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Review

Dynamic changes to the endocannabinoid system in models of chronic pain

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The analgesic effects of cannabinoid ligands, mediated by CB1 receptors are well established. However, the side-effect profile of CB1 receptor ligands has necessitated the search for alternative cannabinoid-based approaches to analgesia. Herein, we review the current literature describing the impact of chronic pain states on the key components of the endocannabinoid receptor system, in terms of regionally restricted changes in receptor expression and levels of key metabolic enzymes that influence the local levels of the endocannabinoids. The evidence that spinal CB2 receptors have a novel role in the modulation of nociceptive processing in models of neuropathic pain, as well as in models of cancer pain and arthritis is discussed. Recent advances in our understanding of the spinal location of the key enzymes that regulate the levels of the endocannabinoid 2-AG are discussed alongside the outcomes of recent studies of the effects of inhibiting the catabolism of 2-AG in models of pain. The complexities of the enzymes capable of metabolizing both anandamide (AEA) and 2-AG have become increasingly apparent. More recently, it has come to light that some of the metabolites of AEA and 2-AG generated by cyclooxygenase-2, lipoxygenases and cytochrome P450 are biologically active and can either exacerbate or inhibit nociceptive signalling.

Keywords: pain; spinal cord; cannabinoid

1. AN OVERVIEW OF THE CANNABINOID RECEPTOR SYSTEM AND PAIN PATHWAYS

The key components of the endocannabinoid system—receptors, ligands and the metabolic enzymes—are present throughout the pain pathway from peripheral nerve terminals up to supraspinal centres. CB1 receptor density is moderate to high in regions involved in pain transmission and modulation, such as dorsal root ganglion (DRG), spinal cord, thalamus, periaqueductal grey (PAG), amygdala and rostroventromedial medulla [1].

Under normal physiological conditions, potentially damaging stimuli are detected by nociceptors expressed on primary afferent fibres, which relay noxious inputs to the spinal cord prior to these inputs being relayed to supraspinal regions. Under pathological conditions, changes in the peripheral, spinal and supraspinal processing of noxious inputs can produce altered nociceptive signalling, leading to aberrant pain responses (for review see [2]).

As discussed in more detail in other chapters, the two classes of endocannabinoids have distinct synthetic pathways. *N*-acylethanolamines (NAEs) such as anandamide (AEA) are produced via the stimulus-dependent hydrolysis of membrane phospholipid precursors of the *N*-arachidonoyl-phosphatidyl-ethanolamine (NAPE) family. Initially, this process was thought to be solely mediated by a specific isoform of phospholipase D known as NAPE-PLD [3]. However, genetic deletion of NAPE-PLD does not alter brain levels of AEA [4], calling into question the relevance of this pathway in the central nervous system (CNS). Two further synthetic pathways for the NAEs have been mapped, involving PLC-PTPN22 [5] and αβ hydrolase D4 (ABHD4)-GDE1 [6]. The expression of these enzymes within the CNS is heterologous, and the relative contributions of each to NAE production have yet to be mapped. The other major endocannabinoid 2-AG is produced through a mechanism involving sequential hydrolysis of phosphatidylinositol by PLA1 and PLC [7] or PLCβ [8] to produce diacyl glycerol (DAG). DAG is then cleaved by diacylglycerol lipase α or β (DAGLα/β) [8,9] to form 2-AG. The synaptic localization of DAGLα has been mapped in various areas of the brain and, relevant to this chapter, the dorsal horn of the spinal cord [10]. The termination of endocannabinoid signalling occurs in two stages: rapid removal of endocannabinoids from the synaptic cleft, and subsequent catabolism via specific enzymes in the intracellular environment; fatty acid amide hydrolase (FAAH) for

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the NAEs [11], and monoacyl glycerol lipase (MAGL) for the MAGs [12,13].

The analgesic properties of cannabinoids have been extensively characterized and reviewed [14–16], but their therapeutic appeal is limited by the plethora of side-effects associated with global activation of CB1 receptors. Endocannabinoids also possess anti-nociceptive properties, and these have been thoroughly explored in animal models of pain (see references in recent studies [17–19]). Many chronic pain states have unknown aetiology and their underlying mechanisms remain unclear. As such, animal models that mimic key aspects of the disease have been developed in order to gain a better understanding of the pain states and to test the potential of therapeutic targets.

A common feature of many chronic pain states arising from a peripheral injury or lesion is the presence of primary hypersensitivity in the area of damage. This arises as a result of the sensitization of peripheral nerve terminals; the infiltration of immune cells such as monocytes, macrophages and neutrophils; and the development of inflammation (see references in McMahon *et al.* [20]). In addition to these peripheral events, chronic pain states are invariably characterized by the presence of central sensitization at both the level of the spinal cord and supraspinally, which leads to aberrant pain responses such as allodynia (see references in Woolf & Salter [21]). Central sensitization is also associated with the activation and/or recruitment of glial cells within the nervous system, which modulate neuronal responses through the initiation of multiple signalling cascades [22]. The activation of microglia and astrocytes in the dorsal horn of the spinal cord plays a critical role in the development of facilitated nociceptive responses and spinal hyperexcitability in chronic pain models [23]. The plasticity of the systems that impact upon nociceptive processing—in particular, the types of cell present at sites of injury, and the activation states of these cells, can have profound influence on the levels of endocannabinoids and their receptors in these discrete regions. Here, we discuss the evidence that the dynamic changes in the endocannabinoid system that are associated with chronic pain states provide new opportunities for cannabinoid-mediated modulation of nociceptive processing (figure 1).

2. CB1 RECEPTOR MODULATION OF PAIN PROCESSING IN MODELS OF CHRONIC PAIN

A number of studies have investigated the effects of systemic and spinal administration of cannabinoid ligands on pain behaviour in models of neuropathic pain (table 1). Early studies demonstrated that systemic administration of WIN55, 212-2 attenuates pain behaviour in neuropathic rats [55]. Subsequently, it was reported that CB1 receptor expression is increased in the spinal cord of neuropathic rats, predominantly in the superficial laminae of the dorsal horn [56]. Increases in the expression of CB1 receptors were significant at day 4 post-injury, with levels continuing to increase until day 14. These increases were mediated in part by tyrosine kinase receptors and by ERK–MAPK signalling pathways. This upregulation of CB1 receptors appeared to be of functional relevance, because prevention of CB1 receptor upregulation diminished the inhibitory effects of WIN55,212-2 in this model [56]. In contrast to this functional enhancement of spinal CB1 receptors, a downregulation of CB1 receptors in the PAG has been described in the chronic constriction injury model of neuropathic pain [57]. Given the complex role of the descending controls in chronic pain states, and the importance of descending facilitations in driving central sensitization [58], these supra-spinal changes in CB1 receptor expression are likely to have important functional consequences.

Another major pain state with an emerging therapeutic role for cannabinoids is cancer pain. Cannabinoid drugs are now used in the symptomatic treatment of pain associated with cancer [59]. Studies in models of cancer pain have revealed differing effects on the expression of cannabinoid receptors within the CNS, with some groups reporting an upregulation of CB1 receptor in the L5 DRG in mice injected with squamous cell carcinoma into the hindpaw [46], while others studying models of bone cancer report no change in expression [48,60]. Despite these differences, administration of cannabinoid agonists produces robust analgesia in all models of cancer pain via activation of both CB1 [46,50,60] and CB2 receptors [46–50]. Interestingly, antinociceptive effects of both intrathecal and systemic administration of the CB2 receptor selective agonist AM1241 were abolished by intrathecal administration of the CB2 receptor antagonist SR144528, indicating a spinal site of action [48], and the effects of systemic AM1241 were blocked by naloxone [48], indicating a role of endogenous opioids, which warrants further investigation. Such interactions have previously been demonstrated in keratinocytes in naive rats [61], and may indicate interplay between the endogenous nociceptive systems at multiple levels of the pain pathways.

3. A NOVEL ROLE OF CB2 RECEPTORS IN CHRONIC PAIN STATES

The expression of CB2 receptors by immune cells is well-established [62]. It is now nearly 10 years since Zhang *et al.* [63] reported the presence of CB2 mRNA in the spinal cord of neuropathic rats, which appeared to be associated with microglia. Since then, a number of other studies have reported CB2 receptor mRNA and protein in the spinal cord in models of neuropathic pain (table 2). This upregulation of CB2 receptors has been shown to have functional consequences, as activation of spinal CB2 receptors attenuates neuronal [37] and behavioural [32,42] nociceptive responses in models of neuropathic pain. In contrast to both mixed CB1 and CB2 agonists and selective CB1 agonists, activation of spinal CB2 receptors attenuated pain responses in neuropathic rats without altering nociceptive processing *per se* in control rats [37]. Studies using CB2 knockout mice report that effects of spinally administered CB2 agonists are absent in these animals [42], further consolidating the evidence for novel functional effects of spinal CB2 receptors in models of neuropathic pain.

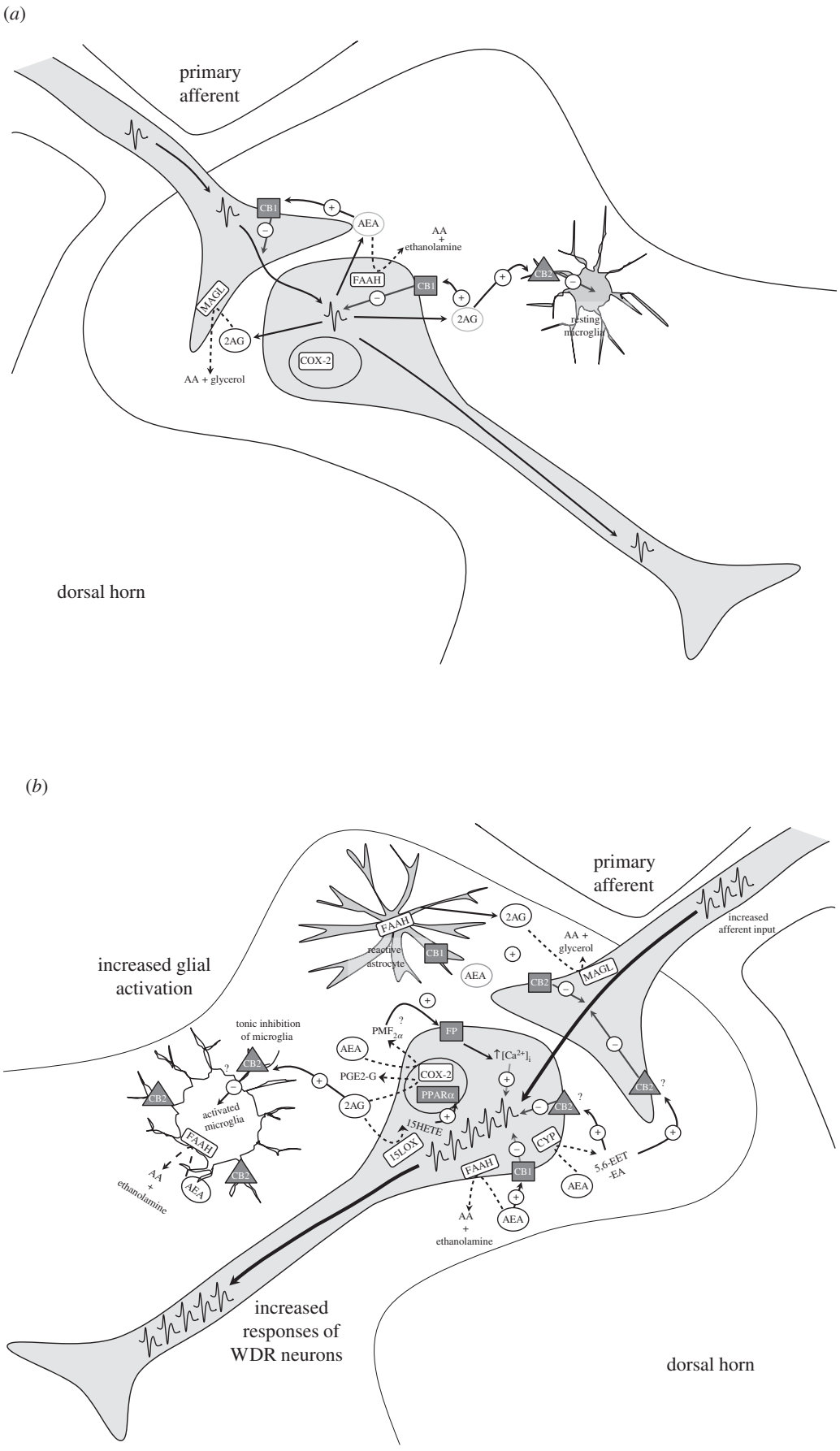

Figure 1. (*Caption opposite.*)

Neuropathic pain responses are often associated with diseases such as diabetes, and can also arise as a result of drug treatments such as chemotherapy [65]. Although less widely studied, the effects of cannabinoids on pain responses have been evaluated in animal models of these conditions. Systemic administration of

Figure 1. (*Opposite.*) (*a*) Under basal conditions, endocannabinoids modulate spinal nociceptive transmission through activation of pre- [10,24] and post-synaptic [25] CB1 receptors expressed on primary afferent fibres. Increased intracellular calcium ($[\uparrow Ca^{2+}]_i$) in postsynaptic neurons can stimulate AEA and 2-AG production and consequently activation of CB1 receptors. AEA and 2-AG are broken down via their respective catabolic enzymes (AEA: FAAH, 2-AG: MAGL) in the pre- and post-synaptic neurons and potentially also by resting microglia, which may express cannabinoid CB2 receptors. (*b*) In chronic pain states, activated spinal microglia and astrocytes release sensitizing factors, which can further facilitate the already-enhanced nociceptive signalling in the spinal cord. Under these conditions, endocannabinoid production is augmented in the spinal cord [26,27], and enzymes such as cytochrome p450 (CYP) and upregulation of cycloxygenase-2 (COX-2) and lipoxygenases (15-LOX) may provide alternative catabolic pathways for these endocannabinoids. These alternative pathways can result in the production of biologically active metabolites that modulate nociceptive transmission [28–30]. The generation of prostaglandin glycerol esters such as PGE2-G and prostaglandin ethanolamides (prostamide) such as prostamide $F_{2\alpha}$ ($PMF_{2\alpha}$) can facilitate neuronal responses, although the mechanisms for these effects are currently unclear. One possibility is activation of prostaglandin FP receptors, producing increased intracellular calcium, leading to enhanced neuronal responses. Cannabinoid receptor expression is upregulated in the spinal cord; however, the exact location is not yet clear. CB1 receptors are also present on astrocytes [31], while CB2 receptors are present on microglia [32] and are thought to be upregulated on pre-synaptic terminals [33] following nerve injury. Spinal administration of CB2 receptor agonists inhibits responses of WDR neurons and thus an increase in postsynaptic expression of CB2 receptors cannot be ruled out. CB2 receptors appear to play a modulatory role in chronic pain state, with exacerbated nociceptive responses present in CB2 null mice [34]. +denotes activation; −denotes inhibition; dashed line denotes enzymatic breakdown.

Table 1. Novel effects of cannabinoids—CB2-mediated effects. i.p., intraperitoneal administration; i.t., intrathecal administration; i.pl., intraplantar administration; i.a., intra-articular administration; ↑, increase; ↓, decrease.

model	species	drug	route	effect	reference
neuropathic pain					
chemotherapy	rat	AM1241, AM1714	i.p.	↓mechanical allodynia	[35,36]
spinal nerve ligation (L5 and L6)	rat	JWH-133	i.pl., spinal	↓ mechanically evoked responses	[37,38]
	rat	AM1241	i.p.	↓ tactile allodynia and thermal hypersensitivity	[39]
	mouse	AM1241	i.p.	↑ tactile withdrawal thresholds to pre-ligation values	[39]
partial sciatic nerve	rat	GW405833	i.p.	↓ mechanical hyperalgesia	[40]
ligation	mouse	GW405833	i.p.	↓ mechanical hyperalgesia	[41]
	mouse	JWH-133	i.t.	↓mechanical allodynia	[42]
L5 nerve transection	rat	JWH-015	i.t.	↓mechanical allodynia	[32]
spared nerve injury	mouse	NES400	i.p.	↓ tactile allodynia and thermal hypersensitivity	[43]
chronic constriction injury	rat	GW405833, JWH-133	i.t.	no effects	[44]
brachial plexus avulsion	mouse	JWH-015	i.p.	↓mechanical allodynia	[45]
cancer pain					
bone cancer	mouse	AM1241,	i.p., i.pl.	↓ spontaneous and evoked pain in inoculated limb, ↓mechanical allodynia	[46,47]
	mouse	JWH-015	i.p., i.t.	↓tactile allodynia and thermal hyperalgesia	[48,49]
	rat	WIN55,212-2	i.t., i.pl.	↓mechanical allodynia	[50,51]
arthritis pain					
MIA-induced OA	rat	GW405833	i.a.	↑ peripheral neuronal responses	[52]
	rat	A-796260	i.p.	↑ hind limb grip strength	[53]
CFA-induced joint	rat	Δ^9-THC	i.p.	↓ mechanical hyperalgesia	[54]

WIN55, 212-2 has been shown to attenuate mechanical allodynia in a model of chemotherapy-induced neuropathy by a number of groups [35,66,67]. The inhibition of mechanical allodynia by spinal WIN55,212-2 was sensitive to both CB1 and CB2 receptor antagonists [36], despite studies showing no change in the expression of either receptor, which may indicate a change in function of CB2 under these conditions. The impact of diabetes on the expression of cannabinoid receptors and the effects of cannabinoid ligands on pain responses have been studied in the rat STZ model of diabetes and diabetic neuropathy. In this model, expression levels of cannabinoid CB1 receptors in the small diameter dorsal root ganglia cell bodies (corresponding to C- and Aδ-fibres) were decreased, which may be related to exposure to high levels of blood glucose [64]. Despite this downregulation in CB1 receptor expression, systemic administration of WIN55,212-2 has been shown to attenuate pain responses in this model [68,69].

In contrast to the previous studies described earlier (tables 1 and 2), a recent study has reported that spinal

Table 2. Changes in cannabinoid CB1 and CB2 expression in models of chronic pain. ↑ denotes increase; ↓ denotes decrease; ↔ denotes no change.

model	species	tissue	CB1	CB2	reference
neuropathic pain					
brachial plexus avulsion	mice	C4-T2 DRG	↑mRNA and protein Ipsilateral	↑mRNA and protein	[45]
		cervical-thoracic spinal cord	↑mRNA and protein	↑mRNA and protein	[45]
		cingulate cortex	↑ protein		[45]
spinal nerve ligation	rat	lumbar spinal cord		↑ protein ipsilateral	[33]
sciatic nerve section	rat	lumbar spinal cord		↑ protein ipsilateral	[33]
	rat	L4-5 DRG		↑ protein ipsilateral	[33]
chronic constriction injury	rat	spinal cord	↑ protein	↑ mRNA ipsilateral	[56,63]
	rat	spinal cord		↔ protein	[44]
L5 spinal nerve transection	rat	lumbar spinal cord	↑ protein		[32]
streptozotocin-induced diabetes	rat	DRG	↓ protein		[64]
spare nerve injury	rat	lumbar spinal cord	↑ protein		[43]
cancer pain					
bone cancer pain	mouse	lumbar spinal cord	↔ protein	↔ protein	[48,60]
	mouse	L4-6 DRG		↔ protein	[48]

administration of the CB2 agonists GW405833 and JWH-133 does not alter mechanical allodynia at 3 or 10 days in the CCI model of neuropathy [44]. Furthermore, this study found no evidence of changes in CB2 receptor protein expression (both using Western blotting and immunohistochemistry) in the spinal cord in the CCI model of neuropathic pain, compared with naive rats [44]. On balance, there are a larger number of studies in support of a novel functional role for spinal CB2 receptors in models of neuropathic pain, although the findings reported by Brownjohn & Ashton [44] suggest that further studies with improved tools are required.

4. CB2 RECEPTOR MODULATION OF SPINAL IMMUNE CELL FUNCTION

To date, studies of the mechanisms underlying the spinal-CB2-receptor-mediated inhibition of neuropathic pain have focused on potential interactions with spinal immune cells (microglia and astrocytes), which play a pivotal role in these chronic pain states. Ipsilateral CB2 receptor upregulation has been demonstrated within 4 days following nerve injury, with CB2 receptors present on microglia and perivascular cells in the spinal cord of neuropathic rats [32]. Spinal administration of the CB2 receptor agonist JWH015 reduced peripheral-nerve-induced hypersensitivity and levels of spinal markers of microglia activation in this model of neuropathic pain. Similarly, treatment with WIN55,212-2 attenuated the expression of markers of microglial activation in the spinal cord in a model of chemotherapy-induced neuropathy [66]. Consistent with these findings, repeated treatment with another selective CB2 ligand was also shown to be antinociceptive. NESS400 administration significantly attenuated mechanical allodynia and thermal hyperalgesia, and also decreased markers of microglia and astrocyte activation in the spinal cord of neuropathic mice at 7 days post-injury [43]. Similarly, repeated treatment with WIN55,212-2 produced a significant attenuation of pain behaviour and levels of markers of astrocytes activation in tumour-bearing mice [70] but had no effect on pain behaviour in neuropathic mice.

Interestingly, the functional upregulation of spinal CB2 receptors in models of neuropathic pain appears to provide an essential brake on the development of central sensitization, as evidenced by the exacerbation of ipsilateral touch-evoked pain (allodynia), and the novel manifestation of contralateral allodynia in CB2 null mice [34]. These changes in pain behaviour in the absence of endogenous CB2 receptors were associated with increased levels of activated microglia and astrocytes in both the ipsilateral and contralateral spinal cord—responses that were strongly attenuated in mice over-expressing CB2 receptors [34]. Gene array studies in neuropathic CB2-null mice revealed a strong interferon response following nerve injury. The involvement of interferon-gamma (IFN-γ) was further implicated by the absence of pain behaviour in neuropathic mice deficient in both IFN-γ and CB2 [71]. Collectively, these data suggest that the presence of spinal CB2 receptors plays a crucial role in dampening down spinal sensitization via modulation of IFN-γ-mediated glial cell activation in these models of neuropathic pain.

The majority of evidence that CB2 receptors modulate microglia cells comes from cell culture studies. Almost 10 years ago, 2-AG was shown to stimulate microglia migration, whereas the CB2 receptor antagonist SR144528 inhibited basal microglia migration [72]. A more recent study [73] showed that a selective CB2 receptor agonist (JWH015) reduced p-ERK1/2 protein expression in LPS-stimulated primary microglia cells, which led to a significant reduction in the expression of tumour necrosis factor-α. In addition, JWH015 inhibited LPS-stimulated microglial migration was shown to be CB2-receptor-mediated as SR144528 blocked the anti-migratory effects of JWH015. Taken together, these reports suggest that CB2 receptors may have an important role in modulating both the activity state of microglia, as well as microglia chemotaxis, particularly during inflammatory conditions.

5. CANNABINOID MODULATION OF ARTHRITIC PAIN

There is increasing evidence that cannabinoid receptors may have clinical potential in other types of chronic pain states—in particular, arthritic pain. Preclinical studies have evaluated their therapeutic potential in models of both rheumatoid arthritis (RA) and osteoarthritis (OA).

RA is an autoimmune disease that is characterized by inflammation of the synovium and swelling of the joints. The cause of OA remains incompletely understood, although its development can be precipitated by damage to the joint structures or ligaments following injury. OA is primarily characterized by a loss of cartilage within the joint, as well as the development of bony outgrowths, or osteophytes, which further alter the structure of the joint. Furthermore, OA is associated with moderate inflammation. Both RA and OA are associated with reduced function of the joint and chronic pain, which dramatically reduces quality of life.

Electrophysiological studies in models of spontaneous and chemically induced arthritis have demonstrated that the facilitated nociceptive responses of peripheral nerves are attenuated in the presence of cannabinoid CB1 receptor agonists [74]. The role of CB2 receptors in modulating peripheral nerves innervating the arthritic joint appear to be complex; close arterial (peripheral) administration of CB2 receptor agonists increased vasodilatation in the inflamed knee joints of rats [75] and facilitated peripheral nerve responses in rats with OA joint damage [52]. Nevertheless, systemic administration of the CB2 receptor agonist A-796260 reversed decreases in grip strength, a surrogate measure of pain, in the monosodium iodoacetate (MIA) model of osteoarthritis pain [76]. Our current lack of knowledge about how arthritis joint pathology impacts on the expression of cannabinoid receptors, both on peripheral nerves and on local cells within the knee joint, hinders the further interpretation of these studies at this point in time. We have demonstrated the expression of cannabinoid CB1 and CB2 receptors in the synovial tissue of patients with RA and OA [77], but the extent by which peripheral cannabinoid receptors present in the synovium modulate arthritis-induced pain remains unknown.

A role of spinal cannabinoid receptors in modulating pain responses has been described in the MIA model of osteoarthritis. We have reported enhanced levels of AEA, 2-AG, PEA and OEA in the spinal cord of MIA-treated rats at 14 and 28 days, and associated elevations in protein levels of the synthetic enzymes DAGLα and NAPE-PLD [26]. These changes in the spinal endocannabinoid receptor system appeared to have functional consequences, as electrophysiological studies demonstrated that blocking spinal CB2 receptors significantly facilitated mechanically evoked responses of spinal neurons in MIA-treated rats, but not in control rats [26]. Furthermore, spinal administration of the FAAH inhibitor URB597 attenuated mechanically evoked responses, to a greater extent, in MIA-treated rats compared with control rats [26], presumably through potentiating inhibitory effects of the elevated levels of spinal endocannabinoids. Collectively, these data are indicative of a tonic modulation of spinal neuronal responses by endocannabinoids in this model of OA pain.

Inhibitory effects of cannabinoids have also been reported in models of inflammatory arthritis. Studies in rats with CFA-induced joint inflammation revealed a novel role of the CB2 receptor in mediating the effects of systemic Δ^9-THC in inflamed, but not in non-inflamed rats [54]. Few papers have addressed the potential role of spinal CB2 receptors in inflammatory arthritis, although studies in models of chronic inflammatory pain may provide information pertinent to these models. The effects of peripheral inflammation on spinal CB2 receptor expression are not conclusive, with evidence both for [78] and against [54,63] changes in the expression levels of spinal CB2 receptors following chronic inflammation. The demonstration that the effects of intrathecal AM1241 were abolished when co-administered with naloxone are suggestive of an interaction between CB2 receptor mechanisms and opioid receptor systems at the level of the spinal cord [78], mirroring that seen in cancer pain models (see above).

6. TONIC CONTROL OF SPINAL NOCICEPTIVE PROCESSING BY ENDOCANNABINOIDS

As discussed earlier, the spinal cord plays a critical role in the integration and modulation of nociceptive inputs prior to messages being sent to the higher brain centres. A number of studies have investigated the role of spinal endocannabinoids in maintaining the balance of neuronal excitability at this level, and how this may be manipulated to harness the therapeutic potential of the endocannabinoids. Exogenous application of endocannabinoids is antinociceptive at the level of the spinal cord [79,80], and the endocannabinoid system is also tonically active during nociceptive processing. Indeed, intrathecal administration of a CB1 receptor antagonist, rimonabant, produces hyperalgesia in mice [81] and enhances C-fibre-mediated firing of WDR neurons in the dorsal horn of the spinal cord [82]. The levels of endocannabinoids in the spinal cord are also elevated in some animal models of acute and chronic pain [26,83,84]. These observations suggest that the endocannabinoids form part of an endogenous brake on the activation of nociceptive pathways, and multiple groups have investigated the effects of pharmacological manipulation of endocannabinoid catabolism. In the case of FAAH, many studies have been discussed at length across a number of reviews [18,85,86] detailing the extensive corroborative evidence that inhibition of FAAH prevents the catabolism of AEA and other NAEs, and produces CB1- and CB2-mediated analgesic effects in models of a variety of pain states. In contrast, the role(s) of 2-AG in modulating nociceptive processing, and the therapeutic potential of MAGL inhibitors have only recently been investigated.

7. 2-AG AND PAIN PROCESSING

In the past few years, a growing number of publications concerning the role of 2-AG in pain processing have been published. Recent contributions to the field have supplied striking evidence that 2-AG may be a key molecular player in endogenous

inhibition at nociceptive synapses. Nyilas *et al.* [10] combined electron microscopy and immunofluorescence techniques to identify the relative positions of DAGLα and CB1 on nociceptive neurons in the dorsal horn of the mouse spinal cord [10]. Both proteins are highly expressed in the superficial laminae, with dense punctate staining revealing a compartmentalized sub-cellular expression. DAGLα is localized on the intracellular surface of cell membranes in dendritic shafts and spine heads post-synaptic to C and Aδ nociceptive afferents. CB1, in contrast, is localized pre-synaptically on excitatory axon terminals corresponding to small excitatory interneurons (e.g. vertical and radial cells) and C and Aδ nociceptor boutons. This expression pattern, along with the discovery that DAGLα co-localized with mGluR5 in the perisynaptic region, is indicative of a role for 2-AG in negative feedback at glutamatergic synapses within nociceptive pathways [87]. These data outline a role for 2-AG in the modulation of spinal nociceptive processing, which could be harnessed by preventing the catabolism of 2-AG.

Attempts to exploit this mechanism for therapeutic effect have been greatly aided by recent advances in this field, including elucidation of crystal structures for MAGL [88,89], and the development of an activity-based protein profiling technique for screening compounds [90]. These have led to the identification of several novel inhibitors of MAGL that produce cannabimimetic effects *in vivo*, including OMDM169 [91] and JZL184 [92]. JZL184 possesses high selectivity for MAGL over FAAH (>300-fold), HSL and other common off-target serine hydrolases and lipases, and shows a nanomolar potency in isolated mouse brain membranes [92]. Systemic administration of 8 mg kg^{-1} JZL184 in mice produced greater than five-fold elevation of brain levels of 2-AG, with minimal inhibition of FAAH. A higher dose of 16 mg kg^{-1} produced significant antinociceptive effects in both thermal and chemical models of acute pain, alleviating both cold and mechanical allodynia in the chronic constriction injury mouse model of neuropathic pain [93]. However, this dose also produced significant hypomotility and FAAH inhibition (>50%), though not elevation of AEA, complicating the interpretation of results. The potency of this compound is markedly reduced (approx. 10-fold) in rat brain membranes [92], but a small number of reports detailing the antinociceptive efficacy of local administration of JZL184 in the rat have been published. Indeed, antinociceptive effects of intra-plantar administration of JZL184 have been described in both phases of the formalin model of inflammatory pain [94], and also in capsaicin-induced acute pain [95], with mechanisms involving both CB1 and CB2 receptors. The effects of spinal or supra-spinal administration of JZL184 on nociceptive processing in control rats, or in models of chronic pain have yet to be reported. In the context of chronic pain states involving the activation of spinal glial cells, it is noteworthy that 2-AG signalling in microglia is thought to be terminated by ABHD12 [96], which is not sensitive to JZL184 [97].

Despite the positive indications for targeting MAGL/2-AG to produce analgesic effects, two recent publications have introduced a note of caution. Studies by Schlosburg *et al.* [98] and Chanda *et al.* [99] have reported that prolonged elevation of 2-AG levels, either by genetic deletion of MAGL or by repeated treatment with the MAGL-selective inhibitor JZL184, produced desensitization and downregulation of brain CB1 receptors. These effects were accompanied by a loss of JZL184-mediated analgesia, and cross tolerance to the antinociceptive and hypothermic effects of the CB1 receptor agonist WIN55,212,2. The development of analgesic tolerance to chronic JZL184 may be overcome, however, as repeated treatment with submaximal doses of JZL184 (<8 mg kg^{-1}) produced sustained analgesic effects [100]. Although these data suggest that MAGL inhibition has therapeutic potential for the treatment of pain, the demonstration that chronic JZL184 administration precipitated withdrawal responses to rimonabant, as indicated by an increase in paw flutters [101], suggests that prolonged JZL184 treatment may be associated with physical dependence.

8. THE POTENTIAL IMPACT OF ALTERNATIVE ENDOCANNABINOID CATABOLISM PATHWAYS ON PAIN PROCESSING

The endocannabinoids are not only subject to metabolism by the major catabolic pathways described earlier, but also by other enzymes, resulting in the production of bioactive metabolites. In particular, AEA and 2-AG can undergo oxidative metabolism by cyclooxygenase 2 (COX-2) [102], 15-, 12- and 5-lipoxygenase (LOX) [28], and some isoforms of cytochrome P450 [29]. Levels of some of these enzymes, which may serve a role in the alternative metabolism of the endocannabinoids, are altered in pain/inflammatory states, which could impact upon the levels of endocannabinoids present under these conditions. It is well established that the expression of COX-2 is elevated both in injured tissue and in the spinal cord in models of chronic pain [103–105]. Similarly, increased spinal expression of 5-LOX and FLAP (5-LOX-activating protein), both in terms of mRNA and protein, has been reported in a model of neuropathic pain [106]. To date, the impact of chronic pain on the expression of cytochrome P450s and αβ hydrolase 6 or 12 has yet to be described.

9. EFFECTS OF NOVEL BIOLOGICAL METABOLITES OF 2-AG AND ANANDAMIDE

The metabolism of AEA and 2-AG by COX-2 results in the generation of biologically active metabolites (prostaglandin ethanolamides and glycerol prostaglandins, respectively [107–109]). These metabolites can modulate synaptic activity with opposing effects to those of AEA and 2-AG [110]. Given that COX-2 expression is upregulated in chronic pain states, it is feasible that under these conditions, COX-2 may play a more prominent role in the catabolism of the endocannabinoids. This could occur both peripherally (at sites of injury) and centrally (at sites involved in the processing and integration of nociceptive inputs, such as the spinal cord). Levels of prostamide $F_{2\alpha}$ ($PMF_{2\alpha}$) are elevated in the spinal cord of mice with knee

inflammation, and spinal application of $PMF_{2\alpha}$ facilitates neuronal responses. This effect is abolished in the presence of the prostamide antagonist AGN 211336 [111]. Similarly, peripheral injection of prostaglandin E2 glycerol ester (PGE_2-G) induces mechanical allodynia and thermal hyperalgesia in rodents [30], and thus it appears that the catabolism of AEA and 2-AG by COX-2 to $PMF_{2\alpha}$ and PGE2-G, respectively, may drive pro-nociceptive mechanisms.

To date, the potential biological consequences of LOX catabolism of the endocannabinoids on nociceptive processing have not been directly investigated either *in vitro* or *in vivo*. The demonstration that, at least in cells, 15-LOX is capable of metabolizing 2-AG to 15-HETE-G (see references in Vandevoorde & Lambert [112]), which is a ligand for the anti-inflammatory nuclear receptor PPARα [28], suggests that changes in LOX expression and metabolism of endocannabinoids via this pathway may also influence nociceptive processing. Indeed, we and others have shown that PPARα ligands can have marked inhibitory effects on inflammatory pain responses (see references in [113–116]).

Most recently, the oxidative metabolism of AEA by cytochrome P450s has been described as another enzymatic pathway, leading to the production of bioactive metabolites. 5,6-epoxyeicosatrienoic acid ethanolamide (5,6-EET-EA) generated by P450-mediated catabolism of AEA is a potent agonist at CB2 receptors [29]. Interestingly, 5,6-EET-EA is also reported to be far more stable than AEA, while having little affinity at CB1 receptors [29], although it does act as a ligand at TRPV4 (see Snider *et al.* [117]). Although the impact of chronic pain states on the expression of P450s (e.g. at the level of the pain-associated regions of the spinal cord or brain) remain unclear, there is evidence that 5,6-EET-EA may modulate microglial activity *in vitro* [29]. Indeed, stimulation of the murine BV2 microglial cell line with IFN-γ increased the expression of CYP3A, and also enhanced the capacity of these cells to metabolize AEA into 5,6-EET-EA [29]. Given the well-documented role of microglia in the development of central sensitization in models of chronic pain, and the role of IFN-γ in mediating the exacerbation of chronic pain responses in CB2-receptor-deficient mice (see earlier), further studies of the effects of 5,6-EET-EA *in vivo* appear warranted.

The complexity of endocannabinoid metabolism via multiple pathways, which are dynamically altered under pathological conditions, is an important consideration when investigating the effects of drugs specifically targeting FAAH or MAGL to elevate AEA and 2-AG levels. Indeed, elevating levels of AEA appears to provide additional substrate for oxidative catabolism via COX-2, LOX and/or cytochrome P450s, depending on the level of enzyme available at the key sites involved in nociceptive processing. It remains to be determined whether FAAH inhibition is associated with an increase in the generation of 5,6-EET-EA or PGE_2-G under control conditions and/or in models of chronic pain. Clearly, it is unlikely that these are the only biologically active metabolites generated by the alternative metabolism of the endocannabinoids that could impact upon nociceptive processing. Thus, further studies are required to advance our understanding of both the role of these enzymatic pathways in the generation of biologically active metabolites of the endocannabinoids and how this may influence the analgesic effects of compounds that elevate levels of endocannabinoids via the inhibition of FAAH or MAGL.

In conclusion, the analgesic effects of cannabinoid ligands mediated by CB1 receptors are well established, but limited by their side-effect profile. Recent studies of models of chronic pain states have revealed complex changes in the expression of cannabinoid receptors and levels of the endocannabinoids, in particular, at the level of the spinal cord. Dynamic changes in levels of the enzymes capable of metabolizing the endocannabinoids AEA and 2-AG, in some cases to biologically active pro-nociceptive metabolites in key areas involved in pain processing, may impact upon pain responses and the therapeutic potential of some endocannabinoid based strategies for novel analgesics.

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