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REVIEW

CB_2: a cannabinoid receptor with an identity crisis

Brady K Atwood[1,2] and Ken Mackie[1]

[1]*The Gill Center and the Department of Psychological & Brain Sciences, Indiana University, Bloomington, IN, USA, and* [2]*University of Washington, Program of Neurobiology and Behavior, Seattle, WA, USA*

CB_2 was first considered to be the 'peripheral cannabinoid receptor'. This title was bestowed based on its abundant expression in the immune system and presumed absence from the central nervous system. However, multiple recent reports question the absence of CB_2 from the central nervous system. For example, it is now well accepted that CB_2 is expressed in brain microglia during neuroinflammation. However, the extent of CB_2 expression in neurons has remained controversial. There have been studies claiming either extreme-its complete absence to its widespread expression-as well as everything in between. This review will discuss the reported tissue distribution of CB_2 with a focus on CB_2 in neurons, particularly those in the central nervous system as well as the implications of that presence. As CB_2 is an attractive therapeutic target for pain management and immune system modulation without overt psychoactivity, defining the extent of its presence in neurons will have a significant impact on drug discovery. Our recommendation is to encourage cautious interpretation of data that have been presented for and against CB_2's presence in neurons and to encourage continued rigorous study.
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Abbrevations: 2-AG, 2-arachidonylglycerol; $CB_{1/2}$, type1/2 cannabinoid receptor; DMNX, dorsal motor nucleus of the vagus; DRG, dorsal root ganglion; FACS, fluorescence activated cell sorting; GFAP, glial fibrillary acidic protein; ICC, immunocytochemistry; IHC, immunohistochemistry; IPSC, inhibitory post synaptic current; ISH, in situ hybridization; MAPK, mitogen-activated protein kinases; NB, northern blot; PGE2, prostaglandin E2; PI3K, phosphoinositide 3-kinase; RLB, radioligand binding; RT-PCR, reverse transcriptase polymerase chain reaction; SB, Southern blot; THC, Δ^9-tetrahydocannabinol; TRPV1, transient receptor potential cation channel V1; WB, western blot

The therapeutic potential of cannabis as well as its psychoactive effects has been known for thousands of years (Adams and Martin, 1996). However, it was not until the discovery of cannabinoid binding sites in brain (Devane *et al.*, 1988; Herkenham *et al.*, 1990; Herkenham *et al.*, 1991; Matsuda *et al.*, 1993) and the subsequent cloning of the CB_1 receptor (Matsuda *et al.*, 1990; Alexander *et al.*, 2008) that cellular mechanisms for these effects began to be elucidated. A second cannabinoid receptor (CB_2) was identified and first cloned from HL60 cells by Munro *et al.* in 1993 (Munro *et al.*, 1993). CB_2 was dubbed the 'peripheral cannabinoid receptor' as a result of *in situ* hybridization analysis that revealed high levels of CB_2 mRNA in spleen and its absence from brain. CB_2 receptors were cloned from mouse and rat in later years (Shire *et al.*, 1996; Griffin *et al.*, 2000; Brown *et al.*, 2002).

Correspondence: Ken Mackie, Department of Psychological and Brain Sciences, Indiana University, 1101 E. 10th Street, Bloomington, IN 47405, USA. E-mail: kmackie@indiana.edu
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CB_2 and cellular signalling

Around the time that CB_1 and CB_2 were cloned, anandamide and 2-arachidonylglycerol (2-AG) were identified as endogenous cannabinoid agonists (Devane *et al.*, 1992; Felder *et al.*, 1993; Sugiura *et al.*, 1995; Hanus *et al.*, 2001). 2-AG is a high efficacy agonist at CB_2 (Lynn and Herkenham, 1994; Slipetz *et al.*, 1995; Gonsiorek *et al.*, 2000; Sugiura *et al.*, 2000; Shoemaker *et al.*, 2005b). However, anandamide has a low efficacy at CB_2, often functioning as a weak partial agonist (Showalter *et al.*, 1996; Gonsiorek *et al.*, 2000; Sugiura *et al.*, 2000).

Similar to CB_1, CB_2 is a $G_{i/o}$ coupled G protein coupled receptor and as such inhibits adenylyl cyclase (Bayewitch *et al.*, 1995; Felder *et al.*, 1995; Slipetz *et al.*, 1995; Gonsiorek *et al.*, 2000; Sugiura *et al.*, 2000; Shoemaker *et al.*, 2005b). Furthermore, it can also promote MAPK activation (p38 and p42/44), PI3K, ceramide production and gene transcription (Bouaboula *et al.*, 1996; Bouaboula *et al.*, 1999a; Bouaboula *et al.*, 1999b; Howlett, 2002; Howlett *et al.*, 2002; Herrera *et al.*, 2005; Herrera *et al.*, 2006; Grimaldi *et al.*, 2009; Romero-Sandoval *et al.*, 2009). A key difference, however, is that unlike CB_1, CB_2 appears to poorly modulate calcium channels or inwardly rectifying potassium channels (Felder *et al.*, 1995). Studies using SR144528, a CB_2 antagonist/inverse agonist, have revealed that the receptor possesses a high degree of constitutive activity in expression systems (Bouaboula *et al.*, 1999a; Bouaboula *et al.*, 1999b). Of further interest is that CB_2 receptors from different species often have different pharmacological responses to identical drugs, complicating the drug discovery process (Mukherjee *et al.*, 2004; Yao *et al.*, 2006; Bingham *et al.*, 2007). Therefore, despite coupling to the same family of G proteins and sharing some ligands, CB_1 and CB_2 appear to differ significantly from one another in their signalling.

CB_2 as a therapeutic target

CB_2 is an attractive therapeutic target. The abundant CB_2 expression in immune cells presents a plausible explanation for cannabinoid immunomodulatory activity (Lynn and Herkenham, 1994; Berdyshev, 2000; Howlett, 2002; Costa, 2007). Indeed, CB_2 activation affects a myriad of immune responses from inflammation to neuroprotection (Cabral and Griffin-Thomas, 2009). Additionally, numerous reports indicate that CB_2 activation is analgesic and CB_2 agonists suppress responses in many animal models of pain, from acute to neuropathic (Anand *et al.*, 2009), although these effects may involve CB_1 activation as well. CB_1 is abundant within the brain, where it appears responsible for mediating the psychoactive effects of cannabis (Mackie, 2005). Thus, the scarcity of central nervous system (CNS) CB_2 receptors makes CB_2 selective drugs attractive as therapeutics as they would presumably lack psychoactivity. In support of this notion, mice with 'knockout' of CB_2 had typical behavioural responses to Δ^9-tetrahydrocannabinol (THC) but lost their normal immune responsiveness to THC (Buckley *et al.*, 2000). CB_2 levels can also be increased under certain conditions and disease states further adding to its attractiveness as a therapeutic target (Zhang *et al.*, 2003; Wotherspoon *et al.*, 2005; Yiangou *et al.*, 2006).

CB_2 agonists and antagonists

As CB_2 is such an attractive therapeutic target, much effort has been made to synthesize selective CB_2 agonists and antagonists. Some of the early cannabinoid agonists such as CP55940, WIN55212-2 and HU210 demonstrate high affinity at CB_2 and are considered full agonists, but are not selective for CB_2 over CB_1 [see Miller and Stella (2008) for a summary of the binding data]. JWH015 was one of the first potential CB_2 selective agonists (Showalter *et al.*, 1996; Griffin *et al.*, 2000), but has since been shown to also be an agonist at GPR55 (Ryberg *et al.*, 2007; Lauckner *et al.*, 2008). Numerous other compounds have been synthesized with the aim of making CB_2 selective agonists. AM1241 (Malan *et al.*, 2001) and JWH133 (Huffman *et al.*, 1999) are two of the most commonly used 'selective' CB_2 agonists. Other early ones include HU308 (Hanus *et al.*, 1999) and GW405833 (L-768242) (Gallant *et al.*, 1996; Valenzano *et al.*, 2005). More recently synthesized compounds include GW833972A (Belvisi *et al.*, 2008), MDA7 (Naguib *et al.*, 2008), A-796260 (Yao *et al.*, 2008) and A-836339 (Yao *et al.*, 2009). Cannabilactones have also been suggested as potential CB_2 selective compounds (Khanolkar *et al.*, 2007). For the interested reader, a review by Whiteside *et al.* contains detailed analysis of many of these compounds as well as numerous others (Whiteside *et al.*, 2007). SR144528 (Rinaldi-Carmona *et al.*, 1998) and AM630 (Pertwee *et al.*, 1995; Ross *et al.*, 1999) are the two of the most commonly used CB_2 selective antagonists and have been frequently used to demonstrate specificity of many of these other CB_2 selective agonists. However, a fundamental problem with designing selective agonists and antagonists is possible interactions with other unforeseen targets. These compounds may exhibit a strong preference for CB_2 over CB_1, but as evidenced by JWH015, other non-CB_1/CB_2 binding sites may still exist. It is extremely difficult to conclusively establish an agonist (or antagonist) is specific for CB_2 and no other receptors. This caveat must be kept in mind when evaluating studies that solely use a pharmacological approach and allege CB_2 involvement in a process. Furthermore, as demonstrated for AM1241, CB_2 agonists may produce very different effects at CB_2 receptors from different species (Bingham *et al.*, 2007). Here, a racemic mixture of AM1241 was an agonist at human CB_2 but functioned as an inverse agonist at rodent CB_2. R-AM1241 has a higher affinity for CB_2 than S-AM1241, but functions similar to the racemate. On the other hand S-AM1241 was an efficacious agonist at both human and rodent CB_2. Furthermore naloxone, a μ opioid receptor antagonist, can block the analgesic effects of AM1241, but this appears not to be the case for other CB_2 agonists (Ibrahim *et al.*, 2005; Yao *et al.*, 2009). It also important to bear in mind that selective agonists and antagonists for a particular receptor may differentially alter coupling to distinct signalling pathways, a concept known as functional selectivity (Urban *et al.*, 2007). Thus, CB_2 agonists that share identical binding characteristics may have different potencies in activating different signalling pathways and evoke substantially different physiological responses. For example, with CB_2 expressed in Chinese hamster ovary cells, 2-AG, CP55940 and noladin ether had different rank orders of potency depending on the signalling pathway analysed: inhibition of adenylyl cyclase, MAPK activation and stimulation of calcium transients (Shoemaker *et al.*, 2005a). This concept of functional selectivity mandates caution in comparing pharmacological studies that employ different agonists and antagonists for CB_2. This may explain the differences previously mentioned between AM1241 and other CB_2 agonists and their ability to produce analgesia and may even extend to other agonist-specific effects obtained with other compounds. Thus, it is important

that functional evidence obtained using CB_2 'selective' agonists and antagonists be balanced with careful controls and supported by additional genetic and anatomical analyses to assure that some other unanticipated target is not the true site of action.

CB_2: the 'peripheral' cannabinoid receptor?

CB_2 and evidence for absence from CNS

In addition to the data obtained during the CB_2 knockout mouse characterization, earlier reports also arrived at the conclusion that CB_2 is absent from the CNS. (Of course, 'absence' just means below the level of detection of the particular assay being employed.) When CB_2 was first cloned, *in situ* hybridization demonstrated a lack of CB_2 mRNA signal in rat brain (Munro *et al.*, 1993). While characterizing CB_1 and CB_2 in immune cells, Schatz *et al.* performed Northern blots on mouse brain and rat cerebellums and could not detect the presence of CB_2 in these tissues (Schatz *et al.*, 1997). However, RT-PCR demonstrated the presence of CB_2 mRNA, at levels too low to be quantified. Northern blot analysis performed by Galiegue *et al.* is in agreement with the Schatz *et al.* data (Galiegue *et al.*, 1995). However, in RT-PCR experiments performed as a part of this study, CB_2 was undetectable in human cortex, cerebellum, whole brain and pituitary gland. McCoy *et al.* also did not detect CB_2 mRNA in mouse brain using RT-PCR/Southern blot analysis (McCoy *et al.*, 1999). As part of the characterization of SR144528 as a CB_2 antagonist, rat brain radioligand binding and GTPγS binding analyses were performed (Griffin *et al.*, 1999). The authors found little SR144528 binding in whole brain and cerebellum and the results of their GTPγS binding analysis supported this. Furthermore, Northern blotting did not detect CB_2 mRNA in cerebellum, cortex or spinal cord. Rat cortex has also been reported to lack CB_2 mRNA (Beltramo *et al.*, 2006). Included in the review by Howlett *et al.* (Howlett *et al.* 2002), Herkenham and Hohmann replicated the *in situ* hybridization results of Munro and colleagues. Derbenev and colleagues did not detect CB_2 mRNA or protein in rat brainstem (Derbenev *et al.*, 2004). As part of an initial characterization of cannabinoid receptors in dorsal root ganglia (DRG), *in situ* hybridization revealed the presence of CB_1 but not CB_2 receptors (Hohmann and Herkenham, 1999a,b). Price *et al.* could also not detect CB_2 mRNA in rat trigeminal ganglia (Price *et al.*, 2003).

Based on all these data, CB_2 was informally referred to as the peripheral cannabinoid receptor. However, a number of more recent reports have suggested that, in contrast to these previous claims of its absence, CB_2 may in fact be expressed in the CNS (see below). This finding has had a significant impact on drug discovery and our understanding of the biology of the endocannabinoid system. This review will focus on reports of CB_2 in neurons and in the brain and the implications of that presence.

CB_2 and the immune system

CB_2 research continues to have a large focus on its role in the immune system. Analysis of the presence and function of CB_2 in the brain necessitates a discussion concerning CB_2 in immune cells. CB_2 mRNA has been identified in many immune tissues (Munro *et al.*, 1993; Lynn and Herkenham, 1994; Galiegue *et al.*, 1995; Schatz *et al.*, 1997; Berdyshev, 2000; Buckley *et al.*, 2000). Of specific immune cell types, the highest levels of CB_2 are found in macrophages, CD4+ T cells, CD8+ T cells, B cells, natural killer cells, monocytes and polymorphonuclear neutrophils (Derocq *et al.*, 1995; Galiegue *et al.*, 1995; Schatz *et al.*, 1997; Carayon *et al.*, 1998; McCoy *et al.*, 1999; Buckley *et al.*, 2000; Carlisle *et al.*, 2002; Maresz *et al.*, 2007; Dittel, 2008). Of particular relevance for the role of CB_2 in the CNS, CB_2 mRNA and protein have been found in microglia (Carlisle *et al.*, 2002; Klegeris *et al.*, 2003; Walter *et al.*, 2003; Beltramo *et al.*, 2006; Maresz *et al.*, 2007). Microglia are derived from macrophages and can be viewed as the resident immune cells of the brain where they monitor the brain for pathological damage. In response to specific signals within the brain they transition between different states of activity (Ashton and Glass, 2007; Hanisch and Kettenmann, 2007). The expression levels of CB_2 in microglia vary depending on the activation state of the cell (Carlisle *et al.*, 2002; Walter *et al.*, 2003; Stella, 2004; Maresz *et al.*, 2007; Cabral *et al.*, 2008; Pietr *et al.*, 2009). CB_2 modulates microglial migration and infiltration into brain areas with active neuroinflammation and degeneration (Walter *et al.*, 2003; Ashton *et al.*, 2007; Fernandez-Ruiz *et al.*, 2008; Miller and Stella, 2008; Price *et al.*, 2009). In healthy brain microglia do not appear to express CB_2 (Stella, 2004). However, in Alzheimer's brain tissue, CB_2 can be detected in neuritic plaque-associated microglia (Benito *et al.*, 2003). Similarly, in models of neuropathic pain (but not inflammatory pain) CB_2 mRNA increases in association with activated microglia in the spinal cord (Zhang *et al.*, 2003). In addition during amyotrophic lateral sclerosis and multiple sclerosis, CB_2 microglial expression increases in the spinal cord (Yiangou *et al.*, 2006). According to this evidence it is clear that under certain conditions brain microglia are capable of expressing CB_2.

CB_2 and tissue distribution

Despite being initially described as an immune cell cannabinoid receptor, CB_2 has been identified molecularly and pharmacologically in numerous other cell types. Evidence for the presence of CB_2 receptors has been found in pulmonary endothelial cells (Zoratti *et al.*, 2003). In these cells, CB_2 activation by anandamide results in phospholipase C-mediated calcium release from smooth ER with subsequent increases in mitochondrial calcium. CB_2 can also be found in bone (in osteocytes, osteoblasts and osteoclasts) where it modulates bone formation and turnover (Ofek *et al.*, 2006). The gastrointestinal system appears to express CB_2 receptors as well (Storr *et al.*, 2002; Hillsley *et al.*, 2007; Duncan *et al.*, 2008). 2-AG affects meiosis in spermatogonia via CB_2 (Grimaldi *et al.*, 2009) as well as a number of other aspects of reproductive function (Maccarrone, 2008; Grimaldi *et al.*, 2009). Keratinocytes release beta-endorphin in response to CB_2 selective agonist stimulation (Ibrahim *et al.*, 2005), although this result is controversial (Whiteside *et al.*, 2007; Anand *et al.*, 2008; Yao *et al.*, 2008; Yao *et al.*, 2009). These cells have also been reported to have CB_2 immunoreactivity. In the eye, trabecular

meshwork cells have been shown to have functional CB_2 receptors (Zhong *et al.*, 2005; He and Song, 2007). Mature and precursor adipocytes express functional CB_2 receptors that are negatively coupled to adenylyl cyclase (Roche *et al.*, 2006). In cirrhotic liver, CB_2 receptors are expressed in hepatic myofibroblasts, but are absent in normal liver (Julien *et al.*, 2005). THC protects cardiomyocytes from hypoxic damage by acting at CB_2 receptors resulting in nitric oxide production (Shmist *et al.*, 2006).

CB_2 and nociception

To better understand the possible presence of CB_2 in neurons it is helpful to consider the role of CB_2 in nociception. Cannabinoids have long been known to possess analgesic activity, but evidence for CB_2 having a role in analgesia was not presented until 1998 (Calignano *et al.*, 1998). Shortly thereafter, HU308, a CB_2 selective agonist, was shown to have analgesic activity without typical cannabinoid CNS side effects (Hanus *et al.*, 1999). AM1241, another CB_2 selective agonist was also shown to promote analgesia when injected peripherally and this did not produce CNS side effects, suggesting that CB_2 receptors modulate nociception (Malan *et al.*, 2001; Ibrahim *et al.*, 2003; Malan *et al.*, 2003; Ibrahim *et al.*, 2006). It has since been shown that a number of different CB_2 agonists can modulate many types of pain: acute, inflammatory, neuropathic, post-surgical and cancer pain (Khanolkar *et al.*, 2007; Whiteside *et al.*, 2007; Jhaveri *et al.*, 2008; Naguib *et al.*, 2008; Ohta *et al.*, 2008; Yao *et al.*, 2008; Anand *et al.*, 2009; Yao *et al.*, 2009). It is still unclear as to where these CB_2 agonists exert their analgesic activity. The site could be microglia, astrocytes, neurons, another cell type or a combination of these. Furthermore, as discussed above, the specificity of these CB_2 'selective' compounds may not be as specific as previously thought. Additional work must be performed to state with confidence that these agonists produce analgesia solely via activation of CB_2 receptors.

CB_2 and peripheral neurons

The first step to determine if CB_2 activation has a direct effect on neural mechanisms is to determine whether CB_2 is expressed in neurons. The existence of functional CB_2 receptors in peripheral neurons has been suggested by a number of studies. The first evidence for CB_2 function in peripheral neurons came in 1997 when CB_2 mRNA was identified in mouse vas deferens tissue (Griffin *et al.*, 1997). In support of a functional role for CB_2, JWH015 and JWH051 (agonists preferring CB_2 over CB_1) produced concentration dependent inhibition of evoked contractions presumably via a prejunctional site. However, a submicromolar concentration of AM630, a CB_2 antagonist, could not block this effect. Further, JWH015 is also an agonist for GPR55 (Ryberg *et al.*, 2007; Lauckner *et al.*, 2008), so the involvement of CB_2 cannot be unequivocally asserted.

CB_2 and sensory neurons

Functional studies have hinted at the presence of CB_2 receptors on sensory neurons. In these studies, it is necessary to consider the possible involvement of CB_2-expressing immune cells as microglia can affect synaptic properties (Cullheim and Thams, 2007; Abbadie *et al.*, 2009). Patel and colleagues provided some of the first functional evidence of CB_2 in sensory neurons (Patel *et al.*, 2003). Using isolated guinea pig and human vagus nerve preparations, they demonstrated that the CB_2 agonist JWH133 inhibited nerve depolarizations in response to capsaicin, PGE_2 and hypertonic saline. These three treatments activate vagal C and/or Aδ fibres. SR144528 blocked the effects of JWH133. A follow-up study with another putative CB_2 agonist, GW833972A, produced similar results (Belvisi *et al.*, 2008). Neither study was designed to determine a specific site or mechanism of action. While CB_2 does not appear to play a role in myenteric contractions, it does seem to play a role in activation of mesenteric sensory nerves. AM1241 administered intravenously inhibits bradykinin induced activation of isolated mesenteric afferents in mice (Hillsley *et al.*, 2007). This effect was blocked by AM630 and absent in CB_2 knockout mice. Interestingly, while CB_2 agonists do not affect normal enteric contractility, JWH133 can prevent lipopolysaccharide (LPS) induced increases in evoked contractions (Mathison *et al.*, 2004; Duncan *et al.*, 2008). JWH133 also blocks LPS stimulation of Fos expression in enteric neurons. AM630 antagonizes these effects. CB_2 receptors in myenteric neurons were identified as the most likely target of this drug effect (see below). CB_2 mRNA has also been identified in rat and mouse retina using RT-PCR as well as within specific layers of the retina (ganglion, inner nuclear and photoreceptor inner layers) using *in situ* hybridization (Lu *et al.*, 2000). Additionally, Burdyga *et al.* identified low, barely detectable levels of CB_2 mRNA in rat nodose ganglion, but were unable to detect CB_2 in the human vagal nerve trunk (Burdyga *et al.*, 2004).

CB_2 and nociceptive neurons

Further functional studies point to a role for CB_2 in sensory neuron function, particularly nociceptive neurons. A study was performed to address AM1241's ability to prevent windup of wide dynamic range (WDR) neurons in spinal cord (Nackley *et al.*, 2004). Here, AM1241 administered locally or systemically reduced the activity of C-fibres synapsing onto WDR neurons and this was reversed by SR144528, but not SR141716A. Significantly, suppression occurred in the presence and absence of inflammation. This, combined with the time course observed suggests long-term changes in presynaptic facilitation, makes the effects less likely to be due to CB_2 targeting immune cells. The authors speculate a direct effect of CB_2 activation on C-fibre neurons. Elmes *et al.* performed a similar study using JWH133 as a CB_2 agonist to test WDR spinal neuron responses in models of inflammatory and neuropathic pain as well as in non-inflammatory and sham-operated conditions (Elmes *et al.*, 2004). Like the Nackley study, they also found that peripherally administered CB_2 agonist inhibits WDR activity in both naïve and inflammatory conditions as well as following neuropathic injury. Once again, the data are suggestive of a non-immune function of CB_2, possibly in peripheral neurons. A follow-up study analysed JWH133's ability to inhibit capsaicin-induced calcium increases in DRG neurons cultured from sham-operated and

neuropathic rats (Sagar *et al.*, 2005). JWH133 slightly inhibited calcium increases in DRG cultured from both neuropathic and sham rats in a SR144528-sensitive fashion, consistent with the presence of functional CB_2 receptors in peripheral neurons. However, spinally administered JWH133 inhibited mechanically evoked responses of dorsal horn neurons from laminae V and VI only in neuropathic rats, but not in sham-operated animals. This points to an up-regulation of CB_2 in intrinsic spinal cord neurons in pain states, although does not provide evidence of the site of up-regulation. AM1241 and L768242 (another CB_2 agonist) can also decrease capsaicin-induced calcitonin gene-related peptide release, a pain biomarker, from neurons in spinal cord slices and this can be blocked by SR144528 (Beltramo *et al.*, 2006). These studies are most consistent with CB_2 participating in neural mechanisms rather than via immune cells, but do not directly answer the question of whether or not CB_2 is expressed in neurons.

The initial support for the presence of CB_2 protein in neurons came from Ross and colleagues. Using fluorescence-activated cell sorting analysis, they determined that DRG cultures and F-11 cells (DRG neuron × neuroblastoma hybrid) express both CB_1 and CB_2, but could not conclude that CB_2 was functionally expressed in DRG neurons (Ross *et al.*, 2001). To further address the site of CB_2 expression in DRG, Wotherspoon and colleagues used immunhistochemistry on DRG and spinal cord of naïve and nerve damaged rats and mice (Wotherspoon *et al.*, 2005). No CB_2 could be detected in normal rat or mouse spinal cord and DRG neurons. However, upon nerve sectioning or ligation, CB_2 immunoreactivity could be detected in the ipsilateral dorsal horn. This immunoreactivity was strongly reduced in CB_2 knockout mice and was blocked by incubation with the immunizing peptide, suggesting specificity of the primary antibody used. Of great interest, and in contrast to what would be expected based on Zhang *et al.*'s (2003) study, was that the CB_2 signal did not co-localize with markers of astrocytes (GFAP) or microglia (OX-42). Instead it co-localized with markers of damaged sensory neuron terminals (GAP-43 and galanin). CB_2 immunoreactivity also accumulated in axons proximal to the ligation site. They could not identify CB_2 in cell bodies in tissue sections, but were able to identify CB_2 in isolated DRG neurons grown in culture from lesioned mice. They also did not observe CB_2 immunoreactivity in skin, in contrast to other studies (Stander *et al.*, 2005; Kress and Kuner, 2009) and Ibrahim *et al.* who found it in keratinocytes (Ibrahim *et al.*, 2005). A few studies have detected CB_2 mRNA in DRG and spinal cord using quantitative RT-PCR. Here levels increased following nerve ligation, but this does not necessarily implicate a neuronal source (Zhang *et al.*, 2003; Beltramo *et al.*, 2006).

A more recent study by Anand and colleagues is consistent with the above findings (Anand *et al.*, 2008). Specifically, they found CB_2 positive, small diameter neurons in human DRG and peripheral nerves using three different CB_2 antibodies. The specificity of the antibodies was assessed using peptide block. CB_2 levels increased following nerve injury. They further extended the analysis by demonstrating CB_2 colocalization with neuronal (GAP-43), axonal (neurofilament) and nociceptive neuronal markers (TRPV1). Similar staining was observed in mouse, rat and guinea pig DRG. This study also replicated the functional data reported by Sagar *et al.* in that a CB_2 agonist (GW833972) inhibited capsaicin-induced calcium increases in DRG sensory neurons. They further sought to identify a mechanism for this activity and determined that CB_2-mediated cAMP depletion attenuated TRPV1 activation. This presumably decreased PKA-mediated phosphorylation of TRPV1, analogous to the effects of μ opioid receptor activation.

CB_2 and the enteric nervous system

Despite earlier findings (Griffin *et al.*, 1997), several studies suggest CB_2 is expressed in the enteric nervous system. Duncan *et al.* and Storr *et al.* found CB_2 mRNA in the enteric nervous system (Storr *et al.*, 2002; Duncan *et al.*, 2008). The site of action of JWH133 in preventing LPS-induced increases in ileum contractility was addressed using RT-PCR and immunohistochemistry (Duncan *et al.*, 2008). CB_2 mRNA was detected in the full-wall thickness ileum, ileal muscle, submucosal and mucosal layers in normal rats. LPS treatment had no effect on the levels of expression. A number of different antibodies and knockout tissue were used for controls. CB_2 protein was detected in all the same tissues in which CB_2 mRNA was found. CB_2 colocalized with markers of enteric ganglia, pan-neuronal markers and synaptic terminals suggesting a strong presence in myenteric neurons. CB_2 immunoreactivity did not colocalize with glial markers.

CB_2: another central cannabinoid receptor?

CB_2 in the cerebellum

One of the earliest reports of the presence of CB_2 in the CNS came from a study performed by Skaper and colleagues (Skaper *et al.*, 1996). *In situ* hybridization revealed the presence of CB_2 mRNA in cultured granule cells. In addition *in situ* hybridization localized CB_2 to the granule and Purkinje cell layers of mouse cerebellum. Radioligand binding analysis of cerebellar membranes revealed the presence of two WIN55212 binding sites. The affinities of WIN55212 at these sites were reported to be close to those of CB_1 and CB_2, although the exact identity of the binding sites could not be specifically determined. RT-PCR analysis has identified CB_2 mRNA in the rat cerebellum and Western blotting has revealed expressed CB_2 protein in rat and ferret cerebellum as well (Van Sickle *et al.*, 2005). Peptide block was used as a control for those Western blots.

Additional studies have also attempted to localize CB_2 protein in the cerebellum (Ashton *et al.*, 2006; Baek *et al.*, 2008). Using an antibody directed against the C-terminus of CB_2, with peptide block control, they identified CB_2 protein expression in the granule, Purkinje and white matter layers of the rat cerebellum. The signal did not overlap with astrocytes markers and the staining pattern in the Purkinje layer and parts of the other layers appeared to be capillary endothelial in nature. There were fine fibres in the white matter and granule cell layers that were CB_2 positive but their origin remains to be determined. These could possibly arise from microglia or neurons. Onaivi *et al.* have also reported CB_2

expression in the Purkinje and molecular layers of the cerebellum using Western blot, immunohistochemistry and *in situ* hybridization techniques (Gong *et al.*, 2006; Onaivi *et al.*, 2008b).

CB_2 in the brainstem

CB_2 has been identified within the brainstem as well. A thorough analysis was performed that investigated CB_2 expression in brain, focusing on mRNA, protein and functional expression within the brainstem (Van Sickle *et al.*, 2005). Quantitative RT-PCR showed that the rat brainstem contains CB_2 mRNA at significantly lower levels than spleen (1.5% of spleen levels). Western blotting confirmed this expression for rat as well as for ferret. Immunocytochemistry identified the dorsal motor nucleus of the vagus nerve (DMNX) of the mouse, rat and ferret as a brainstem nucleus containing CB_2 protein. The CB_2 knockout mouse did not show any immunostaining in the DMNX. The DMNX immunoreactivity colocalized with neuronal markers, but in contrast to what Ashton *et al.* found in the cerebellum (Ashton *et al.*, 2006), the signal did not overlap with glial or blood vessel markers. The authors acknowledged the differences in results between their study and that of Derbenev *et al.* that did not find CB_2 in similar regions (Derbenev *et al.*, 2004) and state that in the latter study a faint signal could be observed in Western blots consistent with low levels of expression. The authors also demonstrated that AM630 blocked the anti-emetic actions of 2-AG treatment in ferrets, suggesting CB_2 receptor involvement. Furthermore a sub-efficacious concentration of anandamide combined with AM1241 treatment produced anti-emetic effects. Another, more superficial study of the brainstem using immunohistochemistry was later performed to look for CB_2 in other brainstem nuclei (Baek *et al.*, 2008). CB_2 immunoreactivity was found in the medial vestibular nucleus as well as the dorsal and ventral cochlear nuclei, but no attempts were made to identify cell types. Peptide block and secondary antibody controls were used to determine CB_2 antibody specificity. Viscomi *et al.* did not find CB_2 protein and only low levels of CB_2 mRNA in inferior olive and pontine nuclei using immunohistochemistry and quantitative PCR in normal rats (Viscomi *et al.*, 2009). However, following a hemicerebellectomy, CB_2 expression dramatically increased in both mRNA and protein levels in these nuclei. The CB_2 immunoreactivity colocalized with neuronal markers but not with microglial or astrocytic ones. Further JWH015 had a neuroprotective effect, preventing cell death due to the hemicerebellectomy. This was likely operating through CB_2, although they did not report block of the neuroprotective effect with a CB_2 antagonist. Gong *et al.* reported the presence of CB_2 in many nuclei of the brainstem using RT-PCR and immunohistochemistry (Gong *et al.*, 2006).

CB_2 and the hippocampal formation

Using several approaches, Onaivi and his collaborators have reported finding CB_2 immunoreactivity in many areas of the hippocampal formation (Gong *et al.*, 2006; Onaivi, 2006; Onaivi *et al.*, 2006; 2008a,b; Brusco *et al.*, 2008). They report a predominately postsynaptic expression and an association with rough endoplasmic reticulum and Golgi structures. They have also demonstrated CB_2 staining in hippocampal cultures. In contrast to their immunohistochemical results, they have had mixed results in finding CB_2 mRNA in the hippocampus (Gong *et al.*, 2006; Onaivi *et al.*, 2008b).

Functional evidence for CB_2 expression in the cortex comes from recording spontaneous inhibitory postsynaptic currents (sIPSCs) in layers II and V of the medial entorhinal cortex. Here, 2-AG mediated suppression of sIPSCs was not blocked by LY320135, a CB_1 antagonist/inverse agonist, whereas they were blocked by AM630 and JTE907 (a structurally distinct CB_2 antagonist) (Morgan *et al.*, 2009). Further JWH133 suppressed sIPSCs in a CB_2 antagonist sensitive fashion. The site of CB_2 agonist action remains to be conclusively demonstrated.

CB_2 and other brain regions

Evidence exists for CB_2 expression in other brain regions. While recording from the ventral posterior nucleus of the thalamus, Jhaveri *et al.* found that after spinal nerve ligation, JWH133 reduced spontaneous and evoked responses in a SR144528-sensitive fashion, but that this effect was absent in sham operated rats (Jhaveri *et al.*, 2008). Gong *et al.* have also reported CB_2 immunoreactivity in many thalamic nuclei, but could not detect CB_2 mRNA using RT-PCR (Gong *et al.*, 2006). Furthermore, this group has reported finding CB_2 mRNA in striatum and hypothalamus, but not in olfactory bulb, cortex and spinal cord and mixed results in midbrain (Gong *et al.*, 2006; Onaivi *et al.*, 2008b). Additionally they report CB_2 immunoreactivity in olfactory bulb, cortex, midbrain as well as the other areas already mentioned (Gong *et al.*, 2006; Onaivi, 2006; Onaivi *et al.*, 2006; 2008b).

CB_2 and neurogenesis

CB_2 also appears to play a role in neurogenesis. Both CB_1 and CB_2 are expressed in stem cells (Jiang *et al.*, 2007; Molina-Holgado *et al.*, 2007). More specifically, RT-PCR, Western blot and immunohistochemical analyses have all revealed the presence of CB_2 in embryonic and adult neural progenitor cells (Palazuelos *et al.*, 2006; Molina-Holgado *et al.*, 2007). CB_2 blockade or genetic disruption impairs neurosphere formation and prevents progenitor cell proliferation, whereas CB_2 agonists promote these activities via ERK and Akt signalling (Palazuelos *et al.*, 2006; Molina-Holgado *et al.*, 2007). However, CB_2 expression seems to diminish as the cells differentiate, being nearly absent by the time neuronal and astrocytic markers appear (Palazuelos *et al.*, 2006). Further, CB_1 agonists and antagonists have similar effects in neurosphere formation and in COR-1 neural stem cell cultures (Molina-Holgado *et al.*, 2007; Goncalves *et al.*, 2008) suggesting either functional interactions or redundant signalling. In contrast to these data, CB_1 agonists and antagonists had no effect on neurogenesis in the subventricular zone (SVZ) of either young or adult mice (Goncalves *et al.*, 2008). On the

other hand, JWH133 and WIN55212 stimulated SVZ neurogenesis, whereas AM630 and JTE907 decreased it (Goncalves *et al.*, 2008).

CB_2: where is its real home and why do we care?

We feel careful analysis of the studies reviewed above allows us to reach the following conclusions: CB_2 is expressed by microglia, with levels increasing as they are activated, and CB_2 is present at detectable and functionally relevant levels in a subset of neurons, with increasing levels following injury. We care where CB_2 is expressed primarily for understanding pathology that involves CB_2 and to develop therapies that target difficult to treat conditions. To this end it is important to have a rigorous understanding of where and under what conditions CB_2 is expressed in the CNS.

Approaches aimed at identifying CB_2 receptor expression in the brain can be divided into functional (pharmacological), biochemical and anatomical techniques. All three have their strengths and weaknesses. The most convincing studies will incorporate a combination of these techniques. Table 1 summarizes the studies presented here, detailing the brain region analysed and whether or not CB_2 was detected and the techniques(s) used to detect it. Pharmacological studies rely on the specificity of the drugs used. When interpreting these studies it is necessary to recall that specificity is never absolute – at sufficiently high concentrations any drug will interact with additional targets. Thus, it is important to relate the concentration of the drug being used to the binding affinity of the CB_2 receptor for that drug. The second consideration is that drugs considered to be 'specific' or 'selective' based on our current understanding may soon be found to interact with other receptors. Examples of this in the cannabinoid system include AM251, often used as a 'selective' CB_1 receptor antagonist, but it is also a GPR55 agonist (Henstridge *et al.*, 2009; Kapur *et al.*, 2009) and JWH015, sometimes used as a 'selective' CB_2 agonist, but it, too, is a GPR55 agonist (Ryberg *et al.*, 2007; Lauckner *et al.*, 2008). Approaches to circumvent this issue include using several structurally diverse agonists and antagonists (presumably decreasing the likelihood of having the same 'off-target' actions) and knockout or 'knockdown' controls, when appropriate.

Biochemical studies include Western blotting and PCR-based approaches. For Western blotting, the key limitations are the sensitivity and specificity of the antibody used. At a minimum, blots from knockout (assuming the antibody is recognizing an epitope present in mouse CB_2) and positive control tissues (e.g. spleen) should be shown. Blindly trusting an antibody to 'work' without concurrent controls is unacceptable. Block with the immunizing antibody is desirable, but will not rule out a fortuitous interaction of the antibody with an unintended epitope on another protein. The sensitivity of Western blotting will depend on the abundance of CB_2 as well as the affinity of the antibody. The lack of detection of CB_2 on the blot can only be interpreted as that the level of CB_2 in the brain is below a certain level. (This level, relative to a CB_2-expressing tissue like spleen, can be determined by serial dilution.) PCR-approached tissues are the most sensitive. Their high sensitivity makes their interpretation subject to several considerations (Suzuki *et al.*, 2000; Lion, 2001). These include amplification of CB_2 mRNA from immune cells trapped in the cerebral vasculature and amplification of CB_2 mRNA from a very small subset of activated microglia. In order to rationally interpret results from PCR-based experiments it is necessary that they be performed in a quantitative fashion, preferably calculating copy number, to facilitate comparisons.

Anatomical studies need to be conducted and interpreted with a similarly critical approach. These studies fall into three categories: autoradiography, *in situ* hybridization and immunocytochemistry. As above the issue of sensitivity needs consideration – it is possible to show CB_2 is present, but it is very hard to conclusively demonstrate that it is not present, just that it is present at a level below the limit of detection. However, this information, coupled with a lack of functional response, can be very valuable in sorting out the role of CB_2 receptors in a particular physiological response. The caveats of autoradiography include the pharmacological considerations discussed above as well as specific technical issues (Frey and Albin, 2001). As this technique has not been widely applied to directly identifying CB_2 receptors in the brain, it will not be further discussed here. *In situ* hybridization studies can yield useful information on which cells express CB_2 and thus can complement PCR-based studies. However, the lower sensitivity of *in situ* hybridization may make this difficult. A necessary control for *in situ* hybridization includes lack of hybridization in knockout tissues (when possible).

Immunocytochemistry studies have the powerful potential to identify the precise localization of CB_2. However, for meaningful information to be drawn from them it is essential that proper controls are followed [as reviewed by Bussolati and Leonardo (2008); Lorincz and Nusser (2008); Saper and Sawchenko (2003)]. Briefly, some of the controls are: adsorption with the immunizing peptide, parallel, blinded staining of wild-type and knockout tissue, the use of two or more antibodies raised against distinct epitopes, antibody titration, omitting the primary antibody from the staining procedure and supporting these findings with RT-PCR, *in situ* hybridization and other such detection methods. Using just one control for one experimental setup is usually insufficient proof of specificity. These basic controls must be remembered when interpreting the data presented from any study cited in this review and future studies as well.

In conclusion, despite originally being thought of as the 'peripheral' cannabinoid receptor, considerable functional and anatomical evidence suggests that CB_2 is expressed in the nervous system – certainly in activated microglia and very likely in some neurons. In addition, this raises the point that any report that identifies CB_2 in neurons of the nervous system must incorporate careful controls to ensure that the CB_2 signal found originates from neurons and not from microglia or immune cells associated with brain blood vessels. Given the importance of determining the functional role of CB_2 in the CNS, under what conditions it is up regulated, and the potential therapeutic applications of CB_2 agonists it is vital to understand where in the CNS CB_2 receptors are expressed. We encourage those working in the field and those reviewing manuscripts to conduct and review these studies in a careful, thoughtful and rigorous fashion.

Table 1 CB_2 distribution in the peripheral and central nervous systems

Location	*Presence*	*Detection method*	*Species*	*Reference*
Whole brain				
Whole brain	Absent	ISH	Mouse	Munro et al., 1993
Whole brain	Absent	NB/RT-PCR	Human	Galiegue et al., 1995
Whole brain	Absent	NB/RT-PCR	Mouse	Schatz et al., 1997
Whole brain	Absent	SB	Mouse	McCoy et al., 1999
Whole brain	Absent	RLB/GTPγS	Rat	Griffin et al., 1999
Whole brain	Absent	ISH	Mouse	Howlett et al., 2002
Whole brain	Present	WB	Rat	Gong et al., 2006
Whole brain	Present	WB/RT-PCR	Mouse	Onaivi, 2006; Onaivi et al., 2008b
Brainstem				
DMNX	Present	RT-PCR/WB/IHC	Rat	Van Sickle et al., 2005
DMNX	Present	ICC	Mouse	Van Sickle et al., 2005
DMNX	Present	WB/ICC/functional	Ferret	Van Sickle et al., 2005
Cochlear nuclei	Present	IHC	Rat	Baek et al., 2008
Medial vestibular nuclei	Present	IHC	Rat	Baek et al., 2008
Inferior olive and pontine nuclei	Present	IHC/ICC/RT-PCR	Rat	Viscomi et al., 2009
Brainstem	Present	RT-PCR/IHC	Rat	Gong et al., 2006
Brainstem	Present	RT-PCR	Mouse	Onaivi et al., 2006; Liu et al., 2009
Cerebellum				
Cerebellum	Absent	NB/RT-PCR	Human	Galiegue et al., 1995
Granule and Purkinje cell layers	Present	ISH/RLB	Mouse	Skaper et al., 1996
Cerebellum	Absent	NB/RT-PCR	Rat	Schatz et al., 1997
Cerebellum	Absent	NB/RLB/GTPγS	Rat	Griffin et al., 1999
Cerebellum	Present	RT-PCR/WB	Rat	Van Sickle et al., 2005
Cerebellum	Present	WB	Ferret	Van Sickle et al., 2005
Cerebellum	Present	ICC	Rat	Ashton et al., 2006
Cerebellum	Present	IHC	Rat	Baek et al., 2008
Cerebellum	Present	IHC/ISH	Rat/mouse	Onaivi, 2006; Onaivi et al., 2008b
Cerebellum	Present	RT-PCR	Human	Liu et al., 2009
Cortex				
Cortex	Absent	RT-PCR/NB	Human	Galiegue et al., 1995
Cortex	Absent	NB	Rat	Griffin et al., 1999
Cortex	Absent	RT-PCR	Rat	Beltramo et al., 2006
Cortex	Present/absent	IHC/RT-PCR	Rat	Gong et al., 2006
Cortex	Present	IHC	Mouse	Onaivi, 2006; Onaivi et al., 2006; 2008b
Cortex	Present	RT-PCR	Mouse/human	Liu et al., 2009
Hippocampus				
Hippocampus	Present/absent	IHC/RT-PCR	Rat	Gong et al., 2006; Brusco et al., 2008
Hippocampus	Present	IHC	Mouse	Onaivi, 2006; Onaivi et al., 2006; 2008b
Hippocampus	Present	IHC	Rat	Onaivi et al., 2008b
Hippocampus	Present	RT-PCR	Human	Liu et al., 2009
Other brain regions				
Thalamus	Present/absent	IHC/RT-PCR	Rat	Gong et al., 2006
Hypothalamus	Present	RT-PCR		Gong et al., 2006
Midbrain	Present/absent	IHC/RT-PCR	Rat	Gong et al., 2006
Olfactory bulb	Present/absent	IHC/RT-PCR	Rat	Gong et al., 2006
VPN of thalamus	Present	Functional	Rat	Jhaveri et al., 2008
Entorhinal cortex	Present	Functional	Rat	Morgan et al., 2009
Peripheral neurons/spinal cord				
Vas deferens	Present	Functional	Mouse	Griffin et al., 1997
Spinal cord	Absent	NB	Rat	Griffin et al., 1999
DRG neurons	Absent	ISH	Rat	Hohmann and Herkenham, 1999b
Retina	Present	ISH/RT-PCR	Mouse	Lu et al., 2000
DRG neurons	Present	FACS	Rat	Ross et al., 2001
Enteric system	Present	RT-PCR	Rat	Storr et al., 2002
Trigeminal ganglia	Absent	ISH	Rat	Price et al., 2003
Spinal cord	Present	ISH	Rat	Zhang et al., 2003
Vagus nerve	Present	Functional	Guinea pig/human	Patel et al., 2003
DRG neurons	Present	Functional	Rat	Nackley et al., 2004
DRG neurons	Present	Functional	Rat	Elmes et al., 2004
Nodose ganglion	Present	RT-PCR	Rat	Burdyga et al., 2004
Vagus nerve trunk	Absent	RT-PCR	Human	Burdyga et al., 2004
DRG neurons	Present	Functional	Rat	Sagar et al., 2005
DRG neurons/spinal cord	Inducible	ICC	Rat/mouse	Wotherspoon et al., 2005
Skin sensory neurons	Present	IHC	Human	Stander et al., 2005
DRG neurons/spinal cord	Present	Functional/RT-PCR	Rat	Beltramo et al., 2006
Mesenteric sensory neurons	Present	Functional	Mouse	Hillsley et al., 2007
Vagus nerve	Present	Functional	Guinea pig	Belvisi et al., 2008
Myenteric neurons	Present	Functional/ICC/RT-PCR	Rat	Duncan et al., 2008
DRG neurons	Present	IHC/WB/functional	Gp/human/rat/mouse	Anand et al., 2008

Table 1 Continued

Location	*Presence*	*Detection method*	*Species*	*Reference*
Neural progenitor cells				
Hippocampal neural progenitor cells	Present	RT-PCR/ICC/functional	Mouse	Palazuelos et al., 2006
Neurospheres	Present	WB/ICC/functional	Mouse	Molina-Holgado et al., 2007
SVZ neural progenitor cells	Present	WB/ICC/functional	Mouse	Goncalves et al., 2008

Summary of studies investigating CB_2 expression in the nervous system. Details the location studied, whether CB_2 was detected or not, the method(s) used to detect it, the species analysed and the reference(s) for the studies.
FACS, fluorescence-activated cell sorting; ICC, immunocytochemistry; IHC, immunohistochemistry; ISH, *in situ* hybridization; NB, Northern blot; RLB, radioligand binding; RT-PCR, reverse transcriptase polymerase chain reaction; SB, Southern blot; WB, Western blot.

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Conflicts of interest

None.

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