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Special Section: Cannabinoid Signaling in Human Health and Disease—Minireview

Analgesic Properties of Next-Generation Modulators of Endocannabinoid Signaling: Leveraging Modern Tools for the Development of Novel Therapeutics

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ABSTRACT

Targeting the endocannabinoid (eCB) signaling system for pain relief is an important treatment option that is only now beginning to be mechanistically explored. In this review, we focus on two recently appreciated cannabinoid-based targeting strategies, treatments with cannabidiol (CBD) and α/β-hydrolase domain containing 6 (ABHD6) inhibitors, which have the exciting potential to produce pain relief through distinct mechanisms of action and without intoxication. We review evidence on plant-derived cannabinoids for pain, with an emphasis on CBD and its multiple molecular targets expressed in pain pathways. We also discuss the function of eCB signaling in regulating pain responses and the therapeutic promises of inhibitors targeting ABHD6, a 2-arachidonoylglycerol (2-AG)-hydrolyzing enzyme. Finally, we discuss how the novel cannabinoid biosensor $GRAB_{eCB2.0}$ may be leveraged to enable the discovery of targets modulated by cannabinoids at a circuit-specific level.

SIGNIFICANCE STATEMENT

Cannabis has been used by humans as an effective medicine for millennia, including for pain management. Recent evidence emphasizes the therapeutic potential of compounds that modulate endocannabinoid signaling. Specifically, cannabidiol and inhibitors of the enzyme ABHD6 represent promising strategies to achieve pain relief by modulating endocannabinoid signaling in pain pathways via distinct, nonintoxicating mechanisms of action.

Analgesic Properties of Cannabinoids

Chronic pain is a prevalent and debilitating condition that profoundly affects the quality of life for millions of individuals worldwide. Current treatment options, including opioids, nonsteroidal anti-inflammatory drugs, and targeted neuropathic pain medications, such as tricyclic antidepressants, gabapentinoids, and serotonin and norepinephrine reuptake inhibitors, often exhibit limited efficacy and induce adverse effects (Szok et al., 2019). Therefore, there is an urgent need to explore novel therapeutic mechanisms and strategies for pain relief.

Cannabis is a plant genus that originated in central Asia, and its use results in a wide range of behavioral changes in humans, with reports of its medical use dating back to 2737 BC in China (Ben Amar, 2006). Behavioral changes induced by *Cannabis* use include 1) recreational effects, including altered perceptions of time, euphoria, hallucinations, and dissociation (altogether referred to as "feeling high"), 2) medicinal effects, in particular analgesic, antiemetic, anticonvulsant, and sedative effects, and

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ABBREVIATIONS: 2-AG, 2-arachidonoylglycerol; ABHD6, α/β-hydrolase domain containing 6; ACC, anterior cingulate cortex; AEA, N-arachidonoylethanolamine; CB_1R, type 1 CBR; CB_2R, type 2 CBR; CBD, cannabidiol; CBR, cannabinoid receptor; cpGFP, circularly permutated green fluorescent protein; eCB, endocannabinoid; GlyR, glycine receptor; GRABeCB2.0, G protein–coupled receptor activation–based eCB2.0; MAGL, monoacylglycerol lipase; NAM, negative allosteric modulator; PAG, periaqueductal gray; Phyto-CB, phyto-cannabinoid; THC, Δ^9-tetrahydrocannabinol; TRPV1, transient receptor potential cation channel subfamily V member 1.

3) select adverse effects, such as paranoia, anxiety, memory impairment, and other symptoms of intoxication (Alexander, 2016; LaFrance et al., 2020; Dos Reis Rosa Franco et al., 2021).

Original studies on the efficacy of *Cannabis* at inducing behavioral changes led to the identification of its bioactive compounds, or phyto-cannabinoids (phyto-CB) (Mechoulam and Gaoni, 1965). Phyto-CBs are a class of lipophilic, 21-carbon polyketides, the most abundant of which are Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD), depending on the strain (Bow and Rimoldi, 2016; Mudge et al., 2018; Dos Reis Rosa Franco et al., 2021; Smith et al., 2022). THC and CBD bind to and modulate the molecular machinery that forms the endocannabinoid (eCB) signaling system, which encompasses the cannabinoid receptors (CBRs) that bind select phyto-CBs at their orthosteric site, the endogenously produced eCBs that also act at the orthosteric site of CBRs, and finally the biosynthetic and degradative enzymes that regulate eCB levels (Di Marzo and Piscitelli, 2015). The best-characterized eCBs are N-arachidonoylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG), which differ from one another in several key characteristics, including their relative abundances in the brain, biosynthetic mechanisms, pharmacodynamic properties at their respective target proteins, and how they are inactivated (Lu and Mackie, 2016). The amount of eCBs that reach and activate CBRs is regulated by at least five mechanisms: 1) eCB production (Di Marzo et al., 1994; Stella et al., 1997), 2) the number and functional coupling of CBRs (McKinney et al., 2008), 3) fatty acid binding proteins that shuttle eCBs from their site of synthesis to CBRs (Fowler, 2013), 4) clearance of extracellular eCBs by uptake through plasma membranes (Nicolussi and Gertsch, 2015), and 5) eCB inactivation by enzymatic hydrolysis (Cao et al., 2019; Stella, 2023). Thus, CBR activation, either with phyto-CBs or by enhancing eCB levels via inhibition of their hydrolysis, modulates multiple physiological processes, inducing pain responses as shown in preclinical mouse models.

Phyto-CBs as Analgesics

It is thought that the original and oldest therapeutic use of THC was for its analgesic properties for the treatment of chronic pain (Buxbaum, 1972; Fisher et al., 2019). We now know that acute THC treatment is 1) analgesic in preclinical rodent models of both pathological and injury-related persistent pain and 2) results in greater analgesia compared with placebo in human patients suffering from chronic pain (Almog et al., 2020). It is important to emphasize that THC binds to the orthosteric binding site of type 1 and type 2 CBRs (CB_1Rs and CB_2Rs, respectively), where it acts as a partial agonist and can compete with the bioactivity of eCBs. Thus, free CB_1R and CB_2R are activated by THC with moderate efficacy, whereas CB_1R and CB_2R signaling stimulated by localized, activity-dependent increases in eCBs may be reduced by THC competing for the same binding site and therefore acting as an antagonist (Shen and Thayer, 1999; Kelley and Thayer, 2004; Straiker and Mackie, 2005; Roloff and Thayer, 2009).

Relevant to the analgesic properties of THC, the CB_1R and CB_2R have distinct roles in pain processing due in part to differences in their expression profiles. The CB_1R is predominantly expressed by neurons, whereas the CB_2R is expressed at low levels in the healthy brain and mostly by endothelial cells forming the blood brain barrier as well as by immune cells, such as microglia (Vendel and de Lange, 2014; Chung et al., 2016). CB_2R expression is greatly upregulated under select pathological states, including in rodent models of chronic pain (Zhang et al., 2003; Atwood and Mackie, 2010; Xu et al., 2023). Although both genetic and pharmacological evidence indicate that CB_2R directly regulates neuronal functions, its expression by neurons and astrocytes remains inconclusive because of the absence of reagents such as selective antibodies (Van Sickle et al., 2005; Eraso-Pichot et al., 2023). CB_2R signals through $G_{i/o}$ and β-arrestin in microglia and oligodendrocytes, and evidence suggests that it can signal through G_q in endothelial cells (Zoratti et al., 2003; Stella, 2010). Thus, THC's bioactivity mediated by CB_2R includes glial cell activation and endothelial cell function, and we still do not know whether modulation of neuronal activity is direct or indirect through these cell types.

CBD also induces analgesia yet through different molecular mechanisms. For example, CBD modulates CB_1R signaling as a negative allosteric modulator (NAM), although its direct binding to CB_1R has still not been demonstrated (Laprairie et al., 2015; Shahbazi et al., 2020; Jakowiecki et al., 2021). In line with this premise, CBD reduces eCB-stimulated CB_1R activity without influencing basal/tonic CB_1R signaling (Thomas et al., 2007; Laprairie et al., 2015). The analgesic properties of CBD are dose dependent and require relatively high doses. For example, in humans, CBD (300 mg/day orally) prevents acute and transient chemotherapy-induced peripheral neuropathy, but CBD (30 mg/kg) does not affect pain linked to irritable bowel syndrome (Nielsen et al., 2022; van Orten-Luiten et al., 2022). Acute CBD in rodents yields mixed results that depend on both the type of pain model studied and the dosing regimen of CBD that was tested. In a meta-review of the literature for animal models, acute CBD significantly attenuates pain-associated behaviors in neuropathic pain models but yields mixed results in inflammatory pain models (Soliman et al., 2021). This is a significant result given that CBD has been showed to reduce inflammation (Burstein, 2015). Chronic or subchronic dosing of CBD also appears to be effective in alleviating pain. Specifically, we showed that 3-week treatment of CBD formulated in gelatin using a voluntary oral consumption paradigm attenuated chronic neuropathic pain–induced allodynia in mice, with a weaker effect measured for thermal hyperalgesia (Abraham et al., 2020). Thus, the pain modality and the dose and duration of CBD administration play important roles in establishing CBD's analgesic efficacy and should be validated in human placebo-controlled trials by measuring specific pain responses. Thus, THC and CBD modulate the eCB signaling system and induce pain relief through different MOAs, which emphasizes the need to better understand their effects in distinct cell types within pain pathways.

Pain Processing in the Central Nervous System

Pain has both sensory and affective components. When a noxious stimulus is presented, nociceptors expressed by multiple peripheral cells send this information to sensory neurons in the dorsal root ganglia, which then synapse onto laminaes in the dorsal spinal cord (Fig. 1) (Kuner, 2010; Yam et al., 2018). Projection neurons in the ascending pain pathway then send this information to many brain areas, including the thalamus, amygdala, and anterior cingulate cortex (ACC), among

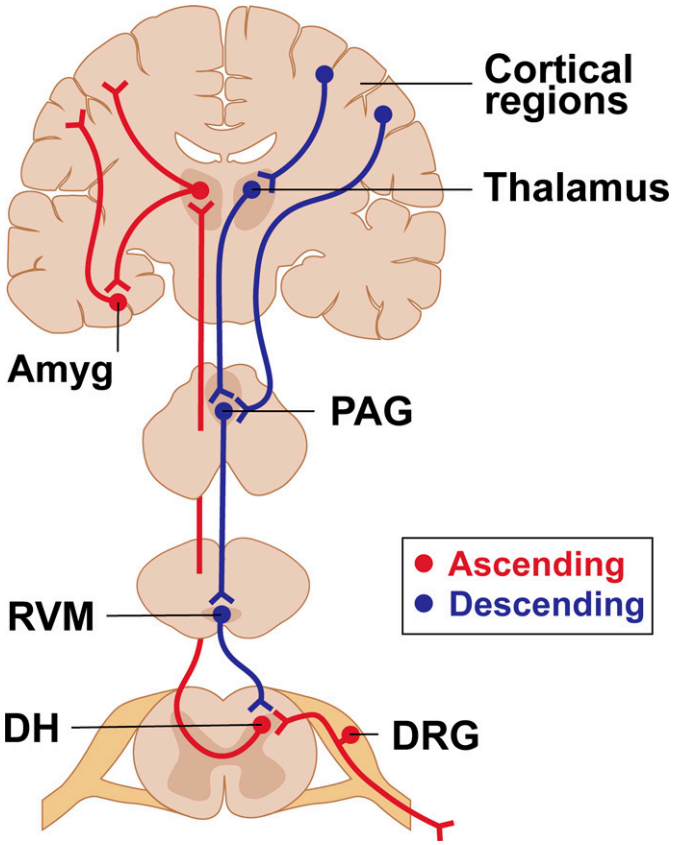

Fig. 1. Anatomical pain pathway. Ascending (red) and descending (blue) pathways involved in pain responses are depicted. Painful stimuli information enters the spinal cord dorsal horn through primary afferent fibers in the dorsal root ganglion (DRG) that synapse onto transmission neurons located in the dorsal horn (DH). Ascending information travels to the thalamus, where it is relayed to the amygdala (Amyg) and cortical regions. Information then travels through the descending pathway, which includes brain regions such as the thalamus, PAG, and rostral ventromedial medulla (RVM). The RVM sends projections back to the DH in the spinal cord. Projections throughout this pathway modulate pain inputs and can be targeted for pain relief.

others. Following higher-level processing within the cortex, neurons that make up the descending pain pathway send the information to the thalamus, periaqueductal gray (PAG), rostroventromedial medulla, and, eventually, back to the dorsal horn. Under chronic pain conditions, both central and peripheral sensitization occurs as well as abnormalities in nociceptive modulation (Cui et al., 2023; Walters et al., 2023). The multiple CBD targets mentioned below are differentially expressed by various cell types throughout this pain pathway and are thought to mediate CBD's ability to modulate and intercept pain signaling at both the spinal and supraspinal level.

Targets of CBD Involved in Pain Modulation

CBD exhibits a far broader poly-pharmacology profile compared with THC, with only a few overlapping molecular targets. In this section, we focus on those most relevant targets bound by CBD and relevant to pain (Fig. 2; Table 1). For example, CBD antagonizes GPR55, a G protein–coupled receptor involved in regulating multiple processes, including cell division, neuronal pathfinding during development, and neurotransmission

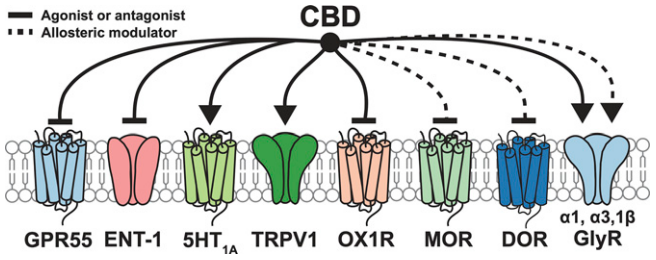

Fig. 2. Receptor targets of CBD that produce analgesia. CBD is an antagonist at G protein-coupled receptor 55 (GPR55) and orexin 1 receptors type 1 (OX1R); a negative allosteric modulator of μ-opioid receptors (MOR) and of δ-opioid receptors (DOR); a blocker of the equilibrative nucleoside transporter-1 (ENT-1) transporters; an agonist at transient receptor potential vanilloid type 1 (TRPV1), serotonin receptor 1 A (5-HT1A), and α1 and 1β GlyRs; and a positive allosteric modulator of α3 GlyR.

in the adult brain (Ross, 2009; Hill et al., 2018). By antagonizing GPR55's endogenous activation via lysophosphatidyl inositol, CBD increases GABAergic inhibitory transmission (Sylantyev et al., 2013; Devinsky et al., 2017; Kaplan et al., 2017; Chuang et al., 2021). GPR55 is highly expressed throughout the central nervous system (Ryberg et al., 2007; Henstridge et al., 2011; Wu et al., 2013), including areas important for pain response processing, such as the PAG (Deliu et al., 2015) and the ACC (Okine et al., 2020). Accordingly, blocking GPR55 action within the PAG activates the descending pain pathway to mitigate neuropathic pain in rats (Armin et al., 2021), whereas activating GPR55 within the PAG reduces the nociceptive threshold in the hot-plate test in rats (Deliu et al., 2015). Additionally, localized microinjections of the GRP55 antagonist CID16020046 into the ACC induces analgesia in a rat formalin test (Okine et al., 2020). GPR55-knockout mice have reduced mechanical pain hypersensitivity in a model of spinal cord compression (Ono et al., 2023), and antagonizing GPR55 in the spinal cord of mice in chronic neuropathic pain has an antiallodynic affect (Zúñiga-Romero et al., 2022). Together, this evidence suggests a predominant role for GPR55 in mediating supraspinal pain processing (Table 1) (Sylantyev et al., 2013; Okine et al., 2020).

CBD modulates the activity of multiple ion channels, including $GABA_AR$ (Rudolph and Knoflach, 2011; Sigel et al., 2011; Golovko et al., 2014; Bakas et al., 2017; Anderson et al., 2019; Cao et al., 2019) and transient receptor potential cation channel subfamily V member 1 (TRPV1), both of which mediate

TABLE 1
Pharmacological profile of CBD at receptors related to analgesia

Receptor	Action	EC_{50} or IC_{50} (μM)	Reference
GPR55	Antagonist	0.445	Ryberg et al., 2007
α1 GlyR	Agonist	12.3	Ahrens et al., 2009
α3 GlyR	PAM	N.D.	Xiong et al., 2012
1β GlyR	Agonist	18.1	Ahrens et al., 2009
ENT-1	Antagonist	0.122	Carrier et al., 2006
OX1R	Antagonist	4.2	Vitale et al., 2021
TRPV1	Agonist	1–3.5	Bisogno et al., 2001; De Petrocellis et al., 2011
$5HT_{1a}$	Agonist	N.D.	Russo et al., 2005
MOR	NAM	8–12	Kathmann et al., 2006
DOR	NAM	8–12	Kathmann et al., 2006

$5\text{-}HT_{1A}$, serotonin receptor 1 A; DOR, δ-opioid receptor; ENT-1, equilibrative nucleoside transporter-1; GlyR, glycine receptor; GPR55, G protein–coupled receptor 55; MOR, μ-opioid receptor; N.D., no data; OX1R, orexin 1 receptor type 1; PAM, positive allosteric modulator; TRPV1, transient receptor potential vanilloid type 1.

pain responses in rat models of acute inflammation (Costa et al., 2004; Campos and Guimarães, 2009). TRPV1 is primarily expressed in sensory neurons involved in the pain pathway and within supraspinal brain regions in the descending pain pathway (Mezey et al., 1999; Szabo et al., 2002; Tóth et al., 2005). Cell culture studies indicate that CBD activates and then desensitizes TRPV1, ultimately reducing nociceptive transmission mediated by these ligand-gated channels (Bisogno et al., 2001; Iannotti et al., 2014). In rodents, CBD exhibits several TRPV1-dependent effects, including reversal of mechanical and thermal allodynia, hyperalgesia, and anxiety behaviors in a neuropathic pain model (De Gregorio et al., 2019; Silva-Cardoso et al., 2021). Because of the bimodal role that TPRV1 channels have in regulating pain (i.e., activation of TRPV1 in the ascending pain pathway is nocifensive, whereas activation of TRPV1 in the descending pain pathway is antinociceptive), it stands to reason that CBD acts primarily on the descending pain pathway and, more likely, is working in concert with other receptors to produce its affects.

CBD is a competitive inhibitor of the equilibrative nucleoside transporter-1, which is responsible for terminating extracellular adenosine signaling (Carrier et al., 2006; Hinton et al., 2014; Pastor-Anglada and Pérez-Torras, 2018). Therefore, inhibition of this transporter by CBD decreases adenosine reuptake by neurons and glia, thereby increasing extracellular adenosine levels and A_{2A} receptor activation (Carrier et al., 2006). A_{2A} receptors are expressed throughout the body and play a significant role in mediating pain (Lee et al., 2003). For example, an A_{2A} receptor agonist delivered intrathecally into the lumbar region of the spinal cord alleviates chronic pain–induced allodynia and hyperalgesia in a rat model (Loram et al., 2013). CBD acts as an anti-inflammatory agent in an acute lung injury model, and this effect is also blocked by an A_{2A} receptor antagonist (here, ZM241385) (Ribeiro et al., 2012). Together, this evidence suggests that CBD's ability to modulate adenosine signaling may involve regulation of adenosine signaling.

Additional targets for CBD include serotonin and glycine receptors. The serotonergic system is involved in mediating pain responses, and serotonin-1a ($5HT_{1a}$) receptor activity is modulated by CBD (Russo et al., 2005). For example, CBD inhibits paclitaxel-induced neuropathic pain and has antiallodynic effects in streptozotocin-induced diabetic rats that are $5HT_{1a}$ dependent (Ward et al., 2015; Jesus et al., 2019). Glycine receptors (GlyRs) within the spinal cord are implicated in regulating chronic pain (Harvey et al., 2004; Ahrens et al., 2009; Chiu et al., 2016; Imlach et al., 2016; Aaltonen et al., 2020). CBD acts as a positive allosteric modulator at the α3 GlyR, enhancing glycine-mediated currents to decrease nociceptive excitability in the spinal cord (Xiong et al., 2012) and as an agonist at the α1 GlyR and 1β GlyR[89].

Additional targets localized at the plasma membrane are modulated by CBD. Computational and cell culture evidence suggests that CBD is an antagonist at orexin 1 receptors (Vitale et al., 2021). CBD acts as a NAM at μ- and δ-opioid receptors, although these effects only occur at high micromolar concentrations (Kathmann et al., 2006; Vitale et al., 2021). CBD inhibits currents mediated by the voltage-gated sodium channel Nav1.4, although these also occur at high concentrations and were only reported in cells in culture (Ghovanloo et al., 2018; Huang et al., 2021).

Most recently, several intracellular targets modulated by CBD were identified. Through elegant molecular and behavioral studies, Wang et al. (2023) demonstrate that CBD blocks FKBP5 and reduces neuroinflammation involved in pain. Furthermore, the voltage-dependent anion channel 1 expressed by mitochondria is inhibited by CBD (Rimmerman et al., 2013).

The large number of molecular targets modulated by CBD that are implicated in analgesia highlight the complexity and diversity of CBD's MOAs and suggest that CBD likely exerts its analgesic effect by simultaneously interacting with multiple targets expressed by distinct cell types. For example, microinjection of CBD into the PAG reduced firing rates of ON and OFF cells in the rostroventromedial medulla as well as the downstream descending pain circuit, and these responses are blocked by antagonists of CB_1R (AM251), adenosine A1 (DPCPX), and TRPA1 (AP18) but not TRPV1 receptors (5′-iodo-resiniferatoxin) (Maione et al., 2011). This complexity represents an exciting challenge in CBD research, and future studies will need to consider the expression profile and involvement of these potential CBD targets in pain management. Further characterization of the mechanisms underlying CBD-induced pain relief will then guide medicinal chemistry efforts to either foster this poly-pharmacology or improve selectivity and potency of CBD at select targets.

eCB Signaling: Focus on 2-AG

Although CBD represents the potential of phyto-CBs in achieving pain relief, there is increasing evidence that enhancing signaling of 2-AG and AEA can also modulate pain responses. In fact, some of CBD's analgesic effects may result from enhanced eCB signaling in a receptor-independent manner, including via CBD's binding to fatty acid binding proteins and impairing the shuttling of eCBs to their targets or by CBD's direct inhibition of AEA hydrolysis by the enzyme fatty acid amide hydrolase (Bisogno et al., 2001; Elmes et al., 2015).

To date, the best-characterized eCBs are 2-AG and AEA. The levels of 2-AG in the brain are approximately 100–1000-fold greater than AEA levels in select brain areas (as determined by mass spectrometry in bulk brain tissue) (Buczynski and Parsons, 2010; Zou and Kumar, 2018). Due to 2-AG's abundance and activity at the CB_1R and CB_2R's, 2-AG is often referred to as the predominant eCB in the brain. 2-AG is a monoacylglycerol derivative of the ω-6 polyunsaturated fatty acid arachidonic acid. It is synthesized by neurons and glial cells in an activity-dependent manner, which refers to its on-demand production stimulated by increases in intracellular calcium (Fig. 3) (Hashimotodani et al., 2013; Shonesy et al., 2015). For example, increasing intracellular calcium using the calcium ionophore ionomycin, which bypasses receptor activation, is sufficient for increasing 2-AG levels in neurons (Stella et al., 1997; Jung et al., 2005). A more physiologically relevant mechanism involves the activation of the group 1 metabotropic glutamate receptors, which increases calcium-dependent phospholipase C and diacylglycerol lipase activities to produce diacylglycerol, which is a precursor for 2-AG synthesis (Shonesy et al., 2015; Baggelaar et al., 2018). Accordingly, decreasing intracellular calcium with BAPTA-AM or by depletion of intracellular calcium stores using the sarco/endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin decreases receptor-dependent 2-AG synthesis (Robbe et al., 2002; Jung et al., 2005; Kellogg et al., 2009).

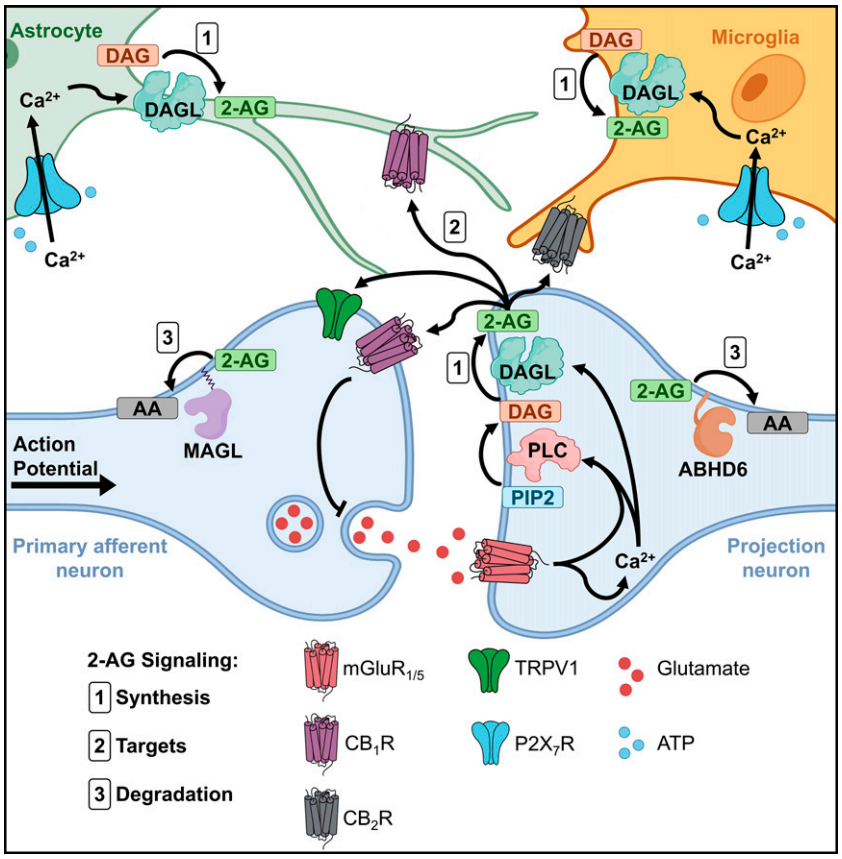

Fig. 3. Mechanisms regulating 2-AG signaling between neurons and glia. 2-AG signaling in regions associated with pain processing, such as the dorsal horn, can be divided into three processes: 1) 2-AG is synthesized by neurons and glia in an activity dependent manner, which relies on increases in cytosolic calcium either by opening of ionotropic receptors, such as $P2X_7$ receptors ($P2X_7R$) in astrocytes and microglia, or through activation of metabotropic receptors, such as group I metabotropic glutamate receptors ($mGluR_{1/5}$). 2) 2-AG activates multiple targets (i.e., CB_1R, CB_2R, and TRPV1) expressed by both neurons and glia to modulate neurotransmission and neuroinflammation. 3) 2-AG signaling is terminated by hydrolases (i.e., ABHD6 and MAGL), which hydrolyze 2-AG to produce arachidonic acid (AA) and glycerol (not shown). ABHD6 and MAGL have distinct subcellular localizations, with ABHD6 predominantly expressed in postsynaptic neurons and MAGL predominantly expressed in presynaptic terminals.

Following its synthesis, 2-AG can act at multiple targets to modulate neurotransmission and inflammation. Both neurons and glial cells express 2-AG synthesis and inactivation enzymes as well as CBRs (Fig. 3). 2-AG acts as a low-affinity full agonist at both the CB_1R and CB_2R, whereas AEA is a high-affinity partial agonist at CB_1R with low activity at the CB_2R (Zou and Kumar, 2018). 2-AG can also act at non-CBR targets expressed throughout the pain pathway, including as an agonist at TRPV1 and GPR55 and as a positive allosteric modulator of $GABA_A$ receptors (Baggelaar et al., 2018). Thus, 2-AG signaling in the pain pathway may involve multiple receptors expressed in neurons, astrocytes, and microglia that regulate neuronal activity and inflammation. Understanding the molecular mechanisms that regulate 2-AG signaling will further establish 2-AG's role in regulating pain responses and help develop novel therapeutic interventions that modulate 2-AG signaling, in particular inhibitors of 2-AG hydrolysis.

2-AG's hydrolysis represents a pivotal mechanism by which 2-AG's activation of CBR signaling is controlled and terminated. The major enzymes involved in 2-AG hydrolysis in the brain are monoacylglycerol lipase (MAGL) and α/β-hydrolase domain containing 6 (ABHD6), both of which hydrolyze 2-AG to produce arachidonic acid and glycerol (Fig. 3) (Blankman et al., 2007). Despite the shared mechanism of 2-AG hydrolysis, MAGL and ABHD6 differ in several key aspects: 1) their relative expression profile and contribution to 2-AG hydrolysis, 2) their subcellular expression that determines the pool of 2-AG that they regulate, and 3) the physiological consequences resulting from their respective inhibition (Cao et al., 2019). Specifically, in neurons, MAGL is expressed in the presynaptic terminal, the same cellular compartment as the presynaptic CB_1Rs (Straiker et al., 2009). Considering that MAGL accounts for ≈85% of 2-AG hydrolysis in brain homogenates, its genetic deletion and/or chronic inhibition strongly increases 2-AG levels in the brain and triggers cannabimimetic responses, including hypolocomotion and catalepsy as well as CB_1R desensitization (Long et al., 2009; Schlosburg et al., 2010; Viader et al., 2015). Thus, although MAGL inhibitors have been shown to induce analgesia (Jiang et al., 2023), widespread and profound CB_1R activation induced by MAGL inhibitors might limit their clinical use.

ABHD6 accounts for 5% of 2-AG hydrolysis in brain homogenate samples, and its subcellular and specific cell type expression results in more selective and restricted modulation of 2-AG signaling (Blankman et al., 2007). In neurons, it is expressed in postsynaptic terminals, the site of 2-AG synthesis (Straiker et al., 2009; Marrs et al., 2010). Significantly, neither inhibition nor genetic deletion of ABHD6 results in CB_1R-mediated cannabimimetic responses and CB_1R desensitization, and yet still induces, therapeutic benefit in several preclinical models associated with increased neuronal activity and neuroinflammation, including chronic pain (Naydenov et al., 2014; Woodhams et al., 2017; Liktor-Busa et al., 2023; Westenbroek et al., 2023). Thus, ABHD6 inhibition allows localized potentiation of 2-AG activation of CB_1R signaling while minimizing the risk of adverse effects and CB_1R desensitization.

Targeting eCB Signaling as an Analgesic Strategy

Mounting evidence supports the theoretical construct of clinical endocannabinoid deficiency, in which decreased AEA and 2-AG tone plays a role in functional pain disorders (Gouveia-Figueira et al., 2017; Leimuranta et al., 2018). In line with this premise, inhibition of MAGL or fatty acid amide hydrolase induces promising analgesic effects in preclinical rodent models of acute and chronic pain (Hohmann et al., 2005; Connell et al., 2006; Guindon et al., 2011; Ghosh et al., 2013; Greco et al., 2018; Finn et al., 2021; Soliman et al., 2021). Further, subcutaneous administration of exogenous 2-AG, or pharmacological inhibition of 2-AG hydrolysis, in mouse models of neuropathic and cancer-induced bone pain induces an antiallodynic effect and attenuates hyperalgesia (Wen et al., 2018; Thomas et al., 2020; Thomas et al., 2020). Both clinical and preclinical studies indicate that neuropathic pain scores correlate with elevated 2-AG levels (Pellkofer et al., 2013). Despite this evidence, the role of 2-AG signaling in pain states and the mechanism underlying the potential therapeutic effects of inhibiting 2-AG hydrolysis mediated by ABHD6 remain understudied.

ABHD6: A Therapeutic Target to Selectively Increase 2-AG Signaling

ABHD6 was first identified as a 2-AG–hydrolyzing enzyme in mouse brain membrane fraction (Blankman et al., 2007). This was confirmed in the BV-2 microglial cell line and in mouse neurons in primary culture, where ABHD6 was found to hydrolyze 2-AG in intact cells (Muccioli et al., 2007; Marrs et al., 2010). In the brain, the highest ABHD6 mRNA expression and activity is found in the hippocampus, cortex, striatum, and cerebellum (Baggelaar et al., 2017). ABHD6 protein is detected in principle glutamatergic cells, GABAergic interneurons, and astrocytes, with low expression in microglia in a healthy state (Marrs et al., 2010). ABHD6's role in regulating 2-AG–dependent neurotransmission was demonstrated by using ABHD6 inhibitors and showing that they enhance CB_1R-dependent long-term depression in mouse cortical slices (Marrs et al., 2010). In neurons, ABHD6 is expressed in the postsynaptic terminal, which is where synaptic 2-AG is produced (Straiker et al., 2009; Marrs et al., 2010). Thus, ABHD6 is a postsynaptic enzyme that regulates activity-dependent 2-AG levels at the site of 2-AG synthesis, whereas MAGL regulates 2-AG's activity at the CB_1R.

ABHD6 belongs to the serine hydrolase superfamily characterized by a conserved catalytic mechanism that relies on three amino acid residues in the active site called the catalytic triad (Long et al., 2009). In the case of ABHD6, these residues are Ser148, Asp278, and His306, and mutating any of these residues abolishes hydrolysis activity of the enzyme (Navia-Paldanius et al., 2012). ABHD6 substrate specificity was established by using a combination of in vitro enzymatic assays and lipidomic analysis of ABHD6 knockout mice. Specifically, ABHD6 hydrolyzes monoacylglycerols with varying acyl chain lengths and levels of saturation, ranging from 8:0 to 20:4 (Navia-Paldanius et al., 2012). ABHD6's substrate selectivity extends beyond the monoacylglycerols as it also degrades lysophospholipids and glycerophospholipids (Thomas et al., 2013; Pribasnig et al., 2015). In part due to this broad substrate specificity, ABHD6 is likely to represent a molecular hub involved in regulating multiple signaling pathways and pathophysiological processes.

ABHD6 inhibitors show promising therapeutic efficacy for the treatment of multiple neurologic diseases associated with chronic increases in neuronal activity (Tchantchou and Zhang, 2013; Zhao et al., 2014; Manterola et al., 2018; Pusch et al., 2022), including epilepsy [see our discoveries (Naydenov et al., 2014; Westenbroek et al., 2023)] and initial evidence for chronic pain (Kiritoshi et al., 2016). Specifically, although the role of 2-AG acting at both CB_1R (to reduce neuronal transmission and regulate neuronal plasticity) and CB_2R (to reduce neuroinflammation) has been well described, only a few studies report the effects of ABHD6 inhibition on 2-AG levels and ensuing potentiation of CB_1R and CB_2R signaling in the context of preclinical pain models (Table 2).

We recently reported the analgesic properties of ABHD6 and MAGL inhibitors in a rat model of headache/migraine induced by cortical administration of KCl to induce cortical spreading depression (Liktor-Busa et al., 2023). Specifically, ABHD6 and MAGL inhibitors both rescue and prevent allodynia in this model of migraine via differential activation of CB_1 R and CB_2R, but ABHD6 inhibition induced more promising, CB_1R-dependent analgesic responses (Liktor-Busa et al., 2023). In all preclinical studies published thus far, a couple of common issues are present: 1) many of these results are reliant on ABHD6 inhibitors, which have limited selectivity and 2) the MOA of ABHD6 inhibition is not yet understood since there is little evidence that ABHD6 inhibition in these disease models affects local 2-AG levels. Addressing these questions requires a combination of novel classes of ABHD6 inhibitors with improved selectivity and new tools that allow for the measurement of rapid dynamic changes in 2-AG levels at the cellular, circuit, and systems levels.

TABLE 2
Effect of decreased ABHD6 activity in rodent models of pain or in pain-related behaviors

Model System	Drug; Dose	Effect	Targets	Reference
KCl-induced periorbital allodynia in rat	KT 182; 2 mg/kg i.p.	Prevented and reversed periorbital allodynia	CB_1R (major target), CB_2R (minor target)	Liktor-Busa et al., 2023
Chronic constriction injury of the sciatic nerve in mouse	WWL70; 5 and 10 mg/kg i.p.	Reduced mechanical allodynia and thermal hyperalgesia	N/A	Wen et al., 2018
ABHD6$^{-/-}$ rat	N/A	Hyperalgesia	N/A	Noguchi et al., 2021
Kaolin- and carrageenan-induced monoarthritis in mouse	WWL70; 10 μM	Rescued impaired infralimbic pyramidal cell mGluR5 signaling in brain slices	CB_1R	Kiritoshi et al., 2016

N/A, not available.

Measuring Activity-Dependent Changes in eCBs Levels

There are multiple ways to detect changes in eCB levels and correlate these changes to alterations in cellular function and behavior (Fig. 4). Directly analyzing tissue and cellular eCB levels is not a trivial task as these lipids are present in multiple subcellular compartments (Saini et al., 2021). The most common technique to quantify eCBs relies on mass spectrometry, which entails multiple steps: 1) lipid extraction along with optional enrichment/purification, 2) chromatographic separation, and 3) detection using specific mass spectrometric instruments and methods (Buczynski and Parsons, 2010). Major advantages of using mass spectrometry to quantify eCBs include sensitivity and identification of specific lipids using their mass-to-charge ratio and retention times. However, this multistep process has key drawbacks that limit the throughput, spatial resolution, and accuracy of the analysis. Another limitation is linked to quantifying eCBs in the brain following decapitation since the ischemic conditions can increase the levels of AEA and 2-AG (Buczynski and Parsons, 2010). These limitations can be somewhat overcome by using microdialysis, which combines the spatial specificity by sampling the interstitial fluid from a discrete brain region in an awake, freely behaving animal, with the sensitivity of mass spectrometry (Buczynski and Parsons, 2010). Furthermore, a common challenge is that 2-AG will isomerize into 1-arachidonoylglycerol during the extraction procedure and has identical molecular weights and must be accounted for during the analysis (Stella et al., 1997; Dócs et al., 2017). Thus, quantification of eCB amounts in complex biological matrices using mass spectrometry involves multiple processing steps to achieve highly sensitive detection of multiple lipids from single samples. However, it often cannot capture rapid and localized changes in eCBs.

Detecting Changes in 2-AG Levels with a Genetically Encoded Fluorescent eCB Sensor

Changes in 2-AG levels are rapid and transient as exemplified by the 2-AG–dependent short-term plasticity termed depolarization-induced suppression of inhibition/excitation, which occurs within seconds after depolarization and recovers within minutes (Diana and Marty, 2004). In fact, much of our understanding about cell-specific, time-resolved effects of 2-AG signaling on neuronal activity has come from electrophysiological studies (Fig. 4). However, when it comes to measuring actual levels of 2-AG, this indirect method has relied on pharmacological and genetic tools to identify which eCBs mediate changes in neuronal plasticity.

The development of the novel, genetically encoded fluorescent sensor G protein–coupled receptor activation–based eCB2.0 ($GRAB_{eCB2.0}$) allows us to answer these questions more directly by measuring changes in eCBs levels within subcellular compartments and with subsecond resolution in cells in culture, brain slices, and select brain regions of behaving mice. $GRAB_{eCB2.0}$ was developed by first incorporating a circularly permutated green fluorescent protein (cpGFP) into the third intracellular loop of the human CB_1R, then performing a randomized single-mutation screen to identify the construct with optimized changes in fluorescent signal induced by 2-AG (Dong et al., 2021). Like other genetically encoded sensors (Labouesse et al., 2020; Sabatini and Tian, 2020; Dong et al., 2022), $GRAB_{eCB2.0}$ couples the active conformational change of CB_1R bound to an agonist to the active/fluorescent conformation of cpGFP. Thus, in the absence of a CB_1R agonist, the sensor has a conformation that elicits a low level of fluorescent signal. Upon binding to a CB_1R agonist, $GRAB_{eCB2.0}$ adopts an active conformation that stabilizes the cpGFP structure to elicit a fluorescent signal (Fig. 4). Such increases in $GRAB_{eCB2.0}$ fluorescent signal are elicited by multiple CB_1R agonists, including 2-AG, AEA, THC, and the synthetic cannabinoids CP55940 and

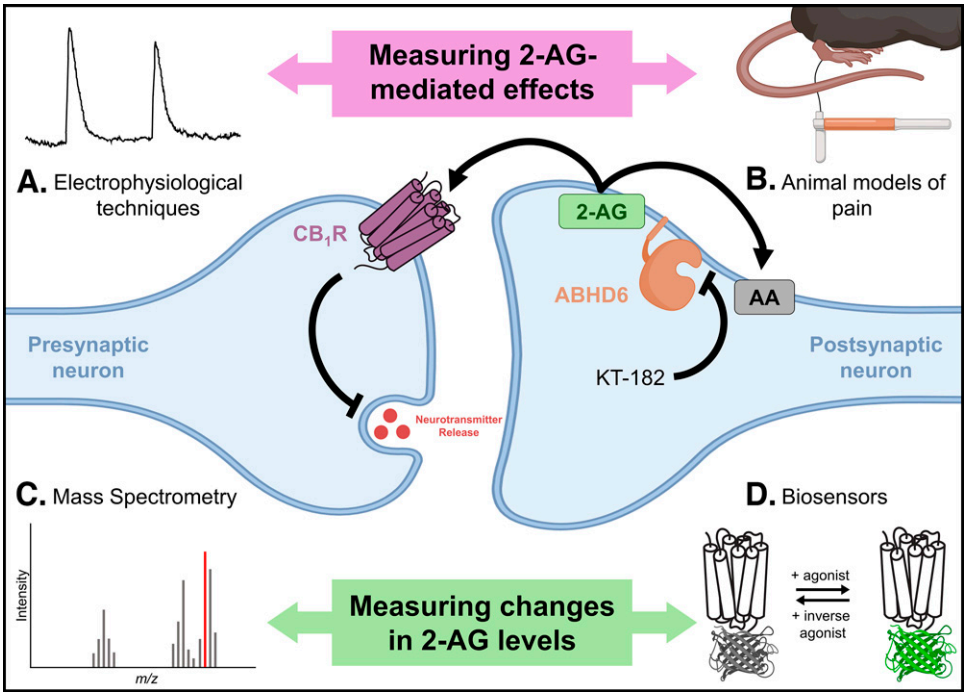

Fig. 4. Methods for measuring changes in 2-AG signaling. Diagram depicts an example of a therapeutic approach that increases 2-AG signaling, here the ABHD6 inhibitor KT-182. Increased 2-AG signaling induces multiple physiological effects, including (A) changes in synaptic transmission and plasticity that can be detected using electrophysiological techniques and (B) modulation of pain responses in animals that can be detected using behavioral assays, such as the von Frey test. Changes in 2-AG levels can also be directly measured by quantifying total 2-AG amounts in bulk tissue using (C) mass spectrometry or by measuring changes in 2-AG levels in specific cells and compartments using a (D) biosensor, such as the genetically encoded eCB sensor $GRAB_{eCB2.0}$. These approaches differ in their sensitivity, spatiotemporal resolution, experimental throughput, and the model systems they can be applied in. This combination of tools, when used in concert, represents an innovative platform that can help evaluate the efficacy and molecular mechanism of potential new analgesics that target the eCB system through alternative mechanisms of action.

WIN55212-2 (Dong et al., 2021; Singh et al., 2023). Importantly, $GRAB_{eCB2.0}$ does not couple to intracellular signaling pathways, including G proteins and β-arrestin, and does not appear to affect the coupling of the native CB_1R when expressed in the same cell (Dong et al., 2021). Although this lack of coupling allows $GRAB_{eCB2.0}$ to act as a sensor without participating in signal transduction, it may also affect agonist affinity for $GRAB_{eCB2.0}$ (Weis and Kobilka, 2018). The increasing use of $GRAB_{eCB2.0}$ and the validation of its function in different model systems can help address these concerns.

$GRAB_{eCB2.0}$ was recently shown to detect changes in 2-AG levels in cells in culture, brain slices, and freely behaving animals and can detect both exogenous application of cannabinoid agonists and endogenous eCB production (Farrell et al., 2021; Liput et al., 2022; Singh et al., 2023). Of note, the in vivo application and interpretation of this sensor requires a thorough characterization of $GRAB_{eCB2.0}$'s selectivity since it can be activated by multiple ligands. Thus, we recently characterized the pharmacological properties of the $GRAB_{eCB2.0}$ expressed in HEK293 cells in culture by testing its activation by 2-AG, AEA, eCB analogs, phyto-CBs, the synthetic cannabinoid agonist CP55940, and CB_1R antagonists (Singh et al., 2023). Our study revealed that in this expression system, the rank order of potency of the CB_1R agonists at the $GRAB_{eCB2.0}$ was 2-AG $\approx$ CP55940 $>$ AEA $>$ THC $\approx$ 1-arachidonoylglycerol (Table 3) and that the $GRAB_{eCB2.0}$ also responded to other endogenous monoacylglycerols, namely 2-linoleoylglycerol and 2-oleoylglycerol, with nanomolar potency.

How do the pharmacological profiles of CB_1R and $GRAB_{eCB2.0}$ compare? We found that nanomolar concentrations of 2-AG and AEA increase $GRAB_{eCB2.0}$ signal in Neuro2a neuronal cells in culture within seconds and with potencies quite comparable to their potencies at native CB_1R (Table 3). The cannabinoid ligands CP55940 and SR141617 increase and decrease $GRAB_{eCB2.0}$ signal, respectively, although with lower potencies compared with the native CB_1R (An et al., 2020). We also noted several similarities and differences with the pharmacological profile of the $GRAB_{eCB2.0}$ sensor expressed in Neuro2a cells compared with when it is expressed by HEK293 cells (Table 3) (Singh et al., 2023). Specifically, although 2-AG induced comparable increases in $GRAB_{eCB2.0}$ signal in both Neuro2a and HEK293 cells, the potencies of AEA, CP55940, and SR141617 at the $GRAB_{eCB2.0}$ revealed cell-specific differences, potentially due to differential endogenous expression of eCB system components, such as CB_1R- and eCB-inactivating enzymes (Graham et al., 2006; Biringer, 2021). Furthermore, CBD retains its NAM activity at the $GRAB_{eCB2.0}$ sensor in HEK293 cells since it does not, by itself, affect the $GRAB_{eCB2.0}$ sensor fluorescent signal but decreased the 2-AG–induced $GRAB_{eCB2.0}$ sensor fluorescent signal in a concentration-dependent manner. Together, these results emphasize the need to characterize the pharmacological profile of $GRAB_{eCB2.0}$ sensor activity in each model system as it may vary depending on sensor trafficking as well as the expression profile of eCB-hydrolyzing enzymes, fatty acid binding proteins, CBRs, and CBR-interacting proteins (Busquets-Garcia et al., 2018).

Furthermore, we detected the presence of intracellular $GRAB_{eCB2.0}$ and showed that agonist treatment also increases its fluorescence signal, though to a lesser extent than $GRAB_{eCB2.0}$, at the plasma membrane (Singh et al., 2023). The possibility that $GRAB_{eCB2.0}$ undergoes internalization was indirectly tested by Dong et al. (2021) in their original publication by quantifying

TABLE 3
Relative potencies of eCBs (2-AG and AEA), the eCB analog 1-AG, the synthetic cannabinoid CP55940, the phytocannabinoid Δ^9-THC, and the CB_1R-selective antagonist SR141716A at targeting the CB_1R, CB_2R, and the $GRAB_{eCB2.0}$

	CB_1R	CB_2R	$GRAB_{eCB2.0}$
Ligand	EC_{50}/IC_{50} (nM)	EC_{50}/IC_{50} (nM)	EC_{50}/IC_{50} (nM)
2-AG	12.59[a]– 96[b]	1.3–8.9[c,d,e]	81[r], 85[g], 2000[h], 3100–9000[i]
1-AG	480–1900[b,j]	N/A	1800[g]
AEA	69–276[a,k,l]	>30,000[e]	58[f], 200–800[i], 815[g]
CP55940	0.75–29.6[k,l,m,n]	0.62–3.2[c,m,o,p]	64[h], 82[g], 193[f]
Δ^9-THC	13–35[m,q]	41.8[m]	1600[g] to 2000[i]
SR141716A	0.33–46[m,r,s]	N/A	3.3[g], 42[t], 449[f]

1-AG, 1-arachidonoylglycerol; HEK, human embryonic kidney cell; N/A, not available; N18TG2, mouse neuroblastoma cell; Neuro2a, mouse neuroblastoma cell.
[a]Inhibition of forskolin-stimulated cAMP accumulation in rat neocortical synaptosomes (Steffens et al., 2005).
[b]Inhibition of forskolin-stimulated cAMP accumulation in rCB_1R-HEK293 cells (Farah et al., 2022).
[c]Inhibition of forskolin-stimulated cAMP accumulation in mCB_2R-HEK293 cells (Dhopeshwarkar and Mackie, 2016).
[d]GTPγS binding in Sf9-hCB_2R membranes (Gonsiorek et al., 2000).
[e]Inhibition of forskolin-stimulated cAMP accumulation and in hCB_2R-CHO cells (Gonsiorek et al., 2000).
[f]$GRAB_{eCB2.0}$-Neuro2a cells with bovine serum albumin (Singh et al., 2024).
[g]$GRAB_{eCB2.0}$-HEK293 cells with bovine serum albumin (Singh et al., 2023).
[h]$GRAB_{eCB2.0}$ expressed in HEKtsA201 cells (Shivshankar et al., 2024).
[i]$GRAB_{eCB2.0}$ expressing-HEK293 cells or-primary cultured rat cortical neurons, in the absence of bovine serum albumin (Dong et al., 2021).
[j]Intracellular Ca^{2+} transients in mCB_1R-COS7 cells (Dócs et al., 2017).
[k]Intracellular Ca^{2+} transients in rat hippocampal neurons in culture (Shen et al., 1996).
[l]GTPγS binding in rat cerebellar membranes (Kearn et al., 1999).
[m]Inhibition of forskolin-stimulated cAMP accumulation in hCB_1R-CHO or hCB_2R-CHO cells (Rinaldi-Carmona et al., 1994; Felder et al., 1995).
[n]GTPγS binding in hCB_1R-HEK293 cells (Ankur et al., 2007).
[o]Arrestin recruitment in mCB_2R-HEK293 cells (Dhopeshwarkar and Mackie, 2016).
[p]Inhibition of forskolin-stimulated cAMP accumulation in hCB_2R-CHO-K1 cells (Valenzano et al., 2005).
[q]Inhibition of forskolin-stimulated cAMP accumulation in rCB_1R-COS7 cells and N18TG2 cells (Bayewitch et al., 1996).
[r]cAMP accumulation in hCB_1R-CHO cells (Bauer et al., 2012).
[s]GTPγS binding and arrestin recruitment in hCB_1R-CHO cells (Liu et al., 2021).
[t]$GRAB_{eCB2.0}$ expressed in HEKtsA201 cells with bovine serum albumin (Shivshankar et al., 2024).

β-arrestin2 binding. They found no increase in β-arrestin2 binding when activating $GRAB_{eCB2.0}$ with cannabinoid agonists in contrast to agonist-induced CB_1R internalization in HEK293 cells that depends on β-arrestin2 (Ibsen et al., 2019; Dong et al., 2021). This suggests that extracellular agonists will slowly cross the plasma membrane and may increase intracellular $GRAB_{eCB2.0}$ signal, although to a lesser extent and with slower kinetics than the increases in plasma membrane $GRAB_{eCB2.0}$ signal (Ahn et al., 2013).

The development and characterization of the $GRAB_{eCB2.0}$ gives us an opportunity to describe the role of eCB signaling in pain processing at the molecular level. For instance, we leveraged the $GRAB_{eCB2.0}$ to interrogate the molecular mechanisms involved in 2-AG production by neuronal cells in culture stimulated with the inflammatory mediator ATP, which is already known to increase 2-AG production in microglia and astrocytes through a $P2X_7$ receptor–dependent mechanism (Walter et al., 2004; Witting et al., 2004; Singh et al., 2024). Significantly, we compared the response induced by ATP on 2-AG levels using liquid chromatography–tandem mass spectrometry and

$GRAB_{eCB2.0}$ and found that ATP increases 2-AG production in Neuro2a cells within minutes through a mechanism mediated primarily by the $P2X_7$ receptor (Singh et al., 2024). This comparison also revealed that the two methods likely detect different pools of 2-AG (Singh et al., 2024), further establishing that the $GRAB_{eCB2.0}$ has an increased spatiotemporal resolution compared with mass spectrometry and can detect changes in plasma membrane 2-AG levels that may not be as evident with a mass spectrometric detection method (Singh et al., 2024). These results should be considered in the context of studies performed in mouse dorsal root ganglia in culture reporting that ATP combined with bradykinin increased 2-AG levels, which can then act at CB1R and TRPV1 receptors to induce analgesia by potentially decreasing the sensitization and excitability of nociceptors (Zygmunt et al., 2013). Together, these studies describe a molecular mechanism by which mediators involved in pain sensation and sensitization, such as extracellular ATP (Masuda et al., 2016; Ren and Illes, 2022), engage eCB signaling.

Of note, despite several behavioral studies that have used $GRAB_{eCB2.0}$ to describe eCB changes during foot shock, locomotion, seizures, and fear extinction, in vivo pain studies using this new sensor have not yet been published (Dong et al., 2021; Gunduz-Cinar et al., 2023). Importantly, there are several limitations to using the sensor, including the need to validate the pharmacological profile of the sensor in each relevant model system and the lack of selectivity for eCB agonists in its current form (although future versions of the sensor may be more selective for specific eCBs) as well as the difficulty in using the sensor for in vivo studies by expressing it in pain processing areas outside of the brain, such as the dorsal horn of the spinal cord and the dorsal root ganglia. Despite these limitations, the $GRAB_{eCB2.0}$, in combination with previously developed tools for studying eCB signaling and pain (Fig. 4), will help unravel the neurocircuitry and molecular mechanism underlying the analgesic effects of elevating 2-AG levels.

Conclusion and Future Directions

From a variety of historical and experimental lines of evidence, it is now well established that phyto-CBs and the eCB signaling system represent a robust venue to develop novel therapeutics for the treatment of pain. Because *Cannabis* induces multiple behavioral changes, the challenge remains to try and focus these responses on just pain relief without intoxicating effects and impairment associated with cannabinoids (Stella, 2023).

New studies on the use of CBD and ABHD6 inhibitors for pain treatment represent two distinct, but related, approaches for pain management. CBD has a broad array of potential pain-related therapeutic targets, so a major question remains as to whether to embrace its poly-pharmacology and the associated combinatorial challenges or to focus research on the optimization of more select targets for therapeutic refinement. ABHD6 has a very clearly defined MOA, and the challenge here remains in properly characterizing potential inhibitors for appropriate effects on 2-AG in pain-relevant circuitry. To accomplish this, accurate detection of eCB dynamics is required. Multiple model systems have been used to describe eCB signaling, yet capturing localized and rapid changes in eCB levels in these models has been technically challenging (Fig. 4). To this end, the $GRAB_{eCB2.0}$ sensor represents a powerful chemogenetic tool to measure changes in eCB levels with high spatial (cellular and subcellular) and temporal (seconds) resolution in vivo and in intact cells in culture. Such related approaches will help the field at large determine how eCB signaling modulates neurotransmission and neuroinflammation. Whether 2-AG levels stimulated by inflammatory mediators play a negative feedback role in the peripheral sensitization of sensory neurons, and if this signaling can be enhanced with ABHD6 inhibitors, also remains to be determined.

Cannabinoid research over the past five decades has resulted in a better understanding of the poly-pharmacology of CBD and its therapeutic potential as a novel analgesic. We also discovered several therapeutic targets within the eCB signaling system, such as ABHD6, that can be inhibited to modulate pain. The current thriving field of *Cannabis* and cannabinoid research has recently been given a long-needed tool, the $GRAB_{eCB2.0}$ biosensor, that has propelled the field's understanding of the dynamics and spatial specificity of activity-dependent changes in eCB levels. This greatly increased momentum will accelerate our optimization of cannabinoids to develop multiple lines of transformative medications devoid of known cannabinoid-induced side effects.

Data Availability

No datasets were generated or analyzed during the present study.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Singh, Ellioff, Bruchas, Land, Stella.

References

Aaltonen N, Singha PK, Jakupović H, Wirth T, Samaranayake H, Pasonen-Seppänen S, Rilla K, Varjosalo M, Edgington-Mitchell LE, Kasperkiewicz P, et al. (2020) High-Resolution Confocal Fluorescence Imaging of Serine Hydrolase Activity in Cryosections - Application to Glioma Brain Unveils Activity Hotspots Originating from Tumor-Associated Neutrophils. *Biol Proced Online* **22**:6.

Abraham AD, Leung EJY, Wong BA, Rivera ZMG, Kruse LC, Clark JJ, and Land BB (2020) Orally consumed cannabinoids provide long-lasting relief of allodynia in a mouse model of chronic neuropathic pain. *Neuropsychopharmacology* **45**:1105–1114.

Ahn KH, Mahmoud MM, Shim J-Y, and Kendall DA (2013) Distinct roles of β-arrestin 1 and β-arrestin 2 in ORG27569-induced biased signaling and internalization of the cannabinoid receptor 1 (CB1). *J Biol Chem* **288**:9790–9800.

Ahrens J, Demir R, Leuwer M, de la Roche J, Krampfl K, Foadi N, Karst M, and Haeseler G (2009) The nonpsychotropic cannabinoid cannabidiol modulates and directly activates alpha-1 and alpha-1-Beta glycine receptor function. *Pharmacology* **83**:217–222.

Alexander SPH (2016) Therapeutic potential of cannabis-related drugs. *Prog Neuropsychopharmacol Biol Psychiatry* **64**:157–166.

Almog S, Aharon-Peretz J, Vulfsons S, Ogintz M, Abalia H, Lupo T, Hayon Y, and Eisenberg E (2020) The pharmacokinetics, efficacy, and safety of a novel selective-dose cannabis inhaler in patients with chronic pain: A randomized, double-blinded, placebo-controlled trial. *European Journal of Pain* **24**:1505–1516.

An D, Peigneur S, Hendrickx LA, and Tytgat J (2020) Targeting Cannabinoid Receptors: Current Status and Prospects of Natural Products. *Int J Mol Sci* **21**:5064.

Anderson LL, Absalom NL, Abelev SV, Low IK, Doohan PT, Martin LJ, Chebib M, McGregor IS, and Arnold JC (2019) Coadministered cannabidiol and clobazam: Preclinical evidence for both pharmacodynamic and pharmacokinetic interactions. *Epilepsia* **60**:2224–2234.

Ankur K, Dow PH, Daniel F, Rob W, Ganesh AT, Alexandros M, Patricia HR, and Mary EA (2007) Mutation studies of Ser7.39 and Ser2.60 in the human CB_1 cannabinoid receptor: evidence for a serine-induced bend in CB_1 transmembrane helix 7. *Mol Pharmacol* **71**:1512–1524.

Armin S, Muenster S, Abood M, and Benamar K (2021) GPR55 in the brain and chronic neuropathic pain. *Behav Brain Res* **406**:113248.

Atwood BK and Mackie K (2010) CB2: a cannabinoid receptor with an identity crisis. *Br J Pharmacol* **160**:467–479.

Baggelaar MP, Maccarrone M, and van der Stelt M (2018) 2-Arachidonoylglycerol: A signaling lipid with manifold actions in the brain. *Prog Lipid Res* **71**:1–17.

Baggelaar MP, van Esbroeck ACM, van Rooden EJ, Florea BI, Overkleeft HS, Marsicano G, Chaouloff F, and van der Stelt M (2017) Chemical Proteomics Maps Brain Region Specific Activity of Endocannabinoid Hydrolases. *ACS Chem Biol* **12**:852–861.

Bakas T, van Nieuwenhuijzen PS, Devenish SO, McGregor IS, Arnold JC, and Chebib M (2017) The direct actions of cannabidiol and 2-arachidonoyl glycerol at GABAA receptors. *Pharmacol Res* **119**:358–370.

Bauer M, Chicca A, Tamborrini M, Eisen D, Lerner R, Lutz B, Poetz O, Pluschke G, and Gertsch J (2012) Identification and quantification of a new family of peptide endocannabinoids (Pepcans) showing negative allosteric modulation at CB1 receptors. *J Biol Chem* **287**:36944–36967.

Bayewitch M, Rhee M-H, Avidor-Reiss T, Breuer A, Mechoulam R, and Vogel Z (1996) (-)-Delta9-tetrahydrocannabinol antagonizes the peripheral cannabinoid receptor-mediated inhibition of adenylyl cyclase. *J Biol Chem* **271**:9902–9905.

Ben Amar M (2006) Cannabinoids in medicine: A review of their therapeutic potential. *J Ethnopharmacol* **105**:1–25.

Biringer RG (2021) The rise and fall of anandamide: processes that control synthesis, degradation, and storage. *Mol Cell Biochem* **476**:2753–2775.

Bisogno T, Hanus L, De Petrocellis L, Tchilibon S, Ponde DE, Brandi I, Moriello AS, Davis JB, Mechoulam R, and Di Marzo V (2001) Molecular targets for cannabidiol and its synthetic analogues: effect on vanilloid VR1 receptors and on the cellular uptake and enzymatic hydrolysis of anandamide. *Br J Pharmacol* **134**:845–852.

Blankman JL, Simon GM, and Cravatt BF (2007) A comprehensive profile of brain enzymes that hydrolyze the endocannabinoid 2-arachidonoylglycerol. *Chem Biol* **14**:1347–1356.

Bow EW and Rimoldi JM (2016) The Structure-Function Relationships of Classical Cannabinoids: CB1/CB2 Modulation. *Perspect Medicin Chem* **8**:17–39.

Buczynski MW and Parsons LH (2010) Quantification of brain endocannabinoid levels: methods, interpretations and pitfalls. *Br J Pharmacol* **160**:423–442.

Burstein S (2015) Cannabidiol (CBD) and its analogs: a review of their effects on inflammation. *Bioorg Med Chem* **23**:1377–1385.

Busquets-Garcia A, Bains J, and Marsicano G (2018) CB(1) Receptor Signaling in the Brain: Extracting Specificity from Ubiquity. *Neuropsychopharmacology* **43**:4–20.

Buxbaum DM (1972) Analgesic activity of Δ9-tetrahydrocannabinol in the rat and mouse. *Psychopharmacologia* **25**:275–280.

Campos AC and Guimarães FS (2009) Evidence for a potential role for TRPV1 receptors in the dorsolateral periaqueductal gray in the attenuation of the anxiolytic effects of cannabinoids. *Prog Neuropsychopharmacol Biol Psychiatry* **33**:1517–1521.

Cao JK, Kaplan J, and Stella N (2019) ABHD6: Its Place in Endocannabinoid Signaling and Beyond. *Trends Pharmacol Sci* **40**:267–277.

Carrier EJ, Auchampach JA, and Hillard CJ (2006) Inhibition of an equilibrative nucleoside transporter by cannabidiol: a mechanism of cannabinoid immunosuppression. *Proc Natl Acad Sci U S A* **103**:7895–7900.

Chiu Y-C, Liao W-T, Liu C-K, Wu C-H, and Lin C-R (2016) Reduction of spinal glycine receptor-mediated miniature inhibitory postsynaptic currents in streptozotocin-induced diabetic neuropathic pain. *Neurosci Lett* **611**:88–93.

Chuang S-H, Westenbroek RE, Stella N, and Catterall WA (2021) Combined Antiseizure Efficacy of Cannabidiol and Clonazepam in a Conditional Mouse Model of Dravet Syndrome. *J Exp Neurol* **2**:81–85.

Chung YC, Shin W-H, Baek JY, Cho EJ, Baik HH, Kim SR, Won S-Y, and Jin BK (2016) CB2 receptor activation prevents glial-derived neurotoxic mediator production, BBB leakage and peripheral immune cell infiltration and rescues dopamine neurons in the MPTP model of Parkinson's disease. *Exp Mol Med* **48**:e205-e205.

Connell K, Bolton N, Olsen D, Piomelli D, and Hohmann AG (2006) Role of the basolateral nucleus of the amygdala in endocannabinoid-mediated stress-induced analgesia. *Neurosci Lett* **397**:180–184.

Costa B, Giagnoni G, Franke C, Trovato AE, and Colleoni M (2004) Vanilloid TRPV1 receptor mediates the antihyperalgesic effect of the nonpsychoactive cannabinoid, cannabidiol, in a rat model of acute inflammation. *Br J Pharmacol* **143**:247–250.

Cui C-X, Liu H-Y, Yue N, Du Y-R, Che L-M, and Yu J-S (2023) Research progress on the mechanism of chronic neuropathic pain. *IBRO Neurosci Rep* **14**:80–85.

De Gregorio D, McLaughlin RJ, Posa L, Ochoa-Sanchez R, Enns J, Lopez-Canul M, Aboud M, Maione S, Comai S, and Gobbi G (2019) Cannabidiol modulates serotonergic transmission and reverses both allodynia and anxiety-like behavior in a model of neuropathic pain. *Pain* **160**:136–150.

De Petrocellis L, Ligresti A, Moriello AS, Allarà M, Bisogno T, Petrosino S, Stott CG, and Di Marzo V (2011) Effects of cannabinoids and cannabinoid-enriched Cannabis extracts on TRP channels and endocannabinoid metabolic enzymes. *Br J Pharmacol* **163**:1479–1494.

Deliu E, Sperow M, Console-Bram L, Carter RL, Tilley DG, Kalamarides DJ, Kirby LG, Brailoiu GC, Brailoiu E, Benamar K, et al. (2015) The Lysophosphatidylinositol Receptor GPR55 Modulates Pain Perception in the Periaqueductal Gray. *Mol Pharmacol* **88**:265–272.

Devinsky O, Cross JH, Laux L, Marsh E, Miller I, Nabbout R, Scheffer IE, Thiele EA, and Wright S (2017) Trial of cannabidiol for drug-resistant seizures in the Dravet syndrome. *N Engl J Med* **376**:2011–2020.

Dhopeshwarkar A and Mackie K (2016) Functional Selectivity of CB2 Cannabinoid Receptor Ligands at a Canonical and Noncanonical Pathway. *J Pharmacol Exp Ther* **358**:342–351.

Di Marzo V, Fontana A, Cadas H, Schinelli S, Cimino G, Schwartz JC, and Piomelli D (1994) Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature* **372**:686–691.

Di Marzo V and Piscitelli F (2015) The Endocannabinoid System and its Modulation by Phytocannabinoids. *Neurotherapeutics* **12**:692–698.

Diana MA and Marty A (2004) Endocannabinoid-mediated short-term synaptic plasticity: depolarization-induced suppression of inhibition (DSI) and depolarization-induced suppression of excitation (DSE). *Br J Pharmacol* **142**:9–19.

Dócs K, Mészár Z, Gonda S, Kiss-Szikszai A, Holló K, Antal M, and Hegyi Z (2017) The Ratio of 2-AG to Its Isomer 1-AG as an Intrinsic Fine Tuning Mechanism of CB1 Receptor Activation. *Front Cell Neurosci* **11**:39.

Dong A, He K, Dudok B, Farrell JS, Guan W, Liput DJ, Puhl HL, Cai R, Wang H, Duan J, et al. (2021) A fluorescent sensor for spatiotemporally resolved imaging of endocannabinoid dynamics in vivo. *Nat Biotechnol* **40**:787–798.

Dong C, Zheng Y, Long-Iyer K, Wright EC, Li Y, and Tian L (2022) Fluorescence Imaging of Neural Activity, Neurochemical Dynamics, and Drug-Specific Receptor Conformation with Genetically Encoded Sensors. *Annu Rev Neurosci* **45**:273–294.

Dos Reis Rosa Franco G, Smid S, and Viegas C (2021) Phytocannabinoids: General Aspects and Pharmacological Potential in Neurodegenerative Diseases. *Curr Neuropharmacol* **19**:449–464.

Elmes MW, Kaczocha M, Berger WT, Leung K, Ralph BP, Wang L, Sweeney JM, Miyauchi JT, Tsirka SE, Ojima I, et al. (2015) Fatty acid-binding proteins (FABPs) are intracellular carriers for Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD). *J Biol Chem* **290**:8711–8721.

Eraso-Pichot A, Pouvreau S, Olivera-Pinto A, Gomez-Sotres P, Skupio U, and Marsicano G (2023) Endocannabinoid signaling in astrocytes. *Glia* **71**:44–59.

Farah SI, Hilston S, Tran N, Zvonok N, and Makriyannis A (2022) 1-, 2- and 3-AG as substrates of the endocannabinoid enzymes and endogenous ligands of the cannabinoid receptor 1. *Biochem Biophys Res Commun* **591**:31–36.

Farrell JS, Colangeli R, Dong A, George AG, Addo-Osafo K, Kingsley PJ, Morena M, Wolff MD, Dudok B, He K, et al. (2021) In vivo endocannabinoid dynamics at the timescale of physiological and pathological neural activity. *Neuron* **109**:2398–2403.e2394.

Felder CC, Joyce KE, Briley EM, Mansouri J, Mackie K, Blond O, Lai Y, Ma AL, and Mitchell RL (1995) Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors. *Mol Pharmacol* **48**:443–450.

Finn DP, Haroutounian S, Hohmann AG, Krane E, Soliman N, and Rice ASC (2021) Cannabinoids, the endocannabinoid system, and pain: a review of preclinical studies. *Pain* **162**:S5–S25.

Fisher E, Eccleston C, Degenhardt L, Finn DP, Finnerup NB, Gilron I, Haroutounian S, Krane E, Rice ASC, Rowbotham M, et al. (2019) Cannabinoids, cannabis, and cannabis-based medicine for pain management: a protocol for an overview of systematic reviews and a systematic review of randomised controlled trials. *Pain Rep* **4**:e741.

Fowler CJ (2013) Transport of endocannabinoids across the plasma membrane and within the cell. *FEBS J* **280**:1895–1904.

Ghosh S, Wise LE, Chen Y, Gujjar R, Mahadevan A, Cravatt BF, and Lichtman AH (2013) The monoacylglycerol lipase inhibitor JZL184 suppresses inflammatory pain in the mouse carrageenan model. *Life Sci* **92**:498–505.

Ghovanloo M-R, Shuart NG, Mezeyova J, Dean RA, Ruben PC, and Goodchild SJ (2018) Inhibitory effects of cannabidiol on voltage-dependent sodium currents. *J Biol Chem* **293**:16546–16558.

Golovko T, Min R, Lozovaya N, Falconer C, Yatsenko N, Tsintsadze T, Tsintsadze V, Ledent C, Harvey RJ, Belelli D, et al. (2014) Control of inhibition by the direct action of cannabinoids on GABAA receptors. *Cereb Cortex* **25**:2440–2455.

Gonsiorek W, Lunn C, Fan X, Narula S, Lundell D, and Hipkin RW (2000) Endocannabinoid 2-Arachidonyl Glycerol Is a Full Agonist through Human Type 2 Cannabinoid Receptor: Antagonism by Anandamide. *Mol Pharmacol* **57**:1045–1050.

Gouveia-Figueira S, Goldin K, Hashemian SA, Lindberg A, Persson M, Nording ML, Laurell K, and Fowler CJ (2017) Plasma levels of the endocannabinoid anandamide, related N-acylethanolamines and linoleic acid-derived oxylipins in patients with migraine. *Prostaglandins, Leukot Essent Fatty Acids* **120**:15–24.

Graham ES, Ball N, Scotter EL, Narayan P, Dragunow M, and Glass M (2006) Induction of Krox-24 by endogenous cannabinoid type 1 receptors in Neuro2A cells is mediated by the MEK-ERK MAPK pathway and is suppressed by the phosphatidylinositol 3-kinase pathway. *J Biol Chem* **281**:29085–29095.

Greco R, Demartini C, Zanaboni AM, Berliocchi L, Piomelli D, and Tassorelli C (2018) Inhibition of monoacylglycerol lipase: Another signalling pathway for potential therapeutic targets in migraine? *Cephalalgia* **38**:1138–1147.

Guindon J, Guijarro A, Piomelli D, and Hohmann AG (2011) Peripheral antinociceptive effects of inhibitors of monoacylglycerol lipase in a rat model of inflammatory pain. *Br J Pharmacol* **163**:1464–1478.

Gunduz-Cinar O, Castillo LI, Xia M, Van Leer E, Brockway ET, Pollack GA, Yasmin F, Bukalo O, Limoges A, Oreizi-Esfahani S, et al. (2023) A cortico-amygdala neural substrate for endocannabinoid modulation of fear extinction. *Neuron* **111**:3053–3067.e3010.

Harvey RJ, Depner UB, Wässle H, Ahmadi S, Heindl C, Reinold H, Smart TG, Harvey K, Schütz B, Abo-Salem OM, et al. (2004) GlyR alpha3: an essential target for spinal PGE2-mediated inflammatory pain sensitization. *Science* **304**:884–887.

Hashimotodani Y, Ohno-Shosaku T, Tanimura A, Kita Y, Sano Y, Shimizu T, Di Marzo V, and Kano M (2013) Acute inhibition of diacylglycerol lipase blocks endocannabinoid-mediated retrograde signalling: evidence for on-demand biosynthesis of 2-arachidonoylglycerol. *J Physiol* **591**:4765–4776.

Henstridge CM, Balenga NAB, Kargl J, Andradas C, Brown AJ, Irving A, Sanchez C, and Waldhoer M (2011) Minireview: recent developments in the physiology and pathology of the lysophosphatidylinositol-sensitive receptor GPR55. *Mol Endocrinol* **25**:1835–1848.

Hill JD, Zuluaga-Ramirez V, Gajghate S, Winfield M, and Persidsky Y (2018) Activation of GPR55 increases neural stem cell proliferation and promotes early adult hippocampal neurogenesis. *Br J Pharmacol* **175**:3407–3421.

Hinton DJ, Lee MR, Jang JS, and Choi D-S (2014) Type 1 equilibrative nucleoside transporter regulates astrocyte-specific glial fibrillary acidic protein expression in the striatum. *Brain Behav* **4**:903–914.

Hohmann AG, Suplita RL, Bolton NM, Neely MH, Fegley D, Mangieri R, Krey JF, Walker JM, Holmes PV, Crystal JD, et al. (2005) An endocannabinoid mechanism for stress-induced analgesia. *Nature* **435**:1108–1112.

Huang C-W, Lin P-C, Chen J-L, and Lee M-J (2021) Cannabidiol Selectively Binds to the Voltage-Gated Sodium Channel Nav1. 4 in Its Slow-Inactivated State and Inhibits Sodium Current. *Biomedicines* **9**:1141.

Iannotti FA, Hill CL, Leo A, Alhusaini A, Soubrane C, Mazzarella E, Russo E, Whalley BJ, Di Marzo V, and Stephens GJ (2014) Nonpsychotropic plant cannabinoids, cannabidivarin (CBDV) and cannabidiol (CBD), activate and desensitize transient receptor potential vanilloid 1 (TRPV1) channels in vitro: potential for the treatment of neuronal hyperexcitability. *ACS Chem Neurosci* **5**:1131–1141.

Ibsen MS, Finlay DB, Patel M, Javitch JA, Glass M, and Grimsey NL (2019) Cannabinoid CB1 and CB2 Receptor-Mediated Arrestin Translocation: Species, Subtype, and Agonist-Dependence. *Front Pharmacol* **10**:350.
Imlach WL, Bhola RF, Mohammadi SA, and Christie MJ (2016) Glycinergic dysfunction in a subpopulation of dorsal horn interneurons in a rat model of neuropathic pain. *Sci Rep* **6**:37104.
Jakowiecki J, Abel R, Orzeł U, Pasznik P, Preissner R, and Filipek S (2021) Allosteric Modulation of the CB1 Cannabinoid Receptor by Cannabidiol-A Molecular Modeling Study of the N-Terminal Domain and the Allosteric-Orthosteric Coupling. *Molecules* **26**:2456.
Jesus CHA, Redivo DDB, Gasparin AT, Sotomaior BB, de Carvalho MC, Genaro K, Zuardi AW, Hallak JEC, Crippa JA, Zanoveli JM, et al. (2019) Cannabidiol attenuates mechanical allodynia in streptozotocin-induced diabetic rats via serotonergic system activation through 5-HT1A receptors. *Brain Res* **1715**:156–164.
Jiang M, Huizenga MCW, Wirt JL, Paloczi J, Amedi A, van den Berg RJBHN, Benz J, Collin L, Deng H, Di X, et al. (2023) A monoacylglycerol lipase inhibitor showing therapeutic efficacy in mice without central side effects or dependence. *Nat Commun* **14**:8039.
Jung K-M, Mangieri R, Stapleton C, Kim J, Fegley D, Wallace M, Mackie K, and Piomelli D (2005) Stimulation of Endocannabinoid Formation in Brain Slice Cultures through Activation of Group I Metabotropic Glutamate Receptors. *Mol Pharmacol* **68**:1196–1202.
Kaplan JS, Stella N, Catterall WA, and Westenbroek RE (2017) Cannabidiol attenuates seizures and social deficits in a mouse model of Dravet syndrome. *Proc Natl Acad Sci USA* **114**:11229–11234.
Kearn CS, Greenberg MJ, DiCamelli R, Kurzawa K, and Hillard CJ (1999) Relationships between ligand affinities for the cerebellar cannabinoid receptor CB1 and the induction of GDP/GTP exchange. *J Neurochem* **72**:2379–2387.
Kathmann M, Flau K, Redmer A, Tränkle C, and Schlicker E (2006) Cannabidiol is an allosteric modulator at mu- and delta-opioid receptors. *Naunyn Schmiedebergs Arch Pharmacol* **372**:354–361.
Kelley BG and Thayer SA (2004) Delta 9-tetrahydrocannabinol antagonizes endocannabinoid modulation of synaptic transmission between hippocampal neurons in culture. *Neuropharmacology* **46**:709–715.
Kellogg R, Mackie K, and Straiker A (2009) Cannabinoid CB1 receptor-dependent long-term depression in autaptic excitatory neurons. *J Neurophysiol* **102**:1160–1171.
Kiritoshi T, Ji G, and Neugebauer V (2016) Rescue of Impaired mGluR5-Driven Endocannabinoid Signaling Restores Prefrontal Cortical Output to Inhibit Pain in Arthritic Rats. *J Neurosci* **36**:837–850.
Kuner R (2010) Central mechanisms of pathological pain. *Nat Med* **16**:1258–1266.
Labouesse MA, Cola RB, and Patriarchi T (2020) GPCR-Based Dopamine Sensors-A Detailed Guide to Inform Sensor Choice for In vivo Imaging. *Int J Mol Sci* **21**:8048.
LaFrance EM, Stueber A, Glodosky NC, Mauzay D, and Cuttler C (2020) Overbaked: assessing and predicting acute adverse reactions to Cannabis. *J Cannabis Res* **2**:3.
Laprairie RB, Bagher AM, Kelly MEM, and Denovan-Wright EM (2015) Cannabidiol is a negative allosteric modulator of the cannabinoid CB1 receptor. *Br J Pharmacol* **172**:4790–4805.
Lee Y, Chien C, Sun C, Huang C, Huang N, Chiang M, Lai H, Lin Y, Chou S, Wang CL, et al. (2003) Characterization of the rat A2A adenosine receptor gene: a 4.8-kb promoter-proximal DNA fragment confers selective expression in the central nervous system. *Eur J of Neuroscience* **18**:1786–1796.
Leimuranta P, Khiroug L, and Giniatullin R (2018) Emerging role of (endo) cannabinoids in migraine. *Front Pharmacol* **9**:420.
Liktor-Busa E, Levine AA, Palomino SM, Singh S, Wahl J, Vanderah TW, Stella N, and Largent-Milnes TM (2023) ABHD6 and MAGL control 2-AG levels in the PAG and allodynia in a CSD-induced periorbital model of headache. *Front Pain Res (Lausanne)* **4**:1171188.
Liput DJ, Puhl HL, Dong A, He K, Li Y, and Lovinger DM (2022) 2-Arachidonoylglycerol mobilization following brief synaptic stimulation in the dorsal lateral striatum requires glutamatergic and cholinergic neurotransmission. *Neuropharmacology* **205**:108916.
Liu Z, Iyer MR, Godlewski G, Jourdan T, Liu J, Coffey NJ, Zawatsky CN, Puhl HL, Wess J, Meister J, et al. (2021) Functional Selectivity of a Biased Cannabinoid-1 Receptor (CB1R) Antagonist. *ACS Pharmacol Transl Sci* **4**:1175–1187.
Long JZ, Nomura DK, and Cravatt BF (2009) Characterization of monoacylglycerol lipase inhibition reveals differences in central and peripheral endocannabinoid metabolism. *Chem Biol* **16**:744–753.
Loram LC, Taylor FR, Strand KA, Harrison JA, Rzasalynn R, Sholar P, Rieger J, Maier SF, and Watkins LR (2013) Intrathecal injection of adenosine 2A receptor agonists reversed neuropathic allodynia through protein kinase (PK)A/PKC signaling. *Brain Behav Immun* **33**:112–122.
Lu H-C and Mackie K (2016) An Introduction to the Endogenous Cannabinoid System. *Biol Psychiatry* **79**:516–525.
Maione S, Piscitelli F, Gatta L, Vita D, De Petrocellis L, Palazzo E, de Novellis V, and Di Marzo V (2011) Non-psychoactive cannabinoids modulate the descending pathway of antinociception in anaesthetized rats through several mechanisms of action. *Br J Pharmacol* **162**:584–596.
Manterola A, Bernal-Chico A, Cipriani R, Ruiz A, Pérez-Samartín A, Moreno-Rodríguez M, Hsu K-L, Cravatt BF, Brown JM, Rodríguez-Puertas R, et al. (2018) Re-examining the potential of targeting ABHD6 in multiple sclerosis: Efficacy of systemic and peripherally restricted inhibitors in experimental autoimmune encephalomyelitis. *Neuropharmacology* **141**:181–191.
Marrs WR, Blankman JL, Horne EA, Thomazeau A, Lin YH, Coy J, Bodor AL, Muccioli GG, Hu SS-J, Woodruff G, et al. (2010) The serine hydrolase ABHD6 controls the accumulation and efficacy of 2-AG at cannabinoid receptors. *Nat Neurosci* **13**:951–957.
Masuda T, Ozono Y, Mikuriya S, Kohro Y, Tozaki-Saitoh H, Iwatsuki K, Uneyama H, Ichikawa R, Salter MW, Tsuda M, et al. (2016) Dorsal horn neurons release extracellular ATP in a VNUT-dependent manner that underlies neuropathic pain. *Nat Commun* **7**:12529.
McKinney DL, Cassidy MP, Collier LM, Martin BR, Wiley JL, Selley DE, and Sim-Selley LJ (2008) Dose-related differences in the regional pattern of cannabinoid receptor adaptation and in vivo tolerance development to Δ9-tetrahydrocannabinol. *J Pharmacol Exp Ther* **324**:664–673.
Mechoulam R and Gaoni Y (1965) Hashish. IV. The isolation and structure of cannabinolic cannabidiolic and cannabigerolic acids. *Tetrahedron* **21**:1223–1229.
Mezey E, Eisenhofer G, Hansson S, Harta G, Hoffman BJ, Gallatz K, Palkovits M, and Hunyady B (1999) Non-neuronal dopamine in the gastrointestinal system. *Clin Exp Pharmacol Physiol Suppl* **26**:S14–22.
Muccioli GG, Xu C, Odah E, Cudaback E, Cisneros JA, Lambert DM, López Rodríguez ML, Bajjalieh S, and Stella N (2007) Identification of a novel endocannabinoid-hydrolyzing enzyme expressed by microglial cells. *J Neurosci* **27**:2883–2889.
Mudge EM, Murch SJ, and Brown PN (2018) Chemometric Analysis of Cannabinoids: Chemotaxonomy and Domestication Syndrome. *Sci Rep* **8**:13090.
Navia-Paldanius D, Savinainen JR, and Laitinen JT (2012) Biochemical and pharmacological characterization of human α/β-hydrolase domain containing 6 (ABHD6) and 12 (ABHD12). *J Lipid Res* **53**:2413–2424.
Naydenov AV, Horne EA, Cheah CS, Swinney K, Hsu K-L, Cao JK, Marrs W, Blankman JL, Tu S, Cherry AE, et al. (2014) ABHD6 blockade exerts antiepileptic activity in PTZ-induced seizures and in spontaneous seizures in R6/2 mice. *Neuron* **83**:361–371.
Nicolussi S and Gertsch J (2015) Endocannabinoid transport revisited. *Vitam Horm* **98**:441-485.
Nielsen SW, Hasselsteen SD, Dominiak HSH, Labudovic D, Reiter L, Dalton SO, and Herrstedt J (2022) Oral cannabidiol for prevention of acute and transient chemotherapy-induced peripheral neuropathy. *Support Care Cancer* **30**:9441–9451.
Noguchi K, Kadekawa K, Nishijima S, Sakanashi M, Okitsu-Sakurayama S, Higa-Nakamine S, Yamamoto H, and Sugaya K (2021) Phenotypic Characterization of the Endocannabinoid-Degrading Enzyme Alpha/Beta-Hydrolase Domain 6 Knockout Rat. *Cannabis Cannabinoid Res* **7**:179–187.
Okine BN, Mc Laughlin G, Gaspar JC, Harhen B, Roche M, and Finn DP (2020) Antinociceptive effects of the GPR55 antagonist CID16020046 injected into the rat anterior cingulate cortex. *Neuroscience* **443**:19–29.
Ono T, Yamashita T, Kano R, Inoue M, Okada S, Kano K, Koizumi S, Iwabuchi K, Hirabayashi Y, Matsuo I, et al. (2023) GPR55 contributes to neutrophil recruitment and mechanical pain induction after spinal cord compression in mice. *Brain Behav Immun* **110**:276–287.
Pastor-Anglada M and Pérez-Torras S (2018) Who is who in adenosine transport. *Front Pharmacol* **9**:627.
Pellkofer HL, Havla J, Hauer D, Schelling G, Azad SC, Kuempfel T, Magerl W, and Huge V (2013) The major brain endocannabinoid 2-AG controls neuropathic pain and mechanical hyperalgesia in patients with neuromyelitis optica. *PLoS One* **8**:e71500.
Pribasnig MA, Mrak I, Grabner GF, Taschler U, Knittelfelder O, Scherz B, Eichmann TO, Heier C, Grumet L, Kowaliuk J, et al. (2015) α/β Hydrolase Domain-containing 6 (ABHD6) Degrades the Late Endosomal/Lysosomal Lipid Bis(monoacylglycero)-phosphate. *J Biol Chem* **290**:29869–29881.
Pusch L-M, Riegler-Berket L, Oberer M, Zimmermann R, and Taschler U (2022) α/β-Hydrolase Domain-Containing 6 (ABHD6)- A Multifunctional Lipid Hydrolase. *Metabolites.* **12**:761.
Ren W-J and Illes P (2022) Involvement of P2X7 receptors in chronic pain disorders. *Purinergic Signal* **18**:83–92.
Ribeiro A, Ferraz-de-Paula V, Pinheiro ML, Vitoretti LB, Mariano-Souza DP, Quinteiro-Filho WM, Akamine AT, Almeida VI, Quevedo J, Dal-Pizzol F, et al. (2012) Cannabidiol, a non-psychotropic plant-derived cannabinoid, decreases inflammation in a murine model of acute lung injury: role for the adenosine A(2A) receptor. *Eur J Pharmacol* **678**:78–85.
Rimmerman N, Ben-Hail D, Porat Z, Juknat A, Kozela E, Daniels MP, Connelly PS, Leishman E, Bradshaw HB, Shoshan-Barmatz V, et al. (2013) Direct modulation of the outer mitochondrial membrane channel, voltage-dependent anion channel 1 (VDAC1) by cannabidiol: a novel mechanism for cannabinoid-induced cell death. *Cell Death Dis* **4**:e949.
Rinaldi-Carmona M, Barth F, Héaulme M, Shire D, Calandra B, Congy C, Martinez S, Maruani J, Néliat G, Caput D, et al. (1994) SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. *FEBS Lett* **350**:240–244.
Robbe D, Kopf M, Remaury A, Bockaert J, and Manzoni OJ (2002) Endogenous cannabinoids mediate long-term synaptic depression in the nucleus accumbens. *Proc Natl Acad Sci U S A* **99**:8384–8388.
Roloff AM and Thayer SA (2009) Modulation of excitatory synaptic transmission by Δ9-tetrahydrocannabinol switches from agonist to antagonist depending on firing rate. *Mol Pharmacol* **75**:892–900.
Ross RA (2009) The enigmatic pharmacology of GPR55. *Trends Pharmacol Sci* **30**:156–163.
Rudolph U and Knoflach F (2011) Beyond classical benzodiazepines: novel therapeutic potential of GABA A receptor subtypes. *Nat Rev Drug Discov* **10**:685–697.
Russo EB, Burnett A, Hall B, and Parker KK (2005) Agonistic properties of cannabidiol at 5-HT1a receptors. *Neurochem Res* **30**:1037–1043.
Ryberg E, Larsson N, Sjögren S, Hjorth S, Hermansson N-O, Leonova J, Elebring T, Nilsson K, Drmota T, and Greasley PJ (2007) The orphan receptor GPR55 is a novel cannabinoid receptor. *Br J Pharmacol* **152**:1092–1101.
Sabatini BL and Tian L (2020) Imaging Neurotransmitter and Neuromodulator Dynamics In Vivo with Genetically Encoded Indicators. *Neuron* **108**:17–32.
Saini RK, Prasad P, Shang X, and Keum Y-S (2021) Advances in Lipid Extraction Methods-A Review. *Int J Mol Sci* **22**:13643.
Schlosburg JE, Blankman JL, Long JZ, Nomura DK, Pan B, Kinsey SG, Nguyen PT, Ramesh D, Booker L, Burston JJ, et al. (2010) Chronic monoacylglycerol lipase blockade causes functional antagonism of the endocannabinoid system. *Nat Neurosci* **13**:1113–1119.
Shahbazi F, Grandi V, Banerjee A, and Trant JF (2020) Cannabinoids and Cannabinoid Receptors: The Story so Far. *iScience* **23**:101301.

Shen M, Piser TM, Seybold VS, and Thayer SA (1996) Cannabinoid receptor agonists inhibit glutamatergic synaptic transmission in rat hippocampal cultures. *J Neurosci* **16**:4322–4334.
Shen M and Thayer SA (1999) Δ^9-Tetrahydrocannabinol acts as a partial agonist to modulate glutamatergic synaptic transmission between rat hippocampal neurons in culture. *Mol Pharmacol* **55**:8–13.
Shivshankar S, Nimely J, Puhl H, and Iyer MR (2024) Pharmacological Evaluation of Cannabinoid Receptor Modulators Using GRAB(eCB2.0) Sensor. *Int J Mol Sci* **25**:5012.
Shonesy BC, Winder DG, Patel S, and Colbran RJ (2015) The initiation of synaptic 2-AG mobilization requires both an increased supply of diacylglycerol precursor and increased postsynaptic calcium. *Neuropharmacology* **91**:57–62.
Sigel E, Baur R, Rácz I, Marazzi J, Smart TG, Zimmer A, and Gertsch J (2011) The major central endocannabinoid directly acts at GABA(A) receptors. *Proc Natl Acad Sci U S A* **108**:18150–18155.
Silva-Cardoso GK, Lazarini-Lopes W, Hallak JE, Crippa JA, Zuardi AW, Garcia-Cairasco N, and Leite-Panissi CRA (2021) Cannabidiol effectively reverses mechanical and thermal allodynia, hyperalgesia, and anxious behaviors in a neuropathic pain model: possible role of CB1 and TRPV1 receptors. *Neuropharmacology* **197**:108712.
Singh S, Sarroza D, English A, McGrory M, Dong A, Zweifel L, Land BB, Li Y, Bruchas MR, and Stella N (2023) Pharmacological Characterization of the Endocannabinoid Sensor $GRAB_{eCB2.0}$. *Cannabis Cannabinoid Res* DOI: 10.1089/can.2023.0036
Singh S, Sarroza D, English A, Whittington D, Dong A, Malamas M, Makriyannis A, van der Stelt M, Li Y, Zweifel L, et al. (2024) P2X7 receptor-dependent increase in endocannabinoid 2-arachidonoyl glycerol production by neuronal cells in culture: Dynamics and mechanism. *Br J Pharmacol* DOI: 10.1111/bph.16348 [published ahead of print].
Smith CJ, Vergara D, Keegan B, and Jikomes N (2022) The phytochemical diversity of commercial Cannabis in the United States. *PLoS One* **17**:e0267498.
Soliman N, Haroutounian S, Hohmann AG, Krane E, Liao J, Macleod M, Segelcke D, Sena C, Thomas J, Vollert J, et al. (2021) Systematic review and meta-analysis of cannabinoids, cannabis-based medicines, and endocannabinoid system modulators tested for antinociceptive effects in animal models of injury-related or pathological persistent pain. *Pain* **162**:S26–S44.
Steffens M, Zentner J, Honegger J, and Feuerstein TJ (2005) Binding affinity and agonist activity of putative endogenous cannabinoids at the human neocortical CB1 receptor. *Biochem Pharmacol* **69**:169–178.
Stella N, Schweitzer P, and Piomelli D (1997) A second endogenous cannabinoid that modulates long-term potentiation. *Nature* **388**:773–778.10.1038/42015] [9285589]
Straiker A and Mackie K (2005) Depolarization-induced suppression of excitation in murine autaptic hippocampal neurones. *J Physiol* **569**:501–517.
Straiker A, Hu SS-J, Long JZ, Arnold A, Wager-Miller J, Cravatt BF, and Mackie K (2009) Monoacylglycerol Lipase Limits the Duration of Endocannabinoid-Mediated Depolarization-Induced Suppression of Excitation in Autaptic Hippocampal Neurons. *Mol Pharmacol* **76**:1220–1227.
Stella N (2010) Cannabinoid and cannabinoid-like receptors in microglia, astrocytes, and astrocytomas. *Glia* **58**:1017–1030.
Stella N (2023) THC and CBD: Similarities and differences between siblings. *Neuron* **111**:302–327.
Sylantyev S, Jensen TP, Ross RA, and Rusakov DA (2013) Cannabinoid-and lysophosphatidylinositol-sensitive receptor GPR55 boosts neurotransmitter release at central synapses. *Proc Natl Acad Sci USA* **110**:5193–5198.
Szabo B, Siemes S, and Wallmichrath I (2002) Inhibition of GABAergic neurotransmission in the ventral tegmental area by cannabinoids. *Eur J Neurosci* **15**:2057–2061.
Szok D, Tajti J, Nyári A, and Vécsei L (2019) Therapeutic Approaches for Peripheral and Central Neuropathic Pain. *Behav Neurol* **2019**:8685954.
Tchantchou F and Zhang Y (2013) Selective inhibition of alpha/beta-hydrolase domain 6 attenuates neurodegeneration, alleviates blood brain barrier breakdown, and improves functional recovery in a mouse model of traumatic brain injury. *J Neurotrauma* **30**:565–579.
Thomas A, Baillie GL, Phillips AM, Razdan RK, Ross RA, and Pertwee RG (2007) Cannabidiol displays unexpectedly high potency as an antagonist of CB1 and CB2 receptor agonists in vitro. *Br J Pharmacol* **150**:613–623.
Thomas G, Betters JL, Lord CC, Brown AL, Marshall S, Ferguson D, Sawyer J, Davis MA, Melchior JT, Blume LC, et al. (2013) The serine hydrolase ABHD6 Is a critical regulator of the metabolic syndrome. *Cell Rep* **5**:508–520.
Thomas A, Okine BN, Finn DP, and Masocha W (2020) Peripheral deficiency and antiallodynic effects of 2-arachidonoyl glycerol in a mouse model of paclitaxel-induced neuropathic pain. *Biomed Pharmacother* **129**:110456.
Thompson AL, Grenald SA, Ciccone HA, BassiriRad N, Niphakis MJ, Cravatt BF, Largent-Milnes TM, and Vanderah TW (2020) The Endocannabinoid System Alleviates Pain in a Murine Model of Cancer-Induced Bone Pain. *J Pharmacol Exp Ther* **373**:230–238.
Tóth A, Boczán J, Kedei N, Lizanecz E, Bagi Z, Papp Z, Edes I, Csiba L, and Blumberg PM (2005) Expression and distribution of vanilloid receptor 1 (TRPV1) in the adult rat brain. *Brain Res Mol Brain Res* **135**:162–168.
Valenzano KJ, Tafesse L, Lee G, Harrison JE, Boulet JM, Gottshall SL, Mark L, Pearson MS, Miller W, Shan S, et al. (2005) Pharmacological and pharmacokinetic characterization of the cannabinoid receptor 2 agonist, GW405833, utilizing rodent models of acute and chronic pain, anxiety, ataxia and catalepsy. *Neuropharmacology* **48**:658–672.
van Orten-Luiten A-CB, de Roos NM, Majait S, Witteman BJM, and Witkamp RF (2022) Effects of Cannabidiol chewing gum on perceived pain and well-being of irritable bowel syndrome patients: a placebo-controlled crossover exploratory intervention study with symptom-driven dosing. *Cannabis Cannabinoid Res* **7**:436–444.
Van Sickle MD, Duncan M, Kingsley PJ, Mouihate A, Urbani P, Mackie K, Stella N, Makriyannis A, Piomelli D, Davison JS, et al. (2005) Identification and functional characterization of brainstem cannabinoid CB2 receptors. *Science* **310**:329–332.
Vendel E and de Lange ECM (2014) Functions of the CB1 and CB2 receptors in neuroprotection at the level of the blood–brain barrier. *Neuromolecular Med* **16**:620–642.
Viader A, Blankman JL, Zhong P, Liu X, Schlosburg JE, Joslyn CM, Liu Q-S, Tomarchio AJ, Lichtman AH, Selley DE, et al. (2015) Metabolic Interplay between Astrocytes and Neurons Regulates Endocannabinoid Action. *Cell Rep* **12**:798–808.
Vitale RM, Iannotti FA, Schiano Moriello A, Tunisi L, Piscitelli F, Savopoulos R, Cristino L, De Petrocellis L, Amodeo P, Gray R, et al. (2021) Identification and Characterization of Cannabidiol as an OX1R Antagonist by Computational and In Vitro Functional Validation. *Biomolecules* **11**:1134.
Walter L, Dinh T, and Stella N (2004) ATP induces a rapid and pronounced increase in 2-arachidonoylglycerol production by astrocytes, a response limited by monoacylglycerol lipase. *J Neurosci* **24**:8068–8074.
Walters ET, Crook RJ, Neely GG, Price TJ, and Smith ESJ (2023) Persistent nociceptor hyperactivity as a painful evolutionary adaptation. *Trends Neurosci* **46**:211–227.
Wang X, Lin C, Jin S, Wang Y, Peng Y, and Wang X (2023) Cannabidiol alleviates neuroinflammation and attenuates neuropathic pain via targeting FKBP5. *Brain Behav Immun* **111**:365–375.
Ward RJ, Pediani JD, Godin AG, and Milligan G (2015) Regulation of oligomeric organization of the serotonin 5-hydroxytryptamine 2C (5-HT2C) receptor observed by spatial intensity distribution analysis. *J Biol Chem* **290**:12844–12857.
Weis WI and Kobilka BK (2018) The Molecular Basis of G Protein-Coupled Receptor Activation. *Annu Rev Biochem* **87**:897–919.
Wen J, Jones M, Tanaka M, Selvaraj P, Symes AJ, Cox B, and Zhang Y (2018) WWL70 protects against chronic constriction injury-induced neuropathic pain in mice by cannabinoid receptor-independent mechanisms. *J Neuroinflammation* **15**:9.
Westenbroek R, Kaplan J, Viray K, and Stella N (2023) The serine hydrolase ABHD6 controls survival and thermally induced seizures in a mouse model of Dravet syndrome. *Neurobiol Dis* **180**:106099.
Witting A, Walter L, Wacker J, Möller T, and Stella N (2004) P2X7 receptors control 2-arachidonoylglycerol production by microglial cells. *Proc Natl Acad Sci U S A* **101**:3214–3219.
Woodhams SG, Chapman V, Finn DP, Hohmann AG, and Neugebauer V (2017) The cannabinoid system and pain. *Neuropharmacology* **124**:105–120.
Wu C-S, Chen H, Sun H, Zhu J, Jew CP, Wager-Miller J, Straiker A, Spencer C, Bradshaw H, Mackie K, et al. (2013) GPR55, a G-protein coupled receptor for lysophosphatidylinositol, plays a role in motor coordination. *PLoS One* **8**:e60314.
Xiong W, Cui T, Cheng K, Yang F, Chen S-R, Willenbring D, Guan Y, Pan H-L, Ren K, Xu Y, et al. (2012) Cannabinoids suppress inflammatory and neuropathic pain by targeting α3 glycine receptors. *J Exp Med* **209**:1121–1134.
Xu K, Wu Y, Tian Z, Xu Y, Wu C, and Wang Z (2023) Microglial Cannabinoid CB(2) Receptors in Pain Modulation. *Int J Mol Sci* **24**:2348.
Yam MF, Loh YC, Tan CS, K, Adam S, Abdul Manan N, and Basir R (2018) General Pathways of Pain Sensation and the Major Neurotransmitters Involved in Pain Regulation. *Int J Mol Sci* **19**:2164.
Zhang J, Hoffert C, Vu HK, Groblewski T, Ahmad S, and O'Donnell D (2003) Induction of CB2 receptor expression in the rat spinal cord of neuropathic but not inflammatory chronic pain models. *Eur J Neurosci* **17**:2750–2754.
Zhao S, Mugabo Y, Iglesias J, Xie L, Delghingaro-Augusto V, Lussier R, Peyot M-L, Joly E, Taïb B, Davis MA, et al. (2014) α/β-Hydrolase domain-6-accessible monoacylglycerol controls glucose-stimulated insulin secretion. *Cell Metab* **19**:993–1007.
Zoratti C, Kipmen-Korgun D, Osibow K, Malli R, and Graier WF (2003) Anandamide initiates Ca2+ signaling via CB2 receptor linked to phospholipase C in calf pulmonary endothelial cells. *Br J Pharmacol* **140**:1351–1362.
Zou S and Kumar U (2018) Cannabinoid Receptors and the Endocannabinoid System: Signaling and Function in the Central Nervous System. *Int J Mol Sci* **19**:833.
Zúñiga-Romero Á, Rivera-Plata Q, Arrieta J, Flores-Murrieta FJ, Rodríguez-Silverio J, Reyes-García JG, Huerta-Cruz JC, Ramírez-Martínez G, and Rocha-González HI (2022) GPR55 and GPR119 Receptors Contribute to the Processing of Neuropathic Pain in Rats. *Pharmaceuticals (Basel)* **15**:67.
Zygmunt PM, Ermund A, Movahed P, Andersson DA, Simonsen C, Jönsson BAG, Blomgren A, Birnir B, Bevan S, Eschalier A, et al. (2013) Monoacylglycerols activate TRPV1–a link between phospholipase C and TRPV1. *PLoS One* **8**:e81618.

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