

Review

Modulation of neuropathic-pain-related behaviour by the spinal endocannabinoid/endovanilloid system

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Neuropathic pain refers to chronic pain that results from injury to the nervous system. The mechanisms involved in neuropathic pain are complex and involve both peripheral and central phenomena. Although numerous pharmacological agents are available for the treatment of neuropathic pain, definitive drug therapy has remained elusive. Recent drug discovery efforts have identified an original neurobiological approach to the pathophysiology of neuropathic pain. The development of innovative pharmacological strategies has led to the identification of new promising pharmacological targets, including glutamate antagonists, microglia inhibitors and, interestingly, endogenous ligands of cannabinoids and the transient receptor potential vanilloid type 1 (TRPV1). Endocannabinoids (ECs), endovanilloids and the enzymes that regulate their metabolism represent promising pharmacological targets for the development of a successful pain treatment. This review is an update of the relationship between cannabinoid receptors (CB1) and TRPV1 channels and their possible implications for neuropathic pain. The data are focused on endogenous spinal mechanisms of pain control by anandamide, and the current and emerging pharmacotherapeutic approaches that benefit from the pharmacological modulation of spinal EC and/or endovanilloid systems under chronic pain conditions will be discussed.

Keywords: transient receptor potential vanilloid type 1; cannabinoid receptor 1; anandamide; fatty acid amide hydrolase; neuropathic pain; spinal cord

1. INTRODUCTION

Pain is a complex sensory and emotional experience determined by an arbitrary interval of time since onset. According to the International Association for the Study of Pain (IASP), pain is defined as ‘an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage’. Pain involves a significant sensory component that results from the activation of nociceptive afferents, followed by extensive processing through the nervous system. This account considers the manners in which the spinal cord, as the first relay in the pathways from the periphery to the brain, can be sensitized by noxious stimuli to facilitate the amplification of minor peripheral input. Pain caused by injury is considered a part of our daily experience, acting as an alarm system that protects against injury or other forms of noxious stimuli to ease the healing process. Some individuals experience severe and recurring pain from various aetiologies, often from peripheral tissue inflammation or destruction. Pain may also originate from activity generated within the nervous system without adequate stimulation

of its peripheral sensory endings. Some of these individuals, and others for whom no peripheral pathophysiology is evident, experience stimulus-independent and/or stimulus-dependent pain, presumably reflecting abnormal spontaneous afferent activity, alterations in central processing and/or increased afferent sensitivity, and resulting in a clinical syndrome of neuropathic pain. The onset of neuropathic pain can be delayed after initial nerve injury; therefore, pain may be present in the absence of a detectable lesion or injury, making proper diagnosis and early treatment difficult. In addition, neuropathic pain may spread beyond the cutaneous distribution of injured nerves and exist bilaterally in mirror image sites, suggesting the involvement of a central mechanism [1]. Neuropathic pain refers to pain as a result of damage (due to injury or disease) to the nervous system, including the nerves, spinal cord and certain CNS regions [2,3]. The IASP introduced the term neuropathic pain as ‘pain caused by a lesion or disease of the somatosensory nervous system’. Patients with neuropathy often suffer from spontaneous pain (typically of a shooting or burning nature), allodynia (pain response to normally innocuous stimuli) and hyperalgesia (aggravated pain evoked by noxious stimuli).

Research examining the understanding of the neurophysiological plasticity of the nervous system in response to neuropathic pain, using a number of

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animal models to simulate human peripheral neuropathic conditions, is based on procedures at or near sciatic nerves. Methods differ in the location and form of injury. The latter includes (i) chronic constriction injury (CCI) by loose ligation of the sciatic nerve [4], (ii) tight ligation of the partial sciatic nerve (PSL) [5] and (iii) tight ligation of the spinal nerves (SNL) [6], as pictured in figure 1. Injury might also be evoked through cryoneurolysis [7], crush [8], perineural inflammation [9] and tumour invasion [10]. The most commonly used models (CCI, PSL and SNL) of peripheral neuropathic pain show behavioural signs of ongoing and evoked pain with similar time courses, and there is a considerable difference in the magnitude of each pain component between models. Signs of mechanical allodynia are greatest in SNL injury and smallest in the CCI model. However, behavioural signs representing ongoing pain are much more prominent in the CCI than in the other two models, suggesting that the three rat models have distinct features. Still, they all are valuable neuropathic pain models [11]. Thus, this review will discuss the literature, with a particular focus on these models.

The mechanisms, symptoms and signs of neuropathic pain are the subject of extensive studies and have been reviewed in detail [12–14]. Persistent pain is an independent disease with devastating effects on the patient's quality of life. Although our understanding of the mechanisms involved in the development of general and chronic pain of various origins has significantly increased in the last decade, the available pharmacological management of chronic pain has not improved considerably. Treating chronic pain—in particular, neuropathic pain—is still a challenge. Although a range of therapeutics to treat pain exists, these drugs are only mildly efficacious in neuropathic pain. Pain responds poorly to opioid analgesics owing to their low effectiveness, the potential and development of tolerance, the risk of addiction and the multiplicity of their side effects [15]. Thus, clinically, antidepressants and anticonvulsants remain the only mainstays of neuropathic pain therapy, but unfortunately, these drugs are only 50 per cent effective. Therefore, developing novel and effective analgesics is of principal importance.

2. SPINAL CORD: FIRST RELAY SITE IN THE NOCICEPTIVE TRANSMISSION

It is important to focus our attention on developing new management strategies for neuropathic pain treatment at the spinal cord level, where the pain stimulus might be altered or combined with other stimuli, thereby controlling neuropathic pain.

The current literature on pain contains details of numerous peripheral and central mechanisms that may provide future drugs for the management of neuropathic pain. The choice of molecular targets in developing treatments for neuropathic pain is currently based on many factors, including preclinical models. In addition, a combination of gene regulation studies, knockouts and pharmacological insights involving sodium [16–18], calcium [19,20] and potassium channels [21,22],

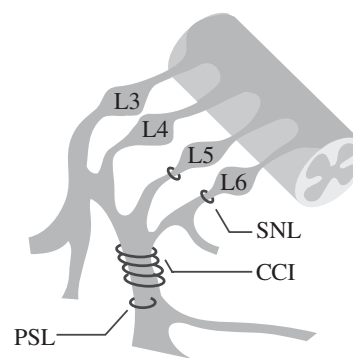


Figure 1. Schematic of some experimental models of neuropathic pain on the basis of injury to peripheral nerve structures. CCI, chronic constriction injury [4]; PSL, partial sciatic nerve ligation [5]; SNL, segmental L5/L6 spinal nerve ligation [6].

neurotrophins [23], cytokines [24–26], N-methyl-D-aspartate (NMDA) and glutamate receptor systems [27–29], the ligand-gated transient receptor potential vanilloid type 1 (TRPV1) and G-protein-coupled cannabinoid receptors [30], among others, have provided a range of new targets for neuropathic pain.

3. ARACHIDONIC ACID-DERIVED SIGNALLING LIPIDS

Many new molecules have reached clinical development, including agents that target cannabinoids and the TRPV1 receptor [31]. Cannabinoids exert antinociceptive effects through complex mechanisms that affect the central nervous system, spinal cord and peripheral sensory nerves. This fact is consistent with the anatomical location of cannabinoid CB1 receptors in areas relevant to pain in the brain: the spinal dorsal horn, dorsal root ganglia and peripheral afferent neurons [32–34]. The analgesic response to exogenous cannabinoids suggests a role for the endocannabinoid (EC) system in regulating pain sensitivity (for details, see [35,36]). However, the cannabinoids that have been tested in clinical trials are psychoactive, causing drowsiness, ataxia, dizziness and confusion. The key challenge for the development of an improved cannabinoid drug treatment for neuropathic pain is the development of compounds that preserve the analgesic effects of cannabinoids and reduce psychoactivity. Therefore, a group of neuromodulatory lipids and their receptors, called ECs, are important in establishing the existence of a new biochemical system and physiological role for these compounds.

4. ENDOCANNABINOIDS

Devane *et al.* [37] described the isolation of a porcine brain lipid arachidonylethanolamide named anandamide (AEA), which bound to the brain cannabinoid receptor and mimicked the behavioural actions of delta-9-tetrahydrocannabinol (Δ^9 -THC) when injected into rodents. Subsequently, Mechoulam *et al.* [38] and Sugiura *et al.* [39] independently identified a second EC, 2-arachidonoylglycerol (2-AG). Although the EC system is relatively 'novel' among

the known signalling systems, it is involved in a number of functions and pathological conditions, including the perception and modulation of pain. The EC system consists of the cannabinoid receptors CB1 and CB2, the endogenous ligands AEA and 2-AG, and their synthetic and metabolic machinery. Several other ECs, including noladin ether [40], O-arachidonylethanolamine, (virodhamine) [41] and N-arachidonol-dopamine [42], have been described (for review, see [43]).

Fatty acid amide hydrolase (FAAH) is the principle catabolic enzyme for fatty acid amides, including AEA and N-palmitoylethanolamine (PEA) [44]. Although PEA does not bind cannabinoid receptors, it has recently been described as an endogenous ligand for peroxisome proliferator receptor- α (PPAR- α) [45]. PEA might indirectly alter levels of ECs by competing with AEA and other fatty acid amides for degradation by FAAH or by suppressing FAAH expression at the transcriptional level [46,47]. Hence, other receptors appear to contribute, along with CB1, to AEA signalling in response to injury. PPAR- α is present in peripheral sensory neurons and immune cells and acts synergistically with CB1 to reduce pain [48–50]. Consequently, the effects of AEA are also mediated through cannabinoids and other receptors.

5. NON-CANNABINOID RECEPTOR 1, NON-CANNABINOID RECEPTOR 2 G-PROTEIN-COUPLED RECEPTORS

Some ECs (Δ^9 -THC and several synthetic CB1/CB2 receptor agonists and antagonists) can also interact with a number of established non-CB1, non-CB2 G-protein-coupled receptor (GPCRs) [51]. Non-CB1 and non-CB2 receptors can be activated or blocked by some CB1/CB2 receptor ligands with similar affinities, as their target CB1 and/or CB2 receptor agonists or antagonists. Some examples include the enhanced activation of glycine receptors by AEA, Δ^9 -THC, HU-210 and R-(+)-WIN55212 [52]; the enhanced NMDA-induced activation of NMDA receptors by anandamide [53]; the inhibition of T-type voltage-gated calcium channels by anandamide and N-arachidonol-dopamine (NADA) [54,55]; and the inhibition of voltage-gated $K_v3.1$ and $K_v4.3$ potassium channels and activation of calcium-activated potassium (BK) channels by anandamide and 2-AG [56,57]. Moreover, some receptors, such as 5-HT₃ [58] and nicotinic acetylcholine receptors [59], may be allosterically targeted by CB1/CB2 receptor ligands.

6. ENDOVANILLOIDS

AEA has been extensively studied, and multiple new receptors for this EC, including the vanilloid (TRPV1) receptor, have been identified. Accordingly, AEA is now frequently referred to as an 'endovanilloid'. AEA [60,61] and other derivatives of long-chain unsaturated fatty acids, such as the N-acyldopamines [42,62], act as endogenous activators for TRPV1 [63]. NADA potently activates TRPV1 in the hippocampus [42]. Notably,

anandamide and N-arachidonol-dopamine appear to interact with TRPV1 at the same intracellular binding site as capsaicin. Hwang *et al.* [64] described other endogenous agonists of TRPV1 and demonstrated that several products of lipoxygenases (LOXs) were able to activate the capsaicin-activated channel in isolated membrane patches of sensory neurons. Of these compounds, 12-(S)-hydroperoxyeicosatetraenoic acid (12-(S)-HPETE), 15-(S)-HPETE and leukotriene B4 (LTB4) exhibited the highest efficacy (summarized in [65]). To qualify as an endogenous activator of TRPV1, the compound should be generated by cells and released in an activity-dependent manner in sufficient amounts to evoke a TRPV1-mediated response through the direct binding and subsequent activation of the channel. Finally, endovanilloid signalling should be terminated within a short time to mediate the strict regulation of its actions. Therefore, biosynthetic and metabolic pathways for a putative endovanilloid should be present in close proximity to TRPV1 [63]. Indeed, these mechanisms have been demonstrated for CNS neurons, and particularly, neurons of the CA3 region of the hippocampus were immunoreactive for 12-LOX, N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD), FAAH and catechol-O-methyltransferase (COMT). Moreover, these enzymes co-expressed TRPV1, suggesting that AEA, NADA and 12-HPETE are endovanilloids in the hippocampus [66]. In Purkinje cells, only AEA and NADA appear to act as endovanilloids, as confirmed by NAPE-PLD, FAAH and COMT co-localization with TRPV1. In summary, the endogenous agonist of TRPV1 and the TRPV1 receptor comprise the 'endovanilloid system'.

Studies correlating the chemical similarities between a canonical TRPV1 ligand, capsaicin and the proposed lipid-based molecules, particularly AEA, initiated a new era of research, suggesting interplay between the cannabinoid and vanilloid systems. However, the cannabinoid and TRPV1 receptors belong to different families of proteins: CB1 and CB2 receptors are seven trans-membrane domain and GPCRs [67], and TRPV1 receptors are six trans-membrane domain cation channels of the large TRP superfamily and more specifically, the TRPV channel subfamily [68]. Moreover, the cannabinoid CB1 and TRPV1 receptors are localized to the same organs, tissues and, in many cases, cells.

7. EXPRESSION OF CANNABINOID RECEPTOR 1 AND TRANSIENT RECEPTOR POTENTIAL VANILLOID TYPE 1 IN THE SPINAL CORD

TRPV1 is both presynaptic and postsynaptic in the superficial laminae of the rat dorsal horn [69]. TRPV1-immunoreactivity (ir) has been primarily localized to lamina I, as the outer part of lamina II is weakly labelled, whereas the inner part is intensely labelled (figure 2) [69–72]. The labelled neuronal profiles in lamina I and II are axons and terminals [71]. TRPV1-ir shows post-synaptic labelling in dendrites and cell bodies in lamina II. TRPV1-ir in the rat dorsal horn is observed in both neuronal and glial cells [71].

CB1 receptors are densely expressed in the superficial dorsal horn of the spinal cord (figure 2) [72].

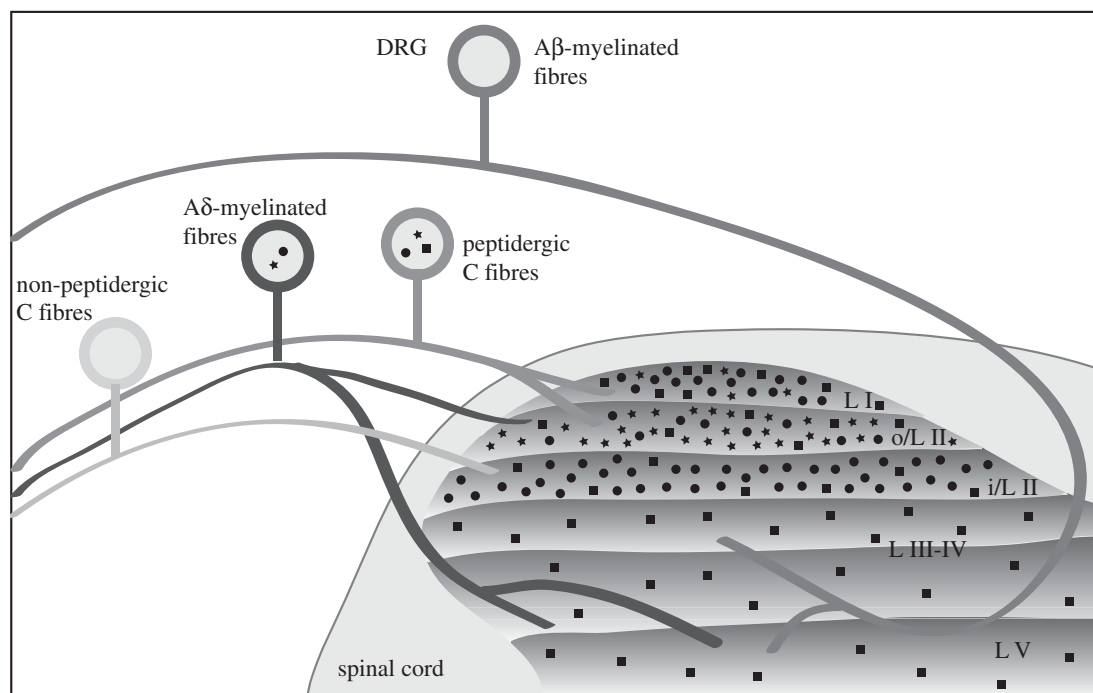


Figure 2. A simplified scheme of the complex interactions between cannabinoid CB1 (stars) and vanilloid TRPV1 receptors in the control of nociception in the grey matter of the dorsal spinal cord. CB1 receptors are densely expressed in the superficial dorsal horn of the spinal cord and both pre- and postsynaptically located in the dorsal horn of the rat spinal cord. TRPV1 (circles) are both pre- and postsynaptic in the superficial laminae of the rat dorsal horn. TRPV1-immunoreactivity (ir) is concentrated primarily in lamina I (L I), as the outer part of lamina II (o/L II) is weakly labelled, whereas the inner part (i/L II) is intensely labelled. TRPV1-ir shows the post-synaptic labelling in dendrites and cell bodies, particularly lamina II. FAAH (squares) immunostaining was observed in transverse sections of the rat lumbar spinal cord in both white and grey matter of both the dorsal and ventral horns.

Initially, Salio *et al.* [73] identified both pre- and postsynaptic CB1 receptors in the dorsal horn of the rat spinal cord. A presynaptic localization of cannabinoids on primary afferent fibres can be inferred from the observation of several CB1-like-receptor-containing cell bodies in the DRG and an intense CB1-ir in the Lissauer's tract. A postsynaptic localization of CB1 receptors was shown in the rat spinal cord, using both light and electron microscopy. In particular, consistent with *in situ* hybridization experiments [74], CB1-immunopositive cell bodies were identified in lamina II of the dorsal horn, where at the ultrastructural level, CB1 receptor immunoreactivity was primarily observed in somatic and dendritic compartments. Numerous CB1-containing neurons in lamina X were also detected [73]. Recently, Pernía-Andrade *et al.* [75] demonstrated the presence of CB1 receptors on the presynaptic terminals of inhibitory mouse superficial dorsal horn neurons using electron microscopy. Peroxidase and immunogold labelling of CB1 receptors and high-resolution electron microscopy showed the presence of CB1 receptors on the presynaptic terminals of symmetrical (inhibitory) synapses and co-localization of CB1 with the vesicular inhibitory amino acid transporter, which is a marker for inhibitory axon terminals.

Essentially, TRPV1 co-localizes with CB1 (e.g. dorsal root ganglia and spinal cord or brain neurons) [76–78] and CB2 receptors in sensory neurons [79]. This co-localization might have important functional consequences for the intracellular crosstalk (for

review see [80]) induced by the endogenous as well as synthetic ligands that activate both cannabinoid and TRPV1 receptors [81,82]. Cannabinoid (particularly CB1) and TRPV1 receptors can exert either opposing or similar functions under similar physiological and pathological conditions, and demonstrate crosstalk when expressed in the same or adjacent cells. Furthermore, as some ECs (AEA and NADA) also act as endovanilloids, the activity of cannabinoid and TRPV1 receptors may be regulated by the same endogenous lipids. These observations represent the basis for an EC–endovanilloid role in neuropathic pain signalling. The data on TRPV1/CB1 distribution in the CNS and their behavioural and neurochemical effects are accumulating (for review, see [83]), and these data clearly indicate that TRPV1 and CB1 receptors are involved in many centrally controlled functions, including pain, hyperalgesia and allodynia.

The co-expression of CB1 and TRPV1 receptors in some sensory afferents [84] suggests a physiological balance between the two receptors. For example, when the two receptors are co-expressed, the stimulation of CB1 can either inhibit or potentiate TRPV1 stimulation by its ligands [82].

Recent evidence has suggested that endovanilloids act as endogenous activators of the brainstem, descending into antinociceptive pain pathways [85]. Thus, not only TRPV1 but also endovanilloids and enzymes that regulate their metabolism [86,87] may represent a promising target for pharmacological research aiming for successful pain treatment. The

best-characterized enzyme involved in EC degradation is the integral membrane protein FAAH [86,88]. FAAH plays a crucial role in the termination of AEA signalling and has been shown to catalyse the degradation of ECs, namely AEA and 2-AG. This membrane-bound serine hydrolase also catalyses the cleavage of other non-cannabinoid fatty acid amides, such as oleylethanolamide (OEA) and palmitoylethanolamide (PEA) [86,89]. FAAH immunostaining was observed in transverse sections of the rat lumbar spinal cord in the white and grey matter of both the dorsal and ventral horns (figure 2) [90]. FAAH immunoreactivity was detected within the soma and dendrites of neurons [90–92]. Lever *et al.* [90] recently showed that CB1 co-localizes with FAAH in the upper laminae of the dorsal horn. An intense labelling of the CB1 receptor within the dorsal lateral funiculus (consistent with [72]) and surrounding FAAH-immunoreactive cells was observed, further implicating the EC system as a therapeutic target in neuropathic pain. In addition, the expression of TRPV1, CB1 and FAAH in the same cells (K. Starowicz, W. Makuch, M. Korostynski, M. Slezak, M. Zychowska, S. Petrosino, L. De Petrocellis, L. Cristino, B. Przewlocka & V. Di Marzo 2012, unpublished results [93,94]), provides an anatomical basis for the modulation of TRPV1 function by compounds previously considered to act solely at CB1 receptors.

Our data suggest that TRPV1-dependent mechanisms mediate the analgesic properties of exogenously and endogenously elevated levels of AEA in neuropathic pain [30]. These elevated levels of AEA do not result uniquely from a direct effect of AEA at TRPV1 because AEA activity is controlled through local metabolism, which can also be exerted by enzymes other than FAAH. Local metabolism can also be influenced through the activity of CB1 receptors and other endovanilloids. For example, 12-LOX metabolites and PEA and OEA—which, unlike anandamide, have no agonist activity at CB1 and CB2 receptors [95,96]—have been reported to potentiate relaxant responses to anandamide through TRPV1 receptors [97].

8. MANIPULATION OF ENDOCANNABINOID LEVELS

Cannabinoids modulate different types of pain through CB1- and/or CB2-receptor-mediated mechanisms of action. CCI of the sciatic nerve is a commonly used neuropathic pain model that causes hypersensitivity to radiant heat stimuli (i.e. hyperalgesia) [98–100] and nociceptive responses to typically non-nociceptive stimuli (e.g. allodynia), including touch [101,102] and cold [102,103].

In recent years, the EC system has attracted the attention of researchers working in neural damage and repair and is being considered a promising target for the development of new therapies [104]. This increasing interest is supported by evidence that the ECs AEA and 2-AG are produced ‘on demand’ [105] rather than being pre-stored and act as neuroprotective and immunomodulatory mediators after

lesions of the nervous system [104,106,107]. However, ECs have relatively short durations of action owing to effective metabolic pathways. In the case of AEA, this is induced through cellular accumulation, followed by hydrolysis to arachidonic acid, which is catalysed by the enzyme FAAH (although it can also act as a substrate for both cyclooxygenase-2 and LOXs; figure 3a). A similar pattern has been observed with 2-AG, although in the brain the enzyme monoacylglycerol lipase (MAGL) is more important. The finding that formalin administration to the hind paw of rats induces AEA release into the periaqueductal grey matter [108] suggests that compounds preventing the metabolism of this EC may be useful agents for the treatment of pain. This effect has subsequently been demonstrated for inflammatory pain. Thus, selective inhibitors of FAAH, such as URB597 and OL-135, produce beneficial effects in a variety of inflammatory pain models, whereas their effects on neuropathic pain are less clear [98,109–112].

ECs can participate in spinal cord function. Previous studies have shown the existence of a basal level of the EC system in the spinal cord that might participate in local circuitry and can be modulated in response to peripheral damages and painful sensations [33,73,113]. Garcia-Ovejero *et al.* [114] demonstrated that the EC system is modulated after spinal cord injury, and this modulation can be observed in at least two different phases: an early phase, when AEA and PEA are strongly upregulated, and a delayed phase, when increases in 2-AG are detected. In sham animals, the levels of 2-AG slightly increase with time, which could be due to the age of the animal or increases in the weight of the animal due to normal growth. This elevation may also depend on experimental factors, such as time after anaesthesia.

Gene targeting is critical to advances in neuroscience, which is evident from the importance of knockout mouse studies in the literature. The availability of genetic mouse models has revolutionized biomedical research; however, given the central magnitude of this method, we must recognize its benefits and also its potential shortcomings. For example, Lichtman *et al.* characterized the behavioural responses of FAAH (–/–) mice in animal models of neuropathic pain (CCI to the sciatic nerve), and no phenotypic difference was observed. CCI-induced allodynia and thermal hyperalgesia were nearly identical in both FAAH (–/–) and FAAH (+/+) genotypes. The animals displayed an equivalent degree of nociceptive behaviour in the CCI model, regardless of whether a low or high intensity of noxious heat was used [115]. The authors concluded that the magnitude of the genotypic differences is much greater in chronic than acute pain states and proposed vague explanations; for example, the deletion of FAAH might lead to a quicker recovery from the hyperalgesic state compared with wild-type mice or that the elevation in AEA levels and/or other FAAs in FAAH (–/–) mice might have been insufficient to block thermal hyperalgesia in this neuropathic pain model. Another explanation could be that nerve ligation may lead to adaptive changes in the nociceptive circuits of FAAH (–/–) mice, thereby reducing the influence

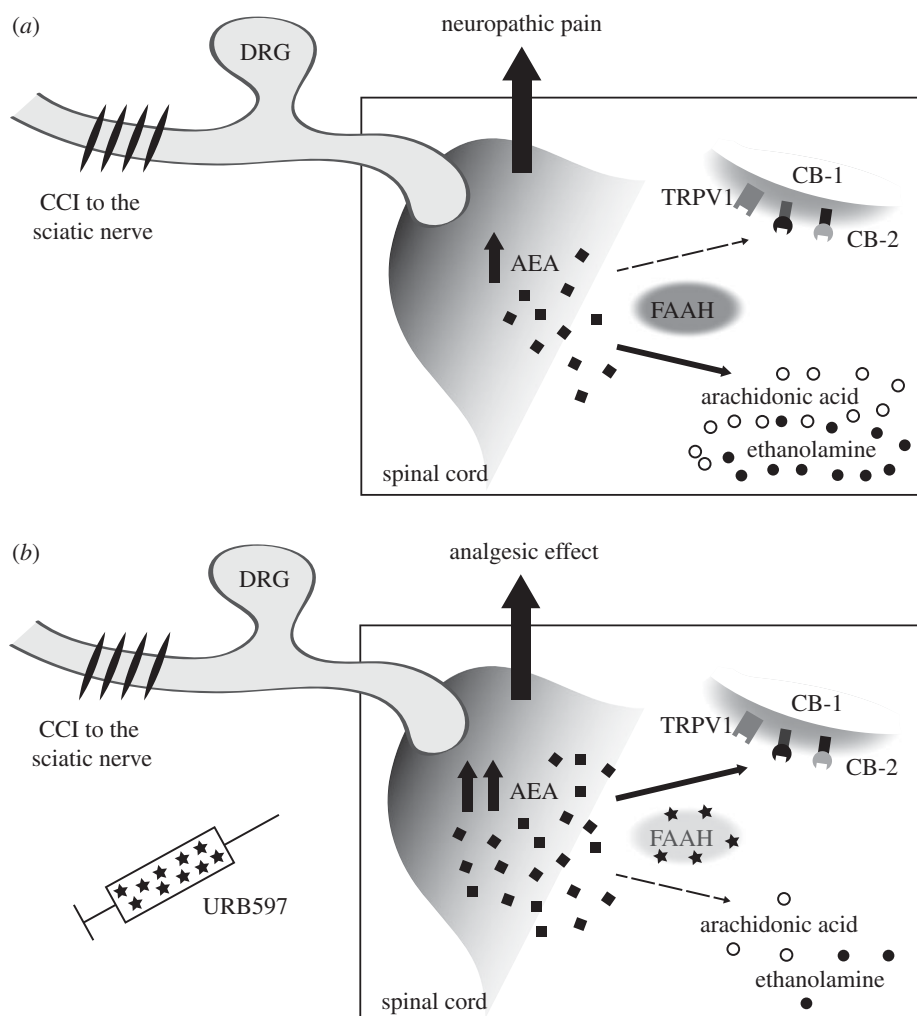


Figure 3. Schematic of the manipulation of spinal anandamide (AEA) levels. (a) The effects of AEA are mediated through the activation of cannabinoid CB1 and CB2 receptors. TRPV1 is also a target for AEA. AEA, internalized by a putative carrier-mediated transporter and hydrolysed by FAAH, produces arachidonic acid and ethanolamine. (b) The injection of URB597, an FAAH inhibitor, results in elevated AEA levels. The augmentation of AEA levels by URB597 corresponds with a behavioural reduction in allodynia and hyperalgesia in CCI rats.

of endogenous FAAs on pain behaviour. Finally, the authors suggested that FAAH might offer a distinctive strategy for the treatment of pain disorders [115].

9. FAAH INHIBITORS: AN ATTRACTIVE DRUG TARGET FOR THE TREATMENT OF PAIN

FAAH ($-/-$) mice represent an effective model system to examine the neurochemical and behavioural consequences of the constitutive inactivation of FAAs catabolism. However, to understand the pharmacological effects of the acute disruption of FAAH activity, potent and selective inhibitors of this enzyme are required. Several FAAH inhibitors have been described, including trifluoromethyl ketones [116], α -keto heterocycles [117], sulphonyl fluorides [118] and fluorophosphonates [119]. Piomelli and co-workers [120] generated a series of carbamate inhibitors of FAAH that were shown to be efficacious *in vivo*. However, some FAAH inhibitors, including carbamate compounds such as URB597, BMS-1, OL-135 and LY2077855, are less selective, displaying multiple

off-targets [121]. Hence the potential drug–drug interactions limits its therapeutic relevance. Further work of Piomelli [122] identified more selective ones. Polar electron-donating O-aryl substituents, which decrease carbamate reactivity, yield compounds, such as URB694, that are highly potent FAAH inhibitors *in vivo* and less reactive, with off-target carboxylesterases significantly improving drug-likeness [122].

Importantly, FAAH inhibitors did not induce the common side-effects of pure CB1 agonists, such as catalepsy, hypothermia or changes in appetite.

In chronic pain typically associated with hyperexcitability in damaged neural pathways [3], FAAH inhibition might increase EC tone selectively in spinal pain circuits (figure 3b), resulting in analgesia without the side effects that accompany the global activation of CB1 receptors; therefore, both FAAH and MAGL represent potential therapeutic targets for the development of pharmacological agents to treat chronic pain resulting from nerve injury. Walker *et al.* [108] reported that inflammatory pain produced by formalin injection into the paw produced a release of

Table 1. Changes in levels of anandamide (AEA) and related fatty acid amides: 2-arachidonoylglycerol (2-AG), palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) in the spinal cord reported for chronic constriction injury (CCI) and spinal nerve ligation (SNL) models of neuropathic pain.

neuropathic pain model	endocannabinoids levels in spinal cord			
	AEA	2-AG	PEA	OEA
CCI	elevated [30,113]	elevated [30,113]	not elevated [30,113]	not elevated [30]
SNL	elevated [125]	not elevated [129]	decreased [129]	not elevated [129]

AEA in the periaqueductal grey matter. The authors concluded that the 'release of AEA in a pain suppression circuit suggests that drugs that inhibit the reuptake of AEA or block its degradation might form the basis of a modern pharmacotherapy for pain' [108]. The distribution of CB1, TRPV1 receptors and EC-hydrolysing enzymes within pain modulatory circuits together with behavioural, neurochemical and neurophysiological studies imply a role for EC signalling in pain modulation [123]. The compound employed, animal model used and, potentially, the level of EC tone resulting from the injury influences antinociception through FAAH, MAGL or AEA uptake inhibitors [124].

Chapman's group demonstrated that the spinal administration of URB597 attenuated spinal neurons' responses and elevated levels of ECs in neuropathic rats [125]. The authors implicated a role for the spinal inhibition of FAAH in nociceptive response modulation in neuropathic rats and determined the contribution of the CB1 receptors to these effects. In addition, they also determined the levels of ECs and related fatty acids in the spinal cord tissue of neuropathic rats. The spinal administration of URB597 attenuated the evoked responses of neurons in neuropathic rats and significantly increased the levels of AEA, PEA and 2AG in the ipsilateral spinal cord. Interestingly, a previous study showed that the systemic administration of URB597 does not alter neuropathic pain behaviour [109], whereas the administration of the FAAH inhibitor OL135 attenuated allodynia in neuropathic rats [110]. These results suggest that there might be tissue-specific changes in the sensitivity to URB597 in neuropathic rats as a result of changes in FAAH activity, metabolic pathways and tissue-specific pH [126].

It has been previously reported that the FAAH inhibitor URB597 increased AEA levels in the spinal cord of control mice [127]. Similarly, the MAGL inhibitor JZL184 significantly increased 2-AG levels in the spinal cord of control mice [127]. Data using the rat SNL model indicated that both AEA and 2-AG levels are increased in pain-relying structures (i.e. dorsal root ganglia, DRG) proximal and ipsilateral to nerve ligation compared with DRG contralateral to injury, sham or naive rats [128]. This localized increase in ECs might be driven by the inflammatory response to nerve insult. In a recent report, Guasti and colleagues indicated that the chronic inhibition of microglial activation with minocycline attenuated SNL-induced increases in 2-AG, but had no effect on AEA in lumbar spinal cord ipsilateral to SNL

compared with tissue contralateral to nerve injury [129]. Although CCI had no effect on AEA or 2-AG levels in the spinal cord of mice [127], these data suggest that regional differences in the pools of these ECs might at least partially mediate the observed anti-allodynic effects of FAAH or MAGL inhibition. In contrast, the results obtained in our laboratory [30] and in others [113] have shown an increase in AEA tissue levels in the lumbar spinal cord of CCI-rats. It is possible that differences in AEA levels occur locally at the site of dorsal horn during injury. Thus, quantifying EC levels from the entire cord might have obscured the detection of these regional changes. Peripheral nerve damage increased both AEA and 2-AG levels in the ipsilateral half of the spinal cord [128].

Notably, Guasti *et al.* provided evidence of a role for activated microglia in the control of ECs levels and related compounds *in vivo* in neuropathic pain. Data from our group [130,131] and others [132,133] indicated that chronic treatment with minocycline significantly attenuates the development of mechanical allodynia and associated increase in activated microglia in the L4-L6 region of the ipsilateral spinal cord in neuropathic rats. Peripheral nerve injury was accompanied by a significant elevation in the levels of activated microglia and AEA in the ipsilateral spinal cord compared with the contralateral spinal cord (unaltered levels in sham operated rats). In contrast, the levels of PEA were significantly decreased in the ipsilateral spinal cord compared with the contralateral spinal cord of neuropathic animals and remained unchanged in both the ipsilateral and contralateral spinal cords of sham animals [129]. Guasti and co-workers identified major differences in the effects of microglia inhibition on the levels of AEA and PEA in neuropathic rats, which suggests that there is variance in the biosynthesis/catabolism of these N-acyl ethanolamines in microglia and that the activated microglia play a major role in modulating the levels of PEA but not AEA or OEA in the spinal cord of neuropathic rats. Furthermore, these data highlight the importance of selective ipsilateral changes in levels of ECs and related compounds, which is not unexpected, given the unilateral nature of the pain behaviour.

However, apparent discrepancies between the changes in the endovanilloid/EC levels in various models of neuropathic pain have been identified (table 1). The levels of AEA and related compounds are differentially regulated, depending on the nature of pain and the tissue under investigation. These

differences might arise as a result of the impact of the pathological condition on levels of the enzymes contributing to the metabolism of AEA and differential roles of these enzymes in the metabolism of different members of the AEA and related family of lipids. Accordingly, the levels of FAAH, COX and LOX might be differentially altered in a manner specific to the pathological condition. It is also likely that changes in the levels of CB1 and TRPV1 receptors, which are modulated in pathological conditions, might also impact the levels of their endogenous ligands. The presence of additional cell types during chronic pain might provide an additional source of EC synthesis and metabolism, which will impact the levels of ECs in the tissue.

Recently, a highly efficacious and selective FAAH inhibitor, N-pyridazin-3-yl-4-(3-{[5-(trifluoromethyl)pyridin-2-yl]oxy}benzylidene)piperidine-1-carboxamide (PF-04457845), has been characterized [134]. Oral administration of PF-04457845 produced potent antinociceptive effects in both inflammatory (complete Freund's adjuvant (CFA)) and non-inflammatory (monosodium iodoacetate) pain models in rats [134]. Significantly, PF-04457845-treated mice at 10 mg kg⁻¹ elicited no effect in motility, catalepsy and body temperature. On the basis of its exceptional selectivity and *in vivo* efficacy, combined with long duration of action and optimal pharmacokinetic properties, PF-04457845 is a clinical candidate for the treatment of pain and other nervous system disorders. Still, in a randomized placebo and active-controlled clinical trial on pain due to osteoarthritis of the knee, PF-04457845 failed to demonstrate efficacy different from placebo-treated group [135]. Currently, the study was stopped at the temporary analysis for ineffectiveness. The lack of analgesic effect of FAAH inhibition in humans is in contrast to data from animal models. This apparent disconnect between species needs further study.

10. TARGETS OTHER THAN CANNABINOID RECEPTOR 1 FOR FATTY ACID AMIDE HYDROLASE INHIBITION

Anandamide is a weak partial agonist of CB1 receptors, has some affinity for CB2 receptors and has lower but significant activity at other receptors or channels implicated in nociceptive transmission, including TRPV1, 5HT₂ and 5HT₃ serotonin receptors and voltage-gated calcium channels [58,60,136–140]. Moreover, evidence on the relationship between CB1 receptors and TRPV1 channels is accumulating (for review, see [80]); however, to date, only a single report has described the analgesic effects of spinal AEA via TRPV1 activation in carrageenan-induced inflammation [141]. The data on the possible role of AEA acting as an endovanilloid via TRPV1 (rather than as an EC via the CB1 receptor) in neuropathic pain are remote. Few cases have demonstrated how the elevation of AEA levels could cause the indirect inactivation of non-cannabinoid receptors [110].

Recent data from our group indicated that in a rat model of neuropathic pain (CCI), AEA-mediated spinal analgesia might also be due to interactions

with TRPV1 channels [30]. Spinal TRPV1 channels facilitate pain transmission; thus, we envision that the effects of AEA are due to TRPV1 activation and subsequent desensitization. Intrathecally delivered AEA alleviates both tactile and cold allodynia and thermal hyperalgesia through a TRPV1-dependent mechanism. Our data also support the hypothesis that AEA exerts analgesia by acting at spinal TRPV1 channels [30]. As previously discussed, AEA degradation by FAAH limits its activity at potential targets; therefore, we elevated the spinal levels of endogenous AEA via the administration of URB597 and investigated how neuropathic pain and the inhibition of spinal FAAH influence the levels of AEA and related fatty acids amides in the lumbar spinal cord tissue of neuropathic animals (CCI model) and the effect on neuropathic-related symptoms in these animals. Depending on the dose of URB597 used, analgesia was mediated via CB1 or TRPV1 receptors [30]. Our findings suggest that intrathecal administration of the FAAH inhibitor produces analgesia by elevating local AEA levels in the spinal cord, where, depending on the extent of its inhibition of AEA degradation, the inhibitory effects on nociception are mediated *via* CB1- or TRPV1-dependent mechanisms. These results support a role for spinal TRPV1 and its endogenous ligands in neuropathic pain and suggest that the enhancement of endogenous AEA signalling through inhibiting its enzymatic degradation is promising for the development of novel multi-target pharmacological treatments [30]. Considering the lower affinity of AEA for TRPV1 compared with CB1 receptors [76,142], as well as its short half-life and its lipophilic nature, AEA is likely to bind to TRPV1 receptors near the site of synthesis inside the same neuron. Thus, co-localization in spinal cord neurons is favourable. TRPV1-mediated currents are activated within individual DRG cells by intracellular AEA produced in response to rising [Ca²⁺] [105] or capsaicin [143]. These data provide functional evidence for the coexistence of FAAH, AEA and TRPV1 in DRG neurons. As previously mentioned, FAAH also hydrolyses PEA, an endogenous lipid implicated in the modulation of pain responses [45,48,144] and a putative ligand for the PPAR- α [49]. PEA could compete with AEA for hydrolysis by FAAH and produce entourage effects at TRPV1 receptors [145]. Hence, by increasing the spinal AEA level via blocking FAAH activity, primary activation of TRPV1 might be followed by receptor desensitization. In CCI rats, the anti-nociception following spinal delivery of URB597 may be partially attributable to the desensitization mechanism hypothesized by McGaraughty *et al.* [146]. Once desensitized, the accumulating levels of AEA would strengthen the CB1-mediated analgesia. Alternatively, when the two receptors are co-expressed, the stimulation of CB₁ could inhibit TRPV1 stimulation through its ligands [82], suppressing the TRPV1-attributed role in generating thermal pain.

We further reported [147] that among different URB597 tested doses, only the highest dose of URB597 elevated the levels of the FAAH non-EC and anti-inflammatory substrates OEA and PEA and

EC FAAH substrate 2-AG, corresponding with thermal and tactile allodynia inhibition, which is blocked almost uniquely by TRPV1 antagonism. Surprisingly, this dose of URB597 decreased spinal AEA levels. We therefore hypothesized that alternative pathways of AEA metabolism, involving AEA lipoxygenation, exist. Indeed, baicalein, an inhibitor of 12/15-LOX activity, significantly reduced URB597 analgesic effects. Moreover, FAAH, 15-LOX and TRPV1 were co-localized in the dorsal spinal horn of CCI rats. We also observed that 15-hydroxy-AEA potently and efficaciously activated the TRPV1 channel [147]. These composite studies suggest that spinal URB597 unmasks a secondary route of AEA metabolism via 15-LOX with possible formation of 15-hydroxy-AEA, which might then cooperate with OEA and PEA, producing TRPV1-mediated analgesic effects in CCI rats. Amadio *et al.* reported that 15(*S*)-hydroxy-AEA stimulates the activity of an acylethanolamide-biosynthesizing enzyme and inhibits FAAH and the activity of a 2-AG-biosynthesizing enzyme [148]. We also observed [147] that the formation of 15(*S*)-hydroxy-AEA might further reinforce and prolong the inhibition of FAAH, contributing to the elevation TRPV1-active acylethanolamide levels by both inhibiting their degradation and stimulating their biosynthesis, sustaining its own biosynthesis from AEA and counteracting CB1 activation by 2-AG, thereby leading to an overall prolonged activation, followed by nociceptor desensitization of TRPV1 signalling.

11. CONCLUDING REMARKS

Here, we reviewed the results of studies indicating that the potential therapeutic effects of ECs can be enhanced by modulating their endogenous tone, i.e. by preventing their metabolism through various enzymes, particularly FAAH, in animal models of neuropathic pain.

URB597 has been considered a selective FAAH inhibitor because of its lack of activities on FAAH-related enzymes and 47 ion channels/receptors; consequently, it has been extensively used as a pharmacological tool to examine the role of FAAH in pain and anxiety [120,149]. These anxiolytic and antinociceptive effects have been attributed exclusively to its ability to inhibit FAAH and augment the level of AEA. However, Niforatos *et al.* [150] show that URB597 has direct gating effects on ion channels. URB597 activated TRPA1 channels endogenously expressed in a population of rat dorsal root ganglion neurons that also responded to allyl isothiocyanate. In contrast to its effect on TRPA1, URB597 inhibited TRPM8 and had no effects on TRPV1 or TRPV4. Thus, URB597 is now considered a novel agonist of TRPA1 and probably activates the channel through a direct gating mechanism. Future study will determine whether and to what extent the observed therapeutic efficacy of URB597 in animal studies is mediated through TRPA1, TRPM8 or other TRP channels.

We also described the latest reports characterizing TRPV1 as an interesting target for the EC anandamide in terms of pharmacotherapy for neuropathic pain. Interestingly, Pernía-Andrade *et al.* suggested

an unexpected role for ECs in dorsal horn neuronal circuits as mediators of spinal activity-dependent pain sensitization. It is likely that ECs produced by strong nociceptive stimulation may activate CB1 receptors on inhibitory dorsal horn neurons to reduce the synaptic release of γ -aminobutyric acid and glycine, rendering nociceptive neurons excitable by non-painful stimuli [75]. Those results suggest that spinal ECs and CB1 receptors located on inhibitory dorsal horn interneurons act as mediators of heterosynaptic pain sensitization and play an unexpected role in dorsal horn pain-controlling circuits. Notably, these results apply to acute but not to chronic pain. Therefore, in the context of the data of Pernía-Andrade *et al.*, it is even more interesting to study the interaction between TRPV1 and CB1, which appears to be more apparent in chronic pain. Owing to the multifaceted interplay between the CB1 and TRPV1 receptors described herein, there are now new potential molecular targets that may be useful in the treatment of neuropathic pain. Although the effort and attempts in our understanding of the molecular basis for neuropathic are promising, a solution to the development of useful analgesic drugs has not been uncovered. Importantly, there is not a single target that is uniquely associated with the establishment of neuropathic pain. However, there are many attractive targets, such as the postulated new pattern of the complex network surrounding AEA signalling and complex consequences of the pharmacological manipulation of the levels of EC/endovanilloid anandamides, which might result in the adaptation of this network, resulting, at least in neuropathic rats, in the counteraction of hyperalgesia and allodynia.

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