV1 antagonism. CB_1 receptor-dependent analgesia was seen at the highest dose of intra-vlPAG URB597

administration (Maione, et al., 2006). The URB597-induced antinociceptive effects (TRPV1-mediated) and pronociceptive effects (CB_1 receptor mediated) were associated with enhanced or reduced RVM OFF cell activity, respectively, suggesting URB597-induced alteration in the activity of excitatory PAG output neurons. This study indicates a role for both CB_1 and TRPV1 receptors in the eCB-mediated control of the descending pain pathway.

Diabetes is frequently associated with neuropathy, with many patients suffering from hyperalgesia or allodynia. A role for TRPV1 and CB_1 receptors in the PAG has been proposed in diabetic thermal hyperalgesia (Mohammadi-Farani, Sahebgharani, Sepehrizadeh, Jaberi, & Ghazi-Khansari, 2010). Intra-vlPAG administration of capsaicin and WIN produced antinociception in the hot plate test of non-diabetic mice (Mohammadi-Farani, et al., 2010). In contrast the anti-nociceptive effects of Intra-vlPAG capsaicin and WIN were reduced in hyperalgesic diabetic mice, an effect associated with CB_1 upregulation and TRPV1 downregulation in the vl-PAG (Mohammadi-Farani, et al., 2010). Taken together, the data demonstrate that diabetic neuropathy is associated with altered eCB signalling in the PAG, effects which may underlie the associated hyperalgesia and allodynia.

Systemic administration of the FAAH inhibitor and TRPV1 antagonist AA-5-HT produced anti-nociceptive effects in both rats and mice treated with formalin and in rats with CCI of the sciatic nerve (Maione, et al., 2007), effects associated with increased levels of AEA in both the PAG and RVM. These anti-nociceptive effects were blocked by both CB_1 receptor and TRPV1 antagonists. Intra-vlPAG injection of AA-5-HT increased eCB levels and induced a pro-nociceptive effect at low doses and an anti-nociceptive effect at higher doses in the rat tail flick test (de Novellis, et al., 2008). These effects were blocked by antagonism of vlPAG CB_1 receptors (AM251) or TRPV1 (I-RTX). Furthermore, administration of the FAAH inhibitor URB597 with the TRPV1 antagonist I-RTX into the vlPAG also induced antin-nociceptive effects in the rat tail flick test and inhibited RVM on and off cell activity (de Novellis, et al.,

2008), thus indicating that the anti-nociceptive effects of FAAH substrates in the vlPAG may be mediated by CB_1 receptors. In the formalin test of inflammatory pain, intra-PAG AA-5-HT prevented the changes in the ON and OFF cell firing activity induced by intra-plantar injection of formalin. Since CB_1 and TRPV1 antagonists blocked the effects of AA-5-HT, (de Novellis, et al., 2008), it suggests that these two eCB receptors in the PAG may be responsible for AA-5-HT-induced analgesia. Furthermore, Intra-PAG administration of the GPR55 agonist LPI reduced the nociceptive threshold in the rat hotplate test, an effect blocked upon pretreatment with the GPR55 antagonist ML-193 (Deliu, et al., 2015). This study suggests that altering GPR55 activity in the PAG may affect pain perception. Taken together these studies suggest that CB_1 receptors, TRPV1 and GPR55 in the PAG all play important roles in modulating pain behaviour.

Orexin (OX) A and B are peptides and endogenous agonists for the OX1 and OX2 receptors which are localized in the lateral and perifornical area of the hypothalamus, (de Lecea, et al., 1998; Sakurai, et al., 1998; Tsujino & Sakurai, 2009). They exert anti-nociceptive effects (Chiou, et al., 2010) including following direct administration into the PAG (Azhdari Zarmehri, et al., 2012). Orexin A decreases GABA release in an eCB~~E~~C-dependant manner in the vlPAG. Activation of OX receptors in the vlPAG leads to antinociception, measured electrophysiologically in brain slices. Intra-vlPAG microinjection of orexin A reduced hot-plate nociceptive responses in rats in a manner blocked AM 251 (Ho, et al., 2011).

SIA:

A number of studies have demonstrated an important role for the eCB system in the PAG in SIA/FCA. Intra-dPAG administration of the CB_1 receptor antagonist rimonabant attenuated SIA, observed as an increase in the tail flick latency following exposure of rats to footshock

stress (Hohmann, et al., 2005). The same dose of this drug administered i.c.v., intra-vlPAG and intra-lPAG had no effect on SIA in this study, highlighting a particular role of CB_1 receptors in the dlPAG in mediating SIA. Increased levels of 2-AG were seen in the dlPAG directly after footshock stress, implicating this eCB in the dlPAG in SIA. Moreover, inhibition of the 2-AG degrading enzyme MAGL in the dlPAG using URB602 enhanced SIA (Hohmann, et al., 2005). A subsequent study by the same group confirmed the CB_1 receptor-dependant attenuation of SIA following intra-dlPAG administration of rimonabant and the CB_1-dependant enhancement of SIA following AA-5-HT administration (Suplita, et al., 2005). These studies provide evidence that the PAG is a key neural substrate for eCB-mediated SIA. Another follow-up study from this group showed that mGlu5 receptor activation mobilizes 2-AG in the dlPAG to produce SIA in rats (Gregg, et al., 2012). Thus, unconditioned SIA mediated by CB_1 receptor stimulation in the PAG is under the control of glutamatergic neurotransmission via mGlu5 receptors.

Our group has reported a role for the eCB system in the PAG in a model of SIA associated with conditioned, learned fear (fear-conditioned analgesia; FCA) (Olango, et al., 2012). FCA in these studies was measured as the reduction of formalin-evoked nociceptive behaviour upon re-exposure of rats to a conditioning arena previously paired with footshock. Systemic administration of the FAAH inhibitor URB597 enhanced FCA, an effect associated with reduced phospho-ERK1/2 expression in the PAG (Butler, et al., 2008). FCA was attenuated by intra-dlPAG administration of rimonabant (Olango, et al., 2012), confirming a role for CB_1 receptors in the dlPAG in mediating both conditioned and unconditioned forms of SIA.

SIH

While there is evidence for a role of the PAG in SIH (for review see Jennings, et al., 2014), there is currently a paucity of studies addressing the role of the eCB system in the PAG in SIH and this is an area that warrants investigation.

See Table 3 for a summary of studies investigating the role of the endocannabinoid system in the PAG in pain and its modulation by stress.

[Table 3 here]

The role of the endocannabinoid system in the amygdala in pain, stress-induced analgesia and stress-induced hyperalgesia

Pain:

The amygdala is a key region of the limbic system located in the medial temporal lobe. It contains a number of different nuclei including, the lateral nucleus (LA), basolateral nucleus (BLA), the central nucleus (CeA), accessory basal nucleus (ABA) and the medial nucleus (MeA). The amygdala plays a key role in the interaction between pain and emotion. The CeA, in particular, is involved in the emotional-affective component of persistent pain (Neugebauer, Galhardo, Maione, & Mackey, 2009; Neugebauer, Li, Bird, & Han, 2004), while the BLA may be involved in the modulation of acute or tonic nociceptive processing (Oliveira & Prado, 1998). The amygdala is a key region of the ascending and descending pain pathways and shares connections with other key regions including the prefrontal cortex and PAG. Pain-related changes have been identified in the amygdala in animals and humans using PET and fMRI neuroimaging studies (Neugebauer, et al., 2004).

All components of the eCB system are expressed in the amygdala, although CB_1 receptors are expressed in highest density in the BLA (Herkenham, et al., 1991; Tsou, Brown, et al., 1998). The amygdala contributes to the anti-nociceptive effects produced by systemically administered CBs. WIN55,212-2 produces dose dependent anti-nociceptive effects in rats characterized as increased tail flick latencies (Manning, Martin, & Meng, 2003). Intra-CeA, but not intra-BLA, administration of muscimol, significantly attenuated these anti-nociceptive effects of systemically administered WIN55,212-2. Moreover, unilateral CeA inactivation via muscimol reduced the suppression of formalin-evoked c-Fos expression by WIN55,212-2 in the superficial dorsal horn of the spinal cord but not in the deeper 'nociceptive' laminae (Manning, et al., 2003). Another study from the same group found that the amygdala also plays a role in antinociception in non-human primates (Manning, Merin, Meng, & Amaral, 2001). WIN55,212-2 produced dose-dependent analgesia in rhesus monkeys. Bilateral lesions to the amygdala of the monkeys significantly reduced CB induced analgesia. Both of these lesion studies indicate that the eCB system in the amygdala, in particular the CeA, can mediate anti-nociceptive effects.

Tail flick latencies have been shown to be increased upon microinjection of WIN55,212-2 into the CeA and BLA in rats (Hasanein, Parviz, Keshavarz, & Javanmardi, 2007; Martin, et al., 1999). Furthermore, intra-BLA administration of WIN55,212-2 has also been shown to reduce formalin-evoked nociceptive behaviour in rats, an effect attenuated by intra-BLA administration of the CB_1 receptor antagonist AM251 (Hasanein, et al., 2007). Interestingly, intra-BLA administration of rimonabant has also been shown to attenuate formalin-evoked nociceptive behaviour and associated increases in c-Fos immunoreactivity in the hippocampus and RVM in rats (Roche, et al., 2010; Roche, O'Connor, Diskin, & Finn, 2007), although intra-BLA administration of a different CB_1 receptor antagonist, AM251, did not exert a similar effect (Rea, et al., 2013).

Using fMRI, it has been shown that the amygdala may play a role in the modulation of pain perception by Δ^9-THC in humans (Lee, et al., 2013). Cutaneous ongoing pain and hyperalgesia induced by capsaicin were monitored in healthy cannabis-naive volunteers. Δ^9-THC reduced 'painfulness' but not the intensity of pain and hyperalgesia an effect positively correlated with amygdala activity. A Δ^9-THC -related reduction in sensory-limbic functional activity was also seen between the amygdala and primary sensorimotor areas (Lee, et al., 2013).

While the evidence points to a clear role for the eCB system in the amygdala in antinociception, there is a paucity of studies investigating its impact on the emotional aspect of pain. As a region with a clear role for the interaction between pain and emotion, it is necessary to further investigate this area and the role of the eCB system therein.

SIA:

The amygdala plays a role in both unconditioned and conditioned SIA (Helmstetter, 1992; Helmstetter & Bellgowan, 1993; Helmstetter, Bellgowan, & Poore, 1995; Werka, 1994, 1997; Werka & Marek, 1990). Intra-BLA microinjection of rimonabant has been shown to suppress unconditioned SIA in rats exposed to footshock stress and then tested in the tail flick test, whereas intra-CeA microinjection had no effect on this form of SIA (Connell, Bolton, Olsen, Piomelli, & Hohmann, 2006). Intra-BLA administration of FAAH and MAGL inhibitors, however, had no effect on SIA (Connell, et al., 2006), suggesting that CB_1 receptors in the BLA, but not CeA, mediate SIA, although the role of the individual eCBs requires further investigation. Roche et al. (2007 and 2010) reported no effect of unilateral or bilateral intra-BLA administration of rimonabant on FCA in rats (Roche, et al., 2010; Roche, et al., 2007). However, a subsequent study showed that the expression of FCA in rats was reduced following

systemic or intra-BLA, but not intra-CeA, administration of a different CB_1 receptor antagonist, AM251 (Rea, et al., 2013).

URB597 enhances the expression of FCA when administered via the intra-peritoneal route, an effect blocked by either CB_1, CB_2 or μ-opioid receptor antagonists (Butler, et al., 2008). Interestingly, FCA in this study was associated with increased expression of phospho-ERK2 in the amygdaloid complex. In contrast, the URB597-induced enhancement of FCA was associated with reduced phospho-ERK1 and phospho-ERK2 expression in the amygdala. This dichotomy is not consistent with a causal role of ERK signalling in FCA (Butler, et al., 2008).

CB_1 receptors are expressed on GABAergic and glutamatergic neurons in the BLA (Herkenham, et al., 1991; Katona, et al., 2001). Expression of FCA in rats was reduced following systemic or intra-BLA, but not intra-CeA, administration of the CB_1 receptor antagonist AM251 (Rea, et al., 2013), an effect attenuated by intra-BLA administration of both the $GABA_A$ receptor antagonist, bicuculline, and the mGlu5 receptor antagonist, MPEP, suggesting that CB_1 receptors in the BLA facilitate the expression of FCA, through a mechanism which is likely to involve the modulation of GABAergic and glutamatergic signalling. FCA was associated with increased levels of AEA in the left BLA (side contralateral to intraplantar formalin injection). Fear-conditioned, formalin-treated rats displayed increased levels of 2-AG and PEA in the left and right BLA, respectively (Rea, et al., 2013).

It is clear, therefore, that the eCB system in the amygdala, and specifically the BLA, plays an important role in mediating both unconditioned and conditioned SIA with likely interactions with GABAergic and glutamatergic signalling.

SIH:

A recent study from our group investigated the effects of repeated exposure to forced swim stress on formalin-evoked nociceptive behaviour in rats in stress normo-responsive (SD) and stress hyper-responsive (WKY) rat strains. Formalin-evoked nociceptive behaviour was increased in SD rats following ten days of forced swim stress (Jennings, Okine, Olango, Roche, & Finn, 2015). AEA levels were reduced in the contralateral amygdala (relative to formalin injection) of SD rats but not WKY rats. There were also strain differences in components of the eCB system within the amygdala. For example, decreased levels of AEA and 2-AG were observed in the ipsilateral amygdala of SD, but not WKY, rats. Lower levels of CB_1 receptor mRNA were seen in the ipsilateral, but not contralateral, amygdala of WKY rats. These data indicate a role for the eCB system in the amygdala in SIH as well as implicating it in the strain differences seen in WKY and SD rats (Jennings, et al., 2015). Additional studies are warranted to fully understand the role of the eCB system in the amygdala in SIH.

See Table 4 for a summary of studies investigating the role of eCB system in the amygdala in pain and its modulation by stress.

[Table 4 here]

The role of the endocannabinoid system in the prefrontal cortex in pain, stress-induced analgesia and stress-induced hyperalgesia

Pain:

The PFC is involved in both the top-down descending modulation of pain and also in the affective dimension of the pain experience. The medial PFC (mPFC) is comprised of the prelimbic cortex (PrL), infralimbic cortex (IL) and anterior cingulate cortex (ACC). Imaging studies have shown that the PFC is consistently activated by noxious stimuli (Casey,

Minoshima, Morrow, & Koeppe, 1996; Davis, Taylor, Crawley, Wood, & Mikulis, 1997; Derbyshire, et al., 1997; May, et al., 1998; Millan, 1999; Neal, Pearson, & Powell, 1990; Svensson, Minoshima, Beydoun, Morrow, & Casey, 1997). CB_1 receptors are expressed in the PFC (Herkenham, et al., 1991; Sim-Selley, Vogt, Vogt, & Childers, 2002; Tsou, Brown, et al., 1998). This, along with its projections to the PAG and amygdala (Diorio, Viau, & Meaney, 1993; Little & Carter, 2013; Marchand & Hagino, 1983), suggest a role for the EC system in the PFC in pain.

CB_1 receptors in the rodent mPFC are expressed on GABAergic interneurons (Marsicano & Lutz, 1999; Wedzony & Chocyk, 2009). CB_1 receptors on presynaptic axon terminals face pyramidal neurons with postsynaptic mGluR5 (Lafourcade, et al., 2007). A rat arthritis pain model, induced via intra-articular injections of kaolin and carrageenan through the patellar ligament, shows hyperactivity in amygdala output neurons and abnormal inhibition of mPFC pyramidal neurons (Ji, et al., 2010). Another study investigated the effect of mGluR5 and CB_1 receptor activation on the activity of the mPFC cells in rats in the previously described arthritis pain model (Ji & Neugebauer, 2014). Co-activation of mGluR5 and CB_1 receptors increased mPFC activity, and inhibited pain-related neuronal activity in the CeA in the arthritis pain model. Thus, there appears to be an inverse link between activation of mPFC neurons and amygdala output and a role for the eCB system in this top-down cortical control (Ji & Neugebauer, 2014). Further evidence for a role of the eCB system in the PFC in arthritic conditions comes from work demonstrating that osteoarthritis pain is associated with increased 2-AG levels in the PFC of mice in the monosodium iodacetate model of arthritis (La Porta, et al., 2015).

CB_1 receptor activity is decreased in the rostral ACC 10 days post CCI in mice, compared with sham controls (Hoot, et al., 2010). CB_1 receptor levels in the rostral ACC of CCI and sham rats remained unchanged and there were no significant differences in the levels of 2-AG or AEA in

the ACC between CCI and sham-operated mice. The ACC is associated with the affective component of pain (Kulkarni, et al., 2005; Kuo, Chiou, Liang, & Yen, 2009; LaBuda & Fuchs, 2005; Treede, Kenshalo, Gracely, & Jones, 1999). It is possible therefore that reduced CB_1 receptor activity in the ACC is associated with the negative affective component of neuropathic pain.

TRPV1 expression is increased, in glutamatergic neurons, in the mPFC (namely the PrL and IL) following spared nerve injury (SNI) (Giordano, et al., 2012). Intra-PL/IL administration of AA-5-HT reduced mechanical allodynia in rats following SNI to a greater extent than that seen with a FAAH inhibitor or TRPV1 antagonist alone (Giordano, et al., 2012). SNI-induced neuropathic pain is also associated with increased levels of endovanilloids and eCBs in the mPFC. Intra-PrL/IL injection of AA-5-HT produced anti-nociceptive effects more efficiently (de Novellis, et al., 2011). These studies suggest that both the eCB and endovanilloid systems in the mPFC may play a role in neuropathic pain. Therapies which target both of these systems may prove useful in the treatment of chronic neuropathic pain.

We have studied the role of PPARα in the mPFC in formalin-evoked nociceptive behaviour in rats. The PPARα antagonist GW6471 delayed the onset of the second phase of formalin-evoked nociceptive behaviour. This reduction in nociceptive behaviour was associated with a reduction in the levels of N-palmitoylethanolamide and N-oleoylethanolamide (PPARα ligands) in the mPFC (Okine, et al., 2014). Together these data suggest a facilitatory role for PPARα in the mPFC in formalin-evoked nociceptive behaviour.

SIA:

Lesion studies have indicated a role for the PFC in acquisition, consolidation and extinction of conditioned fear in rodents (Sierra-Mercado, Corcoran, Lebron-Milad, & Quirk, 2006). This

region has also been shown to project to other regions important in fear neurocircuitry, including the previously discussed amygdala and PAG (LeDoux, 2000). CB_1 receptors in the PrL cortex are involved in the amplification of panic-like aversive reactions and SIA. Thus, microinjection of bicuculline into the dorsomedial and ventromedial hypothalamus induced aversive panic-like behaviour and SIA, an effect attenuated by microinjection of AM251 into the PrL (Freitas, Salgado-Rohner, Hallak, Crippa, & Coimbra, 2013). This work suggests that CB_1 receptor signalling in the PrL may facilitate or augment SIA induced by stimulation of the hypothalamus. Further investigation of the roles of the eCB system in the PrL, IL and ACC in SIA and FCA is warranted.

SIH:

To our knowledge there have been no published studies to date investigating the role of the eCB system in the PFC in SIH.

See Table 5 for a summary of studies investigating the role of the eCB system in the PFC in pain and its modulation by stress.

[Table 5 here]

[Figure 2 here]

Less characterized endocannabinoid mechanisms in pain modulation

Systemic administration of the CB receptor agonist WIN 55,212-2 dose-dependently inhibited stimulus-evoked activity, in the form of graded pressure stimuli to the paw, of nociceptive neurons in the ventroposterolateral thalamus (VPL) of anesthetized rats (Martin, Hohmann, & Walker, 1996). Further evidence for a role of CB_1 receptors in the thalamus in mediating and

modulating nociceptive responding was observed following microinjection of WIN 55,212-2 into the thalamus which resulted in anti-nociceptive effects in the tail flick test in rats (Martin, et al., 1999). Similar effects were observed following microinjection into the alpha part of the gigantocellular reticular nucleus (GiA) and the noradrenergic A5 region. Furthermore, intra-locus coeruleus microinjection of the hypothalamic peptide orexin-A decreased formalin-evoked nociceptive behaviour in rats (Mohammad-Pour Kargar, Azizi, Mirnajafi-Zadeh, Reza Mani, & Semnanian, 2015), an effect reversed following pretreatment with either the OX1 receptor antagonist SB-334867 or the CB_1 receptor antagonist AM251. Intra-locus coeruleus microinjection of SB-334867 and AM251 alone induced hyperalgesia (Mohammad-Pour Kargar, et al., 2015). The results from this study suggest a new mechanism by which orexin-A modulates nociceptive information in the locus coeruleus via interaction with CB_1 receptors.

There is now increasing evidence supporting the role of CB_2 receptors in the supraspinal modulation of pain, (for review see Guindon & Hohmann, 2008). For example, microinjection of the CB_2 receptor agonist JWH-133 into the ventral posterolateral nucleus of the thalamus (VPL) has been shown to reduce noxious activity, recorded with a multichannel electrode array in VPL neurons, in a rat model of neuropathic pain (spinal nerve ligation; SNL) (Jhaveri, et al., 2008). No significant differences in the levels of eCBs in the thalamus of SNL rats compared to sham rats were observed (Jhaveri, et al., 2008). The results from this study suggest that CB_2 receptors in the thalamus may contribute to the modulation of neuropathic pain responses.

The eCB system has also been proposed to play a role migraine-related pain (for review see Greco, Gasperi, Maccarrone, & Tassorelli, 2010; Russo, 2004; Smith & Wagner, 2014). FAAH deficient mice (FAAH (-/-)) express less nitroglycerin-induced migraine-like pain, with similar effects observed following pahramcological inhibition of FAAH inhibitors using URB597 and PF3945. Administration of the CB_1 receptor antagonist rimonabant blocked these anti-

nociceptive effects in this migraine model, demonstrating a key role for CB_1 receptors in mediating the effects of the FAAH substrates (i.e. AEA) (Nozaki, Markert, & Zimmer, 2015). Similarly, several other studies have demonstrated that genetic and/or pharmacological inhibition of FAAH is associated with increased AEA levels in the brain, and associated with anti-nociceptive effects in several pain models (Kwilasz, Abdullah, Poklis, Lichtman, & Negus, 2014; Lichtman, Shelton, Advani, & Cravatt, 2004). For example, URB597 (intra-peritoneal), a selective FAAH inhibitor, produced anti-nociception in the form of CB_1 dependant decreases in acid-stimulated stretching in a lactic acid model of pain, an effect associated with increased AEA levels in the brain (Kwilasz, et al., 2014). Increased FAAH activity and an increased density of CB binding sites have also been found in the hypothalamus in animal models of migraine (nitroglycerin-induced hyperalgesia), (Greco, Gasperi, Sandrini, et al., 2010). It is clear that elevation of brain eCB levels produces robust modulatory effects in mouse models of pain anti-nociception (Cravatt, et al., 2001; Holt, Comelli, Costa, & Fowler, 2005; Jayamanne, et al., 2006; Lichtman, Leung, et al., 2004; Lichtman, Shelton, et al., 2004), suggesting supraspinal CB_1 receptor-dependant anti-nociception.

PET imaging with a CB_1 receptor radioligand demonstrated that patients with in functional dyspepsia have higher CB_1 receptor availability in the hypothalamus and anterior cingulate cortex (Ly, et al., 2015). It is possible that eCB dysfunction and abnormal brain activity in these areas may be related to the pain felt in patients with functional dyspepsia, however further work is warranted (Ly, et al., 2015)

As previously mentioned, eCBs act on other non-CB_1/non-CB_2 receptors, such as the ligand-gated ion channel, TRPV1. TRPV1 on primary afferent neurons plays a key role in the sensation of pain and thermal hyperalgesia (Caterina, et al., 2000). However increasing evidence suggests a role for TRPV1 in pain modulation in supraspinal regions (Madasu, Roche, & Finn, 2015). Central administration of the TRPV1 antagonist A-784168 induced potent

analgesia in the rat sodium monoiodoacetate model of osteoarththitic pain and reduced thermal hyperalgesia and mechanical allodynia in the complete Freund's adjuvant model of inflammatory pain (Cui, et al., 2006). Moreover, i.c.v. administration of TRPV1 antagonists reduced nociceptive behaviour in the rat formalin test (Santos & Calixto, 1997). Following spinal cord injury, CB_1 and TRPV1 receptors interact and play a role in the plastic changes that occur in the rat brain (Knerlich-Lukoschus, et al., 2011). In the same study, seven days following spinal cord lesion, CB_1 receptor immunoreactivity was increased in the thalamus and hippocampus and downregulated in the anterior cingulate cortex (ACC), amygdala and PAG, brain regions related to pain, emotion, learning, and memory in rats. Double labelling studies revealed that TRPV1 was co-expressed with CB_1 (Knerlich-Lukoschus, et al., 2011). Thus alterations in CB1-TRPV1 expression/activity may underlie the emotional-affective and somatosensory pain responses following spinal cord lesion.

Paracetamol (acetaminophen) is a well-recognised and potent analgesic drug (Toms, McQuay, Derry, & Moore, 2008) and a number of recent studies have demonstrated that paracetamol is metabolized to the TRPV1 agonist and AEA transport blocker AM404, which contributes to the anti-nociceptive activity of paracetamol (Hogestatt, et al., 2005; Mallet, et al., 2010; Zygmunt, Chuang, Movahed, Julius, & Hogestatt, 2000). The breakdown of paracetamol to AM404 occurs in the brain and is dependent on FAAH (Hogestatt, et al., 2005). The de-acetylated paracetamol metabolite 4-aminophenol and 4-hydroxy-3-methoxybenzylamine (HMBA), produces anti-nociception in a variety of rodent (both mice and rats) pain tests (Barriere, et al., 2013) and is metabolised in the brain to form AM404 plus HPODA or arvanil plus olvanil. The anti-nociceptive effects of arvanil were dependent on FAAH, TRPV1 and CB_1 receptors (Barriere, et al., 2013). FAAH-dependent generation of TRPV1-active analgesic drug metabolites may be useful in the production of novel pain therapeutics (Barriere, et al., 2013).

GPR55, a putative novel CB receptor, has recently been shown to be involved in the development of hyperalgesia in models of inflammatory and neuropathic pain. Inflammatory mechanical hyperalgesia was absent in GPR55(-/-) knockout mice (Castane, et al., 2006; Staton, et al., 2008). Furthermore, following partial sciatic nerve ligation, GPR55(-/-) mice failed to express mechanical hyperalgesia (Staton, et al., 2008). Together, these results suggest a pro-nociceptive role for GPR55. However, as discussed below, only one study to date has investigated the role of this novel target supraspinally in the modulation of pain or stress (Deliu, et al., 2015).

The PPARs are also targets for eCBs, and may play a role in eCB-induced analgesia. PPARγ agonists produced anti-inflammatory and anti-hyperalgesic effects in carrageenan-treated rats, effects which were supra-spinally mediated (Morgenweck, et al., 2010). Similarly, i.c.v. administration of PPARα ligands produced anti-inflammatory and anti-hyperalgesic effects in mice and rats in the carrageenan model of inflammation, (D'Agostino, et al., 2009; D'Agostino, et al., 2007; Taylor, Kriedt, Nagalingam, Dadia, & Badr, 2005). Thus, central PPARs play an important role in inflammatory nociceptive processing and responding.

See Table 1 for a synthesis of the studies described above.

Concluding remarks:

This review has provided a detailed overview of the role of the supraspinal eCB system in pain, SIA and SIH. Work in animal models has provided clear evidence that activation of supraspinal CB receptors (particularly CB_1) or elevation of supraspinal eCB levels can induce anti-nociception. Although our understanding of the physiological, biochemical and molecular mechanisms mediating these processes has become much clearer in recent years, there is still

a need for further work to provide full details on the neurobiological mechanism underlying these effects.

We have highlighted a few areas where further investigation is warranted. In particular, studies investigating the role of the supraspinal eCB system in SIA and SIH are still relatively sparse, and particularly for SIH. Further studies in this area would enhance our understanding of pain-stress interactions. Pain is processed via many inter-connected receptor-mediated pathways utilizing different neurotransmitter systems including GABA, glutamate, monoamines, opioids and the eCB system, among others. In order to fully understand the role of the supraspinal eCB system in pain, and its modulation by stress, it is imperative that we look at the interactions between the supraspinal eCB system and other neurotransmitter systems. To date there has been a relative paucity of studies investigating these interactions.

The majority of studies discussed in this review and the bulk of our understanding on this topic has come from laboratory animal studies. While there are many clinical trials investigating the effects of CBs in pain and psychiatric disorders (International Clinical Trials Registry Platform), to our knowledge there are no studies that specifically target the supraspinal eCB system to evaluate its effect on pain or co-morbid pain and stress disorders, likely owing to the technical and ethical challenges that would be involved. It will be necessary to develop therapeutic approaches relevant to the clinical setting without overt side effects and these may include sub-region specific targeting of CB_1 receptors, elevation on eCB levels rather than potent CB_1 receptor agonism and targeting of CB_2 receptors or other non-CB_1 receptor targets of relevance within the eCB system – all of which have been reviewed in this manuscript. Given the high incidence of pain disorders and their co-morbidity with stress-related disorders, there is an urgent need to fully understand the neurobiological mechanisms underpinning supraspinal modulation of pain, SIA and SIH and develop new, more effective treatments with more favourable adverse side effect profiles.

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Conflict of Interest:

None.

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Class of pharmacological compound	Pharmacological or genetic intervention	Route of administration	Species	Model	Effect	Reference
CB_1 / CB_2 Agonist	WIN 55,212-2	i.v.	Rat	Spinal Transection; Tail Flick	Inhibits noxious activity; blocked by spinal transection	(Hohmann, Tsou, & Walker, 1999)
CB_1 / CB_2 Agonist	THC; CP 55,940	i.c.v.	Rat	Spinal Transection; tail flick	Antinociception; blocked by rimonabant	(Lichtman & Martin, 1991, 1997)
CB_1 / CB_2 Agonist	THC	i.c.v.	Mouse	Tail Flick	Enhances the antinociceptive effects of morphine	(Welch, Thomas, & Patrick, 1995)
CB_1 / CB_2 Agonist	WIN 55,212-2; CP-55,940	i.c.v.	Rat	Tail Flick	Antinociception	(Martin, Lai, Patrick, Tsou, & Walker, 1993)
CB_1 / CB_2 Agonist	WIN 55,212-2; THC	i.c.v.	Mouse	Tail Flick	Dose-related antinociception	(Raffa, Stone, & Hipp, 1999)
CB_1 / CB_2 Agonist CB_1 / CB_2 Agonist	WIN 55,212-2	i.v.	Rats	Pressure stimulus to hindpaw; Electrophysiological recording of nociceptive neurons in thalamus	Decrease in nociceptive transmission in the thalamus	(Martin, Hohmann, & Walker, 1996)

	WIN 55,212-2	Microinjection; GiA, Thalamus, noradrenergic A5 region	Rats	Tail Flick	Antinociception when administered to each region	(Martin et al., 1999)
CB_1 / CB_2 Agonist	WIN 55,212-2	i.c.v.	Mice	Tail Flick; Paw Withdrawal in the lamp-foot-flick assay	Antinociception	(Fang et al., 2012)
CB_1 / CB_2 Agonist	Rimonabant	i.c.v.; i.t.; intraperitoneal	Mouse	Tail Flick	Rimonabant exhibits greater efficacy supraspinally rather than spinally.	(Welch, Huffman, & Lowe, 1998)
CB_1Antagonist/ inverse agonist	JWH-133	Intra-VPL	Rats	Spinal Nerve Ligation	Reduced noxious activity in SNL rats; blocked by SR144528 (CB_2 antagonist)	(Jhaveri et al., 2008)
CB_2 Agonist	FAAH (-/-); URB597 PF3945		Mice	Nitroglycerin-induced migraine-like pain	FAAH (-/-), URB597 and PF3945 reduce nociceptive behaviour; blocked by rimonanbant	(Nozaki, Markert, & Zimmer, 2015)
FAAH Inhibitor	FAAH (-/-)		Mice	Tail immersion,	FAAH (-/-)	

				hot plate, formalin tests, CCI and carageenan	increased response latency in tail immersion and hot plate test; reduced formalin-evoked nociceptive behaviour. Anti-hyperalgesic in carrageenan model	(Lichtman, Shelton, Advani, & Cravatt, 2004)
FAAH Inhibitor; MAGL Inhibitor	URB597; JZL184	i.p.	Mice	CUS; NGF hyperalgesia; tail flick; hot plate	Effects in CUS mice - Enhanced levels of AEA and 2-AG; decreased thermal hyperalgesia; URB597 decreased NGF hyperalgesia	(Lomazzo et al., 2015)
	A-784168	i.c.v.	Rats	Model of osteoarthritis (Sodium monoiodoacetate); Complete Freund's Adjuvant (chronic inflammatory pain)	Decreased weight bearing; decreased chronic inflammatory thermal hyperalgesia	(Cui et al., 2006)
TRPV1 Antagonist	Capsazepine	i.c.v.	Mice	Formalin	Attenuation of nociceptive	

					behaviour	(Santos & Calixto, 1997)
TRPV1 Antagonist	GPR55(-/-)		Mice	Inflammatory mechanical hyperalgesia (von Frey; Complete Freuds Adjuvent)	Hyperalgesia absent in GPR55(-/-)	(Castane et al., 2006; Staton et al., 2008)
	GPR55(-/-)		Mice	Nerve ligation; mechanical hyperalgesia	Mechanical hyperalgesia absent following nerve ligation in GPR55(-/-)	(Staton, et al., 2008)
PPARγ agonist	Rosiglitazone; 15d-PGJ(2)	i.c.v.	Rats	Plantar carageenan model of inflammatory pain	Anti-inflammatory and anti-hyperalgesia effects	(Morgenweck et al., 2010)
CB_1 Antagonist/inverse agonist; Opioid Antagonist	Rimonabant; naloxone; CCK_2 knockout mice	i.p.	Mice	SIA – footshock; tail flick	Rimonabant prevented SIA, an effect not seen in CCK_2 knockout mice; naloxone weakened SIA in wild type and CCK_2 knockout mice	(Kurrikoff, Inno, Matsui, & Vasar, 2008)
	URB597	i.p.	Rats	FCA - Conditioned fear (footshock) and formalin test	URB597 enhances FCA; attenuated by	

FAAH Inhibitor					rimonabant, SR144528 and naloxone	(Butler, Rea, Lang, Gavin, & Finn, 2008)

Table 1: Summary of studies investigating the role of the brain's endocannabinoid system in pain and its modulation by stress (excluding studies on RVM, PAG, amygdala and PFC which are summarised in Tables 2-5). $CB_{1/2}$ - Cannabinoid receptor type 1/2; i.v. – Intravenous; i.c.v. – Intracerebroventricular; GiA - nucleus reticularis gigantocellularis pars alpha; i.t. – intra thecal; Intra-VPL - Ventral posterolateral nucleus; FAAH – Fatty acid amide hydrolase; SNL – spinal nerve ligation; (-/-) – knock out; CCI – chronic constriction injury ; MAGL - Monoacylglycerol lipase; PPARα - Peroxisome proliferator-activated receptor alpha; TRPV1 - transient receptor potential cation channel subfamily V member 1; CUS – chronic unpredictable stress; NGF – nerve growth factor; AEA – anandamide; 2-AG - 2-Arachidonoylglycerol; GPR55 - G protein-coupled receptor 55; CCK2 – Cholecystokinin 2; SIA – Stress induced analgesia; FCA – fear-conditioned analgesia

Class of pharmacological compound	Pharmacological or genetic intervention	Route of administration	Species	Model	Effect	Reference
CB_1/CB_2 Agonists	WIN 55,212-2; HU210	Intra-RVM	Rat	Tail Flick	Nociceptive behaviour suppression	(Martin, Tsou, & Walker, 1998)
CB_1/CB_2 Agonist	WIN 55,212-2	Intra-RVM	Rat	Tail Flick	Increased tail flick latency; inhibition of on-cell activity; increase in off-cell activity; effects blocked by rimonabant	(Meng & Johansen, 2004)
CB_1/CB_2 Agonist	WIN 55,212-2	Intra-GiA	Rat	Tail Flick	Increased antinociception; blocked by rimonabant	(Monhemius, Azami, Green, & Roberts, 2001)
CB_1/CB_2 Agonist	WIN 55,212-2	Intra-GiA	Rat	Partial nerve ligation; formalin test	Decrease in formalin-evoked nociceptive behaviour following nerve	(Monhemius, et al., 2001)

					ligation; reversed by rimonabant	
CB_1 Antagonist/ Inverse agonist; Dual FAAH/TRPV1 Inhibitor	Rimonabant; AA-5-HT	Intra-RVM;	Rats	SIA –Footshock, Formalin	Suppression of SIA by rimonabant; enhancement of SIA by AA-5-HT	(Suplita Ii, Farthing, Gutierrez, & Hohmann, 2005)
CB_1 Antagonist/ Inverse agonist; FAAH Inhibitor	AM251; URB597	intraperitoneal (AM251 and URB597); Intra-RVM AM251	Wistar-Kyoto and Sprague Dawley rats	Formalin	Systemic AM251 potentiates hyperalgesia in WKY, URB597 attenuates hyperalgesia in WKY associated with impaired pain-related mobilization of ECs in RVM of WKY rats as seen from Intra-RVM AM251.	(Rea et al., 2014)

Table 2. Summary of studies investigating the role of the endocannabinoid system in the RVM in pain and its modulation by stress. $CB_{1/2}$ - Cannabinoid receptor type 1/2; RVM - Rostral ventromedial medulla; GiA - nucleus reticularis gigantocellularis pars alpha; SIA – Stress-induced analgesia; WKY – Wistar Kyoto; TRPV1 - transient receptor potential cation channel subfamily V member 1; FAAH – Fatty acid amide hydrolase

Class of pharmacological compound	Pharmacological or genetic intervention	Route of administration	Species	Model	Effect	Reference
CB_1/CB_2 Agonist	HU-210	vlPAG; systemic	Rat	Hot late test	HU210 enhanced antinociceptive effect of morphine and morphine enhanced the antinociceptive effect of HU210	(Wilson-Poe, Pocius, Herschbach, & Morgan, 2013).
CB_1/CB_2 Agonist	HU210	Intra-dPAG	Rat	Formalin	Reduced formalin-evoked nociceptive behaviour; blocked following rimonabant administration	(Finn et al., 2003)
CB_1/CB_2 Agonist	CP-55,940	Intra-vlPAG; Intra-dPAG;	Rat	Tail Flick	Intra-vlPAG microinjection produced antinociception; intra-dPAG had no effect	(Lichtman, Cook, & Martin, 1996)
CB_1/CB_2 Agonist	WIN55,212-2	Intra-dPAG	Rats	Tail Flick		

					Increased tail flick latency	(William J. Martin, Patrick, Coffin, Tsou, & Walker, 1995)
CB_1 Antagonist/ Inverse agonist	AM251	Intra-PAG; Intra-RVM	Rat	Metazinol induced antinociception in a carrageenan model of inflammation	Reverses metazinol-induced analgesia	(Escobar et al., 2012)
TRPV1 Agonist	Capsaicin (low dose); Capsaicin (high dose)	Intra-dlPAG	Rat	Plantar test	Low dose – Antinociception; High dose – Blocked antinociception	(Palazzo et al., 2002)
TRPV1 Agonist; TRPV1 Antagonist	Capsaicin; Capsazepine	Intra-dlPAG	Rat	Tail Flick	Capsaicin – hyperalgesia followed by antinociception; Capsazepine – blocked hyperalgesic effect of capsaicin	(McGaraughty et al., 2003)
FAAH Inhibitor	URB597	Intra-vlPAG	Rat	Plantar test	Low dose – Hyperalgesia – coadministration with AM251 converted	(Maione et al., 2006)

					hyperalgesia to analgesia – coadministration with capsazepine and AM251 abolished any effect of URB597; High dose – Antinociception; Intermediate dose – biphasic response – blocked by AM251, hyperalgesic following coadministration of URB597 with capsazepine	
Dual FAAH/TRPV1 Inhibitor; CB_1 Antagonist/Inverse agonist; TRPV1 antagonist; FAAH Inhibitor	AA-5-HT; AM251; I-RTX; URB597	Intra-PAG	Rat	Tail Flick;	AA-5-HT induced antinociception – blocked by AM251 and I-RTX; URB597 and I-RTX induced analgesia	(de Novellis et al., 2008)
CB_1 Antagonist/Inverse agonist; MAGL Inhibitor	Rimonabant; URB602	Intra-dlPAG	Rat	SIA – Footshock and Tail Flick	Rimonabant- Attenuation of SIA; URB602 – Enhances SIA.	(Hohmann et al., 2005)
CB_1 Antagonist/Inverse	Rimonabant; AA-5-HT	Intra-dlPAG;	Rat	SIA – Footshock and Tail Flick		

agonist; Dual FAAH/TRPV1 Inhibitor CB_1 Antagonist/Inverse agonist	Rimonabant	Intra-dlPAG	Rat	FCA - Formalin and Footshock	Rimonabant- Suppression of SIA; AA-5-HT – enhances SIA Rimonabant attenuated FCA	(Suplita Ii, et al., 2005) (Olango, Roche, Ford, Harhen, & Finn, 2012)

Table 3. Summary of studies investigating the role of the endocannabinoid system in the PAG in pain and its modulation by stress. $CB_{1/2}$ - Cannabinoid receptor type 1/2; TRPV1 - transient receptor potential cation channel subfamily V member 1; FAAH – Fatty acid amide hydrolase; MAGL - Monoacylglycerol lipase; PAG – periaquaductal grey; vlPAG – ventrolateral periaquaductal grey; dPAG – dorsal periaquaductal grey; RVM - Rostral ventromedial medulla; dlPAG – dorsolateral periaquaductal grey; SIA – stress-induced analgesia; FCA – fear-conditioned analgesia

Class of pharmacological compound	Pharmacological or genetic intervention	Route of administration	Species	Model	Effect	Reference
CB_1/CB_2 Agonist	WIN55,212-2	Intra-BLA; Intra-CeA	Rat	Tail Flick	Increased tail flick latency	(Martin et al., 1999)
CB_1/CB_2 Agonist	WIN55,212-2	Intra-BLA	Rat	Formalin; Tail flick	Increased tail flick latency; decreased formalin evoked nociceptive behaviour - attenuated via AM251	(Hasanein, Parviz, Keshavarz, & Javanmardi, 2007)
CB_1/CB_2 Agonist	WIN 55,212-2;	Intra-CeA, Intra-BLA.	Rats	Tail Flick	Antinociception	(Manning, Martin, & Meng, 2003)
CB_1/CB_2 Agonist	WIN 55,212-2	Intramuscularly	Rhesus monkey	Warm-water tail-withdrawal assay	Dose-dependent antinociception; attenuated via bilateral amygdala lesions	(Manning, Merin, Meng, & Amaral, 2001)
	Rimonabant	Intra-BLA	Rat			

CB_1 Antagonist/Inverse agonist				SIA - Tail Flick, Footshock	Suppression of SIA	(Connell, Bolton, Olsen, Piomelli, & Hohmann, 2006)
	Rimonabant	Intra-BLA	Rat	FCA – Formalin, Footshock	Reduced formalin-evoked nociceptive behaviour; no effect on FCA	(Roche et al., 2007, 2010)
FAAH Inhibitor	URB597	intraperitoneal	Rat	FCA – Formalin, Footshock	URB597 enhances FCA; weakened by rimonabant and SR144528; FCA associated with enhanced phospho-ERK in the amygdala	(Butler, et al., 2008)
CB_1 Antagonist/Inverse agonist	AM251	intraperitoneal; Intra-BLA; Intra-CeA	Rat	FCA – Formalin, Footshock	AM251 prevents expression of FCA following intraperitoneal and intra-BLA but not intra-CeA injection	(Rea et al., 2013)

Table 4. Summary of studies investigating the role of the endocannabinoid system in the amygdala in pain and its modulation by stress. CB_1 - Cannabinoid receptor type 1; FAAH – Fatty acid amide hydrolase; BLA – basolateral amygdala; CeA - central nucleus of the amygdala; SIA – stress-induced analgesia; FCA – fear-conditioned analgesia

Class of pharmacological compound	Pharmacological or genetic intervention	Route of administration	Species	Model	Effect	Reference
Dual FAAH/TRPV1 Inhibitor	AA-5-HT	Intra-PrL; Intra-IL	Rats	Noxious mechanical stimuli (Von Frey); SNI rats	Reduced mechanical allodynia in rats following SNI	(Giordano et al., 2012)
Dual FAAH/TRPV1 Inhibitor; FAAH Inhibitor; TRPV1 Antagonist	AA-5-HT; URB597; I-RTX	Intra-PrL; Intra-IL	Rats	SNI rats	All drugs produced antinociception; AA-5-HT produced antinociception more efficiently than URB597 or I-RTX	(de Novellis et al., 2011)
PPARα antagonist; PPARα agonist	GW6471; GW7647	Intra-mPFC	Rats	Formalin	GW6471, but not GW7647, delayed onset of the second phase of formalin-evoked nociceptive behaviour	(Okine et al., 2014)

CB_1 Antagonist/Inverse agonist	AM251	Intra-PrL	Rat	FCA – Tail Flick, Footshock	Attenuated FCA	(Freitas, Salgado-Rohner, Hallak, Crippa, & Coimbra, 2013)

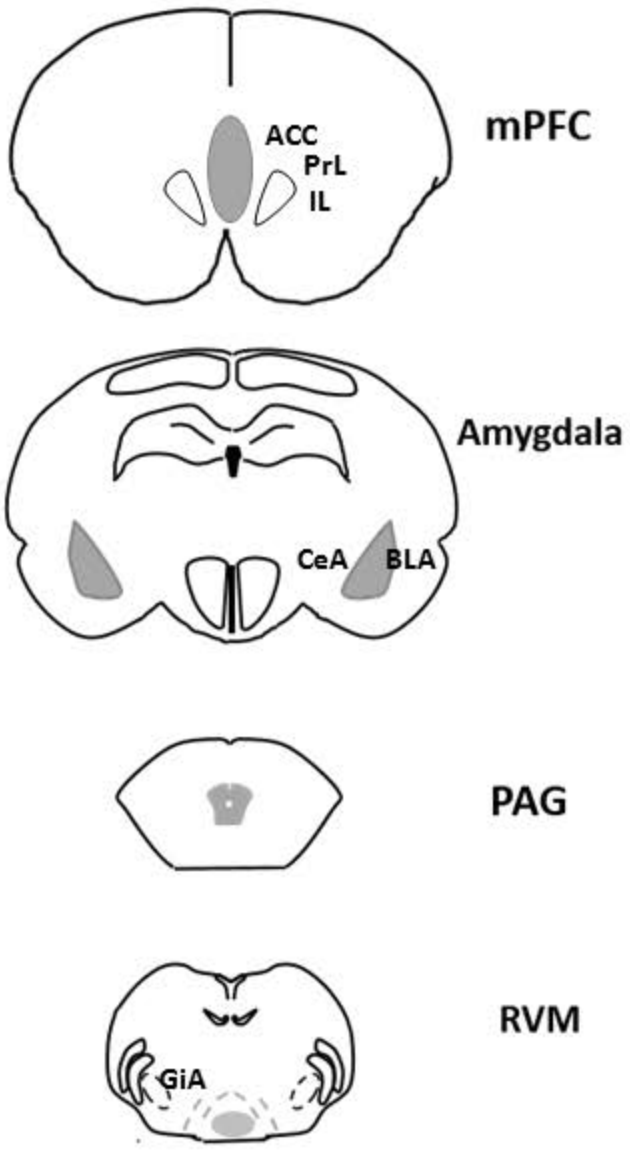
Table 5. Summary of studies investigating the role of the endocannabinoid system in the PFC in pain and its modulation by stress. CB_1 - Cannabinoid receptor type 1; TRPV1 - transient receptor potential cation channel subfamily V member 1; FAAH – Fatty acid amide hydrolase; PPARα - Peroxisome proliferator-activated receptor alpha; SNI – spared nerve injury; mPFC – medial prefrontal cortex; IL – infralimbic cortex; PrL – prelimbic cortex; FCA – fear conditioned analgesia

Figure legends:

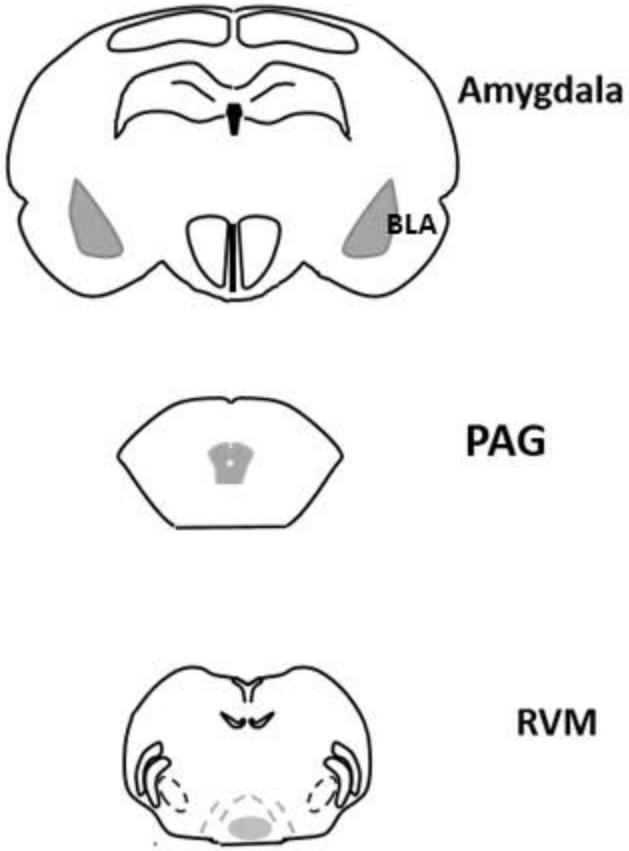
Figure 1. A synthesis of the literature reviewed herein on the role of the supraspinal endocannabinoid system in discrete brain regions in pain. mPFC: medial prefrontal cortex; ACC: anterior cingulate cortex; PrL: prelimbic cortex; IL: infralimbic cortex; BLA: basolateral amygdala; CeA: central nucleus of the amygdala; PAG: periaquaductal grey; RVM: rostral ventromedial medulla; GiA: gigantocellular reticular nucleus; TRPV1: transient receptor potential vanilloid 1; PPARs: peroxisome proliferator-activated receptors.

Figure 2. A synthesis of the literature reviewed herein on the role of the supraspinal endocannabinoid system in discrete brain regions in stress-induced analgesia (SIA) and stress-induced hyperalgesia (SIH). eCB: endocannabinoid; BLA: basolateral amygdala; PAG: periaquaductal grey; dlPAG: dorsolateral PAG; RVM: rostral ventromedial medulla; TRPV1: transient receptor potential vanilloid 1; WKY: Wistar-Kyoto rat.

Brain Region	Receptor Event	Functional Consequence
ACC	CB_1 receptor activation	Decreased pain-related behaviour/nociception
rACC	CB_1 receptor inhibition	Increased pain-related behaviour/nociception
IL/PrL	CB_1 receptor activation coupled with TRPV1 inhibition	Decreased pain-related behaviour/nociception
ACC	PPARα inhibition	Decreased pain-related behaviour/nociception
BLA	CB_1 receptor inhibition	Decreased pain-related behaviour/nociception
CeA	CB_1 receptor inhibition	Decreased pain-related behaviour/nociception
CeA/BLA	CB_1 receptor activation	Decreased pain-related behaviour/nociception
PAG	CB_1 receptor activation	Decreased pain-related behaviour/nociception
PAG	TRPV1 activation, opposite at higher dose	Decreased pain-related behaviour/nociception, opposite at higher dose
PAG	CB_1 receptor activation coupled with TRPV1 inhibition	Increased pain-related behaviour/nociception
RVM	CB_1 receptor activation	Decreased pain-related behaviour/nociception
GiA	CB_1 receptor activation	Decreased pain-related behaviour/nociception



Brain Region	Receptor Event	Functional Consequence
BLA	CB_1 receptor inhibition	Decreased SIA
Amygdala	eCB dysfunction in WKY rats	Decreased SIH
dlPAG	CB_1 receptor inhibition	Decreased SIA
dlPAG	CB_1 receptor activation	Increased SIA
dlPAG	CB_1 receptor activation coupled with TRPV1 inhibition	Increased SIA
RVM	CB_1 receptor inhibition	Decreased SIA
RVM	CB_1 receptor activation coupled with TRPV1 inhibition	Increased SIA
RVM	eCB dysfunction in WKY rats	Decreased SIH



Class of pharmacological compound	Pharmacological or genetic intervention	Route of administration	Species	Model	Effect	Reference
CB_1 / CB_2 Agonist	WIN 55,212-2	i.v.	Rat	Spinal Transection; Tail Flick	Inhibits noxious activity; blocked by spinal transection	(Hohmann, Tsou, & Walker, 1999)
CB_1 / CB_2 Agonist	THC; CP 55,940	i.c.v.	Rat	Spinal Transection; tail flick	Antinociception; blocked by rimonabant	(Lichtman & Martin, 1991, 1997)
CB_1 / CB_2 Agonist	THC	i.c.v.	Mouse	Tail Flick	Enhances the antinociceptive effects of morphine	(Welch, Thomas, & Patrick, 1995)
CB_1 / CB_2 Agonist	WIN 55,212-2; CP-55,940	i.c.v.	Rat	Tail Flick	Antinociception	(Martin, Lai, Patrick, Tsou, & Walker, 1993)
CB_1 / CB_2 Agonist	WIN 55,212-2; THC	i.c.v.	Mouse	Tail Flick	Dose-related antinociception	(Raffa, Stone, & Hipp, 1999)
CB_1 / CB_2 Agonist CB_1 / CB_2 Agonist	WIN 55,212-2	i.v.	Rats	Pressure stimulus to hindpaw; Electrophysiological recording of nociceptive neurons in thalamus	Decrease in nociceptive transmission in the thalamus	(Martin, Hohmann, & Walker, 1996)

	WIN 55,212-2	Microinjection; GiA, Thalamus, noradrenergic A5 region	Rats	Tail Flick	Antinociception when administered to each region	(Martin et al., 1999)
CB_1 / CB_2 Agonist	WIN 55,212-2	i.c.v.	Mice	Tail Flick; Paw Withdrawal in the lamp-foot-flick assay	Antinociception	(Fang et al., 2012)
CB_1 / CB_2 Agonist	Rimonabant	i.c.v.; i.t.; intraperitoneal	Mouse	Tail Flick	Rimonabant exhibits greater efficacy supraspinally rather than spinally.	(Welch, Huffman, & Lowe, 1998)
CB_1Antagonist/ inverse agonist	JWH-133	Intra-VPL	Rats	Spinal Nerve Ligation	Reduced noxious activity in SNL rats; blocked by SR144528 (CB_2 antagonist)	(Jhaveri et al., 2008)
CB_2 Agonist	FAAH (-/-); URB597 PF3945		Mice	Nitroglycerin-induced migraine-like pain	FAAH (-/-), URB597 and PF3945 reduce nociceptive behaviour; blocked by rimonanbant	(Nozaki, Markert, & Zimmer, 2015)
FAAH Inhibitor	FAAH (-/-)		Mice	Tail immersion,	FAAH (-/-)	

				hot plate, formalin tests, CCI and carageenan	increased response latency in tail immersion and hot plate test; reduced formalin-evoked nociceptive behaviour. Anti-hyperalgesic in carrageenan model	(Lichtman, Shelton, Advani, & Cravatt, 2004)
FAAH Inhibitor; MAGL Inhibitor	URB597; JZL184	i.p.	Mice	CUS; NGF hyperalgesia; tail flick; hot plate	Effects in CUS mice - Enhanced levels of AEA and 2-AG; decreased thermal hyperalgesia; URB597 decreased NGF hyperalgesia	(Lomazzo et al., 2015)
	A-784168	i.c.v.	Rats	Model of osteoarthritis (Sodium monoiodoacetate); Complete Freund's Adjuvant (chronic inflammatory pain)	Decreased weight bearing; decreased chronic inflammatory thermal hyperalgesia	(Cui et al., 2006)
TRPV1 Antagonist	Capsazepine	i.c.v.	Mice	Formalin	Attenuation of nociceptive	

					behaviour	(Santos & Calixto, 1997)
TRPV1 Antagonist	GPR55(-/-)		Mice	Inflammatory mechanical hyperalgesia (von Frey; Complete Freuds Adjuvent)	Hyperalgesia absent in GPR55(-/-)	(Castane et al., 2006; Staton et al., 2008)
	GPR55(-/-)		Mice	Nerve ligation; mechanical hyperalgesia	Mechanical hyperalgesia absent following nerve ligation in GPR55(-/-)	(Staton, et al., 2008)
PPARγ agonist	Rosiglitazone; 15d-PGJ(2)	i.c.v.	Rats	Plantar carageenan model of inflammatory pain	Anti-inflammatory and anti-hyperalgesia effects	(Morgenweck et al., 2010)
CB_1 Antagonist/inverse agonist; Opioid Antagonist	Rimonabant; naloxone; CCK_2 knockout mice	i.p.	Mice	SIA – footshock; tail flick	Rimonabant prevented SIA, an effect not seen in CCK_2 knockout mice; naloxone weakened SIA in wild type and CCK_2 knockout mice	(Kurrikoff, Inno, Matsui, & Vasar, 2008)
	URB597	i.p.	Rats	FCA - Conditioned fear (footshock) and formalin test	URB597 enhanced FCA; attenuated by	

FAAH Inhibitor					rimonabant, SR144528 and naloxone	(Butler, Rea, Lang, Gavin, & Finn, 2008)

Table 1: Summary of studies investigating the role of the brain's endocannabinoid system in pain and its modulation by stress (excluding studies on RVM, PAG, amygdala and PFC which are summarised in Tables 2-5). $CB_{1/2}$ - Cannabinoid receptor type 1/2; i.v. – Intravenous; i.c.v. – Intracerebroventricular; GiA - nucleus reticularis gigantocellularis pars alpha; i.t. – intra thecal; Intra-VPL - Ventral posterolateral nucleus; FAAH – Fatty acid amide hydrolase; SNL – spinal nerve ligation; (-/-) – knock out; CCI – chronic constriction injury ; MAGL - Monoacylglycerol lipase; PPARα - Peroxisome proliferator-activated receptor alpha; TRPV1 - transient receptor potential cation channel subfamily V member 1; CUS – chronic unpredictable stress; NGF – nerve growth factor; AEA – anandamide; 2-AG - 2-Arachidonoylglycerol; GPR55 - G protein-coupled receptor 55; CCK2 – Cholecystokinin 2; SIA – Stress induced analgesia; FCA – fear-conditioned analgesia

Class of pharmacological compound	Pharmacological or genetic intervention	Route of administration	Species	Model	Effect	Reference
CB_1/CB_2 Agonists	WIN 55,212-2; HU210	Intra-RVM	Rat	Tail Flick	Nociceptive behaviour suppression	(Martin, Tsou, & Walker, 1998)
CB_1/CB_2 Agonist	WIN 55,212-2	Intra-RVM	Rat	Tail Flick	Increased tail flick latency; inhibition of on-cell activity; increase in off-cell activity; effects blocked by rimonabant	(Meng & Johansen, 2004)
CB_1/CB_2 Agonist	WIN 55,212-2	Intra-GiA	Rat	Tail Flick	Increased antinociception; blocked by rimonabant	(Monhemius, Azami, Green, & Roberts, 2001)
CB_1/CB_2 Agonist	WIN 55,212-2	Intra-GiA	Rat	Partial nerve ligation; formalin test	Decrease in formalin-evoked nociceptive behaviour following nerve ligation; reversed by rimonabant	(Monhemius, et al., 2001)
	Rimonabant; AA-5-HT	Intra-RVM;	Rats	SIA –Footshock, Formalin	Suppression of SIA by	(Suplita Ii, Farthing,

CB_1 Antagonist/ Inverse agonist; Dual FAAH/TRPV1 Inhibitor CB_1 Antagonist/ Inverse agonist; FAAH Inhibitor	AM251; URB597	intraperitoneal (AM251 and URB597); Intra-RVM AM251	Wistar-Kyoto and Sprague Dawley rats	Formalin	rimonabant; enhancement of SIA by AA-5-HT Systemic AM251 potentiates hyperalgesia in WKY, URB597 attenuates hyperalgesia in WKY associated with impaired pain-related mobilization of ECs in RVM of WKY rats as seen from Intra-RVM AM251.	Gutierrez, & Hohmann, 2005) (Rea et al., 2014)

Table 2. Summary of studies investigating the role of the endocannabinoid system in the RVM in pain and its modulation by stress. $CB_{1/2}$ - Cannabinoid receptor type 1/2; RVM - Rostral ventromedial medulla; GiA - nucleus reticularis gigantocellularis pars alpha; SIA – Stress-induced analgesia; WKY – Wistar Kyoto; TRPV1 - transient receptor potential cation channel subfamily V member 1; FAAH – Fatty acid amide hydrolase

Class of pharmacological compound	Pharmacological or genetic intervention	Route of administration	Species	Model	Effect	Reference
CB_1/CB_2 Agonist	HU-210	vlPAG; systemic	Rat	Hot late test	HU210 enhanced antinociceptive effect of morphine and morphine enhanced the antinociceptive effect of HU210	(Wilson-Poe, Pocius, Herschbach, & Morgan, 2013).
CB_1/CB_2 Agonist	HU210	Intra-dPAG	Rat	Formalin	Reduced formalin-evoked nociceptive behaviour; blocked following rimonabant administration	(Finn et al., 2003)
CB_1/CB_2 Agonist	CP-55,940	Intra-vlPAG; Intra-dPAG;	Rat	Tail Flick	Intra-vlPAG microinjection produced antinociception; intra-dPAG had no effect	(Lichtman, Cook, & Martin, 1996)
CB_1/CB_2 Agonist	WIN55,212-2	Intra-dPAG	Rats	Tail Flick	Increased tail flick latency	(William J. Martin, Patrick, Coffin, Tsou, & Walker, 1995)

CB_1 Antagonist/ Inverse agonist	AM251	Intra-PAG; Intra-RVM	Rat	Metazinol induced antinociception in a carrageenan model of inflammation	Reverses metazinol-induced analgesia	(Escobar et al., 2012)
TRPV1 Agonist	Capsaicin (low dose); Capsaicin (high dose)	Intra-dlPAG	Rat	Plantar test	Low dose – Antinociception; High dose – Blocked antinociception	(Palazzo et al., 2002)
TRPV1 Agonist; TRPV1 Antagonist	Capsaicin; Capsazepine	Intra-dlPAG	Rat	Tail Flick	Capsaicin – hyperalgesia followed by antinociception; Capsazepine – blocked hyperalgesic effect of capsaicin	(McGaraughty et al., 2003)
FAAH Inhibitor	URB597	Intra-vlPAG	Rat	Plantar test	Low dose – Hyperalgesia – coadministration with AM251 converted hyperalgesia to analgesia – coadministration with capsazepine and AM251	(Maione et al., 2006)

					abolished any effect of URB597; High dose – Antinociception; Intermediate dose – biphasic response – blocked by AM251, hyperalgesic following coadministration of URB597 with capsazepine	
Dual FAAH/TRPV1 Inhibitor; CB_1 Antagonist/Inverse agonist; TRPV1 antagonist; FAAH Inhibitor	AA-5-HT; AM251; I-RTX; URB597	Intra-PAG	Rat	Tail Flick;	AA-5-HT induced antinociception – blocked by AM251 and I-RTX; URB597 and I-RTX induced analgesia	(de Novellis et al., 2008)
CB_1 Antagonist/Inverse agonist; MAGL Inhibitor	Rimonabant; URB602	Intra-dlPAG	Rat	SIA – Footshock and Tail Flick	Rimonabant- Attenuation of SIA; URB602 – Enhances SIA.	(Hohmann et al., 2005)
CB_1 Antagonist/Inverse agonist; Dual FAAH/TRPV1 Inhibitor	Rimonabant; AA-5-HT	Intra-dlPAG;	Rat	SIA – Footshock and Tail Flick	Rimonabant- Suppression of SIA; AA-5-HT – enhances SIA	(Suplita Ii, et al., 2005)

CB_1 Antagonist/Inverse agonist	Rimonabant	Intra-dlPAG	Rat	FCA - Formalin and Footshock	Rimonabant attenuated FCA	(Olango, Roche, Ford, Harhen, & Finn, 2012)

Table 3. Summary of studies investigating the role of the endocannabinoid system in the PAG in pain and its modulation by stress. $CB_{1/2}$ - Cannabinoid receptor type 1/2; TRPV1 - transient receptor potential cation channel subfamily V member 1; FAAH – Fatty acid amide hydrolase; MAGL - Monoacylglycerol lipase; PAG – periaquaductal grey; vlPAG – ventrolateral periaquaductal grey; dPAG – dorsal periaquaductal grey; RVM - Rostral ventromedial medulla; dlPAG – dorsolateral periaquaductal grey; SIA – stress-induced analgesia; FCA – fear-conditioned analgesia

Class of pharmacological compound	Pharmacological or genetic intervention	Route of administration	Species	Model	Effect	Reference
CB_1/CB_2 Agonist	WIN55,212-2	Intra-BLA; Intra-CeA	Rat	Tail Flick	Increased tail flick latency	(Martin et al., 1999)
CB_1/CB_2 Agonist	WIN55,212-2	Intra-BLA	Rat	Formalin; Tail flick	Increased tail flick latency; decreased formalin evoked nociceptive behaviour - attenuated via AM251	(Hasanein, Parviz, Keshavarz, & Javanmardi, 2007)
CB_1/CB_2 Agonist	WIN 55,212-2;	Intra-CeA, Intra-BLA.	Rats	Tail Flick	Antinociception	(Manning, Martin, & Meng, 2003)
CB_1/CB_2 Agonist	WIN 55,212-2	Intramuscularly	Rhesus monkey	Warm-water tail-withdrawal assay	Dose-dependent antinociception; attenuated via bilateral amygdala lesions	(Manning, Merin, Meng, & Amaral, 2001)
CB_1 Antagonist/Inverse agonist	Rimonabant	Intra-BLA	Rat	SIA - Tail Flick, Footshock	Suppression of SIA	(Connell, Bolton, Olsen, Piomelli, & Hohmann, 2006)
CB_1 Antagonist/Inverse agonist	Rimonabant	Intra-BLA	Rat	FCA – Formalin, Footshock	Reduced formalin-evoked nociceptive	

					behaviour; no effect on FCA	(Roche et al., 2007, 2010)
FAAH Inhibitor	URB597	intraperitoneal	Rat	FCA – Formalin, Footshock	URB597 enhances FCA; weakened by rimonabant and SR144528; FCA associated with enhanced phospho-ERK in the amygdala	(Butler, et al., 2008)
CB_1 Antagonist/Inverse agonist	AM251	intraperitoneal; Intra-BLA; Intra-CeA	Rat	FCA – Formalin, Footshock	AM251 prevents expression of FCA following intraperitoneal and intra-BLA but not intra-CeA injection	(Rea et al., 2013)

Table 4. Summary of studies investigating the role of the endocannabinoid system in the amygdala in pain and its modulation by stress. CB_1 - Cannabinoid receptor type 1; FAAH – Fatty acid amide hydrolase; BLA – basolateral amygdala; CeA - central nucleus of the amygdala; SIA – stress-induced analgesia; FCA – fear-conditioned analgesia

Class of pharmacological compound	Pharmacological or genetic intervention	Route of administration	Species	Model	Effect	Reference
Dual FAAH/TRPV1 Inhibitor	AA-5-HT	Intra-PrL; Intra-IL	Rats	Noxious mechanical stimuli (Von Frey); SNI rats	Reduced mechanical allodynia in rats following SNI	(Giordano et al., 2012)
Dual FAAH/TRPV1 Inhibitor; FAAH Inhibitor; TRPV1 Antagonist	AA-5-HT; URB597; I-RTX	Intra-PrL; Intra-IL	Rats	SNI rats	All drugs produced antinociception; AA-5-HT produced antinociception more efficiently than URB597 or I-RTX	(de Novellis et al., 2011)
PPARα antagonist; PPARα agonist	GW6471; GW7647	Intra-mPFC	Rats	Formalin	GW6471, but not GW7647, delayed onset of the second phase of formalin-evoked nociceptive behaviour	(Okine et al., 2014)
CB_1 Antagonist/Inverse agonist	AM251	Intra-PrL	Rat	FCA – Tail Flick, Footshock	Attenuated FCA	(Freitas, Salgado-Rohner, Hallak, Crippa, & Coimbra, 2013)

Table 5. Summary of studies investigating the role of the endocannabinoid system in the PFC in pain and its modulation by stress. CB_1 - Cannabinoid receptor type 1; TRPV1 - transient receptor potential cation channel subfamily V member 1; FAAH – Fatty acid amide hydrolase; PPARα - Peroxisome proliferator-activated receptor alpha; SNI – spared nerve injury; mPFC – medial prefrontal cortex; IL – infralimbic cortex; PrL – prelimbic cortex; FCA – fear conditioned analgesia