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CB2 cannabinoid receptors as a therapeutic target—What does the future hold?

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Abbreviations used:

2-AG, 2 arachidonoyl glycerol; AM1241, 2-iodo-5-nitrophenyl)-(1-(1-methylpiperidin-2-ylmethyl)-1H-indol-3-yl)methanone; cAMP, cyclic adenosine monophosphate; CB2, cannabinoid receptor 2; CP55940, (-)-cis-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexane; CREB/ATF, cAMP response element-binding protein/Activating transcription factor; ERK1/2, extracellular signal regulated kinases 1/2; GIRK, inwardly rectifying potassium channel; HEK293, Human Embryonic Kidney cells 293; JAK/STAT1, Janus kinase/ Signal Transducer and Activator of Transcription; KO, knock-out; JNK, c-Jun N-terminal Kinases; MAPK, Mitogen activated protein kinases; NF-AT, Nuclear factor of activated T-cells; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; PI3K, phosphoinositide 3-kinase.

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Abstract:

The past decades have seen an exponential rise in our understanding of the endocannabinoid system, comprised of CB1 and CB2 cannabinoid receptors, endogenous cannabinoids (endocannabinoids), and the enzymes that synthesize and degrade endocannabinoids. The primary focus of this review is the CB2 receptor. CB2 receptors have been the subject of considerable attention, primarily due to their promising therapeutic potential towards treating various pathologies while avoiding the adverse psychotropic effects, which can accompany CB1 receptor-based therapies. With the appreciation that CB2 selective ligands show marked functional selectivity, there is a renewed opportunity to explore this promising area of research both from a mechanistic as well as therapeutic perspective. In this review we will summarize our present knowledge of CB2 receptor signaling, localization and regulation. We will discuss the availability of genetic tools (and their limitations) to study CB2 receptors and also provide an update on preclinical data using CB2 agonists in pain models. Finally, we suggest possible reasons for the failure of CB2 ligands in clinical pain trials and offer possible ways to move the field forward in a way that can help reconcile the inconsistencies between preclinical and clinical data.

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Introduction:

The endocannabinoid system consists of endogenous cannabinoids (endocannabinoids), cannabinoid receptors (primarily CB1 and CB2), and the enzymes that synthesize and degrade endocannabinoids. A complete (receptor(s), enzymes, endocannabinoids) endocannabinoid system appears to be present in all vertebrates (Elphick and Egertová, 2005). Delta-9-Tetrahydrocannabinol(Δ^9 THC), the primary psychoactive component of Cannabis, produces many of its psychoactive effects by engaging CB1 cannabinoid receptors. In addition to its psychoactivity, Cannabis has been shown (or suggested) to be efficacious for multiple therapeutic indications and maladies (Borgelt et al., 2013; Grotenhermen and Müller-Vahl, 2012). Most of these appear to be mediated by Δ^9THC's activation of either CB1 or CB2 receptors, though cannabidiol can be an important factor in the therapeutic efficacy of *Cannabis*-based medicines (Campos et al., 2012). These potential therapeutic effects of Δ^9THC have motivated a great deal of drug development over the past forty years. Most of these efforts have taken the form of targeted manipulation of endocannabinoid engagement with cannabinoid receptors or inhibition of the enzymes that degrade endocannabinoids.

A major limitation for the therapeutic development of compounds that directly activate CB1 receptors is unwanted psychotropic effects (Volkow et al., 2014). These CB1-mediated psychotropic actions produce both practical and administrative hurdles that has severely curtailed the development of direct-acting CB1 agonists. In contrast, activation of CB2 receptors does not appear to produce these psychotropic effects (Deng

et al., 2014). Thus, the observation that CB2 receptor activation produces desirable actions in a range of preclinical models (Han et al., 2013; Leleu-Chavain et al., 2012) attracted considerable interest and generated much activity in both academic and commercial laboratories. For example, agonists targeting CB2 receptors have been proposed as therapies for the treatment or management of a range of painful conditions including acute pain, chronic inflammatory pain and neuropathic pain (Ehrhart et al., 2005). They may also be helpful in treating diseases that have a neuroinflammatory or neurodegenerative component such as multiple sclerosis (Pertwee, 2007; Dittel, 2008), amyotrophic lateral sclerosis (Kim et al., 2006; Shoemaker et al., 2007), Huntington's disease (Sagredo et al., 2012), and stroke (Zhang et al., 2007; Pacher and Hasko, 2008). CB2 agonists have also been proposed as therapeutics in peripheral disorders that involve inflammation including atherosclerosis (Mach et al., 2008), inflammatory bowel diseases (Izzo and Camilleri, 2008; Wright et al., 2008), ischemia/reperfusion injury (Bátkai et al., 2007), renal fibrosis (Barutta et al., 2011) and liver cirrhosis (Mallat et al., 2007; Izzo and Camilleri, 2008; Lotersztajn et al., 2008). Both epidemiological and preclinical data suggest that activation of CB2 receptors may be protective in osteoporosis (Ofek et al., 2006). Finally, CB2 agonists have shown efficacy in preclinical cancer models (Guzman, 2003; Izzo and Camilleri, 2008; Wright et al., 2008). However, despite very favorable efficacy in a range of preclinical models, CB2 agonists have fared poorly in the clinic. In this review we will summarize our current state of knowledge of CB2 receptor signaling, preclinical and clinical studies using CB2 agonists, discuss the mismatch between preclinical and clinical results, and suggest possible ways forward. As mentioned above, CB2 agonists may be beneficial for a variety of ailments. However, this minireview will

primarily focus on CB2 agonists for treating chronic pain. Nonetheless, many of the concepts discussed will apply to the use of CB2 agonists for other therapeutic indications.

CB2 receptors:

Like CB1 receptors, CB2 receptors are class A serpentine receptors primarily coupling to $G_{i/o}$ proteins to modulate an array of signalling pathways: adenylyl cyclase, MAPK(p44/42 and p38), JNK, AKT kinase/Protein kinase B, PI3K/AKT, NFkB, NF-AT, CREB/ATF, JAK/STAT1, sphingomylinase, caspase, as well as some potassium and calcium ion channels (Sugiura et al., 2000,Howlett et al., 2002; Bouaboula et al., 1996; Pertwee, 1997, Macllister et al., 1999;Atwood et al., 2012a, Pertwee et al., 2010, Ehrhart et al., 2005, Herrera et al., 2005, Herrera et al., 2006),Molina-Holgado et al.,2002) (Figure 1). Despite activation of a wide range of signalling pathways by CB2 receptors, characterization of CB2 receptor ligands has primarily focused on modulation adenylyl cyclase and ERK1/2, while other pathways such as those involving arrestin, Akt, ceramide and ion channel modulation, and the physiological processes they mediate, are much less well-studied.

Adenylyl cyclase:

CB2 receptor- mediated pertussis toxin sensitive Gi/o protein stimulation leads to inhibition of adenylyl cyclase and decreased cAMP levels (Slipetz et al., 1995; Felder et al., 1995; Mukherjee et al., 2004). However, expression levels and the environment of

expression influences strongly influence the coupling of CB2 to adenylyl cyclase inhibition. For example, stimulation of CB2 receptors on human lymphocytes endogenously expressing CB2 receptors poorly inhibits forskolin-stimulated adenylyl cyclase when compared to CB2 transfected HEK or CHO cells (Schatz et al., 1997; Bouaboula et al., 1996; Herring et al., 1998; Gardner et al., 2002; Massi et al., 2003; Pertwee, 1997, 1999). Similarly, activation of CB2 receptors in mouse spleen cells endogenously expressing CB2 receptors does not inhibit forskolin-stimulated adenylyl cyclase at physiologically relevant agonist concentrations (Kaminiski, 1993, 1994). However, activation of CB2 receptors in the BV2 microglial cell line inhibits adenylyl cyclase (Franklin et al., 2003). A particularly interesting example is the natural product, 4'-*O*-methylhonokiol, which shows inverse agonism for cAMP production, and agonism for release of intracellular calcium (Schuehly et al., 2011).

MAP kinase:

MAP kinases are enzymes involved in a widely variety of vital signalling cascades in many cellular responses including cell proliferation, migration, transformation and cell death. Bouaboula and co-workers (1996) were the first to report the time and dose dependent activation of ERK1/2 by CB2 agonists in CHO cells transfected with receptors. They found this activation to be pertussis-toxin sensitive, indicating involvement of Gi/o protein, but was adenylyl cyclase independent. They further showed that activation of this signalling cascade results in phosphorylation of transcription factor, krox-24 thus indicating potential control of gene transcription by

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CB2 receptors. Unlike inhibition of adenylyl cyclase, ERK1/2 activation is routinely observed in both recombinant as well as non-recombinant cells/systems (Beltramo, 2009). For example, robust ERK1/2 activation was also reported in immune cells as well as microglia and macrophages, thus confirming the likely physiological relevance of this pathway (Beltramo, 2009; Merighi et al., 2012).

p38 MAPK activation by non-selective CB2 receptor agonist (Δ^9THC) was found to have pro- apoptotic effect in human leukaemia Jurkat cell line (Herrera et al., 2005) and cytotoxicity in J774-1 macrophages(Yamaori et al., 2013). This effect was exclusively mediated by CB2 receptors (Herrera et al., 2005, Kauppinen et al., 2014, Yamaori et al., 2013). Interestingly, Yamaori and co-workers (2013), in the same cells, also found a JNK-mediated cytoprotective effect mediated by Δ^9THC activation of CB2 receptors. Thus, the same CB2 receptor ligand can activate different MAPKs with varied responses and outcomes (Lopez-Ilasaca, 1998).

Ion channels:

Although initial experiments failed to detect functional coupling of CB2 receptors to inwardly rectifying potassium channels (GIRK channels) and calcium channels (Felder et al., 1995; Pertwee, 1997), other reports suggest that CB2 receptors can modulate activity of these channels (Ho et al., 1999; Macllister et al., 1999; Atwood et al., 2012b). Atwood et al. (2012b) showed CB2 receptor-mediated inhibition of voltage gated calcium channels (VGCC) in AtT20 cells. CP55940 effectively inhibited VGCC's, while WIN55212-2 was inactive on its own and antagonized CP55940 inhibition. Thus, the

reason why some of the earlier studies failed to find ion channel modulation can likely be attributed to the functional selectivity of the ligands used in the earliest studies (see below) (Atwood et al., 2012b).

Internalization:

Most G protein-coupled receptors (GPCR's) undergo some degree of internalization following agonist binding. Internalization can play a role in down regulation of the GPCR's ability to signal at the membrane (Ferguson, 2001). Additionally, internalized GPCR's can engage novel signalling pathways, inaccessible to GPCR's residing on the surface membrane (Miller and Lefkowitz, 2001). Thus, internalization of a GPCR in response to a ligand can be considered a form of signalling. CB2 receptors exhibit variable internalization in response to agonist, with some agonists promoting marked internalization, and others being inactive (Grimsey et al., 2011; Atwood et al., 2012b; Petrov et al., 2013).

Beta-arrestin signalling:

Beta-arrestins are multifunctional proteins that down regulate G protein signalling through direct interactions with GPCR's (Ferguson et al., 1996) as well as serving as scaffolds to recruit other signalling complexes to GPCR's (Miller and Lefkowitz, 2001). Beta-arrestin activation is conventionally measured by enzyme complementation, bioluminescence resonance energy transfer (BRET) or optical imaging of the recruitment of fluorescently-labeled arrestin molecules to the plasma membrane. Two studies have systematically evaluated arrestin recruitment by CB2 receptors and found that the limited

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number of CB2 ligands tested recruited arrestin to the plasma membrane (Atwood et al., 2012b; McGuinness et al., 2009).

CB2 receptor dimerization:

While class A GPCR's can signal as monomers (Milligan, 2013), much GPCR signalling appears to involve homo- or heterodimerization of GPCR's (Milligan, 2013). Heterodimerization can greatly enrich the range of intracellular responses elicited by a ligand (Rozenfeld et al., 2012) and alter the pharmacology of receptor ligands (Rozenfeld and Devi, 2007). The capacity of CB2 receptors to dimerize and potential implications of CB2 heterodimerization have not been deeply explored (Callén et al., 2012), but are likely to contribute to the heterogeneity of CB2 receptor signalling reported in various preparations. A particularly fascinating possibility is that dimerization of CB1 and CB2 receptors might produce novel receptor pharmacology as well as introducing a confound in interpreting the results of experiments using CB1/CB2 antagonist or deletion of one of the CB1 or CB2 receptor gene.

Functional selectivity of CB2 signalling:

Functional selectivity is the phenomenon where different agonists will activate distinct (or overlapping) intracellular signalling pathways (Kenakin, 2011) and is a concept that has important implications for drug development (Kenakin and Miller, 2010; Violin et al., 2014). Functional selectivity, also known as biased agonism or stimulus trafficking, is often noted as different agonists activating signalling pathways with

different rank order potencies (Kenakin et al., 2012). A *balanced agonist* will activate all pathways similarly, while a *biased agonist* will show bias towards a subset of pathways. In the most extreme example of functional selectivity an agonist may maximally activate some signalling pathways and not others.

Theoretically, functional selectivity offers the opportunity to "fine-tune" receptor stimulation. Functional selectivity could facilitate the discovery of agonists that will stimulate signalling pathways to elicit desirable therapeutic benefits, while avoiding activation of signalling pathways that may lead to undesirable side effects. However, if endogenous ligands are present at significant levels and are balanced agonists, which appears to be the case for endocannabinoids (particularly for 2-AG), functionally selective ligands may actually antagonize some signalling pathways activated by endocannabinoids, which could be detrimental.

Interestingly, CB2 ligands show significant functional selectivity. For example, Shoemaker et al. (2005) found that endocannabinoids activated distinct signalling pathways with varied rank order potencies in Chinese hamster ovary (CHO) cells transfected with CB2 receptors. The endocannabinoid, 2-AG, was most potent in activating the ERK1/2-MAPK pathway but higher concentrations were needed to inhibit adenylyl cyclase and induce calcium transients. On other hand, noladin ether displayed higher potency in inhibiting adenylyl cyclase as compared to activating ERK1/2 and calcium transients. An even more extreme example of functional selectivity occurs for several commonly used CB2 agonists. Certain CB2 agonists (e.g., the

aminoalkylindoles) inhibit adenylyl cyclase and activate ERK1/2, but fail to induce CB2 internalization or inhibition of voltage sensitive calcium channels (Atwood et al., 2012b). This striking functional selectivity of CB2 receptor agonists must be considered during the therapeutic development of CB2 agonists and enriches the possibilities for developing drugs targeting CB2 receptors (Atwood et al., 2012b; Han et al., 2013). Arrestin-biased signalling has been found to produce useful therapeutic effects for other GPCR's (Wisler et al., 2007). To date no CB2 arrestin-biased CB2 agonists have been described, so it will be interesting to screen the rich repertoire of CB2 ligands synthesized to determine if arrestin-biased signalling exists for CB2 ligands and if arrestin-biased signaling is necessary or dispensable for CB2 actions in preclinical models (e.g., analgesia, anti-inflammatory, etc.).

Regulation of CB2 receptor expression and CB2 receptor localization:

An interesting biological property of CB2 receptors is their high inducibility, with CB2 mRNA levels often increasing as much as 100-fold following nerve injury or during inflammation (Hsieh et al., 2011; Maresz et al., 2005). If these increases in mRNA are followed by a corresponding increase in functional receptor protein, and activation of the receptor is therapeutically beneficial, theoretically, this leads to a therapeutically desirable situation where agonists will stimulate CB2 receptors primarily where its activation will be beneficial. In healthy organisms, CB2 receptors are most abundant in cells of macrophage lineage, though they are also found in other immune cells (Galiègue et al., 1995). The extent of their expression in healthy CNS tissue is quite controversial.

This topic has been recently reviewed. Caveats of the existing literature and suggestions for resolving the controversy can be found there (Atwood and Mackie, 2010). The bottom line is that immunocytochemical studies purporting to show CB2 expression are many, but are often flawed, and the inclusion of concurrent and careful controls are mandatory before accepting any claim of CB2 expression in a particular tissue. These conditions have been met for many immune cells, and possibly in neurons following pathological insult, but remain to be established for most other tissues. In this regard, carefully conducted pharmacology has much to offer in discussions on CB2 receptor localization as the key question is often whether or not CB2 receptors are functionally involved in a response. The anatomical demonstration of CB2 receptors in this style of experiment are a secondary concern.

CB2 ligands:

A full consideration of the range of CB2 receptor ligands that have been synthesized and characterized is beyond the scope of this minireview. Several recent reviews have comprehensively considered this topic (Han et al., 2013, 2014) and can be consulted by the interested reader. An interesting development in the identification of naturally occurring ligands for CB2 is the existence of a number of abundant phytochemicals that engage CB2 receptors. Perhaps the best example of this is beta-caryophyllene (Gertsch et al., 2008) which offers a starting point for novel compounds that will influence endocannabinoid signalling (Chicca et al., 2014). A key concept to keep in mind when evaluating experiments conducted with CB2 ligands is that many of the commonly used CB2 ligands are only relatively selective with regards to CB1. This

is because most of the commonly encountered CB2 ligands were evolved from molecules that have appreciable affinity for CB1 receptors. Therefore, the concentrations of CB2 preferring agonist that are commonly encountered in the literature (low micromolar) can result in significant occupancy of CB1 receptors with subsequent signalling (Murataeva et al., 2012). Similarly, CB2 preferring antagonists at micromolar concentrations can substantially antagonize CB1-mediated responses (Murataeva et al., 2012). Thus, when interpreting the results of experiments conducted solely using a pharmacological approach, careful attention needs to be paid to the controls and a healthy scepticism maintained.

Genetic tools to study CB2 receptor signalling:

Because of the typically low specificity of cannabinoid ligand pharmacology, the complementary use of mice lacking CB2 receptors is desirable to implicate CB2 receptors in a specific response. There are two CB2 receptor KO lines in wide use. The first line was developed by Nancy Buckley and Andreas Zimmer (Buckley et al., 2000). This mouse was made by replacing the sequence coding for the receptor from the middle of its third intracellular loop through its stop codon, corresponding to amino acids 217 through 347, with a PGK Neomycin resistance cassette. While this receptor is non-functional when tested for classical CB2 activity (Buckley et al., 2000), these mice make mRNA for the proximal part of the receptor (Liu et al., 2009) so there is the concern that this mRNA might be translated. Since a large portion of expressed partial receptor could interact with other GPCRs to form partial dimers. Interestingly, if the protein corresponding to the truncated CB2 receptor is heterologously expressed in HEK293

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cells it is trafficked to a sub-plasma membrane compartment (Brady Atwood and K. Mackie, unpublished). In addition, background strain can strongly affect the immune phenotype of this mouse, necessitating caution in using this line to determine the utility of therapeutic manipulations of the endocannabinoid system in immune disorders, and presumably chronic pain, which often has a substantial immune component (Sisay et al., 2013). A second CB2 KO line in common use is one that was made by Deltagen and is available from JAX through the KOMP (Wotherspoon et al., 2005). This mouse was constructed by deleting the sequence corresponding to amino acids 26-140, which comprise to a portion of the amino terminus and the first three transmembrane domains (Yao and Mackie, 2009). While the deletion is more proximal in this mouse, the presence of mRNA for the residual receptor still remains to be carefully examined. Thus, interpretation of experiments using either line of CB2 receptor knockout mice must be interpreted with the caveats that variable portions of the CB2 receptor may be present. Recently, the generation and characterization of a CB2 conditional knockout was reported. This mouse has the additional feature of the insertion of an IRES sequence following by GFP downstream of the CB2 coding sequence, which facilitates localization of CB2 expression (Vazquez et al., 2014). An additional genetic resource that would be very helpful for the field is a knockin mouse expressing human CB2 in the mouse CB2 locus.

Preclinical studies of CB2 agonists in pain:

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The initial studies by Ibrahim and colleagues (Malan et al., 2001; Ibrahim et al., 2003) showing that the CB2 agonist AM1241 reversed tactile and thermal hyperalgesia in neuropathic rats and mice, independent of CB1 receptors (that is, the effect persisted in CB1 KO's), stimulated a great deal of excitement. These findings offered the possibility of producing significant analgesia through the endogenous cannabinoid system in the absence of CB1-mediated psychoactivity. Subsequent work by a number of groups have confirmed and extended these studies with a variety of structurally distinct CB2 agonists (Murineddu et al., 2013). Generally, the field has moved away from AM1241 as AM1241 appears to engage a unique endogenous-opioid mediated component for its analgesia (Ibrahim et al., 2005; Whiteside et al., 2005), its enantiomers have different actions and its efficacy is species dependent (Bingham et al., 2007), and is a low efficacy agonist, as it exhibits protean agonism (Yao et al., 2006).

Significant issues in developing GPCR agonists for therapy include the potential for tolerance (i.e., more drug needed for the desired effect) or physical dependence (i.e., physical signs and symptoms upon cessation of drug administration) (Williams et al., 2013). When given in preclinical neuropathic pain models, tolerance develops quickly to efficacious doses of CB1 agonists (Deng et al., 2014). Similarly, physical dependence can be demonstrated following repeated administration of low doses of a CB1 agonist (Deng et al., 2014). In comparison to CB1-mediated analgesia, tolerance to CB2-mediated analgesia in neuropathic pain models does not appear to develop, at least over the course of ~7 days of treatment (Deng et al., 2014). This lack of tolerance to the analgesic effects of a CB2 agonist raises the question if the immune modulation by CB2

agonists will also be persistent, and, if so, will this be detrimental. Importantly, abrupt antagonism of CB2 receptors after chronic dosing with an effective dose of a CB2 agonist does not appear to elicit signs of physical or autonomic withdrawal (Deng et al., 2014). Finally, while CB2 agonists do not appear to be rewarding by themselves (Ignatowska-Jankowska et al., 2013), rats will only self-administer CB2 agonists and will only show preference for the environment in which they received a CB2 agonist, if they are experiencing a painful stimulus (e.g., neuropathy) (Gutierrez et al., 2011).

Reconciling preclinical and clinical studies:

Conventional drug development relies heavily on preclinical models to evaluate efficacy of compounds towards potential targets. The high failure rate of agents in clinical trials has prompted a re-evaluation of the predictive reliability of this approach (Baxter et al., 2013; Paul et al., 2010). There are many potential reasons why an agent that appears effective in preclinical models fails in the clinic. These include: (1) irreproducible preclinical studies, (2) irrelevance of the preclinical model being evaluated towards the clinical condition being treated, (3) an inability in humans to reach tissue concentrations that were necessary for efficacy in animal models because of dose-limiting side effects, (4) species differences in how a ligand engages and activates its targets, (5) off target negative effects that were not detectable in animal toxicology tests, and (6) lack of selectivity of the ligands used to validate the target in preclinical models. Which of these might explain with CB2 agonists have failed in the preclinical to clinical transition? As numerous groups have demonstrated efficacy of CB2 agonists in diverse preclinical models of pain, and CB2 agonists as used in clinical trials appear to have

minimal side effects, reasons 1, 3, and 5 are unlikely to explain the failure of CB2 agonists in clinical trials, so we will focus on possibilities 2, 4, and 6. In the case of possibility 6, many CB2 "selective" agonists still have significant affinity for CB1. Since CB1 receptors are highly abundant, low occupancy still results in the activation of a significant number of CB1 receptors, potentially producing CB1-mediated effects, including significant analgesia. Analgesia produced by these agonists activating CB1 receptors will be absent in mice lacking functional CB2 receptors (but see above for the caveats in using existing CB2 knockout lines).

The outcome of clinical trials in pain is strongly affected by the type of pain being treated, study design, patient population, and many related factors (Gewandter et al., 2014). While preclinical studies are typically conducted in a genetically and environmentally (usually un-enriched) uniform population of often young (and male) rodents, human populations enrolled in clinical pain trials tend to be older, of both sexes, and are invariably more heterogeneous. In addition, practical concerns on adequate subject enrolment and the ease of evaluating subject response often result in clinical pain trials being performed on populations whose disease may be quite distinct from those represented in the preclinical models. For example, third molar extraction and osteoarthritis patients are often examined in early phase analgesic trials, and commonly used preclinical pain models do not faithfully recapitulate these conditions. Indeed, this appears to be the case as the reported trials where CB2 agonists have failed include third molar extraction (GSK, GW842166X) (Ostenfeld et al., 2011), topical capsaicin (Pharmos, PRS211375, Cannabinor) (Roche and Finn, 2010), and osteoarthritis (Lilly,

LY2828360) (Pereira et al., 2013). An additional complicating issue is that early stage clinical trials are often not reported, or only reported in abstract form. The paucity of details and lack of peer review can make it very difficult to determine why a clinical trial has been considered to "fail" as the decision not to pursue a clinical target may be a commercially-, rather than an efficacy-, driven decision (Hay et al., 2014).

Species differences in receptors and signalling pathways are additional potential confounds in moving from exploratory and preclinical studies to human therapies. Differences in sequence between receptors from different species can lead to different pharmacologies. Thus, a ligand for a rodent receptor may be inactive, or have different signalling properties than the human receptor. An example of this is the H3 histamine receptor where ligands switch from agonism to antagonism, depending on the species of receptor (Ireland-Denny et al., 2001; Yao et al., 2003). Thus, while *in vitro* pharmacology can be done in cell lines expressing receptors of the correct species (or possibly in iPSC's), preclinical testing is often done in rodents. While mice expressing the human version of the appropriate receptor can be generated and used for testing (e.g., Jun et al., 2014), this is often not done. Furthermore, even if a "humanized" mouse is used, coupling between the receptor and downstream signalling pathways may be species-dependent. This concern is especially significant in situations where there are substantial species differences in non-ligand binding portions of a receptor, for examples in domains that may be important for signal transduction or receptor regulation, such is the case for the highly divergent carboxy termini of the CB2 receptor (Brown et al.,

2002). The developmental and characterization of a mouse expressing humanized CB2 receptors will be very useful to address these and related questions.

Concluding comments:

The development of CB2 receptor as a therapeutic target has gained significant momentum over the past decade due to the identification of CB2-specific synthetic and natural product ligands, a better understanding of the range of physiological processes mediated by CB2 receptors, the regulation of CB2 receptors, and promising preclinical studies. However, the publically available clinical data has thus far been disheartening. One reason for this appears to be discrepancies in the fundamental pain mechanisms of the preclinical models, where CB2 agents have been shown to be efficacious, and the types pain studied in the clinical trials. Thus efforts to examine the clinical efficacy of CB2 agonists in (neuro) inflammatory conditions and neuropathic pain syndromes (e.g., chemotherapy or diabetic) may be more productive. A second potential reason for the lack of translation is that CB2 agonists show very strong functional selectivity, and this functional selectivity may significantly affect agonist efficacy across species and types of pain. With the availability of increasingly precise and selective pharmacological, genetic, preclinical and clinical tools and a more complete understanding of the importance of CB2 agonist functional selectivity, CB2 receptors still appear to be promising targets for drug development, both for chronic pain and other targets.

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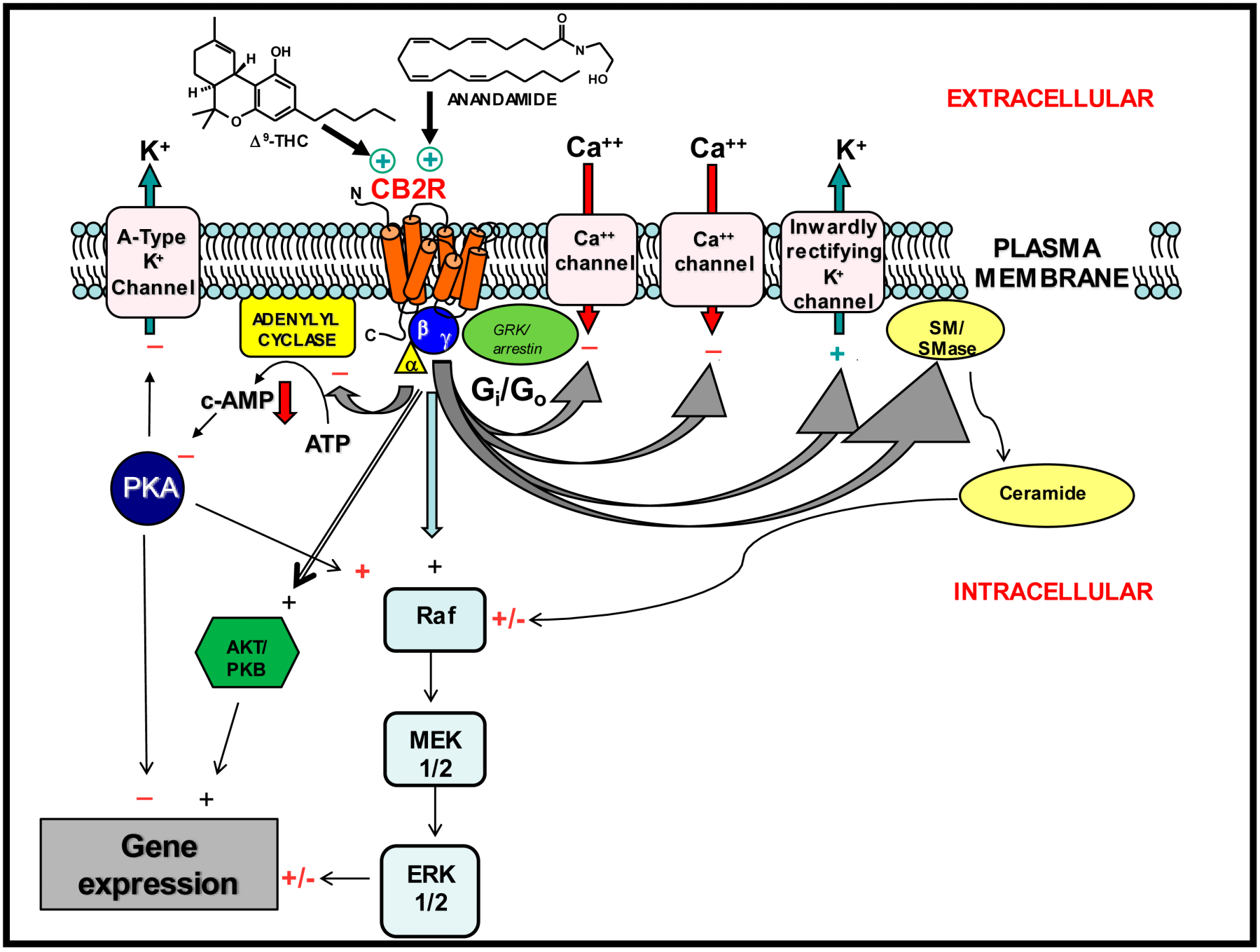
Figure legend

Figure 1: Activation of CB2 receptors by natural or synthetic ligands favors a range of receptor conformations that can variably affect different signaling pathways in the following ways:

- Inhibition of adenylyl cyclase, decreased cAMP production and less activation of cAMP-dependent protein kinase (PKA), culminating in inhibition of A-type potassium channels and inhibition of some gene expression.
- Activation of AKT/protein Kinase B, stimulating cell survival, migration and growth.
- Activation of MAP kinase cascade favouring cell survival and modulating gene expression
- Inhibition of specific calcium channels and enhanced opening of GIRK channels
- Stimulating *de novo* synthesis of ceramide and inhibition of the MAP kinase, cascade promoting apoptosis.
- Recruitment of β arrestin to the activated CB2 receptor resulting in desensitization and /or internalization of the receptor and the potential activation of arrestin-specific signalling.
- Decreased PKA activity increases Raf-1 to stimulate the MAP kinase cascade, positively regulating the expression of many genes.

  \+ indicates activation of pathway by CB2 receptor agonists, – indicates inhibition/down regulation of pathway by CB2 receptor agonist while +/- indicates a variable outcome


Δ9-THC
ANANDAMIDE
EXTRACELLULAR
K+
Ca++
Ca++
K+
CB2R
A-Type K+ Channel
Ca++ channel
Ca++ channel
Inwardly rectifying K+ channel
PLASMA MEMBRANE
ADENYLYL CYCLASE
GRK/ arrestin
SM/ SMase
c-AMP
ATP
Gi/Go
PKA
Ceramide
INTRACELLULAR
Raf
+/-
AKT/ PKB
MEK 1/2
Gene expression
+/-
ERK 1/2