THERAPEUTIC POTENTIAL OF THE CANNABINOID CB2 RECEPTOR

EDITED BY: Reem Smoum, Uwe Grether, Meliha Karsak, Andrea Vernall, Frank Park, Cecilia Hillard and Pal Pacher

PUBLISHED IN: Frontiers in Pharmacology

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-83250-568-7
DOI 10.3389/978-2-83250-568-7

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

THERAPEUTIC POTENTIAL OF THE CANNABINOID CB2 RECEPTOR

Topic Editors:
Reem Smoum, Hebrew University of Jerusalem, Israel
Uwe Grether, Roche (Switzerland), Switzerland
Meliha Karsak, University Medical Center Hamburg-Eppendorf, Germany
Andrea Vernall, University of Otago, New Zealand
Frank Park, University of Tennessee Health Science Center (UTHSC), United States
Cecilia Hillard, Medical College of Wisconsin, United States
Pal Pacher, National Institute on Alcohol Abuse and Alcoholism (NIH), United States

Citation: Smoum, R., Grether, U., Karsak, M., Vernall, A., Park, F., Hillard, C., Pacher, P., eds. (2022). Therapeutic Potential of the Cannabinoid CB2 Receptor. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83250-568-7

Table of Contents

frontiers | Frontiers in Pharmacology

TYPE Editorial
PUBLISHED 07 October 2022
DOI 10.3389/fphar.2022.1039564

OPEN ACCESS

EDITED AND REVIEWED BY
Heike Wulff,
University of California, Davis,
United States

*CORRESPONDENCE
Reem Smoum,
reems@ekmd.huji.ac.il

SPECIALTY SECTION
This article was submitted to Experimental Pharmacology and Drug Discovery,
a section of the journal
Frontiers in Pharmacology

RECEIVED 08 September 2022
ACCEPTED 21 September 2022
PUBLISHED 07 October 2022

CITATION
Smoum R, Grether U, Karsak M, Vernall AJ, Park F, Hillard CJ and Pacher P (2022), Editorial: Therapeutic potential of the
cannabinoid CB2 receptor.
Front. Pharmacol. 13:1039564.
doi: 10.3389/fphar.2022.1039564

Editorial: Therapeutic potential of the cannabinoid CB2 receptor

Reem Smoum[1]*, Uwe Grether[2], Meliha Karsak[3], Andrea J. Vernall[4], Frank Park[5], Cecilia J. Hillard[6] and Pal Pacher[7]

[1]School of Pharmacy, The Institute for Drug Research, The Hebrew University of Jerusalem, Jerusalem, Israel, [2]Roche Innovation Center Basel, F. Hoffman-La Roche Ltd., Basel, Switzerland, [3]Neuronal and Cellular Signal Transduction, Center for Molecular Neurobiology Hamburg (ZMNH), University Medical Center Hamburg-Eppendorf, Hamburg, Germany, [4]Department of Chemistry, University of Otago, Dunedin, New Zealand, [5]Department of Pharmaceutical Sciences, College of Pharmacy, University of Tennessee Health Science Center, Memphis, TN, United States, [6]Department of Pharmacology and Toxicology, Neuroscience Research Center, Medical College of Wisconsin, Milwaukee, WI, United States, [7]Laboratory of Cardiovascular Physiology and Tissue Injury, National Institute of Health/NIAAA, Rockville, MD, United States

KEYWORDS

CB2R, CB2R ligands, inflammation, neurodegenarative diseases, addiction

Editorial on the Research Topic
Therapeutic potential of the cannabinoid CB2 receptor

The cannabinoid receptor type 2 (CB2R) has emerged as a promising therapeutic target for treating various pathologies. Under normal conditions, CB2R is primarily expressed in the immune system, but there is emerging evidence that various states of disease can lead to robust induction of this receptor. This suggests that CB2R is a viable therapeutic target and for this reason, molecules interacting with CB2R have been tested as potential treatments in a wide array of chronic conditions, including cardiovascular and gastrointestinal/inflammatory bowel disease; liver, kidney, lung, neuro-degenerative and psychiatric disorders; reproductive system and skin pathologies; inflammation; pain; cancer; and osteoporosis (Whiting et al., 2022). Through the years, researchers have designed and synthesized novel ligands targeting CB2R with a preference to be highly selective over the cannabinoid receptor type 1 (CB1R) to avoid undesirable CB1-dependent psychotropic effects. However, the clinical results using these CB2R ligands have been largely ineffective (Morales et al., 2016; An et al., 2020).

Greater knowledge of ligand-target binding kinetics, CB2R biased signaling and allosterism, and additional structures of antagonist- and agonist bound CB2R will likely enable more selective drug design (Soethoudt et al., 2017). This will bring new hope for the therapeutic potential of CB2R and a better understanding of the endocannabinoid system (ECS).

This Research Topic provides more insight into our current understanding of the CB2R field and its therapeutic potential and highlights new findings.

Four comprehensive reviews cover diverse aspects of the therapeutic potential of CB2R. Hashiesh et al. provide a full overview of the pharmacological properties, molecular

and signaling mechanisms, and therapeutic potential of the CB2R specific agonist JWH133 in various pathological conditions. This review provides confirmation that CB2R is a viable therapeutic target, but that more preclinical pharmacokinetic and safety data is needed to develop effective human treatments. Young and Denovan-Wright thoroughly review the role of microglia and the ECS in neuroinflammation. Observed variations regarding components of the ECS in microglia together with the potential of CB2R as a therapeutic target are discussed. In the review by Liu et al., the authors propose that specific agonists of CB2R may serve as disease modifiers in type 1 diabetes. They demonstrate the involvement of CB2R in regulating the inflammasome and controlling intracellular autophagy, governing the secretion of extracellular vesicles from adipocytes and thus, dysregulating which induces chronic inflammation and obesity. In this regard, CB2R activation may play a similar role in the islets of Langerhans. Naturally occurring CB2R selective agonists or selective, peripherally restricted synthetic cannabinoids that work by intervening in both CB1R and CB2R signaling needs further investigation. The review by Franco et al. discusses the binding mode at orthosteric sites and/or exosites underlying the therapeutic potential of drugs targeting CB2R. According to the authors, a drug in a specific CB2R conformation leads to a signaling cascade that differs qualitatively and/or quantitatively from that triggered by another drug. A given drug may lead to different signaling outputs in a cell- or tissue-dependent manner due to potentially distinct allosteric effects from unique interactions with other proteins or with membrane lipids on the receptor. This highlights the pharmacological complexity of this receptor and the need to further unravel the binding mode of CB2R ligands in order to fine-tune signaling effects and therapeutic propositions.

A research article by Simard et al. provided data on the expression of both CB1R and CB2R in human blood leukocytes. The expression of CB2 mRNA can be detected in eosinophils, neutrophils, monocytes, and B and T lymphocytes, with the highest abundance in human eosinophils and B lymphocytes. The authors also review the evidence obtained from primary human leukocytes and immortalized cell lines regarding the regulation of their functions by CB2R, which highlights the urgent need to deepen the understanding of CB2R as an immunoregulator in humans.

Previous research proved that CB2R expression in the CNS is low under physiological conditions and is elevated in chronic neuroinflammatory states associated with neurodegenerative diseases. Esteban et al. analyzed the expression of CB2R in cortical areas of the brain of an AD mouse model (5xFAD/$CB_2^{EGFP/f/f}$) and showed that CB2Rs are expressed in the dystrophic neurite-associated microglia and their modulation modifies the number and activity of microglial cells as well as the metabolism of the insoluble form of the amyloid peptide. Thus, microglial CB2Rs can be potential targets for the development of amyloid-modulating therapies.

Brain CB2Rs were shown to be involved in drug reward and addiction. Indeed, He et al. reported that β-caryophyllene (BCP), a natural CB2R agonist, has therapeutic effects on methamphetamine (METH) abuse and dependence. Systematic administration of BCP dose-dependently inhibited METH self-administration in rats, indicating that BCP reduces METH reward, METH intake, and incentive motivation to seek and take METH.

A study by Reichenbach et al. demonstrated that CB2R ligands can influence the antinociceptive effects of morphine. The authors provide evidence of interactions between the CB2R selective agonist O-1966 and morphine that are probably mediated in part by the direct binding activity of O-1966 on the mu-opioid receptor. This interaction results in decreased potency of morphine to produce acute thermal antinociceptive effects, but can also lead to the potentiation of morphine antinociceptive tolerance, suggesting complex alterations in morphine signaling. However, O-1966 co-administration also blocked morphine hyperalgesia, and led to an attenuation of morphine tolerance when administration followed each morphine injection, perhaps due to well-documented and anti-inflammatory effects of CB2R agonism.

Keller et al. focused their study on p62 (sequestosome 1, SQSTM1) as an interaction partner for CB2R. In their research, JWH133 resulted in a weak osteoanabolic function in mice. Furthermore, this CB2R agonist modulated the bone cell differentiation in p62 KO animals comparable to Paget´s disease of bone indicating that p62 influences the function of CB2R. The authors emphasize the need for more studies to explore the possibility that this molecular link affects bone processes under pathological conditions or at older ages and is thus involved in disorganized bone turnover or osteoclast activity.

Ribeiro et al. demonstrated in their research article that the antidepressant-like behavior and the pro-neurogenic effect promoted by escitalopram (Esc) in stressed mice are in part mediated by CB2Rs. The chronic reduction of endogenous CB2R activity by the CB2 inverse agonist, AM630, attenuated the neuroplastic, the antidepressant- but not the anxiolytic-like effects of Esc.

Jayarajan et al. found that O-1966 inhibits allogeneic skin graft rejection *in vivo* supporting the fact that CB2R selective agonists may have the potential to act as a new class of compounds to prolong graft survival in transplant patients.

A theoretical study by El-Atawneh and Goldblum was used to build activity models for CB2R and other targets such as CB1R, peroxisome proliferator-activated receptor gamma (PPARγ), and 5-hydroxytryptamine receptor 4 (5-HT4R) for combinations that could be used for various indications such as Inflammatory Bowel Disease (IBD). Many dual CB2R/CB1R agonists were found together with CB2R agonists that acted also as 5-HT4R agonists. The authors also performed CB2R docking studies and found lower statistical performance of the docking ("structure-

based") compared to "Iterative Stochastic Elimination" ISE modeling ("ligand-based") suggesting that ISE modeling may be a better starting point for molecular discovery than docking.

Despite significant progress in CB2R research, including the studies reported in this Research Topic, several hurdles toward a CB2R-based therapy remain to be cleared. Detection of CB2R protein still represents a major challenge for researchers. There is an essential need for simultaneous use of multiple approaches to confirm the expression of CB2R in cells/tissues (e.g. RNA sequencing, digital droplet PCR, RT-PCR, RNA-scope, new fluorescent probes, radioligand binding, PET-CT with radioligands, etc) including proper positive and negative controls. The use of CB2R antibodies is not recommended in tissues. Thus, it is very difficult to do proper target validation of CB2R in diseases and consequently in clinical trials. Regarding CB2 agonists, most of the CB2-related therapeutic conclusions are based on the effects of nonselective and nonspecific first-generation ligands (JWH133, AM1241, and AM630, etc) and have not been confirmed with more selective ligands. Numerous problems exist with the first generation of commercially available ligands: 1) Selectivity and specificity issues (numerous off-targets and potential effect on CB1R *in vivo*), 2) Few of the ligands used were tested on mouse CB2Rs where the binding is often decreased (compared to humans); in some cases, the ligands, may even exert opposite effects on human vs. mouse receptors (e.g. agonist vs. inverse agonists), 3) These ligands have less than optimal bioavailability (e.g. short half-life, rapid degradation in the liver, etc), which is often ignored in the study designs, making the conclusions questionable, 4) The quality control is not good (degradation and contamination with endotoxins, and organic solvents are possible), and 5) Some of the ligands have biased signaling on CB2R, hence, introducing another layer of complexity in understanding the therapeutic effects/potential of these ligands. Furthermore, many studies conclude a role for CB2R in behavioral or other CNS-mediated effects based upon antagonism by SR144528. However, this compound has very poor brain penetrance, which complicates the interpretation of these studies.

Thus, better tools and multiple approaches using proper positive and negative controls are required to evaluate the CB2R expression in normal and pathological tissues in order to succeed with the target validation in preclinical and clinical studies/trials. Development of more selective and specific ligands with better PK properties and known effects on CB2R signaling (in mice, rats, primates, or humans) are required. The use of multiple validated approaches for CB2R detection, in concert with the new generation of CB2R ligands and genetic tools (e.g. tissue and cell specific CB2R knockouts, and GFP mice, etc) could enhance our understanding of the role of CB2R signaling in health and disease and facilitate development of successful therapies to ease human suffering.

Author contributions

All authors listed have made an equal, substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

UG is a full employee of F. Hoffmann-La Roche Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

An, D., Peigneur, S., Hendrickx, L. A., and Tytgat, J. (2020). Targeting cannabinoid receptors: Current status and prospects of natural products. *Int. J. Mol. Sci.* 21, 5064–5095. doi:10.3390/ijms21145064

Morales, P., Hernandez-Folgado, L., Goya, P., and Jagerovic, N. (2016). Cannabinoid receptor 2 (CB2) agonists and antagonists: A patent update. *Expert Opin. Ther. Pat.* 26, 843–856. doi:10.1080/13543776.2016.1193157

Soethoudt, M., Grether, U., Fingerle, J., Grim, T. W., Fezza, F., de Petrocellis, L., et al. (2017). Cannabinoid CB_2 receptor ligand profiling reveals biased signalling and off-target activity. *Nat. Commun.* 8, 13958–13971. doi:10.1038/ncomms13958

Whiting, Z. M., Yin, J., de la Harpe, S. M., Vernall, A. J., and Grimsey, N. L. (2022). Developing the cannabinoid receptor 2 (CB2) pharmacopoeia: Past, present, and future. *Trends Pharmacol. Sci.* 43 (9), 754–771. doi:10.1016/j.tips.2022.06.010

frontiers in Pharmacology

REVIEW
published: 30 July 2021
doi: 10.3389/fphar.2021.702675

Pharmacological Properties, Therapeutic Potential and Molecular Mechanisms of JWH133, a CB2 Receptor-Selective Agonist

Hebaallah Mamdouh Hashiesh[1], *Charu Sharma*[2], *Sameer N. Goyal*[3], *Niraj Kumar Jha*[4] *and Shreesh Ojha*[1,5]*

[1]Department of Pharmacology and Therapeutics, College of Medicine and Health Sciences, United Arab Emirates University, Al Ain, United Arab Emirates, [2]Department of Internal Medicine, College of Medicine and Health Sciences, United Arab Emirates University, Al Ain, United Arab Emirates, [3]Shri Vile Parle Kelavani Mandal's Institute of Pharmacy, Dhule, India, [4]Department of Biotechnology, School of Engineering and Technology (SET), Sharda University, Greater Noida, India, [5]Zayed Bin Sultan Center for Health Sciences, United Arab Emirates University, Al Ain, United Arab Emirates

The endocannabinoid system has attracted attention as a pharmacological target for several pathological conditions. Cannabinoid (CB2)-selective agonists have been the focus of pharmacological studies because modulation of the CB2 receptor (CB2R) can be useful in the treatment of pain, inflammation, arthritis, addiction, and cancer among other possible therapeutic applications while circumventing CNS-related adverse effects. Increasing number of evidences from different independent preclinical studies have suggested new perspectives on the involvement of CB2R signaling in inflammation, infection and immunity, thus play important role in cancer, cardiovascular, renal, hepatic and metabolic diseases. JWH133 is a synthetic agonist with high CB2R selectivity and showed to exert CB2R mediated antioxidant, anti-inflammatory, anticancer, cardioprotective, hepatoprotective, gastroprotective, nephroprotective, and immunomodulatory activities. Cumulative evidences suggest that JWH133 protects against hepatic injury, renal injury, cardiotoxicity, fibrosis, rheumatoid arthritis, and cancer as well as against oxidative damage and inflammation, inhibits fibrosis and apoptosis, and acts as an immunosuppressant. This review provides a comprehensive overview of the polypharmacological properties and therapeutic potential of JWH133. This review also presents molecular mechanism and signaling pathways of JWH133 under various pathological conditions except neurological diseases. Based on the available data, this review proposes the possibilities of developing JWH133 as a promising therapeutic agent; however, further safety and toxicity studies in preclinical studies and clinical trials in humans are warranted.

Keywords: cannabinoid receptor agonist, cannnabinoids, JWH133, synthetic cannabinoids, cannabinoid agonists

OPEN ACCESS

Edited by:
Meliha Karsak,
University Medical Center Hamburg-Eppendorf, Germany

Reviewed by:
Paula Morales,
Instituto de Química Médica (IQM), Spain
Sabatino Maione,
University of Campania Luigi Vanvitelli, Italy

***Correspondence:**
Shreesh Ojha
shreeshojha@uaeu.ac.ae

Specialty section:
This article was submitted to Experimental Pharmacology and Drug Discovery, a section of the journal Frontiers in Pharmacology

Received: *29 April 2021*
Accepted: *02 July 2021*
Published: *30 July 2021*

Citation:
Hashiesh HM, Sharma C, Goyal SN, Jha NK and Ojha S (2021) Pharmacological Properties, Therapeutic Potential and Molecular Mechanisms of JWH133, a CB2 Receptor-Selective Agonist. Front. Pharmacol. 12:702675. doi: 10.3389/fphar.2021.702675

INTRODUCTION

The endocannabinoid system comprises cannabinoid receptors (CB1R and CB2R), which play pivotal roles in various human biological and pathological conditions. Substantial effort has been focused on developing ligands for CB1R and CB2R, leading to hundreds of phyto- and synthetic cannabinoids with variable affinities linked to the treatment of several disorders (An et al., 2020). The

endocannabinoid signaling pathway restores homeostasis after damage; thus, it is the basis of therapeutic approaches to pain, inflammation, cancer, cardiovascular, and metabolic and neurodegenerative disorders (Fulmer and Thewke, 2018; Cristino et al., 2020). CB1R and CB2R also mediate the bioactivities of several phytocannabinoids (Morales et al., 2017), suggesting the importance of these receptors in the pharmacological functions of the cannabis plant. These findings encouraged the ongoing development of diverse synthetic cannabinoids with similar or different structures compared with endo- and phytocannabinoids.

The CB2R is a G protein-coupled receptor that regulates intracellular mechanisms by coupling with Gi/o proteins (Howlett, 2005). CB2R inhibits adenylyl cyclase activity to produce cyclic adenosine monophosphate (cAMP) and mediates mitogen-activated protein kinase (MAPK) activation (Bouaboula et al., 1996). As a therapeutic target, CB2R has significant advantages. First, CB1R is primarily localized in the human brain and is primarily responsible for the psycho-activity of D9-tetrahydrocannabinol (THC) and the harmful psychiatric adverse effects of CB1R ligands (Zou and Kumar, 2018). Conversely, CB2R is mainly expressed in the peripheral tissues, including the immune system, and regulates immunologic function, cell migration, and cytokine secretion (Jordan and Xi, 2019). CB2R is expressed to a lesser degree in the brain, although at lower levels than CB1R (Zou and Kumar, 2018). Despite the lower levels of CB2R expression in the peripheral and central nervous system, CB2R plays a key role in nociception and neuroinflammation (Morales et al., 2016). Researchers have developed selective CB2R agonists with remarkable *in vitro* and *in vivo* effectiveness and no undesired psychotropic effects. Examples of such CB2R selective agonists are JWH015, HU308, JWH133 and GW-405833 (Hanuš et al., 1999; Valenzano et al., 2005; Verty et al., 2015; Çakır et al., 2019b). Animal studies have shown that CB2R stimulation modulates several pathophysiological processes (Aghazadeh Tabrizi et al., 2016) and is implicated in controlling different pathological conditions, including pain (Shang and Tang, 2017), inflammation (Turcotte et al., 2016), atherosclerosis (Carbone et al., 2014), diabetes (Basha and Sankaranarayanan, 2014), cancer (Elbaz et al., 2017), and cardiovascular disease (Steffens and Pacher, 2012). A clinical study of a CB2R agonist demonstrated effective mitigation of neuropathic pain (Gertsch et al., 2008). The neuroprotective effects of JWH133 will be comprehensively reviewed in another review. Thus, CB2R-specific therapeutic targeting is promising for discovering new therapies without adverse psychoactive effects associated with CB1R.

Synthetic Cannabinoids

Synthetic cannabinoids are diverse in chemical structure and function. They were initially used as pharmacological tools for delineating the cannabinoid receptor-induced activity (Howlett and Abood, 2017). Thus, their structural features allow them to bind to one of the recognized cannabinoid receptors found in human cells, CB1 and/or CB2 (Hervás, 2017). Some of these synthetics appeared on the market as substitutes to phytocannabinoids for recreational drug use. Diverse synthetic cannabinoids have been developed recently with subtle structural changes (Morales et al., 2016; Hervás, 2017). These synthetic cannabinoids are structurally classified as classical, non-classical, amino-alkyl indoles, and eicosanoids (Badal et al., 2017), and many have been used in pharmacological studies, including those on structure-activity relationships, receptor binding, and drug mechanisms of action.

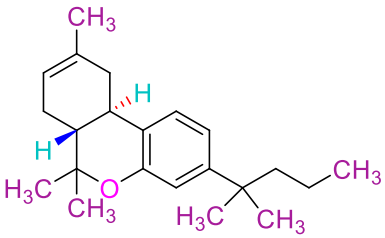
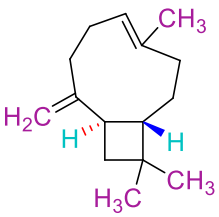
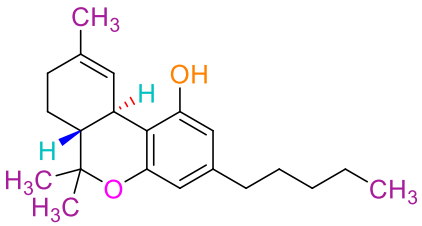
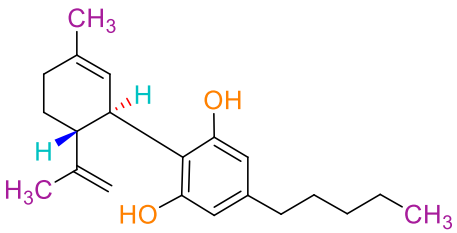
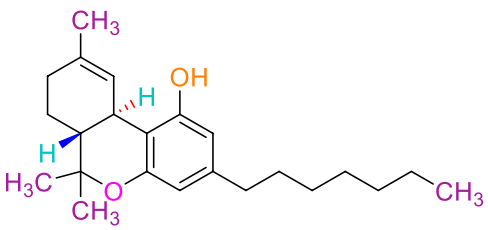
New selective CB2 agonists are now the focus of academic and commercial efforts, and a growing number of preclinical and *in vitro* studies have yielded encouraging findings. However, there has been limited success in clinical trials owing to a lack of translation from animal models to humans and differences among species (Morales et al., 2016; Ghonim et al., 2019; Mugnaini et al., 2019). The most extensively used pharmacological agent is the classical CB2R-selective cannabinoid JWH133 produced by Dr John Huffman Huffman et al, (1999). JWH133 binds with greater affinity to CB2R than CB1R and acts as a potent CB2R-selective agonist (Huffman et al., 1999).

JWH133

JWH133 is a synthetic agonist devoid of psychogenic activity, with 200-fold greater CB2R selectivity than CB1R, with Ki of 3.4 nM and inhibitor constant of 677 nM (Huffman et al., 1999). JWH133 had no CB1R activity, such as antinociceptive, cataleptic, and hypothermic activities, in mouse cannabinoid triads (Soethoudt et al. (2017)). JWH133 is a highly selective full agonist of mCB2R but functionally inactive on hCB1R, with a maximum activity of only 20% at 10 mM, without off-target activities at active concentrations. Moreover, it has a moderate volume of distribution ($1–3\ l\ kg^{-1}$), with a half-life of only 1 h.

JWH133 belongs to the class of Δ8-tetrahydrocannabinol derivatives, which resembles the Δ9-tetrahydrocannabinol. Particularly, the research team of Huffman et al. revealed that the deletion of the phenolic OH group from HU210, non-selective CBRs agonist (Mechoulam et al., 1990), to obtain JWH051, did not markedly affect affinity for CB1R, but significantly increased CB2R affinity and selectivity (Huffman et al., 1996). The additional removal of alcoholic group and further modifications of the alkyl chain resulted in more CB2R-selective ligands, among them, JWH133 is remarkable: it is a potent CB2R agonist, with a Ki of 3.4 nM and a high selectivity for CB2R (around 200 folds over CB1R) (Huffman et al., 1999; Pertwee, 1999). A Comparision of the binding type and affinity of JWH133 with main phytocannabinoids are summarized in **Table 1**. The most significant plant-derived cannabinoid is Δ9-tetrahydrocannabinol (Δ9-THC). The psychogenic effects of cannabis are mostly attributed to partial agonistic activity of Δ9-THC at CB1Rs (Turner et al., 2017; Amin and Ali, 2019). In addition, Δ9-THC is also featured as a partial agonist at CB2Rs (Pertwee, 2008; Turner et al., 2017). Moreover, it has been shown that cannabidiol (CBD) has a very low affnity for CB1R and CB2R (Turner et al., 2017). CBD acts as an antagonist/inverse agonist at certain concentrations below which it binds to both CB1 and CB2 orthosteric sites (Badal et al., 2017). Lately, various studies have displayed that CBD acts as a negative allosteric modulator of CB1R, which modifies the potency and effcacy of the orthosteric ligands but does not activate the receptor itself (Chung et al., 2019; Tham

TABLE 1 | A comparision of JWH133 and main phytocannabinoids in terms of binding type and binding affinity.

Cannabinoids	Binding type/CB	CB1 K_i value (nM)	CB2 K_i value (nM)	References
 JWH 133	Full agonist/CB2	677	3.4	Pertwee et al. (2010)
 ß-caryophyllene (BCP)	Full agonist/CB2	NA	155	Gertsch et al. (2008)
 Δ^9 Tetrahydrocannabinol	Partial agonist/CB1,CB2	5 to 80	1.7 to 75	Turner et al. (2017)
 Cannabidiol (CBD)	Antagonist/inverse agonist, negative allosteric modulator/CB1 Partial agonist/CB2	73 to >10,000	370 to >10,000	Turner et al. (2017)
 Δ^9-tetrahydrocannabiphorol (D9- THC)	Agonist/CB1,CB2	1.2	6.2	Citti et al. (2019)

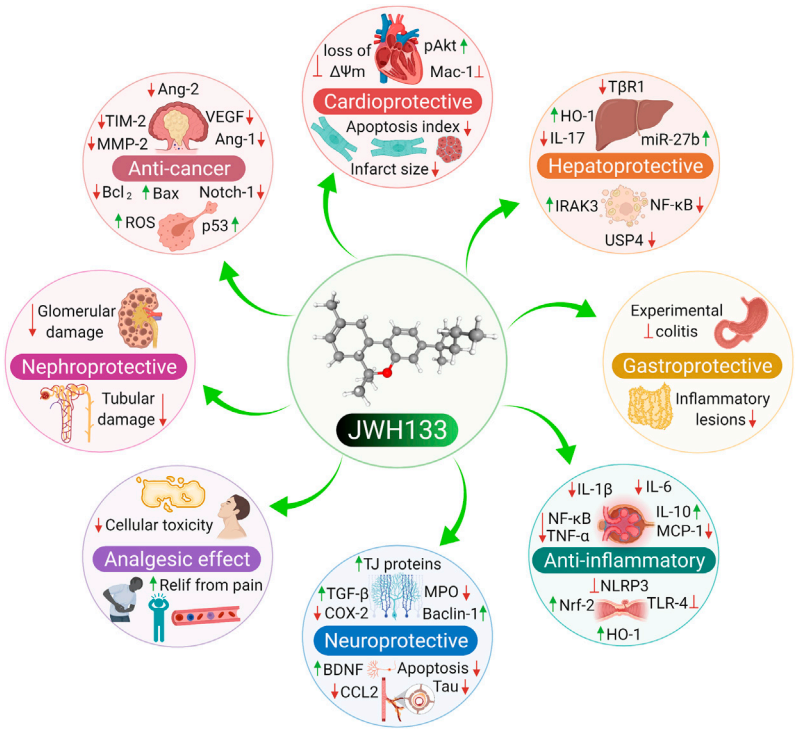


FIGURE 1 | The Pharmacological properties of JWH133.

et al., 2019). For CB2R, CBD acts as a partial agonist (Tham et al., 2019). Comparing with another natural cannabinoid with high selectivity to CB2R, β-caryophyllene (BCP), which selectively and competitively interact with the CP55,940 binding site (i.e., THC binding site) of the CB2R, with 165-fold selectivity over CB1R, where it showed a weak partial agonism (Gertsch et al., 2008).

JWH133 exhibits anticancer (Sánchez et al., 2001; Qamri et al., 2009), cardioprotective (Yu et al., 2019), hepatoprotective (Wu et al., 2019), gastroprotective (Tartakover Matalon et al., 2020), nephroprotective (Feizi et al., 2008), anti-inflammatory (Çakır et al., 2020), antihyperalgesic (Cabañero et al., 2020), and immunomodulatory activities (Zhu et al., 2019). It has also been demonstrated to exert neuroprotective effects in Parkinson's disease, ischemic stroke, depression, anxiety, Alzheimer's disease, epilepsy, and neuropathic pain (Kruk-Slomka et al., 2015; Sheng et al., 2019; Cao et al., 2020; Ivy et al., 2020; Jia et al., 2020; Jing et al., 2020; Zhao et al., 2020). The neuroprotective role of JWH133 has been well demonstrated in a large number of experimental studies and currently not included in the present study due to space constraints. The neuroprotective effects of JWH133 will be reviewed comprehensively in another successive review. Several *in vitro* and animal studies have verified the biological properties of JWH133. The pharmacological properties of JWH133 are depicted in **Figure 1**. The pharmacological activities, mechanism and therapeutic potential of JWH133 in the *in vivo* studies and in the *in vitro* studies are summarized in **Tables 2** and **3**, respectively.

Collectively, the modulation of CB2R signaling represents a promising, nonpsychoactive pharmacological target that can be harnessed to treat a wide number of disorders. This review emphasizes the polypharmacological properties and therapeutic potential of JWH133, its molecular mechanism, and signaling pathways in different pathological conditions except neuronal diseases as the neuroprotective effects of JWH133 are discussed in another review. The neuroprotective role of JWH133 has been well demonstrated in a large number of experimental studies and is not included in the present study.

THERAPEUTIC POTENTIAL OF JWH133

JWH133 in Inflammation

Increasing evidence suggests that CB2R stimulation has anti-inflammatory effects in various inflammatory diseases (Storr et al., 2009; Hao et al., 2010; Gui et al., 2015). CB2R stimulation also inhibits the production of inflammatory cytokines and chemokines and induces the secretion of anti-inflammatory cytokines (He et al., 2019). Indeed, CB2R-deficient mice have an exaggerated inflammatory response (Turcotte et al., 2016). Thus, therapeutic approaches that target the modulation of CB2R signaling might hold promise for the treatment of inflammatory pathologies. The anti-inflammatory activity and mechanisms of JWH133 are displayed in **Figure 2**.

Local application of JWH133 to the joints of normal rats induced a dose-dependent increase in synovial blood flow. This effect was abolished by pretreatment with AM630 or the TRPV1 antagonist SB366791, indicating that TRPV1 is necessary for CB2R-mediated activity (McDougall et al., 2008). CB2R primarily

TABLE 2 | Pharmacological activities, mechanism and therapeutic potential of JWH133 in the *in vivo* studies.

Experimental model	JWH133 doses	Indication/Disease	Demonstrated actions and mechanisms	References
Compound 48/80-induced inflammation in BALB/cJBom mice	20 and 20 μg/mouse i.p	Inflammation		Jonsson et al. (2006)
Cecal ligation and puncture (CLP)-induced polymicrobial sepsis model in Sprague-Dawley rats	0.0, 1, and 5 mg/kg, i.p	Inflammation	Inhibits the apoptosis and NF-κB signaling	Çakır et al. (2020)
C57Bl/6J mice injected with LPS-induced vascular inflammation	10 mg/kg ip	Atherosclerosis	Attenuates the TNF-α- and/or endotoxin induced expression of ICAM-1 and VCAM-1 and vascular endothelium adhesion	Rajesh et al. (2007)
Shear stress-induced atherogenesis and plaque vulnerability in apoE$^{-/-}$ mice	5 mg/kg, i.p. for 5 days/week	Atherosclerosis	Suppresses neutrophil production of MMP-9 via attenuation of ERK1/2 phosphorylation	Montecucco et al. (2012)
Balloon-induced neointima in WT, ApoE$^{-/-}$, CB2$^{-/-}$ mice	5 mg/kg, i.p. 1 h before surgery and for 28 days after	Atherosclerosis	Modulates neointima formation via decreasing of proliferation, macrophage infiltration, and smooth muscle cell content	Molica et al. (2012)
Monosodium iodoacetate-induced osteoarthritis pain in Sprague-Dawley rats	1 mg/kg, s.c, for 28 days post-MIA injection	Osteoarthritis	Stimulation of CB2R diminished central sensitization process, leading to mitigation of pain behavior	Burston et al. (2013)
Subcutaneous xenografts mice and male PyMT transgenic mice	5 mg/kg, i.p. for 4 weeks	Breast cancer	Modulates COX-2/prostaglandin E2 signaling pathways -Induces cell cycle arrest and apoptosis	Qamri et al. (2009)
MMTV-neu mice, a model of ErbB2-driven metastatic breast cancer	0.05 mg/animal/day, twice a week for 90 days	Breast cancer	Suppression of the pro-tumorigenic Akt pathway	Caffarel et al. (2010)
Rag-2$^{-/-}$ mice, a mouse model of glioma	50 μg for 8 days, intratumoral	Brain cancer	Induces apoptosis via ceramide synthesis and ERK1/2 activation	Sánchez et al. (2001)
Glioma and astrocytoma xenografts	50 μg/d for 8 days or 25 days, intratumoral	Brain cancer	inhibition of vascular endothelial cell migration and survival as well as the decrease in expression of proangiogenic factors (VEGF and angiopoietin-2) and MMP-2 in the tumors	Blázquez et al. (2003)
Glioma xenografts mice	50 μg/d for 8 days, peritumorally	Brain cancer	Downregulates MMP-2 via inhibiting sphingolipid ceramide synthesis	Blázquez et al. (2008)
Nude mice inoculated with glioma cells	1.5 mg/kg, s.c	Brain cancer	Decreases efficiency of glioma stem cells and glioma formation due to reduced neurosphere formation and cell growth	Aguado et al. (2007)
SCID CB-17 mice inoculated with A549 cells	1 mg/kg, peritumorally for 28 days	Lung cancer	Decreases tumor proliferation and neo-vascularization along with enhanced apoptotic death	Preet et al. (2011)
Nude mice inoculated with PDV.C57 epidermal tumor cells	1,580 μg for 11 days locally infused at a rate of 0.52 μl/h	Skin cancer	Interferes with the tumor angiogenic switch together with the direct stimulation of apoptosis on tumor cells, which in turn inhibits tumor proliferation Abolishes EGFR function	Casanova et al. (2003)
Nude mice bearing B16 melanoma cells	50 μg/day, daily for 8 days	Skin cancer	Rise in apoptosis and reduction of tumor vascularization, and vascular density	Blázquez et al. (2006)
Quetiapine-induced cardiotoxicity in Balb/C mice	5 mg/kg, i.p. for 21 days	Cardiotoxicity	Modulates necroptosis process	Li et al. (2019b)
Ethanol-induced cardiotoxicity in C57BL/6J mice	3 mg/kg, i.p. 1 h before ethanol administration for 30 or 45 days	Cardiotoxicity	Attenuates RIP1/RIP3/MLKL-mediated necroptosis	Liu et al. (2020b)
Clozapine-induced cardiotoxicity in C57BL/6J mice	2 mg/kg, i.p. before clozapine administration for 14 days	Cardiotoxicity	Attenuates myocardial inflammation, fibrosis, and myocardial injury	Li et al. (2019a)
I/R injury of the C57Bl/6 mouse heart	20 mg/kg, i.p. 5 min before reperfusion	Myocardial infarction	Inhibition of oxidative stress and neutrophil recruitment and activation of ERK 1/2 and STAT3 pathway	Montecucco et al. (2009)
I/R injury of the Sprague-Dawley rats heart	20 mg/kg, I.V. 5 min before ischemia	Myocardial infarction	Prevents apoptotic cell death via suppressing the intrinsic mitochondrial apoptotic process and implication of the PI3K/Akt signaling pathway	Li et al. (2013a)
I/R injury of the C57Bl/6 WT and CB2$^{-/-}$ mice heart	3 mg/kg, I.V. 5 min before reperfusion	Myocardial infarction	Prevention of oxidative stress-induced cardiac myocyte and fibroblast apoptosis and the suppression of myofibroblast activation	Defer et al. (2009)
I/R injury of the C57Bl/6 mouse heart	1, 3, and 10 mg/kg, i.p. 5 min before ischemia	Myocardial infarction	Modulation of NLRP3 inflammasome pathway	Yu et al. (2019)

(Continued on following page)

TABLE 2 | (*Continued*) Pharmacological activities, mechanism and therapeutic potential of JWH133 in the *in vivo* studies.

Experimental model	JWH133 doses	Indication/Disease	Demonstrated actions and mechanisms	References
HFD-induced obese mice model (60% kcal fat content) for 10 weeks	5, 10 mg/kg, i.p. for 21 days	Obesity	Attenuates pro-inflammatory M1 macrophage cytokines through the Nrf2/HO-1 mechanism	Wu et al. (2020)
db/db mice	0.15, 0,5, 1 and 3 mg/kg, s.c	Diabetic neuropathy	-Activation of antioxidant Nrf2/HO-1pathway potentiated the antiallodynic effects	McDonnell et al. (2017)
Seven-day-old swiss CD-1 mice	1.5 mg/kg for 5 h (acute treatment) or for 5 consecutive days per week for 2 and 3 weeks (chronic treatment)	Spermatogenesis	Accelerates the spermatogenesis process and regulates transcription of the c-Kit and Stra8 genes at meiotic entry through specific alterations of histone modifications	Di Giacomo et al. (2016)
Trinitrobenzene sulfonic acid (TNBS)-induced colitis in wildtype and $CB2^{-/-}$ mice	20 mg/kg, i.p. 30 min before the induction of colitis and then twice daily for 3 days	Colitis	Reduces intestinal inflammation via a decrease in colonic adhesions and myeloperoxidase activity	Storr et al. (2009)
Oil of mustard-induced model of colitis in CD-1 mice Dextran sulfate sodium (DSS)-induced colitis in BALB/c mice	20 mg/kg, i.p. 30 min before the induction of colitis and then twice daily for 3 days	Colitis	Reductions in overt inflammatory damage and bowel dysmotility	Kimball et al. (2006)
IL-$10^{-/-}$ mice model of colitis Dextran sulfate sodium (DSS)-induced colitis in BL/6 mice	1, 2.5, 5 mg/kg i.p. for 7 weeks	Colitis	Anti-inflammatory activities through inhibiting activated T cells, and inducting apoptosis in T cells	Singh et al. (2012)
Oil of mustard-induced model of colitis in CD-1 mice	1 mg/kg s.c	Colitis	Modulation of GI motility attenuating the associated diarrhea	Kimball et al. (2010)
LPS-stimulated transit in Sprague–Dawley rats	1 mg/kg s.c	Colitis	Suppresses GI transit via inhibition of cyclooxygenase	Mathison et al. (2004)
Cerulein-induced acute pancreatitis in WT and $MK2^{-/-}$mice	5 μg/g, i.p. 30 min before the induction of acute pancreatitis	Acute pancreatitis	Suppression of JNK, stimulation of p38 and MK2-signaling pathway reducing the pancreatic injury	Michler et al. (2013)
GalN/LPS-induced acute liver injury in C57BL/6 mice	20 mg/kg i.p, two doses administered 24 and 2 h before the GalN/LPS injection	Acute liver injury	Mediates an M1 to M2 transition in macrophages and modulates the expression of miR-145 to hamper the TLR4 signaling stimulation	Tomar et al. (2015)
Alcohol-fed WT and $CB2^{-/-}$ mice induced fatty liver	3 mg/kg, i.p. for 10 days	Alcoholic liver disease	Anti-inflammatory effects via upregulating of HO-1 in macrophages	Louvet et al. (2011)
Ethanol-fed WT and $CB2Mye^{-/-}$, and $ATG5Mye^{-/-}$ mice	3 mg/kg, i.p. for 10 days	Alcoholic liver disease	Stimulates autophagic process via upregulating of HO-1 in macrophage that mediates the anti-inflammatory and anti-steatogenic activities of CB2 receptors	Denaës et al. (2016)
CCl4-Induced liver cirrhosis in Wistar rats	1 mg/kg, s.c. for 9 days	Liver cirrhosis	Mitigates hepatic fibrosis via decreasing collagen content, α-SMA, and increasing the proteolytic enzyme MMP-2	Muñoz-Luque et al. (2008)
Bile duct ligation (BDL)-induced cirrhotic rats	1 mg/kg, i.p. from days 35–42 days of BDL	Liver cirrhosis	Suppresses mesenteric blood flow leading to mitigation of liver fibrosis	Huang et al. (2012)
Thioacetamide or bile duct ligation-induced cirrhotic rats	1 mg/kg, orally for 2 weeks	Liver cirrhosis	Improves phagocytosis of peritoneal macrophages through suppressing the TNFα signaling, pro-inflammatory cytokines secretion and oxidative stress	Yang et al. (2014)
CCl4-Induced liver cirrhosis in sprague–Dawley rats	10 mg/kg, i.p. 2 h prior to the start of portal pressure measurements	Liver cirrhosis	Mediates HO-1 pathway which decreases vasoconstrictor production and portal hypertension related to $PPAR\gamma$ and CB2R	Steib et al. (2013)
CCl4-Induced liver injury in WT and $CB2^{-/-}$ mice	3 mg/kg, i.p. before CCl4	Liver fibrosis	Mitigates hepatic injury and promotes hepatic regeneration through a paracrine mechanism including hepatic myofibroblasts and antifibrogenic effects	Teixeira-Clerc et al. (2010)
CCL4 plus clodronate- induced liver injury in C57BL/6 mice	10 mg/kg, i.p. before CCl4	Liver fibrosis	Transcriptional regulation of the CB2 receptor gene in hepatocytes by LXRα resulting in inhibition of USP4-stabilizing TβRI through miR-27b	Wu et al. (2019)
Hepatic ischemia/reperfusion in WT and $CB2^{-/-}$ mice	i.p. 60 min prior to the occlusion of the hepatic artery and the portal vein	Hepatic ischemia/reperfusion	Attenuates oxidative stress and the infilteration of inflammatory cells	Bátkai et al. (2007)
Hepatic ischemia/reperfusion in C57BL/6 mice	0.2 mg/kg, i.p. 24 h before the experiment	Hepatic ischemia/reperfusion	Selective depletion or deactivation of HSCs through CB2R activation reduces CD4+ T cell–dependent I/R injury	Reifart et al. (2015)

(Continued on following page)

TABLE 2 | (*Continued*) Pharmacological activities, mechanism and therapeutic potential of JWH133 in the *in vivo* studies.

Experimental model	JWH133 doses	Indication/Disease	Demonstrated actions and mechanisms	References
Collagen-induced arthritis (CIA) mice	1, 4 mg/kg, i.p. from day 15 to day 35	Rheumatoid arthritis	Inhibits production of pro-inflammatory cytokines, and prevents formation of bone-resorbing cells	Fukuda et al. (2014)
Collagen-induced arthritis (CIA) in mice	10 mg/kg, i.p. from day 22 to day 45	Rheumatoid arthritis	Inhibits osteoclastogenesis and inflammation-mediated bone destruction via inhibiting NF-kB signaling pathway	Zhu et al. (2019)
Experimental autoimmune uveoretinitis in B10.RIII mice and BALB/c mice	0.015–1.5 mg/kg, i.p	Autoimmune uveoretinitis	Anti-inflammatory activity through suppressing the stimulation and function of autoreactive T cells and averting leukocyte trafficking into the inflamed retina	Xu et al. (2007)
Hypochlorite-induced systemic sclerosis in BALB/c, C57BL/6 $CB2^{-/-}$ mice	1,1.5, 2, 2.5, 3, and 4 mg/kg, i.p. for 6 weeks	Systemic sclerosis	Inhibits systemic fibrosis, skin fibroblast proliferation and autoimmune reaction	Servettaz et al. (2010)
I/R Injury of albino NMRI mice kidney	0.2, 1 and 5 mg/kg, i.p. 30 min prior initiation of reperfusion-induced ischemia	Renal ischemia reperfusion	Suppression of inflammatory cytokines secretion by NF-κB and mitigates apoptosis	Feizi et al. (2008)
Cyclophosphamide-induced cystitis in C57BL/6J mice	1 mg/kg, i.p. 30 min before cyclophosphamide	Cystitis	Activates autophagy via AMPK-mTOR pathway mitigating bladder inflammatory responses and severity of cystitis	Liu et al. (2020a)
Bleomycin-induced dermal fibrosis in WT and $CB2^{-/-}$ mice	2.5 mg/kg, i.p. for 4 weeks	Dermal fibrosis	-Antifibrotic effects by preventing the infiltration of leukocytes into skin lesion	Akhmetshina et al. (2009)
Paraquat-induced lung injury in Sprague-Dawley rats	5 and 20 mg/kg, i.p. before paraquat administration	Lung injury	Mitigates lund injury via suppressing the stimulation of MAPKs and NF-kB signaling	Liu et al. (2014)
Bleomycin-induced pulmonary fibrosis in C57BL/6 mice	2.5 mg/kg, i.p. for 21 days	Pulmonary fibrosis	Anti-fibrotic activity via repressing TGF-β1/Smad2 signaling pathway	Fu et al. (2017)
Nicotine-induced lung fibrosis in swiss mice	1 mg/kg, i.p. before nicotine administration	Pulmonary fibrosis	Anti-fibrotic activity via downregulating the expression of connective tissue growth factor, and α-SMA	Wawryk-Gawda et al. (2018)
Lung ischemia/reperfusion Injury in C57BL/6 mice	5 mg/kg, i.p. 5 min before occlusion	Lung ischemic reperfusion injury	Attenuates the inflammation and oxidative stress relies on activation of PI3K/Akt signaling	Zeng et al. (2019)
Lung ischemia/reperfusion Injury in C57BL/6 mice	5 mg/kg, i.p. 5 min before occlusion	Lung ischemic reperfusion injury	Inhibits oxidative stress via downregulation of NOX2	Huang et al. (2020)
Respiratory syncytial virus challenged Balb/c mice	i.p. for 5 days	Acute respiratory tract infections	Anti-inflammatory activity via reducing the influx of BAL cells, leukocyte migration into the lungs, and cytokines/chemokines	Tahamtan et al. (2018)
Skeletal muscle contusion model in Sprague-Dawley rat	10 mg/kg, i.p. injected 30 min after contusion and once a day for 13 days	Skeletal muscle contusion	Inhibits fibrosis and improves muscle regeneration via reducing TGF-β1, fibronectin-EIIIA and α-SMA, decreases production of myofibroblasts, and concurrently upregulation of MMP-1/2	Yu et al. (2015)
Incised skin wound model in BALB/c mice	3 mg/kg, i.p. for 1–9 days	Skin wound healing	Inhibition of inflammatory process by attenuating infiltrated M1 macrophage cells and enhancing M2 macrophage phenotype	Du et al. (2018)

localizes on immunocytes, suggesting that these cells mediate the vasomotor activities of JWH133. However, JWH133-induced vasodilation was markedly reduced in acute and chronically arthritic knees, suggesting that the expression and sensitivity of articular CB2R are altered in inflamed joints. CB2R activation in the knee joint may yield vasodilation via vanilloid TRPV1 channels. Further studies are needed to characterize the molecular and biochemical pathways linking TRPV1 and CB2R.

Plasmacytoid dendritic cells (pDC) play a pivotal role in initiating host immunity. Enhanced and chronic pDC stimulation is a characteristic of autoimmune disorders such as systemic lupus erythematosus and rheumatoid arthritis (Colonna et al., 2004). Treatment of pDC with JWH133 suppressed CpG-stimulated IFNα and TNFα responses (Henriquez et al., 2019). JWH133 also suppressed key markers of pDC stimulation, including phosphorylated levels of IRF7, TBK1, NFκB, and IKKγ. Similarly, AKT phosphorylation at S473 and T308 was differentially modified by treatment with JWH133. Thus, CB2R activation represents a potential target for treating inflammatory pathologies caused by aberrant pDC activity.

Tissue mast cells are involved in several inflammatory conditions and play a key role in multiple sclerosis and rheumatoid arthritis (Puxeddu et al., 2003). In a mouse model, JWH133 injection reversed inflammation induced by injecting the compound 48/80 into the ear pinna (Jonsson et al., 2006). Interpreting the CB2-agonist effect of JWH133 is complicated because CB2R antagonism by SR144528 also produced an anti-inflammatory effect in mice. *In vitro* results were discordant because JWH133 did not affect mast cell degranulation induced by compound 48/80 in mouse skin slices, perhaps owing to

TABLE 3 | Pharmacological activities, mechanism and therapeutic potential of JWH133 in the *in vitro* studies.

Experimental model	JWH133 concentration	Indication/Disease	Demonstrated actions and mechanisms	References
Plasmacytoid dendritic cells stimulated with CpGODN Type A 2216	0.001, 0.01, and 0.1 μM	Inflammation	Suppresses CpG-stimulated IFNα and TNFα dependent on modifying the phosphorylation of AKT	Henriquez et al. (2019)
LPS/IFN-γ or Theiler's virus (TMEV)-activated macrophages	10 nM 100 nM, 1 μM, and 5 μM	Inflammation	Inhibits IL-12p40 production and enhances IL-10 biosynthesis via activation of ERK1/2 MAP kinase	Correa et al. (2005)
Human coronary artery endothelial cells (HCAECs) activated with TNF-α	0.5, 2.5, and 4 μM	Atherosclerosis	Attenuates TNF-α-triggered NF-κB and RhoA activation, upregulates of adhesion molecules ICAM-1 and VCAM-1, decreases expression of monocyte chemoattractant protein, TEM of monocytic THP-1 cells, and monocyte-endothelial adhesion	Rajesh et al. (2007)
Human coronary artery smooth muscle cells (HCASMCs) activated with TNF-α	0.5–4 μM	Atherosclerosis	Mitigates the activation of induced Ras, mitogen-activated protein kinases (p38 MAPK, ERK ½), stress-activated protein kinases (SAPK)/Jun amino-terminal kinases (JNK) and Akt	Rajesh et al. (2008)
Human neutrophils	0.3 and 1 μM	Atherosclerosis	Suppresses neutrophil production of MMP-9 via attenuation of ERK1/2 phosphorylation	Montecucco et al. (2012)
Normal-cultured and oxidative low-density lipoprotein (OxLDL)-loaded RAW264.7 and primary macrophages	0.1, 1, and 10 μM	Atherosclerosis	Improves efferocytosis via increasing expression of tyrosine kinase family phagocytic receptors, inhibition of RhoA GTPase stimulation, and alleviation of oxidative/inflammation responses	Jiang et al. (2016)
Human osteoblastic hFOB 1.19 cells	1, 2, 5 10, and 20 μM	Osteoporosis	Osteogenic differentiation mediated by CB2R dependent mechanism involved autophagy activation and p62- mediated Nrf2 degradation	Xu et al. (2020)
Methylprednisolone-induced osteoclast overactivity from healthy donors	100 nM from day 14 to day 21	Osteoporosis	Reduces bone resorption dependent on PKC βII signaling	Bellini et al. (2017)
MDA-MB231 and MDA-MB468 cells	0.1–10 μmol/L	Breast cancer	Inhibits cell proliferation and migration	Qamri et al. (2009)
Rat glioma C6 cell	100 nM	Brain cancer	Induces apoptosis via ceramide synthesis and ERK1/2 activation	Sánchez et al. (2001)
Human umbilical vein endothelial cells (HUVECs)	25 nM	Brain cancer	Direct inhibition of vascular endothelial cell migration and survival as well as the decrease of the expression of proangiogenic factors (VEGF and angiopoietin-2) and MMP-2 in the tumors	Blázquez et al. (2003)
Glioma stem-like cells and glioma cell lines U87MG and U373MG	30 nM	Brain cancer	Stimulates glia cell differentiation in a CB2R-related mechanism	Aguado et al. (2007)
A2058 melanoma cells	10 μM for 4 h	Brain cancer	Reduces adhesion and transmigration of melanoma cells through the cerebral endothelium	Haskó et al. (2014)
A549 cells and HUVECs	10^{-4}–10^{-8} mol/l	Lung cancer	Anti-proliferative and anti-angiogenic potential Downregulates MMP-2 activity	Vidinský et al. (2012)
A549 cells co-cultured with huvec	3 μM	Lung cancer	Increases tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) production from lung cancer cells and a consequent stimulation of ICAM-1 expression, thereby modifying the tumor cells microenvironment and inhibiting the angiogenesis	Ramer et al. (2014)
Human lung macrophage stimulated with LPS	1 μM	Lung cancer	Modulates tumor vascularization via reduction of macrophage-derived angiogenic and lymphangiogenic factors	Staiano et al. (2016)
T-ALL patients and Jurkat cell line	100 nM	Leukemia	Anti-proliferative, pro-apoptotic and cell cycle arrest	Punzo et al. (2018a)
ARO/IL-12, ARO and ARO/CB2 thyroid carcinoma cells	2 μM for 24 h	Thyroid carcinoma	IL-12-mediated CB2 upregulation rendered the thyroid cancer cells more responsive to CB2 agonist-induced apoptosis and remission of the tumors	Shi et al. (2008)
Saos-2, MG-63, MNNG/HOS, KHOS/NP, Hs888Lu and U-2 OS Osteosarcoma cells	100 nM for 24 h	Osteosarcoma	Anti-proliferative, pro-apoptotic, anti-invasive effect with downregulation of Notch-1 and MMP-2	Punzo et al. (2017)
Isolated perfused rat hearts subjected to 30 min global ischemia followed by 120 min reperfusion	1,10, and 100 nmol/L for 15 min before I-R treatment	Myocardial infarction	Increases phosphorylated ERK1/2 and preventing MPTP opening	Li et al. (2014)

(Continued on following page)

TABLE 3 | (*Continued*) Pharmacological activities, mechanism and therapeutic potential of JWH133 in the *in vitro* studies.

Experimental model	JWH133 concentration	Indication/Disease	Demonstrated actions and mechanisms	References
Adult cardiac myocytes from WT or CB2/ mice	1 μM	Myocardial infarction	Prevention of oxidative stress-induced cardiac myocyte and fibroblast apoptosis and the suppression of myofibroblast activation	Defer et al. (2009)
Mice cardiomyocytes under oxygen-glucose deprivation (ODG)	1, 10, and 100 nM 10 min before OGD challenge	Myocardial infarction	Modulation of NLRP3 inflammasome pathway	Yu et al. (2019)
Mouse RAW264.7 macrophages and 3T3-L1 fibroblasts	1 or 3 μM for 24 h	Obesity	Attenuates pro-inflammatory M1 macrophage cytokines through the Nrf2/HO-1 mechanism	Wu et al. (2020)
Obese-derived white adipocyte (ADP)	100 nM	Obesity	Mitigates the obesity-associated inflammation, and the excess lipid storage in white adipose tissue WAT through modulating perilipin expression, up-regulating IL-4, and stimulating UCP-1 signaling	Rossi et al. (2016)
Rat m5F insulinoma β-cells	10^{-6} M	Diabetes mellitus	CB2R stimulation is linked to Ca2+ mobilization from the endoplasmic reticulum stores leading to insulin release in pancreatic β-cells	De Petrocellis et al. (2007)
Isolated uterus from female ICR mice stimulated with exogenous PGE2	10^{-8}–10^{-5} M, for 20 min	Female reproduction	Mitigation of myometrial contractility dependent on the suppression of prostaglandin release/ synthesis	Pagano et al. (2017)
SPG germ cells obtained from testes of immature 7-day-old swiss CD-1 mice	10^{-6} M for 0–60 min	Spermatogenesis	Pro-differentiated effect via induction of the phosphorylated ERK 1/2 MAPK in spermatogonia and their progression toward meiosis	Grimaldi et al. (2009)
SPG germ cells obtained from testes of immature 7-day-old swiss CD-1 mice	1 μM for 24 h	Spermatogenesis	Accelerates the spermatogenesis process and regulates transcription of the c-Kit and Stra8 genes at meiotic entry through specific alterations of histone modifications	Di Giacomo et al. (2016)
Mucosal samples from areas of inflamed/ uninflamed colon from IBD patients and Caco-2 cell line	10 μM for 6 h	Colitis	Enhances colon cells proliferation and migration and affects secretome characteristics that facilitate mucosal healing	Tartakover Matalon et al. (2020)
Isolated ileum from Sprague-Dawley rats injected with LPS	10^{-2} M	Colitis	Reduces the accelerated contraction induced by LPS via downregulation of the FOS expression in enteric glial and neurons	Duncan et al. (2008)
RAW264.7 macrophages activated with LPS	5 μM for 24 h	Alcoholic liver disease	Anti-inflammatory effects via upregulating of HO-1 in macrophages	Louvet et al. (2011)
RAW264.7 macrophages from CB2Mye$^{-/-}$ mice activated with LPS	5 μM for 6 h	Alcoholic liver disease	Stimulates autophagic process in macrophage mediated the anti-inflammatory and anti-steatogenic activities of CB2R	Denaës et al. (2016)
Isolated kupffer cells activated with zymosan A and LPS	5 μM for 3 h	Liver cirrhosis	Mediates HO-1 pathway which decreases vasoconstrictor production and portal hypertension related to PPARγ and CB2R	Steib et al. (2013)
Cultured Th17 lymphocytes IL-17-induced inflammatory Response on macrophages and hepatic myofibroblasts	5 μM	Liver fibrosis	Decreases IL-17 production by Th17 lymphocytes relies on STAT5 pathway, and by dampening the proinflammatory activity of IL-17, while conserving IL-22 production	Guillot et al. (2014)
AML12 cells exposed to TGF-β1	1, 3, and 10 μM for 1 h	Liver fibrosis	Transcriptional regulation of the CB2 receptor gene in hepatocytes by LXRα that in turn inhibits USP4-stabilizing TβRI through miR-27b	Wu et al. (2019)
Human liver sinusoidal endothelial cells (HLSECs) treated with TNF-α	0–4 μM for 4 h	Hepatic ischemia/ reperfusion	Mitigates the TNF-α-stimulated ICAM-1 and VCAM-1 expression and decreases the adhesion of human neutrophils	Bátkai et al. (2007)
Fibroblast-like synoviocytes activated with TNF-α	1, 10, and 50 μM for 24 h	Rheumatoid arthritis	Inhibits production of pro-inflammatory cytokines, and prevents formation of bone-resorbing cells	Fukuda et al. (2014)
Bone marrow-derived macrophages cultured with TNF-α	1 μM for 24 h	Rheumatoid arthritis	Inhibits osteoclastogenesis and inflammation-mediated bone destruction via inhibiting NF-kB signaling pathway	Zhu et al. (2019)
Mesenchymal stromal cells from ITP patients	2.5 μM for 24 h	Immune thrombocytopenia	CB2 stimulation attenuates apoptosis via Bcl-2 signaling, and restores the immune-modulatory properties of MSCs	Rossi et al. (2019a)
Mice lung fibroblasts exposed to TGF-β1	10 μM for 48 h	Pulmonary fibrosis	Inhibited firbosis via repressing TGF-β1/Smad2 signaling pathway	Fu et al. (2017)
Human Adipose tissue mesenchymal stromal cells (atMSCs)	1, 3, 10, and 30 μM	Wound healing	Enhances secretion of VEGF, TGF-β1 and HGF, which in turn enhances the regenerative activity of at MSCs	Ruhl et al. (2020)

(Continued on following page)

TABLE 3 | (*Continued*) Pharmacological activities, mechanism and therapeutic potential of JWH133 in the *in vitro* studies.

Experimental model	JWH133 concentration	Indication/Disease	Demonstrated actions and mechanisms	References
Mesenchymal stem cells	3 μM for 1 h or 6 h	Bone healing	Induction of p42/44 MAPK that mediates migration of mesenchymal stem cells	Schmuhl et al. (2014)
Human Tenon's fibroblasts exposed to TGF-β1	0.5 μM for 24 h before TGF-β1	Wound healing	Suppresses ECM synthesis and MAPKs (ERK1/2, p38, and JNK) induced by TGF-β1 and reduces the contractility of HTFs	Guan et al. (2017)
Corneal epithelial cells	300 nM	Wound healing	Exerts chemorepulsive activity Stimulates *p*-ERK and cAMP production	Murataeva et al. (2019)
Differentiating oligodendrocyte progenitor cells	0.1, 0.5, and 1 μM for 48 h	Brain repair	Enhances oligodendrocyte differentiation dependent on stimulation of *p*-Akt and mTOR signaling	Gomez et al. (2011)

an unrecognized CB2R deficiency in the skin of the experimental mouse strain. JWH133 also failed to inhibit [3H] pyrilamine binding to histamine H1 receptors *in vitro*. Therefore, the capacity of JWH133 to influence mast cell-mediated inflammatory responses *in vivo* could be mediated by an indirect action on the mast cells.

In a rat model of cecal ligation and puncture (CLP)-induced polymicrobial sepsis, JWH133 reduced injury in the brain, heart, lung, and liver and attenuated the expression of caspase-3, p-NF-κB, TNF- α, IL-1β, and IL-6 levels while enhancing the expression of the anti-inflammatory cytokine IL-10 levels (Çakır et al., 2020). Thus, CB2R activation reduced inflammatory mediator expression by inhibiting apoptotic and NF-κB signaling, suggesting that JWH133 has therapeutic benefit in sepsis. JWH133 suppressed LPS/IFN-γ or Theiler's virus -activated macrophage-mediated IL-12p40 release in a dose-dependent manner, whereas cotreatment with SR-144528 reversed this effect (Correa et al., 2005). The effect of JWH133 on IL-12p40 release was mediated by ERK1/2 signaling, as indicated by a significant increase in ERK1/2 kinase. Suppression of ERK1/2 by the selective inhibitor PD98059 amplified LPS-induced IL-12p40 release, suggesting that persistent stimulation of ERK1/2 inhibits the release of IL-12p40. CB2R stimulation by JWH133 boosted IL-10 release from LPS/IFN-γ-stimulated macrophages. The effect was abrogated by SR144558 or ERK inhibitor PD98059. Blocking IL-10 with neutralizing antibody led to enhanced IL-12p40 production by LPS-activated macrophages in the absence or presence of JWH133, suggesting that endogenous IL-10 is involved in mediating the inhibitory effect of JWH133 on IL-12p40 secretion by activated macrophages. Thus, CB2R specific ligands could be beneficial for treating chronic inflammatory disorders.

In a carrageenan-induced inflammatory model, systemic administration of JWH133 3 h after carrageenan markedly ameliorated ipsilateral hindpaw weight-bearing and paw volume (Elmes et al., 2005). Similarly, pretreatment with JWH133 had the same effect on weight-bearing. The post-treatment effects of JWH133 on weight-bearing and paw volume were analogous to the systemic post-treatment effects of morphine and rofecoxib. Thus, CB2R activation by JWH133 mitigated inflammatory reaction and swelling, indicating that CB2R agonists might be a beneficial target for treating inflammatory pain responses. In contrast, JWH133 increased intracellular Ca^{2+} levels in human retinal pigment epithelial cells, indicating their responsiveness to JWH133 (Hytti et al., 2017). However, JWH133 did not inhibit oxidative stress-induced apoptosis mediated by reactive aldehyde 4-hydroxynonenal. Furthermore, JWH133 triggered cell death and increased the production of proinflammatory cytokines IL-6 and IL-8 via an ERK1/2-related mechanism. Contrary to the previous findings, CB2R activation increased inflammation instead of reducing it in human retinal pigment epithelial cells.

JWH133 in Atherosclerosis

Atherosclerosis is a chronic inflammatory disease and the leading cause of cardiac disorders and stroke worldwide (Libby, 2002). A significant link has been established among inflammatory processes, oxidative stress, nitrosative stress, and fat metabolism in the pathophysiology of atherosclerosis and vascular remodeling after injury (Patel et al., 2000; Hansson and Libby, 2006). The cannabinoid system has been identified to be associated with a growing number of chronic inflammatory diseases such as atherosclerosis (Pacher et al., 2006; Pacher and Mechoulam, 2011). CB2R stimulation has been specifically proposed to regulate atherosclerosis (Steffens et al., 2005). In this latter study, oral treatment with low-dose D9-tetrahydrocannabinol (THC, 1 mg kg^{-1} per day) markedly decreased plaque development in ApoE-knockout mice. Another study showed that administration of a CB2R/CB1R agonist ameliorated atherosclerosis in ApoE-deficient mice via a proposed CB2R-dependent mechanism (Zhao et al., 2010). TNF-α activates NF-κB and RhoA and upregulates adhesion molecules ICAM-1 and VCAM-1 in human coronary artery endothelial cells (HCAECs), thereby enhancing the expression of monocyte chemoattractant protein and promoting transendothelial migration of monocytes and monocyte–endothelial cell adhesion (Rajesh et al., 2007). All these effects were mitigated by pretreating HCAECs with JWH133.

JWH133 attenuated TNF-α- and/or endotoxin-induced expression of ICAM-1 and VCAM-1 in isolated aortas and prompted monocyte-aortic vascular endothelium adhesion. The protective effect of JWH133 was abolished by CB2R blockers (SR-144528 and AM-630) but not by CB1R (SR-141716 and AM-251) blockers. Thus, CB2R stimulation might alleviate endotoxin-driven vascular inflammation. Similarly, pretreatment of human coronary artery smooth muscle cells with JWH133 resulted in dose-dependent inhibition of proliferation and migration of vascular smooth muscle cells, which was reversed by SR2/AM630 but not by the CB1 blocker SR1 (Rajesh et al., 2008). Moreover, JWH133 mitigated the TNF-α activation of Ras, MAPKs (p38 and ERK 1/2), stress-activated protein kinases (SAPK)/Jun amino-terminal kinases (JNK), and Akt. These effects were abolished by

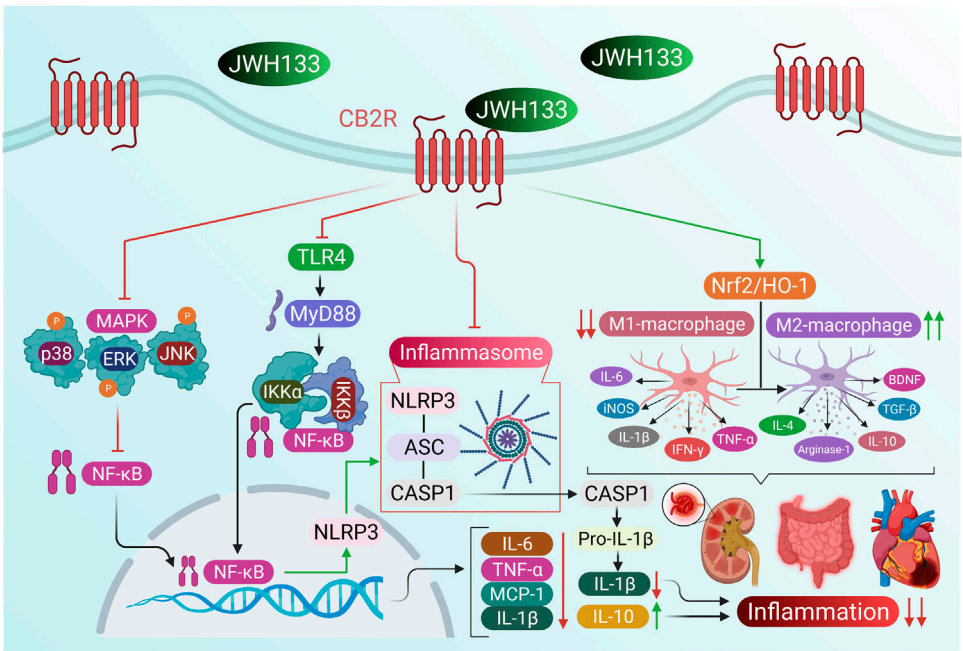


FIGURE 2 | The anti-inflammatory activity and mechanisms of JWH133.

AM630, indicates that CB2R activation counteracted TNF-α-induced pathways.

In another study, JWH133 significantly decreased MMP-9 content in ApoE2/2 mouse aortic root and carotid plaques (Montecucco et al., 2012). *In vitro*, preincubation of human primary neutrophils with JWH133 significantly reduced TNF-α-induced MMP-9 release, and this effect was abrogated by coincubation with AM630. The CB2R-mediated protective effect occurred via attenuation of TNF-α-induced ERK1/2 phosphorylation. Because CB2R stimulation suppressed neutrophil production of MMP-9 *in vivo* and *in vitro*, this treatment strategy could specifically diminish carotid atherosclerotic susceptibility in humans.

JWH133 induced dose-dependent phagocytosis of apoptotic cells in normal-cultured and oxidative low-density lipoprotein (OxLDL)-loaded RAW264.7 and primary macrophages (Jiang et al., 2016). JWH133 also induced the expression of tyrosine kinase family phagocytic receptors MerTK, Tyro3, and Axl. Efferocytosis of macrophages is mainly mediated by tyrosine kinase family phagocytic receptors (Seitz et al., 2007). JWH133 also decreased OxLDL-induced TNF-α and reactive oxygen species (ROS) production and blocked RhoA GTPase stimulation. Thus, selective CB2R activation improved efferocytosis of normal-cultured and OxLDL-loaded macrophages via induction of the tyrosine kinase family phagocytic receptors, inhibition of RhoA GTPase stimulation, and alleviation of oxidative/inflammation responses, thereby reducing the risk and promoting the stability of atherosclerotic plaques. Administration of JWH133 to ApoE/− mice fed on a high-cholesterol diet caused significant reduction of proliferation, decreased smooth muscle cell content, and reduced macrophage infiltration (Molica et al., 2012). Complete endothelial repair was observed after 14 days in both JWH133 and vehicle-treated mice, indicating that the CB2 agonist does not inhibit endothelial repair. CB2 deficiency resulted in increased intima formation compared with WT, whereas JWH133 did not affect intimal formation in $CB2^{-/-}$ mice. Genetic CB2R deletion increases neointima formation and *in situ* apoptosis after carotid balloon injury; enhances macrophage adhesion and migration; and enhances smooth muscle cell proliferation *in vitro*. In conclusion, pharmacological activation or genetic deletion of CB2R modulates neointima formation via smooth muscle cells and macrophages. Treatment of $ApoE^{-/-}$ hypercholesterolemic mice with JWH133 mitigated ROS release and NADPH-oxidase expression in mice penis (Fraga-Silva et al., 2013).

Furthermore, JWH133 upregulated endothelial NO synthase in the corpus cavernosum and increased nitric oxide bioavailability. The reduction in oxidative stress levels was associated with a decrease in collagen content. Therefore, CB2R stimulation attenuated ROS production and fibrosis associated with erectile dysfunction in hypercholesterolemic mice. In contrast, intraperitoneal injections of JWH133 in $LDLR^{-/-}$ mice on a high-cholesterol diet resulted in no significant difference in intimal lesion size in sections of the aortic roots and arches, indicating that CB2R stimulation did not modulate atherogenesis in mice (Willecke et al., 2011). Further, JWH133 treatment did not mitigate the contents of lipids, macrophages, collagen, T cells, and smooth muscle cells and the rate of cell apoptosis in atherosclerotic mice. However, JWH133 reduced intraperitoneal macrophage numbers after 72 h of intraperitoneal injection in a model of thioglycollate-induced peritonitis but not after 4 h. Neither genetic deficiency nor pharmacologic stimulation of the CB2R caused a change in the expression of proinflammatory cytokines (IL-6, MCP-1, IL-10, IFNγ, or IL-12p70) in mice challenged with intraperitoneal TNF-α or inflammatory cell adhesion in murine endothelial cells

isolated from LDLR$^{-/-}$ mice. Therefore, neither CB2R activation nor its genetic deficiency modulated atherogenesis.

JWH133 in Bone Disorders

CB2Rs are highly expressed in bone cells compared with CB1Rs and have a crucial role in controlling the balance between bone resorption and osteogenesis (Whyte et al., 2012). CB2Rs are upregulated during bone remodeling (Idris, 2012). CB2R activation improves osteoblast proliferation and function by enhancing the expression of osteogenic factors such as RUNX2, bone sialoprotein, osteopontin, alkaline phosphatase, and osteocalcin (Qian et al., 2010).

Preclinical studies revealed that CB2R-knockout mice developed osteoporosis at 12 months, reduced osteoblast production and function, and enhanced osteoclast production (Sophocleous et al., 2011). Clinical studies of postmenopausal women demonstrated that the gene encoding the CB2R (*CNR2*) is responsible for low bone mineral density (Zheng et al., 2019). Thus, CB2Rs may be a translational target for pharmacologic agents that augment bone regeneration, but quality clinical trials are warranted.

Osteoarthritis

In developed nations, osteoarthritis (OA) is the most common chronic joint disease with a social cost of approximately 0.5% of gross domestic product (Puig-Junoy and Ruiz Zamora, 2015). It is marked by pain and frequent disability and is correlated with anxiety, depression (Axford et al., 2010), and cognitive changes (Moriarty et al., 2011). Spinal CB2R expression is correlated with knee joint damage (macroscopic chondropathy score) in human post mortem samples (Burston et al., 2013). Systemic administration of JWH133 mitigated OA pain induced by monosodium iodoacetate, decreased the expression of inflammatory cytokines (IL-1β and TNFα), and increased the levels of anti-inflammatory IL-10. Spinal administration of JWH133 suppressed noxious mechanically evoked responses of spinal neurons in animal model of OA pain, but not in naive rats, indicating great potential of this treatment route. SR144528 abrogated the effect of JWH133. Systemic administration reduced the expression of glial fibrillary acidic protein (GFAP; a marker of reactive gliosis) and MMP-2 and MMP-9 in the spinal cord. These findings suggest that CB2R stimulation diminished central sensitization, thereby mitigating pain behavior.

In another study, JWH133 improved the alternations in nociception and anxiety behaviors but did not ameliorate memory impairment in an animal model of OA pain (La Porta et al., 2015); this was probably owing to a direct result of the pain-relieving effect mediated by CB2R. The absence of a memory-protective effect suggests that the JWH133-mediated improvement of these symptoms is owing to the direct effect of JWH133 on emotion and cognition.

Osteoporosis

Osteoporosis is a systemic skeletal disease characterized by low bone mass, damage of bone tissue, and decreased bone mineral density and is considered a silent disease until a fracture occurs (Pisani et al., 2016). In human osteoblast hFPB1.9 cells, JWH133 produced a dose-dependent increase in autophagy, as measured by the conversion of LC3I to LC3II, increased beclin-1 expression, and enhanced p62 degradation (Xu et al., 2020). Furthermore, JWH133 inhibited mTOR signaling by reducing the levels of phosphorylated mTOR, P70S6K, and 4EBP1 in hFOB 1.19 cells. However, CB2R-knockdown abrogated the effect of JWH133 on autophagy. JWH133 also increased alkaline phosphatase activity and bone mineralization and increased the expression of osteogenic markers osteopontin and osteocalcin. Interestingly, the osteogenic activities mediated by CB2R stimulation were significantly attenuated by the autophagic inhibitor 3-MA, indicating that the stimulation of autophagy is needed for CB2R-mediated osteoblast differentiation. Moreover, JWH133 decreased nuclear Nrf2 accumulation and upregulated Keap1 and re-expression of p62 prevented CB2R agonist-mediated Nrf2 deactivation. In summary, osteogenic differentiation mediated by CB2R involves autophagy activation and p62-mediated Nrf2 degradation.

Antagonism of vanilloid receptor 1 (TRPV1) and/or activation of CB2R reduces the number and activity of osteoclast cells (Rossi et al., 2019b). Methylprednisolone-induced telomerase activity was markedly decreased by JWH133 and the TRPV1 antagonist I-RTX in healthy subject-derived osteoclasts (Bellini et al., 2017). Additionally, JWH133 and I-RTX reverted methylprednisolone-induced osteoclast hyperactivity, evidenced by a significant reduction in osteoclast numbers. Furthermore, CB2R activation by JWH133 hampered resorption and modulated protein kinase C beta II (PKC βII) signaling induced by methylprednisolone, suggesting that JWH133 reduced PKC βII signaling-dependent bone resorption. Conversely, JWH133 stimulated osteoclast formation in mouse osteoblast–bone marrow cocultures (Idris et al., 2008). It produced a dose-dependent increase in RANKL-induced osteoclast formation and increased osteoclast size and nuclearity with no remarkable effect on apoptotic cell death. The conflicting results on bone resorption and osteoclast function require further investigation.

JWH133 in Cancer

A previous study suggested that endocannabinoids possess anticancer activity by demonstrating that oral administration of D9-THC, D8-THC, and cannabinol prevented the proliferation of Lewis lung adenocarcinoma cell growth *in vitro* and *in vivo* (Munson et al., 1975). Many other cannabinoids have since been demonstrated to inhibit proliferation, metastasis, angiogenesis, and apoptosis in different cancer types *in vitro* and *in vivo* (Casanova et al., 2003; Carracedo et al., 2006; Cianchi et al., 2008). Growing evidence suggests that the anticancer effects of phyto-, endo-, and synthetic cannabinoids are attributed to their ability to modulate cellular signaling mechanisms controlling cell proliferation and survival (Guzmán, 2003; Bifulco et al., 2008). The anticancer properties, effects and mechanisms of JWH133 are presented in **Figure 3**.

Breast Cancer

Breast cancer is the most prevalent cancer type, constituting approximately 30% of newly diagnosed cancers yearly. Almost

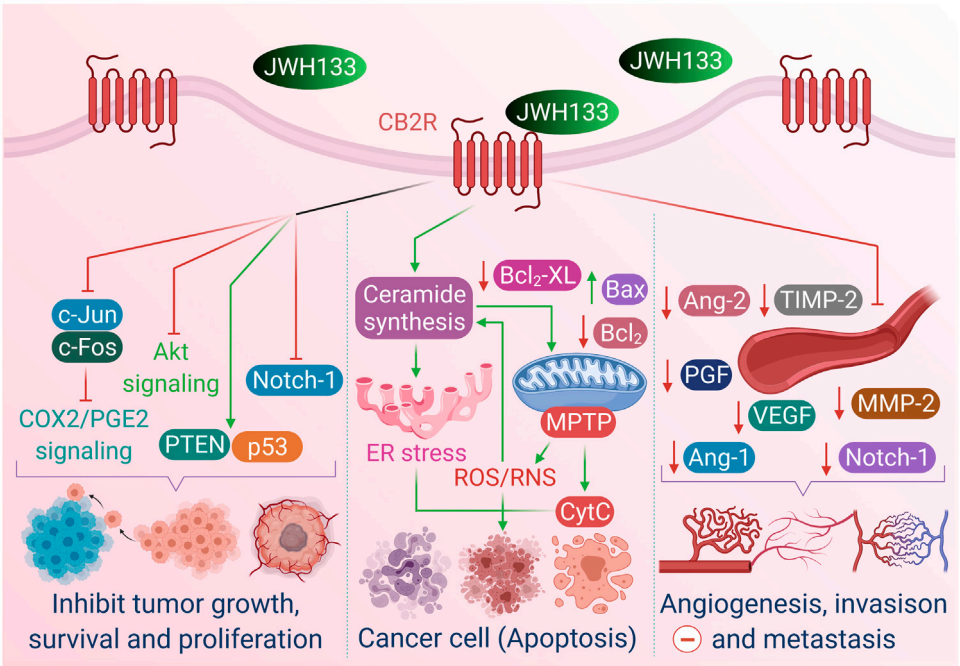


FIGURE 3 | The anticancer properties, effects and mechanism of JWH133.

one-third of breast cancers overexpress the ErbB2 tyrosine kinase receptor (Her2 in humans, Neu in rats) (Baselga and Swain, 2009). Qamri et al. (2009) showed that JWH133 provided a dose-dependent inhibition of the proliferation and migration of MDA-MB231 and MDA-MB468 cells. JWH133 resulted in a significant decrease in tumor growth and lung metastasis and markedly attenuated angiogenesis in mice. However, these effects were abolished by SR144528, suggesting that the anticancer activities were mediated by CB2R. CB2R activation by JWH133 also delayed and reduced mammary gland tumor growth in a PyMT transgenic mouse model by modulating COX-2/prostaglandin E2 signaling. COX-2 expression was inhibited by the downregulation of transcription factors *c-Fos* and *c-Jun* by JWH133 in breast cancer cells. Synthetic cannabinoids may block tumor growth by inducing cell cycle arrest and apoptosis in human breast cancer tumors. Therefore, CB2Rs might provide a clinical therapeutic approach for treating breast cancer proliferation and metastasis.

Caffarel et al. (2010) reported that JWH133 mitigated tumor growth, tumor number, and the severity of lung metastases in MMTV-neu mice, a clinically relevant model of ErbB2-driven metastatic breast cancer. JWH133 inhibited tumor cell proliferation, as indicated by a decreased number of Ki67-positive cells in cannabinoid-treated tumors, stimulated apoptosis in cancer cells by inducing caspase 3, and prevented angiogenesis. In addition, JWH133 induced a CB2R-dependent reduction in N202.1A cell proliferation and xenograft growth. The effect of JWH133 was blocked by SR144528 but not by SR141716, demonstrating the CB2R specificity of JWH133 and subsequent suppression of the protumorigenic AKT pathway.

Low micromolar concentrations of JWH133 decreased the cell viability of MDA-231, 4T1 and MCF7 (Sophocleous et al., 2015). However, nanomolar concentrations augmented human and mouse breast cancer cell-mediated osteoclastogenesis and enhanced osteolysis, and these effects were reversed by CB2-knockout or treatment with AM630, indicating that inactivation of CB2R suppressed osteoclastogenesis in bone metastasis. In addition, JWH133 did not impact osteoblast differentiation in the presence of breast cancer cells-conditioned medium. In contrast, it increased osteoblast differentiation induced by parathyroid hormone, and the ability to stimulate osteoclast formation supported the fact that CB2R stimulation enhanced osteoblast differentiation in a metastatic environment. Furthermore, JWH133 promoted PI3K/AKT activity in a CB2-specific mechanism in the presence of osteolytic and osteoblastic factors such as RANKL and parathyroid hormone. These findings suggest that breast cancer and bone cells respond differently to CB2R agonists depending on cell type and concentration.

Colon Cancer

Colon cancer is the second leading cause of cancer mortality in developed nations and the fourth worldwide, with greater than one million newly diagnosed patients yearly (Jemal et al., 2009a).

JWH133 inhibited the adrenaline-driven migration of SW480 colon and MDA-MB-468 breast cancer cells and attenuated T lymphocyte migration induced by chemokine stromal cell-derived factor 1. This effect was not diminished by the selective CB1R agonist docosatetraenoyl ethanolamide (Joseph et al., 2004).

Martínez-Martínez et al. (2016) reported that sub-micromolar doses of JWH133 enhanced cell proliferation of the human colon cancer cell lines HT29, SW480, and LS174T and in nude mice by stimulating the AKT/Protein kinase B pathway. Consequently, JWH133 activated AKT, which induced the phosphorylation and suppression of glycogen synthase kinase-3β (GSK3β), leading to a more aggressive cell phenotype with increased levels of SNAIL, the Snail family zinc-finger transcription factor which induces the initiation of the epithelial–mesenchymal transition (Bachelder et al., 2005) as well as downregulation of E-cadherin and β-catenin delocalization from the cell membrane. Cumulatively, CB2 stimulation with submicromolar concentrations of JWH133 activated PI3K/AKT signaling, thereby promoting colon tumor cell proliferation and aggressiveness. These results must be considered when exploring cannabinoid therapy for patients with colon cancer because of the dose-dependent response and the challenges of delivering the drug to the tumor site.

Brain Cancer

Malignant gliomas are considered the most common malignant brain tumors with poor prognosis (Maher et al., 2001). The first study to investigate the antitumor activity of JWH133 was conducted by Sánchez et al. (2001), who demonstrated that incubating rat glioma C6 cells with JWH133 significantly reduced cell viability by approximately 50% owing to the activation of apoptotic cell death via ceramide synthesis and ERK1/2 stimulation. Intratumoral administration of JWH133 in a Rag-$2^{-/-}$ mouse model of glioma resulted in a remarkable reduction in tumor growth by approximately 71%. This antitumor effect was abrogated by SR144528 but not by SR141716. JWH133 prevented the growth of highly malignant human astrocytoma in Rag-$2^{-/-}$ mice. Cumulatively, CB2R activation exerted antitumor activity by inducing apoptosis via ceramide synthesis and ERK1/2 activation.

In a similar study by the same group using the same mouse model, intratumoral treatment with JWH133 significantly downregulated the expression of proangiogenic factors, including vascular endothelial growth factor (VEGF) and angiopoietin 2 (Ang 2), revealing another significant feature of JWH133-mediated tumor inhibition (Blázquez et al., 2003). These results were confirmed in glioma and astrocytoma xenografts, in which JWH133 inhibited VEGF, Ang1, Ang2, MMP-2, and TIMP-2 (Blázquez et al., 2003).

Cotreatment with the ceramide biosynthesis inhibitor Fumonisin B1 reversed the antitumor effect of JWH133, and its inhibitory effect on MMP-2 suggested that JWH133 downregulated MMP-2 expression underlying CB2R-mediated suppression of glioma cell invasion that occurred by inhibiting sphingolipid ceramide synthesis. These results were compared to findings with the mixed agonist Δ9-THC, suggested a critical role of CB2R in the Δ9-THC mediated effect.

The discovery of brain tumor stem cells has significant implications in developing new therapeutic approaches for managing malignant glioma and evaluating the benefits of currently available therapeutic medications (Maher et al., 2001). Aguado et al. (2007) reported that JWH133 stimulated glial cell differentiation in a CB2R-related manner, as shown by an increase in S-100β and GFAP and neuronal marker β-tubulin III in human glioma stem cells. Moreover, JWH133 reduced the cell population expressing the neuroepithelial progenitor marker nestin, causing a marked decrease in the efficiency of glioma formation *in vivo*, linked with reduced neurosphere formation and cell growth in secondary xenografts.

During parenchymal brain metastasis, cancer cells migrate through the brain endothelial cells that form the morphological basis of the blood–brain barrier (Wilhelm et al., 2013). Haskó et al. (2014) showed that CB2R stimulation by JWH133 decreased the adhesion of A2058 melanoma cells to the layer of hCMEC/D3 brain endothelial cells, indicating that CB2R activation on both endothelial and melanoma cells contributed to the adhesion-decreasing property of JWH133. JWH133 also reduced the rate of transmigration of melanoma cells, whereas coincubation with SR-144528 reversed these effects, verifying the CB2R-dependent effect of JWH133.

Lung Cancer

Non–small-cell lung cancer (NSCLC) is one of the common causes of cancer mortality worldwide. Despite this, only limited anticancer medications are available in current clinical practice (Jemal et al., 2009b). Preet et al. (2011) reported that JWH133 suppressed tumor growth and lung metastasis in SCID CB-17 mice inoculated with A549 cells. These antitumor effects were abolished by pretreatment with SR144528, indicating the direct involvement of CB2R in effect of JWH133. Moreover, JWH133 decreased tumor proliferation and neovascularization and enhanced apoptotic cell death in SCID CB-17 mice.

In another study, JWH133 exhibited cytotoxic activity in A549 cells and human umbilical vein endothelial cells (HUVECs) when used at the highest concentration (10^{-4} mol/L), whereas colony formation was prevented at non-toxic concentrations (10^{-5}–10^{-8} mol/L) (Vidinský et al., 2012). Furthermore, JWH133 weakly induced DNA fragmentation in A549 cells. Furthermore, non-toxic concentrations of JWH133 inhibited some processes involved in angiogenesis and suppressed endothelial cell migration. JWH133 at 10^{-4} mol/L suppressed MMP-2 secretion. Thus, the antitumor activity of JWH133 occurred at micromolar concentrations in A549 cells.

Ramer et al. (2014) demonstrated that JWH133 in A549/huvec cocultures mitigated migration and tube and sprout formation in huvec. Inhibition was associated with the upregulation of tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) and its upstream trigger ICAM-1, the intercellular adhesion molecule-1. The antiangiogenic effects of JWH133 are site-specific and limited to the tumor tissue. Indeed, conditioned media from JWH133-treated BEAS-2B cells, a normal bronchial epithelial cell line, did not prevent huvec migration. Therefore, JWH133 increased TIMP-1 production in lung cancer cells and induced ICAM-1 expression, thereby modifying the tumor cell microenvironment and inhibiting angiogenesis.

Considering the important role of macrophage-mediated vascular remodeling in several cancers, JWH133 significantly inhibited lipopolysaccharide-induced release of VEGF-A, VEGF-C, Ang1, and Ang2 and modestly affected IL-6 release in human lung macrophages (Staiano et al., 2016). However,

JWH133 did not modulate the release of TNF-α or IL-8/CXCL8, and production of VEGF-A by human monocyte-derived macrophages was observed. CB2R activation by JWH133 inhibited the production of VEGF-A and VEGF-C from human lung macrophages but not from monocyte-derived macrophages. Stimulation of CB2R on tissue-derived macrophages could be a critical approach for the modulation of macrophage-mediated vascular remodeling in tumors and chronic inflammation.

Leukemia

Leukemias account for 30% of all pediatric cancers, and acute lymphoblastic leukemia (ALL) is the most prevalent pediatric leukemia, representing 75% of all pediatric leukemia cases (Terwilliger and Abdul-Hay, 2017). Punzo et al. (2018a) showed that JWH133 promoted apoptosis in patients with T-ALL and a Jurkat cell line via enhanced caspase-3 expression and Bax/Bcl-2 ratio. Moreover, JWH133 prevented tumor cell growth and survival via reduced expression of AKT, ERK, and Notch-1, while increasing the expression of PTEN and p53. This antitumor effect correlated with a remarkable inhibition of cell cycle progression by reducing the expression of cyclin-dependent kinase 2. Therefore, CB2R activation downregulated genes implicated in cell cycle progression and proliferation and upregulated genes implicated in apoptosis and cell cycle arrest in Jurkat cells.

Thyroid Carcinoma

Thyroid carcinoma is a malignant tumor of the endocrine system, which encompasses the majority of mortalities from endocrine tumors (Farid et al., 1994). Shi et al. (2008) reported that JWH133 induced a significantly greater apoptosis rate in ARO/IL-12 than in ARO thyroid carcinoma cells. Moreover, their findings were similar to those obtained when ARO cells were transfected with CB2 transgene (ARO/CB2). Intratumoral injection of JWH133 caused remission of thyroid tumors in nude mice inoculated with ARO/CB2 cells. CB2R was overexpressed after IL-12 expression in thyroid carcinoma cells. Thus, the upregulation of CB2R rendered thyroid cancer cells more responsive to CB2 agonist-induced apoptosis and led to tumor remission. Thus, the discovery of IL-12-mediated CB2 upregulation in thyroid tumors might provide a translational target for treating thyroid carcinoma.

Skin Cancer

The incidence of skin tumors has been increasing at a startling rate for several years. Various therapeutic agents have been identified, including cryotherapy, topical chemotherapeutic agents, and photodynamic therapy. However, these strategies have many limitations, including poor penetration of substances into the skin and difficulty accessing whole tumors (Leber et al., 1999). Casanova et al. (2003) found that incubating the tumorigenic mouse epidermal cell line PDV. C57 with JWH133 reduced cell viability by approximately 40%. JWH133 administration in nude mice inoculated with PDV. C57 cells caused approximately 60% reduction of tumor volume by inhibiting tumor vascularization as indicated by modified blood vessel morphology and downregulation of proangiogenic factors, including VEGF, placental growth factor, and angiopoietin-2, and inducing apoptosis. Activation of CB2R in tumor cells abolished EGFR function. It is possible that JWH133 interfered with the tumor angiogenic switch and directly stimulated tumor cell apoptosis, which in turn inhibited tumor proliferation. Therefore, both CB2R and EGFR might be critical for initiating signaling events that lead to tumor regression.

A similar study by Blázquez et al. (2006) demonstrated that JWH133 treatment resulted in tumor regression in nude mice bearing B16 melanoma cells, reducing tumor volume by approximately 75%, accompanied by an increase in apoptosis and reduced tumor vascularization and vascular density. Conversely, Luca et al. (2009) found that Kaposi sarcoma cells-treated JWH133 did not show remarkable inhibition of tumor proliferation and survival.

Osteosarcoma

Osteosarcoma (OS) is considered the most common bone cancer; it mainly affects children and teenagers and has a high rate of invasion and metastasis (Anderson, 2016). Punzo et al. (2017) reported that incubation with JWH133 induced apoptosis, upregulated caspase-3, and downregulated *p*-AKT in all OS cell lines studied (Saos-2, MG-63, MNNG/HOS, KHOS/NP, Hs888Lu, and U-2 OS). The antiproliferative activity of JWH133 was associated with the downregulation of Notch-1 and MMP-2, suggesting that JWH133 suppressed invasion/migration. Low-dose JWH133 decreased tumor growth and induced apoptosis, whereas higher doses had the opposite effect. Thus, CB2R stimulation exerted antiproliferative, proapoptotic, and antiinvasive effects; however, the dose should be considered while shifting to clinical setting.

In another study by the same group, activation of CB2R by JWH133 increased the efficacy of bortezomib in mediating apoptosis and decreasing invasion, arresting cell cycle progression, and modulating bone balance. Thus, they proposed that combining bortezomib with CB2R ligands in osteosarcoma therapy enables optimal dosing and reduces adverse effects (Punzo et al., 2018b).

JWH133 in Cardioprotection

CB1 and CB2Rs are widely found in many tissues, including cardiac myocytes (Pertwee, 1997). The first indication that cannabinoids can be effective in ischemia was reported by Lagneux and Lamontagne (2001), who showed that cannabinoid receptors provided cardioprotection against lipopolysaccharide-triggered damage in isolated rat heart. Emerging evidence indicates that the CB2R acts during the early stages of ischemia–reperfusion, as shown by the decrease in infarct size in the presence of CB2 agonists before ischemia or during reperfusion in *ex vivo* preparations (Lépicier et al., 2006; Pacher and Haskó, 2008). The cannabinoid receptors have been involved in different cardiovascular disorders, including myocardial infarction, cardiomyopathy, arrhythmias, stroke, and cardiac arrest (Pacher et al., 2018).

Drug-induced Cardiotoxicity

Preincubation of myocardial HL-1 cells with JWH133 mitigated the histological alterations mediated by quetiapine (Li et al., 2019). JWH133 administration in mice resulted in a significant decrease in the ratio of heart weight to tibia length (HW/TL) and inhibited inflammatory cell infiltration and fibrosis. CB2R activation attenuated cell necroptosis by downregulating the expression of MLKL, phosphorylated MLKL, and attenuated RIP1 and RIP3. Thus, CB2R protected against quetiapine-induced cardiac toxicity by modulating necroptosis.

In another study by the same group, JWH133 reversed the elevated expression levels of p-RIP1, p-RIP3, and *p*-MLKL induced by ethanol in mice, indicating that CB2R may be the upstream signal molecules in necroapoptosis. Moreover, CB2R activation significantly ameliorated heart dysfunction, as indicated by increased left ventricular ejection fraction and fractional shortening and attenuated levels of cardiac injury markers (BNP, COL1A1, TGF-β1, IL-1B, and IL-6). The cardioprotective effect was associated with remarkable inhibition of inflammatory cell infiltration and fibrosis (Liu et al., 2020).

Pretreating mice with JWH133 suppressed clozapine-induced cardiotoxicity in mice, with a significant improvement in heart function and attenuation of infiltration index, fibrotic cardiac tissue, and serum cTnI levels (Li et al., 2019). Therefore, these findings proved the protective effects of CB2R activation against drug-induced cardiotoxicity.

Heart Failure

Myocardial hypertrophy is the increased myocyte mass elicited by hemodynamic stress or myocardial injury and is linked with a markedly increased risk of heart failure (Tanai and Frantz, 2015). Lu et al. (2014) found that low micromolar concentrations of JWH133 mitigated endothelin-1-elicited myocardial enlargement but did not attenuate endothelin-1-induced brain natriuretic peptide activation in isolated neonatal rat ventricular myocytes. Thus, CB2R stimulation might be a novel antihypertrophic cannabinoid therapy, which could improve the side effects of unopposed stimulation of CB1R alone.

The cardioprotective effects were validated by Maggo and Ashton (2018), who demonstrated that JWH133 did not influence atrial chronotropy in isolated rat atria, suggesting that CB2R activation did not induce tachycardia, whereas a CB1/CB2 agonist (WIN) and selective CB1 agonist (methanandamide) increased atrial chronotropy. Therefore, cannabinoid cardiotoxicity might include activation of CB1R in the heart, and CB2R agonists were not likely to have remarkable effects on the myocardium.

Myocardial Infarction

Acute myocardial infarction is the leading cause of death worldwide. Despite significant advances in restoring blood flow in the infarct area, reperfusion can damage the ischemic cardiac tissue (Yellon and Hausenloy, 2007). Montecucco et al. (2009) reported that JWH133 decreased the infarct size and severity of the cardiac injury, evidenced by reduced serum cTnI levels in mice. The cardioprotective effect of JWH133 was abrogated by pretreatment with AM630. JWH133 also attenuated ROS production and neutrophil infiltration in the infarcted myocardium, activated ERK1/2, which counteracted cardiac reperfusion injury, and enhanced STAT-3 expression. Pretreatment with the PI3K inhibitor LY294002, MEK1/2 inhibitor U0126, and JAK-2 inhibitor AG-490 partially blocked the JWH133-mediated mitigation of infarct size. JWH133 also inhibited human neutrophil migration in response to TNF-α by suppressing CD11b/CD18 (Mac-1) expression. Therefore, JWH133-mediated cardioprotection depends on the inhibition of oxidative stress and neutrophil recruitment and activation of the ERK 1/2 and STAT3 pathways.

JWH133 treatment significantly reduced the infarct size and apoptosis index of rat myocardium (Li et al., 2013). JWH133 maintained mitochondrial membrane potential (ΔΨm), downregulated the expression of caspases−3 and −9, inhibited the release of mitochondrial cytochrome c, and increased the expression of phosphorylated AKT. These effects were reversed by the PI3K inhibitors wortmannin and AM630. Thus, CB2R stimulation by JWH133 prevented apoptotic cell death during ischemia–reperfusion by suppressing intrinsic mitochondrial apoptosis via the PI3K/AKT signaling pathway.

In a similar study, JWH133 pretreatment remarkably improved ventricular function recovery during reperfusion, enhanced coronary flow, and decreased infarct size (Li et al., 2014). CB2R activation inhibited the loss of ΔΨm and mitochondrial permeability transition pore (MPTP) opening, decreased cytochrome c release into the cytosol, and upregulated *p*-ERK1/2 expression. These effects on the myocardium were abrogated by pretreatment with AM630 or the ERK1/2 inhibitor PD98059. Moreover, JWH133 counteracted atractyloside-induced MPTP opening. Thus, the cardioprotective effects of JWH133 during ischemia–reperfusion likely occur via phosphorylated ERK1/2 and preventing MPTP opening.

Defer et al. (2009) showed increased infarct size in CB2 knockout mice but reduced infarct size in wild-type mice treated with JWH133 at the time of reperfusion. Incubation with JWH133 protected cardiac myocytes from apoptosis induced by H_2O_2. However, the protective effect of JWH133 was diminished in $CB2^{-/-}$ cardiac myocytes, and preincubation with AM630 confirmed the involvement of the CB2-dependent pathway. CBR2-mediated protection against apoptosis correlated with increased AKT phosphorylation and a reduced late apoptotic signal. Degradation of 45-kDa actin in cardiac myocytes suggested that CB2R stimulation increased cardiac myocyte resistance to oxidative damage by enhancing AKT signaling. In addition, JWH133 protected cardiac fibroblasts from H_2O_2-mediated apoptosis, limiting the release of TNF-α and α-SMA and inducing MMP-2 secretion. This protective effect was reversed in $CB2^{-/-}$ fibroblasts. Therefore, CB2R activation provided cardioprotection by preventing oxidative stress-induced apoptosis in cardiac myocytes and fibroblasts and suppressing myofibroblast activation.

In another mouse model, JWH133 mitigated the severity of myocardial infarction by reducing infarct size, limiting myocardial enzyme expression (CK-MB and LDH), and improving cardiac function (Yu et al., 2019). Additionally,

JWH133 protected primary cardiomyocytes as demonstrated by improved cell viability and LDH release. JWH133 attenuated the release of inflammatory cytokines (IL-1β, IL-18, IFN-γ, and TNF-α), and this effect was markedly reversed by AM630. JWH133 administration significantly inhibited the NLRP3 inflammasome in cardiac tissues of mice and in primary cardiomyocytes as indicated by the downregulation of NLRP3, casp1, and proIL-1β. Thus, the cardioprotective effect of CB2R activation relied on the modulation of NLRP3 inflammasome pathway.

JWH133 in Metabolic Disorders

Metabolic syndrome is a complex pathological condition that involves several cardiovascular diseases, insulin resistance, and abdominal obesity (Kaur, 2014). Obesity is a potentially fatal metabolic disorder resulting from excessive calorie intake (Haase et al., 2014). Chronic inflammation associated with obesity is a core mechanism underlying obesity-related complications, including type 2 diabetes, non-alcoholic fatty liver disease, hypertension, atherosclerosis, and myocardial infarction (Van Gaal et al., 2006).

The cannabinoid system has a pivotal role in controlling energy metabolism (Engeli, 2012; Watkins and Kim, 2014). Several studies have demonstrated CB2R expression in peripheral metabolic tissues such as adipose tissue (Lin et al., 2017), the liver (Romero-Zerbo et al., 2012), pancreatic islet cells (Verty et al., 2015). Further, Ishiguro et al. (2010) found that Q63R, a common CB2R variant, causing decreased CB2 function, has been linked with eating disorders in humans. CB2 ligands reduce dietary intake in lean mice (Ishiguro et al., 2010) and ameliorate body weight and obesity-related inflammation in diet-induced obese mice (Verty et al., 2015). Moreover, CB2 genetic deficiency causes adiposity (Schmitz et al., 2016). This evidence suggests that CB2R ligands are a clinically viable therapeutic target for obesity.

Wu et al. (2020) examined the anti-inflammatory activities of CB2R and JWH133 in a diet-induced mouse model of obesity and cultured macrophages. They showed that JWH133 decreased body weight gain and adipocytic cell size, alleviated glucose intolerance, and enhanced insulin resistance. It also decreased the expression levels of M1 macrophage biomarkers (TNF-α, IL-6, iNOS, IL-1β, CCL2, and CXCL-10) while enhancing the expression of M2 macrophage biomarkers (IL-10 and arginase-1) in both mice and RAW264.7 macrophages. In both cases, the effects of JWH133 were blocked by pretreatment with AM630. JWH133 also inhibited the translocation of NF-κB p65 into the nucleus, enhanced the nuclear translocation of Nrf2, and upregulated the expression of HO-1 in cultured macrophages preincubated with LPS. However, the effect of JWH133 was reversed by an HO-1 inhibitor, Sn (IV) protoporphyrin IX dichloride. Thus, JWH133 exhibited antiobesity activity that attenuated proinflammatory M1 macrophage cytokines via Nrf2/HO-1.

In a clinical study, Rossi et al. (2016) found that the less-functional CB2-R63 variant was markedly correlated with a high z-score body mass index. Treatment of obese mouse-derived adipocytes with JWH133 showed decreased levels of PPARɣ, leptin, IL-6, and TNFα and increased expression of IL-4. The authors also observed a significant decrease in lipid droplet size and perilipin levels via CB2R-related modulation of PPARɣ. In addition, treatment of obese mouse-derived adipocytes with JWH133 resulted in significant upregulation of uncoupling protein-1 (UCP-1); this effect was abrogated by AM630 pretreatment. The evidence suggests that CB2R activation is a therapeutic target for mitigating obesity-associated inflammation and excess lipid storage in white adipose tissue by modulating perilipin expression, upregulating IL-4, and stimulating UCP-1 signaling.

In another study on the role of CB in controlling binge eating and obesity, it was reported that systemic administration of JWH133 produced a dose-dependent reduction in sucrose self-administration in wild-type and $CB1^{-/-}$ mice, but not in $CB2^{-/-}$ mice (Bi et al., 2020). However, pretreatment with AM251 accelerated and AM630 reversed the JWH133-mediated decrease in sucrose self-administration in wild-type mice, suggesting that cannabinoids inhibited this behavior by CB1R antagonism and CB2R agonism. Thus, JWH133 could decrease food rewarding and the motivation to seek sweetened food.

In contrast, Deveaux et al. (2009) reported that administration of JWH133 enhanced adipose tissue inflammation in HFD-fed mice. Moreover, exposure of cultured fat pads isolated from ob/ob mice to JWH133 showed increased expression of EMR1, TNF-α, and CCL2 (encoding MCP-1) in epididymal fat cells. Intraperitoneal administration of JWH133 enhanced HFD-induced insulin and hepatic steatosis in mice. These conflicting results suggest that CB2R activation mediates adipose tissue inflammation and enhances obesity-related insulin resistance and fatty liver.

JWH133 in Diabetes

Diabetes mellitus (DM), one of the most common metabolic diseases, is caused by a lack of insulin (T1DM) or reduced sensitivity and increased insulin resistance (T2DM) (Choi et al., 2015). DM often leads to numerous microvascular and macrovascular complications (Gruden et al., 2016).

Endocannabinoids modulate food consumption, glucose homeostasis, redox-inflammatory changes, and insulin release (Gruden et al., 2016). CB2Rs expressed in the islets of Langerhans mediate endocannabinoid signaling and endocrine secretion. CB2R stimulation increases insulin release from β-cells, inducing Ca^{2+} signalling (Juan-Picó et al., 2006). De Petrocellis et al. (2007) reported that treating rat insulinoma β-cells with JWH133 increased $[Ca^{2+}]i$ in the absence of extracellular Ca^{2+}, whereas the inhibitor of phosphoinositide-specific phospholipase C (PI-PLC) U73122 resulted in a dose-dependent inhibition of intracellular Ca^{2+}, which is the primary insulin release regulator in pancreatic β-cells. This observation may indicate that CB2R is coupled with enhanced $[Ca^{2+}]i$ via Gq/11-type G-proteins and stimulation of the phosphoinositide-specific phospholipase C cascade. Moreover, incubating rat insulinoma β-cells with JWH133 elevated $[Ca^{2+}]i$ independent of extracellular Ca^{2+}, whereas preincubation with inhibitors of Ca^{2+} channels in the endoplasmic reticulum blocked the effect of JWH133. Thus, CB2R stimulation is associated with Ca^{2+} mobilization from endoplasmic reticulum stores.

In another study, McDonnell et al. (2017) found that JWH133 administration suppressed mechanical allodynia in db/db mice in a dose-dependent manner, whereas pretreatment with AM630 abrogated this effect. Stimulation of antioxidant Nrf2/HO-1 signaling by cobalt protoporphyrin IX (CoPP), a HO-1 inductor, and sulforaphane potentiated the antiallodynic effects of JWH133 and could be beneficial for the treatment of T2DM-associated neuropathic pain.

JWH133 in the Reproductive and Hormonal System

Female Reproductive System

The endocannabinoid system is expressed in the female reproductive system of various species from sea urchins to humans, indicating its likely role in female reproduction (Sun and Dey, 2012). Components of the endocannabinoid system have been observed in the rodent and human uterus, and alterations in anandamide synthesis and expression of CB receptors in the uterus have been associated with early pregnancy failure or female infertility (Schmid et al., 1997). The expression and localization of cannabinoid receptors and enzymes in human oocytes and granulosa cells suggest that the endocannabinoid system plays a role in oocyte maturation (Agirregoitia et al., 2015).

Pagano et al. (2017) showed that JWH133 attenuated spontaneous uterine contraction induced by prostaglandin during the diestrus phase, whereas pretreatment with a CB2R blocker eliminated the spasmolytic effect of JWH133. JWH133 also reduced uterine contraction induced by exogenous PGE2 during the estrus phase, suggesting that the mechanism of action of JWH133 depends on the suppression of prostaglandin release and synthesis rather than on the selective effects on receptors present on smooth muscle. CB2R stimulation resulted in specific mitigation of myometrial contractility. These findings could be of interest to designers of tocolytic agents.

Ernst et al. (2016) found that CB2R activation by JWH133 significantly reduced basal but not FSH-activated estradiol and cytochrome P450 aromatase in the immortalized human granulosa cell line KGN. However, basal progesterone level and its FSH-induced stimulation remained unaffected after treatment with JWH133. Therefore, the intrinsic ovarian endocannabinoids showed modulatory effects in regulating estradiol synthesis.

Male Reproductive System

The cannabinoid system stimulates the mitotic–meiotic switch in male germ cells (Grimaldi et al., 2009). Active endocannabinoids have been reported in the testes and spermatozoa from mammals, sea urchins, and the frog *Rana esculenta* (Maccarrone et al., 2005; Schuel and Burkman, 2005). CB2R may also stimulate *in vitro* meiotic entry of postnatal male germ cells and sustain spermatogenesis progression *in vivo* (De Domenico et al., 2017). Thus, endocannabinoid agonism of CB2R may regulate meiotic entry and progression in germ cells.

Grimaldi et al. (2009) reported that CB2Rs were highly expressed throughout spermatogenesis with higher expression levels in spermatocytes (SPC). CB2R activation by JWH133 induced phosphorylation of ERK 1/2 MAPK in spermatogonia and their progression toward meiosis, as evidenced by an increase of synaptonemal complex protein (SCP3), a marker of meiotic prophase, and upregulation of early meiotic prophase genes (c-Kit, Dmc1, and Stra8). However, this effect was abrogated by pretreatment with AM630, indicating a prodifferentiation function of CB2Rs in male germ cells. A similar study by Di Giacomo et al. (2016) demonstrated that JWH133 stimulated the expression of the meiotic genes c-Kit and Stra8 through upregulation of H3K4me3 and downregulation of H3K9me2 in isolated spermatogonia (SPG). Moreover, JWH133 upregulated the Prdm9 gene, which encodes a meiosis-specific histone, H3K4me3 methyltransferase. Chronic administration of JWH133 to immature 7 dpp CD-1 mice accelerated spermatogenesis, whereas CB2 blockade retarded it, suggesting that CB2R hyper- and hypoactivation disrupted the progression of the spermatogenic cycle. The contribution of CB2Rs to the physiological control of spermatogenesis might provide novel therapeutic strategies for treating infertility in humans.

De Domenico et al. (2017) reported that CB2R activation by JWH133 triggered meiosis by elevating SCP3 populations, including preleptotene and leptotene spermatocytes but not in more advanced stages, and indicated that CB2R stimulation facilitated entry and progression of the early stages of meiosis in fetal and postnatal male germ cells. However, they did not repress meiotic checkpoints to move toward the end of prophase I. Additionally, JWH133 upregulated the expression of the meiotic genes Stra8, Kit, Scp1, Scp3, and Dmc1 and downregulated Nanos2—these effects were reversed by pretreatment with AM630. The effect of JWH133 was accompanied by induction of apoptosis, indicating that meiosis facilitation by JWH133 was not followed by DNA repair, thus enhancing oocyte apoptotic rate. Interestingly, JWH133 treatment of pregnant females from E12.5 to E16.5 attenuated primordial and primary follicles in ovaries of newborns with subsequent exhaustion of ovarian store and decreased fertility in adulthood, without affecting spermatogenesis in the offspring' testis. These results highlight the promeiotic function of CB2R in male and female germ cells and suggest that the use of cannabis during pregnancy is a risk for fertility and reproduction in female offspring.

In contrast, Innocenzi et al. (2019) reported that prolonged treatment of P7 CD-1 male mice with JWH133 reduced sperm count, inhibited placental development, and decreased offspring growth, suggesting an overall negative effect on embryo growth. These deformities were accompanied by modified DNA methylation/hydroxymethylation at imprinted genes in sperm that was preserved in the placenta. Thus, overactivated CB2Rs altered sperm DNA methylation patterns that might be inherited and induced negative consequences on offspring growth, underlining the possible risks of recreational use of cannabinoids.

JWH133 in Gastrointestinal Disorders

The gastrointestinal endocannabinoid system is implicated in regulating motility, sensation, and intestinal inflammation (Unal et al., 2020). CBR2 is found throughout the GI tract, with

expression dependent on the presence of inflammation (Ambrose and Simmons, 2019). Moreover, CB2Rs have been identified on enteric neurons, where they are implicated in the regulation of intestinal motility during inflammation (Duncan et al., 2008).

Inflammatory Bowel Disease

Crohn's disease and ulcerative colitis are chronic intestinal inflammatory pathologies collectively known as inflammatory bowel disease (IBD), which is considered a significant health problem currently (Loftus, 2004). Storr et al. (2009) reported that JWH133 treatment mitigated trinitrobenzene sulfonic acid-induced colitis in mice was associated with a remarkable attenuation of inflammation, as demonstrated by reduced macroscopic damage score, colonic adhesions, and myeloperoxidase activity. However, cotreatment with AM630 and JWH133 abrogated the protective effects of JWH133, indicating CBR dependence.

Furthermore, Kimball et al. (2006) found that prophylactic low-dose of JWH133 mitigated colon weight gain, colon shrinkage, macroscopic inflammatory damage score, diarrhea, and pathological damage in a mustard oil-induced model of colitis in CD-1 mice. JWH133 ameliorated microscopic and macroscopic inflammatory damage scores when administered in a prophylactic dose to mice with dextran sulfate sodium-induced colitis, although relatively higher doses of 10 or 20 mg/kg were needed, indicating that JWH133 was less efficient than the CB1R agonist ACEA.

In another study by Singh et al. (2012), JWH133 mitigated colitis-related pathogenesis and decreased body weight in IL-$10^{-/-}$ mice. This was accompanied by a significant decrease in the percentage of $CD4^+$ T cells, neutrophils, mast cells, natural killer cells, and activated T cells in the intestinal lamina propria. Thus, JWH133 abrogated colitis through inhibition of Th cell stimulation by facilitating apoptotic cell death, thereby reducing the production of other inflammatory cells at inflamed sites in the colon. In addition, JWH133 improved dextran sodium sulfate-induced colitis, indicated by a significant reduction in macrophage number and percentage and IFN-γ expression. JWH133 administration stimulated T-cell apoptosis *in vivo* and *in vitro*, whereas AM630 abrogated the protection mediated by JWH133. Cumulatively, CB2R activation by JWH133 mediated anti-inflammatory activities by inhibiting T-cell activation and inducing apoptosis.

In a clinical study, mucosal samples were obtained from the inflamed/uninflamed colon of patients with IBD and Caco-2 cells (Tartakover Matalon et al., 2020). JWH133 did not influence epithelial apoptosis but augmented epithelial/stromal cell proliferation, indicating that enhanced epithelial cell growth could result from the direct action of JWH133 on the epithelial cells or because of a cross-link between CB2R-expressing stromal cells and epithelial cells. Moreover, CB2R stimulation decreased secretome MMP9 and IL-8 levels in inflamed areas. Secretomes of JWH133-treated biopsies showed enhanced Caco-2 number, migration, proliferating cell nuclear antigen, and autophagic LC3IIB expression but did not affect permeability. Therefore, CB2R activation might stimulate mucosal healing in patients with IBD.

Gastrointestinal Motility

Kimball et al. (2010) reported that JWH133 exerted dose-dependent attenuation of small intestinal transit in mustard oil-induced colitis in mice. A dose of 1 mg/kg JWH133, alone or in combination with a CB1R-specific agonist, ACEA, significantly decreased the small intestinal transit in colitis mice compared with that in control mice. CB2R was highly expressed in the lamina propria on day 28 after colitis induction. Therefore, CB2R remodeling occurred during GI inflammation and continued throughout the recovery phase, resulting in increased JWH133 efficacy. Thus, CB2R-specific agonists might improve GI motility in patients suffering from diarrhea-predominant IBS. However, a study by Baldassano et al. (2008) showed that JWH133 at 0.1–10 μM did not cause a dose-dependent decrease in spontaneous contraction in mouse ileal longitudinal muscle; therefore, it did not modulate intestinal motility. Indeed, CB2R in the rat intestine has contributed to GI transit mitigation only following inflammatory stimulus (Mathison et al., 2004). In mice, CB2R function depends on the region of the digestive tract in which it is expressed. CB2R stimulation is ineffective in the colon (Mulè et al., 2007); however, its activation by JWH133 attenuates cholinergic contraction in the stomach, an effect that is reversed by AM630 (Mulè et al., 2007).

Mathison et al. (2004) showed that JWH133 did not affect basal transit but suppressed LPS-mediated GI transit, which was reversed by AM630. JWH133 seemingly acted via cyclooxygenase and independent of iNOS and platelet-activating factor. Thus, CB2R stimulation in response to LPS reestablished regular GI transit following inflammation. This observation was confirmed by Li et al. (2010), who revealed that JWH133 decreased myoelectrical activity, whereas AM630 did not, indicating that CB2Rs do not modulate myoelectrical activity under normal conditions. They also noted that CB2 agonists did not affect upper GI transit under basal conditions.

Similarly, Duncan et al. (2008) identified CB2Rs on enteric neurons. JWH133 did not influence the twitch response of electrically stimulated ileum under physiological conditions but exerted a dose-dependent reduction in LPS-accelerated contraction in rats. Further, JWH133 downregulated the Fos expression induced by LPS in both enteric glia and neurons. This action was blocked by AM630; thus, CB2R stimulation in the enteric neurons of the GI tract decreased the endotoxin-induced accelerated intestinal contractility.

Pancreatitis

Michler et al. (2013) found that JWH133 ameliorated cerulein-induced acute pancreatitis, thereby reducing trypsin activity in pancreatic tissue, myeloperoxidase activity in lung tissue, and IL-6 levels in serum as well as mitigating histological alternations. This was accompanied by inhibition of intra-acinar JNK stimulation and suppression of apoptosis. Pretreatment with JWH133 enhanced p38 phosphorylation in both wild-type and $MK2^{-/-}$ mice. However, the protective effects of JWH133 were reversed after pretreatment with AM630 or in MK2 knockout mice, validating the dependence of JWH133 on CB2R.

Suppression of JNK and stimulation of p38 as well as the MK2-signaling pathways may be responsible for mediating the beneficial effects of CB2R stimulation during acute pancreatitis. Moreover, Xia et al. (2019) showed that JWH133 prevented acetylcholine-induced Ca^{2+} oscillations in mouse pancreatic acinar cells, whereas CB2R-knockout or AM630 blocked the suppressive effects of JWH133. Thus, CB2R activation might play a novel role in modulating the physiology and pathophysiology of pancreatic acinar cells.

JWH133 in Hepatic Diseases

Endocannabinoids are expressed at lower levels in the liver under normal basal conditions and are markedly increased after hepatocyte injury (Caraceni et al., 2010). CB2R stimulation has anti-inflammatory and antifibrogenic activities. It mitigates paracetamol-induced liver injury (Rivera et al., 2020), cirrhosis (Dibba et al., 2018), non-alcoholic fatty liver disease (Mendez-Sanchez et al., 2007), and alcoholic liver disease (Louvet et al., 2011) in experimental models. Thus, targeting the cannabinoid system might attenuate liver injury and reduce the incidence of complications. The hepatoprotective effects and mechanisms of JWH133 are presented in **Figure 4**.

Acute Liver Failure

Acute liver injury (ALI) is characterized by sudden onset of severe dysfunctional hepatocytes and has been correlated with viral hepatitis, drug toxicity, exposure to toxins, and unknown reasons (Zhan et al., 2014). Tomar et al. (2015) reported that JWH133 attenuated GalN/LPS-induced elevation of mortality rate; release of alanine transaminase and inflammatory cytokines (TNF-a, MCP-1, and IL-6), histological alterations, hepatic apoptotic damage, and liver infiltration of mononuclear cells in ALI mice. These effects were accompanied by a significant increase in the production of anti-inflammatory cytokine IL-10 in M1 macrophages, and upregulation of M2 markers (Arg-1 and Chi3L3) in M2 macrophages suggested that JWH133 suppressed M1 stimulation while potentiating the M2 phenotype. Similarly, JWH133 treatment of ALI mice inhibited ALF-mediated expression of M1 markers (TNF-α and IL-12) while upregulating M2 markers (Arg1, IL-10) in liver mononuclear cells. JWH133 downregulated miR-145 expression, which in turn led to a significant upregulation of interleukin-1 receptor-associated kinase 3 (IRAK3), a negative regulator of TLR4 signaling. Cumulatively, CB2 activation could mitigate GalN/LPS-induced ALF by mediating the M1 to M2 transition in macrophages and modulating miR-145 expression to hamper TLR4 signaling following LPS-triggered inflammation.

Killilea et al. (2020) showed that pretreatment with low-dose JWH133 did not attenuate LPS/GalN-induced ALI in Sprague–Dawley or WKY rats at 6 h. These results indicated a lack of CB receptor-mediated protection in ALI SD or WKY rats, and protective effects could be noted with higher doses of JWH133 over different time intervals (e.g., 24 h) after prolonged administration. Further studies are needed to determine whether CB2R activation stimulates or mitigates severe liver injury in stress-sensitive rats.

Alcoholic Liver Disease

Alcoholic liver disease (ALD), a principal cause of morbidity and mortality globally, involves a broad spectrum of diseases, ranging from the relatively benign fatty liver to more severe liver injury (Gao and Bataller, 2011).

Louvet et al. (2011) demonstrated that treatment of alcohol-fed mice with JWH133 mitigated hepatic M1 gene expression (TNF-α and the chemokines CCL3, CCL4, and IL-6) without influencing M2 macrophages, indicating that endogenous or exogenous stimulation of CB2R suppressed alcohol-mediated M1 polarization of Kupffer cells. Further, CB2R activation by JWH133 led to significant modulation of alcohol-induced fatty liver, as demonstrated by the attenuation of liver steatosis in mice and its acceleration in $CB2^{-/-}$ mice. Additionally, JWH133 inhibited M1 polarization and mediated the shift to M2 macrophages in isolated Kupffer cells and cultured macrophages, thereby protecting against lipid accumulation in hepatocytes via paracrine effects. In cultured macrophages and alcohol-fed mice, JWH133 also upregulated the expression of heme oxygenase-1, whereas the HO-1 inhibitor zinc protoporphyrin blocked the preventive effect of JWH133 on LPS-induced NF-κB stimulation and M1 polarization, indicating that CB2R activation affords anti-inflammatory effects by upregulating HO-1 in macrophages.

Furthermore, Denaës et al. (2016) showed that CB2R stimulation by JWH133 augmented autophagy, as evidenced by increased accumulation of LC3-II and reduced SQSTM1/p62 levels via HO-1 pathway in cultured RAW264.7 macrophages. Moreover, JWH133 mitigated the release of LPS-induced proinflammatory genes (CCL4, IL-1, CCL3, and IL-6, iNOS) in cultured macrophages but not in ATG5-deficient cells. Confirming these results *in vivo*, they found that JWH133 protected wild-type mice from alcohol-induced hepatic inflammation and steatosis; however, this was not noted in $ATG5Mye^{-/-}$ mice, demonstrating that autophagic process in macrophages mediate the anti-inflammatory and antisteatogenic activities of CB2Rs.

Liver Cirrhosis

Muñoz-Luque et al. (2008) reported that JWH133 ameliorated arterial pressure, reduced the infiltration of inflammatory CD68 cells, and reduced activated stellate cells while enhancing apoptotic cell death in myofibroblastic and monocytic cells and reducing fibrosis in cirrhotic rats with ascites induced by CCl4. The authors also observed reduced α-SMA and collagen I and enhanced MMP-2 expression in cirrhotic rat liver. Therefore, selective stimulation of hepatic CB2R caused a significant decrease in hepatic collagen levels in cirrhotic rats, indicating that selective CB2 agonists might be a therapeutic agent for liver fibrosis.

Huang et al. (2012) found that JWH133 reduced mesenteric vascular density, mesenteric angiogenesis, and portosystemic shunting in cirrhotic rats induced by bile duct ligation. Because mesenteric blood flow is the major contributor of portal blood inflow, its suppression by JWH133 mitigates liver fibrosis. Yang et al. (2014) reported that prolonged JWH133 treatment alleviated portal hypertension, systemic/intestinal

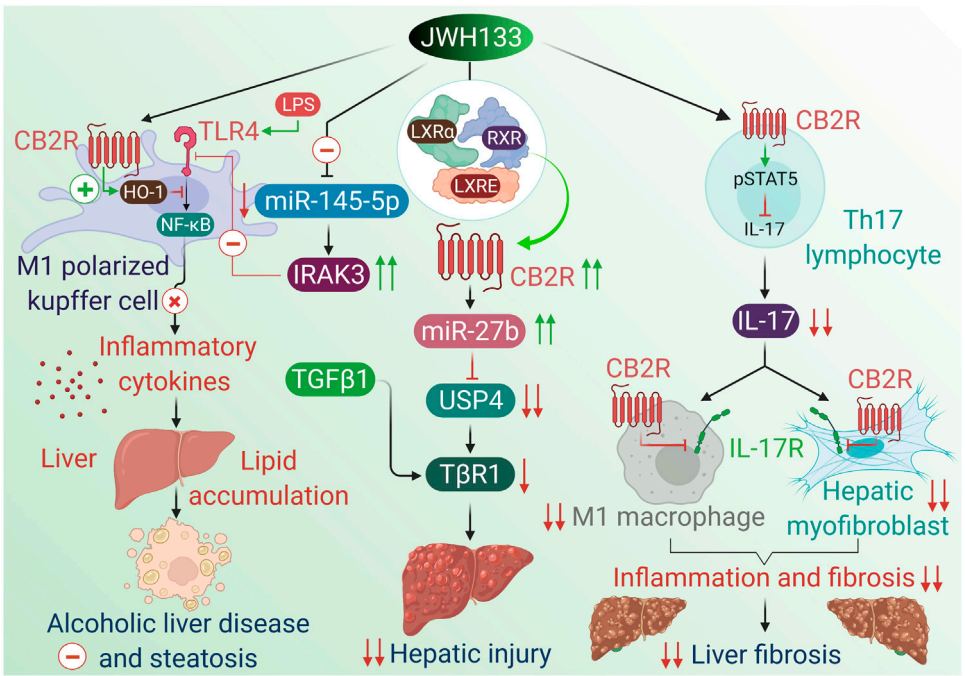


FIGURE 4 | The hepatoprotective effects and mechanism of JWH133.

oxidative damage, associated inflammation, infection, intestinal mucosal damage, and hyperpermeability in cirrhotic ascitic rats. The authors observed a significant reduction in bacterial overgrowth and adhesion; decrease in spontaneous bacterial peritonitis; upregulation of intestinal tight junction genes, namely, occludin, claudin, and ZO-1; and downregulation of TNF-α-receptor/NF-kBp65 protein expression in peritoneal macrophages. Additionally, acute and chronic JWH133 treatment protected against the TNFα-mediated inhibition of phagocytosis of peritoneal macrophages in cirrhotic rats, an effect that was abrogated by cotreatment with AM630, suggesting that chronic CB2R stimulation by JWH133 markedly improved the phagocytosis of peritoneal macrophages in cirrhotic rats by suppressing TNF-α signaling, proinflammatory cytokine secretion, and oxidative stress. Therefore, CB2R ligands might be beneficial for treating bacterial translocation in cirrhosis.

Steib et al. (2013) found that pretreatment with JWH133 mitigated portal hypertension following Kupffer cell activation in cirrhotic rats induced by BDL. Further, JWH133 upregulated the expression of HO-1, whereas treatment with the HO-1 inhibitor ZnPP IX accelerated portal hypertension, indicating the beneficial role of HO-1 signaling. In isolated Kupffer cells activated by either Zymosan or LPS, JWH133 treatment significantly increased the expression of CB2 and HO-1, while reducing the expression of the vasoconstrictor TXB2. HO-1 reduces portal pressure via its anti-inflammatory activity (Angermayr et al., 2006), leading to decreased TXB2 production. Pretreatment with the PPARγ inhibitor GW9662 blocked JWH133-induced attenuation of portal hypertension and upregulation of HO-1. Therefore, PPARγ might be the link between CB2R and HO-1. CB2R activation mediating the HO-1 pathway could be a beneficial target for patients with liver cirrhosis-associated portal hypertension.

Liver Fibrosis

Teixeira-Clerc et al. (2010) reported that CB2R activation by JWH133 led to a significant reduction in liver apoptosis and acceleration of hepatic regeneration measured by the increased onset of PCNA induction in CCl4-treated mice. Incubating hepatic myofibroblasts with JWH133 enhanced the expression of TNF-α and IL-6 and reduced the expression of MMP-2 as myofibroblasts secrete bioactive cytokines with antiapoptotic and mitogenic effects, such as TNF-α and IL-6 (Lotersztajn et al., 2005). Thus, CB2R mitigated hepatic injury and promoted regeneration through a paracrine mechanism, including hepatic myofibroblasts, suggesting that CB2 ligands demonstrate hepatoprotective activities as well as antifibrogenic effects.

Guillot et al. (2014) showed that incubation of T-helper (Th17) lymphocytes with JWH133 reduced the differentiation of CD41-naive T cells into Th17 lymphocytes and was accompanied by decreased Th17 marker expression and IL-17 secretion. IL-17 is a proinflammatory and fibrogenic cytokine mainly produced by Th17 lymphocytes. It did not alter the release of antifibrogenic IL-22. However, the suppressive effects of JWH133 were abrogated in Th17 lymphocytes obtained from CB2-knockout mice. Further, JWH133 increased the phosphorylation and translocation of STAT5 into the nucleus, a function that was blocked by adding a STAT5 inhibitor. Finally, CB2R stimulation in macrophages and hepatic myofibroblasts showed blunted IL-17-induced expression of proinflammatory

genes. Cumulatively, CB2R stimulation decreased liver fibrosis by specifically decreasing IL-17 production by Th17 lymphocytes in a STAT5-dependent manner and decreasing the proinflammatory activity of IL-17 while conserving IL-22 production.

Wu et al. (2019) found that treating mice with JWH133 and CCL4 plus clodronate inhibited toxicant-mediated hepatic injury, as demonstrated by reduced levels of ALT, AST, apoptotic cells, caspase-3, and CREB. JWH133 also attenuated protein kinase A activity except in CB2-deficient mice, demonstrating that hepatocytic cells express functionally active CB2 downstream of the liver X receptors (LXRα). Additionally, JWH133 administration suppressed TGF-β1-mediated cleavage of caspase-3 in AML12 cells and reduced ubiquitin-specific peptidase 4 (USP4), indicating that LXRα stimulation of CB2 destabilized TGF-β receptor 1 (TβRI), an upstream sensing molecule via USP4 suppression. This result was associated with significant upregulation of miR-27b, an inhibitor of USP4. Thus, LXRα could exert a protective effect against TGF-β by transcriptional regulation of the CB2R gene in hepatocytes, and then CB2 might inhibit USP4-stabilizing TβRI via miR-27b.

Hepatic Ischemia-Reperfusion

Bátkai et al. (2007) reported that CB2R activation by JWH133 markedly decreased transaminase levels, attenuated oxidative stress, and reduced the infiltration of inflammatory cells, as demonstrated by reduced levels of MPO activity, TNF-α, MIP-1α, MIP-2, and ICAM-1 following ischemia–reperfusion in mice. Furthermore, JWH133 mitigated TNF-α-stimulated ICAM-1 and VCAM-1 expression in human liver sinusoidal endothelial cells (HLSECs) and decreased the adhesion of human neutrophils to HLSECs. However, this protective effect was completely abolished by cotreatment with SR144528 or in $CB2^{-/-}$ mice, indicating the dependence on CB2R.

Reifart et al. (2015) found that JWH133 pretreatment downregulated α-SMA in I/R mice, and hepatic stellate cell activity was negatively affected by CB2R activation. HSC deactivated by JWH133 exhibited markedly reduced $CD4^+$ T-cell migration in the postischemic liver. JWH133 resulted in significant improvement of postischemic perfusion and decreased liver injury. Thus, the deactivation of hepatic stellate cells by JWH133 attenuated $CD4^+$ T-cell recruitment and decreased microvascular and hepatocellular injuries. Thus, hepatic stellate cells could be a clinical target for novel therapeutic approaches for T-cell-induced I/R injury during liver transplantation.

JWH133 in Autoimmune Disorders

CB2Rs are expressed by all immune cells with varying expression between immune cells and activation conditions (Suárez-Pinilla et al., 2014). CB2Rs from hematopoietic cells promote cannabinoid-induced immune modulation (Munro et al., 1993). Synthetic CB2R agonists significantly suppressed autoimmunity in different animal models, including collagen-induced arthritis (Malfait et al., 2000), experimental autoimmune encephalomyelitis (Sánchez et al., 2006), and virus-mediated demyelinating disease (Arévalo-Martín et al., 2003).

Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic autoimmune disorder characterized by prolonged inflammation of the synovium, resulting in bone and cartilage destruction (McInnes and Schett, 2017). Fukuda et al. (2014) found that JWH133 prevented the secretion of IL-6, MMP-3, and CCL2 from TNF-α-activated fibroblast-like synoviocytes obtained from the rheumatoid joints. Further, coincubating peripheral blood $CD14^+$ monocytes with JWH133 caused a dose-related suppression of osteoclast formation and inhibition of M-CSF and RANKL-mediated calcium resorption. Additionally, JWH133 treatment of mice with collagen-induced arthritis (CIA) decreased the arthritis score and reduced inflammatory cells' infiltration, bone destruction, and antiCII IgG1 release. Thus, CB2R activation might be a beneficial target for RA by inhibiting the production of proinflammatory cytokines from fibroblast-like synoviocytes and preventing the formation of bone-resorbing cells.

Zhu et al. (2019) showed that JWH133 mitigated synovial hyperplasia, associated inflammation, cartilage damage, and bone destruction in CIA mice, indicating the remarkable protective activity of JWH133 against arthritis and local bone loss in the CIA mice. JWH133 injection decreased the infiltration of proinflammatory M1-like macrophages and promoted macrophage repolarization from the M1 to M2 phenotype. The authors also observed upregulation of the anti-inflammatory cytokine IL-10 and downregulation of inflammatory mediators, such as TNF-a, IL-1b, and IL-6. Moreover, JWH133 treatment alleviated osteoclast formation and bone resorption and downregulated the expression of RANKL-induced NF-kB activation, MMP-9, tartrate-resistant acid phosphatase, cathepsin K, and nuclear factor of activated T cells 1 (NFAT-1) in CIA mice and osteoclast precursors. These effects were abolished by cotreatment with SR144528. JWH133 also downregulated the expression of p65-positive cells in CIA mice. Thus, JWH133 inhibited osteoclastogenesis and inflammation-mediated bone destruction by inhibiting NF-kB signaling, thereby highlighting its clinical potential as a therapeutic agent for human RA.

Conversely, Fechtner et al. (2019) reported that pretreatment of RA synovial fibroblasts (RASFs) with JWH133 did not attenuate IL-1β-mediated IL-6 and IL-8 production and upregulated the expression of COX-2. However, these effects were reversed in CB2-deficient mice. Further, MMP-2 and MMP-9 activities were decreased in CB2-deficient mice. In contrast, activation of CB2 in RASFs augmented the IL-1β-induced IL-6, IL-8, RANTES, and ENA-78. They also found that JWH133 coordinated the CB2R association with TGFβ-activated kinase 1, a key signaling molecule, increasing the IL-1β-induced nuclear translocation of NF-κBp65 and activation protein-1. This conflicting data showed that pharmacological activation of CB2R mediated IL-1β-induced inflammation in RASFs, whereas genetic deletion of CB2R in mice alleviated the inflammation induced by IL-1β, thus highlighting the role of the CB2R in managing RA pain and inflammation.

Immune Thrombocytopenia

Immune thrombocytopenia (ITP) is a complex autoimmune disease marked by antibody-stimulated platelet destruction

(Khan et al., 2017). Rossi et al. (2019a) reported that incubating ITP-mesenchymal stromal cells with JWH133 and Dexa, alone or in combination, significantly reduced the expression of the inflammatory mediator IL-6 and induced the expression of IL-4. These effects were reversed by AM630, thereby verifying the dependence of these effects on CB2R. CB2R activation by JWH133 and Dexa attenuated apoptosis by stimulating Bcl2 signaling and restored mesenchymal stromal immunomodulation. This effect was blocked by AM630, indicating the dependence of mesenchymal stromal cell immunosuppression on CB2R. These findings suggest that the combination of Dexa with JWH133 is beneficial in ITP, decreasing the dose requirements and incidence of adverse effects.

Autoimmune Uveoretinitis

Experimental autoimmune uveoretinitis (EAU) in rats and mice is a prototypic T-cell-induced autoimmune disorder targeting the neural retina and associated tissues (Caspi, 2003). Xu et al. (2007) showed that JWH133 inhibited EAU in mice by suppressing disease induction and effector stages. JWH133 also abolished cytokine/chemokine production (TNF-α, IL-6, IL-10, INF-γ, CCL2). Additionally, treating EAU mice with JWH133 inhibited leukocyte trafficking in the inflamed retina because of its effect on attenuating adhesion molecules CD162 (P-selectin glycoprotein ligand 1) and CD11a (LFA-1) expression on T cells. Leukocytes isolated from JWH133-treated mice exhibited a reduced response to activation by retinal peptide and mitogen Concanavalin A. Downregulation of TLR4 via Myd88 signaling may be responsible for the inhibitory effects on antigen presentation. Taken together, CB2R activation by JWH133 produced an anti-inflammatory effect by suppressing the stimulation and function of autoreactive T cells and averting leukocyte trafficking into the inflamed retina.

Systemic Sclerosis

Systemic sclerosis (SSc) is an autoimmune connective tissue disease marked by inflammation and intensive fibrosis of the skin and visceral organs (LeRoy and Medsger, 2001). Servettaz et al. (2010) found that CB2R activation by JWH133 suppressed the development of skin fibrosis, with significantly reduced dermal thickness and collagen content in the skin and lungs of hypochlorite-induced SSc mice. JWH133 also reduced pulmonary T-cell infiltration and counteracted the increase in splenic B cell numbers, decrease in fibroblast growth, and the development of autoantibodies (antiDNA topoisomerase1 IgG Abs). However, these effects were blocked in CB2R-knockout mice, confirming the impact of CB2R in systemic fibrosis and autoimmunity.

JWH133 in Renal Disorders

CB2Rs are expressed in glomeruli and tubules in human and rat kidneys (Jenkin et al., 2010; Barutta et al., 2011). CB2R also localizes to the bladder tissue of different species, such as humans, rodents, and monkeys (Gratzke et al., 2010; Li et al., 2013). Earlier studies have revealed that the levels of endocannabinoids and CB2Rs in renal ischemia are linked with renal damage (Moradi et al., 2016; Pressly et al., 2018). CB2 stimulation reduced renal damage and CB2 antagonism increased kidney damage in various experimental models of nephropathy (Jenkin et al., 2016; Zoja et al., 2016).

Renal Ischemia-Reperfusion Injury

Kidney IR injury is a pathological condition that leads to acute kidney failure (Hsu et al., 2007). Feizi et al. (2008) reported that JWH133 administration resulted in dose-dependent inhibition of reperfusion-induced ischemia-mediated lesions in mouse kidneys. These results were confirmed by Çakır et al. (2019a), who found that treatment with three different doses of JWH133 significantly mitigated the glomerular and tubular injury in rats. This was accompanied with a significant reduction in the levels of renal NF-κB, TNF-α, IL-1β, and caspase-3.

Likewise, JWH133-treated rats showed a remarkable decrease in the serum levels of TNF-alpha, blood urea nitrogen, creatinine, kidney injury molecule-1, neutrophil gelatinase-associated lipocalin, cystatin C, IL-18, IL-1β, IL-6, and IL-10. Therefore, CB2R activation by JWH133 ameliorated pathological kidney damage by suppressing inflammatory cytokine secretion and apoptosis. JWH133 could be a novel therapeutic agent in the prevention of renal IR injury.

Interstitial Cystitis/Bladder Pain Syndrome

Furthermore, Liu et al. (2020a) showed that JWH133 diminished mechanical hyperalgesia, reduced urine spot numbers, and enhanced the micturition frequency mediated by cyclophosphamide-induced cystitis in mice. They also observed a reduction in bladder tissue inflammation and oxidative damage as indicated by reduced levels of proinflammatory mediators, including IL-1β, TNF-α, and IL-8, and enhanced activities of cellular GSH and SOD, while lowering MDA levels. CB2R stimulation by JWH133 induced autophagy via upregulation of LC3-II/LC3-I and downregulation of SQSTM1/p62 in mouse bladder tissue. However, treatment with AM630 abolished these protective effects. Cotreatment with the autophagy inhibitor 3-methyladenine also blocked the influence of JWH133 on inflammation and oxidative injury. Furthermore, JWH133 upregulated *p*-AMPK expression and downregulated *p*-mTOR expression, whereas pretreatment with 3-methyladenine blocked this effect. Thus, CB2 stimulation in the bladder mitigated the severity of cyclophosphamide-induced cystitis and improved bladder inflammatory responses by activating autophagy and AMPK-mTOR signaling.

JWH133 in Skin Diseases

CB2Rs are localized in the skin, indicating that CB2 signaling could have a role in dermal fibrosis (Blázquez et al., 2006; Karsak et al., 2007). Akhmetshina et al. (2009) reported that JWH133 mitigated the profibrotic activity of bleomycin and reduced dermal thickening in bleomycin-induced fibrosis in mice. CB2 mediated its antifibrotic effects in mice by preventing leukocyte infiltration into skin lesions in mice treated with JWH133. Thus, CB2 signaling could indirectly influences dermal fibrosis by modulating leukocyte

infiltration rather than direct action on the collagen synthesis in fibroblasts. However, preventing CB2 signaling by gene inactivation or CB2R blockade enhanced the vulnerability to bleomycin-induced dermal fibrosis. These findings suggest CB2 activation is a promising strategy for treating the early inflammatory stages of systemic sclerosis. Norooznezhad and Norooznezhad (2017) suggested using oral or topical JWH133 for psoriasis owing to its ability to inhibit keratinocyte proliferation and prevent angiogenesis and inflammation. However, further *in vivo* studies and clinical trials are needed.

JWH133 in Respiratory and Lung Diseases

CB receptors in rat and human pulmonary artery endothelial cells can be stimulated to reduce oxidative damage and inflammation (Luchicchi and Pistis, 2012). Previous studies have demonstrated that smoking marijuana or ingestion of 9-tetrahydrocannabinol (THC) results in bronchodilation (Tashkin et al., 1974; Tashkin et al., 1975). In the lung tissue, activation of CB1 or CB2Rs can suppress C-fiber-induced responses, such as neurogenic inflammation, bronchoconstriction, and cough (Patel et al., 2003; Fukuda et al., 2010). Thus, treatments targeting CB receptors could help manage airway hyperresponsiveness and asthma (Pini et al., 2012).

Lung Injury

Paraquat (PQ) poisoning is one of the greatest clinically important herbicides causing morbidity and mortality. Respiratory failure resulting from lung injury is the most common cause of death from PQ (Dinis-Oliveira et al., 2008). Liu et al. (2014) found that JWH133 mitigated PQ-induced lung edema and pathology. JWH133 also inhibited the release of inflammatory cytokines (TNF-α and IL-1β) in bronchoalveolar lavage fluid, PaO_2 in arterial blood, and myloperoxidase levels in the lung tissue. This was associated with a remarkable inhibition of phosphorylation of p38MAPK, ERK1/2, JNK1/2, and MAPK and stimulation of NF-kB. CB2R activation in lung tissue protected against PQ-induced acute lung injury by suppressing the stimulation of MAPKs and NF-kB signaling.

Asthma

Asthma is a chronic inflammatory airway disease linked with bronchospasm and airway hyperresponsiveness (Groot Kormelink et al., 2009). Frei et al. (2016) found that JWH133 stimulated a moderate migratory response in mice eosinophils. However, short-term treatment with JWH133 augmented chemoattractant-mediated eosinophil shape changes and upregulated adhesion molecules such as CD11b and ICAM-1 as well as increased the release of ROS. However, the effects of JWH133 were abrogated in CB2 knockout mice and after treatment with SR144528. Systemic treatment with JWH133 intensified the eotaxin-2/CCL24-induced eosinophil recruitment into the airways of IL-5Tg mice and aggravated ovalbumin-induced asthma by enhancing eosinophil migration into the lungs and deteriorating airway hyperreactivity in a CB2-dependent manner. This effect was completely reversed in eosinophil-deficient ΔdblGATA mice, indicating that eosinophils could be the main target of JWH133 in allergic inflammation. This CB2-induced triggering of eosinophil influx could be independent of Gi/o/adenylyl cyclase but includes the Gaq/MEK/ROCK pathway. Thus, the cannabinoid/CB2 axis might influence allergic inflammation and indicate possible unwanted inflammatory effects of continuing cannabinoid use.

Similarly, a study by Yeh et al. (2016) found that perivagal treatment with JWH133 did not attenuate H_2O_2-induced vagal lung C-fiber hypersensitivity in rats, reflecting the pathophysiology of airway hyperresponsiveness in asthmatic patients (Kuo and Lai, 2008), suggesting that CB2R activation does not reduce the hypersensitivity in vagal lung C-fibers. Furthermore, Bozkurt et al. (2016) reported that JWH133 did not modify serotonin-induced hyperreactivity in tracheas obtained from dinitrofluorobenzene (DNFB) group of non-atopic asthmatic mice. Further, JWH133 did not inhibit the increase in macrophage number in bronchoalveolar lavage fluid. Therefore, CB2R stimulation did not mitigate airway inflammation in DNFB-treated mice.

Gastroesophageal reflux is a prevalent clinical disease linked with several respiratory symptoms, such as bronchoconstriction and chronic cough, and it is more common in patients with asthma (Leggett et al., 2005). Contrary to the nonprotective effect of JWH133 in airway inflammation, a study by Cui et al. (2007) found that JWH133 suppressed microvascular airway leakage and bronchoconstriction induced by intraoesophageal HCl in guinea pigs. However, the protective effect of JWH133 was reversed by SR144528, indicating that the effect was mediated by CB2R stimulation. This finding was consistent with a study conducted by Yoshihara et al. (2004), who found that JWH133 resulted in a dose-dependent inhibition of electrical field stimulation and capsaicin-induced contraction of bronchi obtained from guinea pigs.

Further, JWH133 prevented capsaicin-mediated production of substance P-like immunoreactivity from guinea pig airway tissues, which indicated that CB2R decreased the stimulation of capsaicin-sensitive afferent sensory nerves (C-fibers) in airways. Moreover, Patel et al. (2003) showed that JWH133 administration suppressed cough reflex induced by citric acid in guinea pigs. They also found that JWH133 repressed sensory nerve depolarization of the guinea pig and human vagus nerve induced by hypertonic saline, capsaicin, or the prostaglandin PGE2, whereas this effect was blocked by treatment with SR 144528. Furthermore, Grassin-Delyle et al. (2014) found that the highest concentrations of JWH133 resulted in the suppression of electrical field stimulation-induced contraction of human bronchi with a longer time to onset of action of 167 min.

Lung Fibrosis

Pulmonary fibrosis is a group of lung diseases that comprises a combination of inflammation and fibrosis of the lung parenchyma (Gutsche et al., 2012). Fu et al. (2017) reported that preincubation of TGF-β1-activated lung fibroblasts with JWH133 counteracted the induction of collagen I and α-SMA and suppressed fibroblast growth and migration, all of which were reversed by coincubation with SR144528. Preventive dosing with JWH133 reduced lung fibrosis in bleomycin-treated mice

and was associated with significant inhibition of the inflammation and extracellular collagen accumulation and reduced hydroxyproline content.

Notably, JWH133 decreased the serum levels of TGF-β1 and repressed TGF-β1/Smad2 signaling *in vitro* and *in vivo*. These data suggest that activation of the CB2R by a pharmacological agent is a promising strategy for pulmonary fibrosis. Wawryk-Gawda et al. (2018) found that JWH133-treated mice showed normal lung tissue structure and thinner alveolar septum compared with nicotine mice. JWH133-treated mice also showed septum thickness and collagen accumulation. JWH133 downregulated the expression of connective tissue growth factor, an essential inducer of pulmonary fibrosis, and α-SMA, suggesting its beneficial function in preventing interstitial fibrosis.

Lung Ischemia-Reperfusion Injury

Lung ischemia–reperfusion injury (IRI) is a common and severe postoperative complication after cardiopulmonary bypass, lung transplantation, pulmonary thrombosis, and cardiac arrest (den Hengst et al., 2010). Zeng et al. (2019) reported that pretreatment with JWH133 mitigated lung edema and infiltration of inflammatory cells and lung histopathological alternations induced by IRI in mice. Further, JWH133 administration ameliorated the PaO2/FiO2 ratio, reduced lung TNF-α, IL-6, MDA levels, myeloperoxidase activities, and enhanced superoxide dismutase activity. However, the beneficial effects of JWH133 were abrogated by pretreatment with AM630, indicating that CB2R stimulation prevented IR-mediated oxidative injury and inflammatory response and improved lung IRI.

Likewise, pretreatment with a PI3K inhibitor reversed the protective effect of JWH133 and decreased the expression of *p*-AKT without altering JWH133-driven CB2R expression. Thus, CB2R activation could protect against IR-mediated lung injury by attenuating inflammation and oxidative stress in mice via PI3K/AKT signaling. Huang et al. (2020) showed that pretreatment with JWH133 markedly mitigated the lung injury induced by I/R and reduced oxidative stress in mice. It also led to a significant upregulation of expression of CB2R and downregulation of NOX2. In contrast, cotreatment with AM630 or a NOX2 inhibitor reversed the effects of JWH133. Therefore, CB2R stimulation alleviated lung IRI by inhibiting oxidative stress via NOX2 in mice.

JWH133 in Viral Infections

Respiratory syncytial virus (RSV) causes severe lower respiratory tract symptoms, mainly bronchiolitis and pneumonia, in infants and young children (Borchers et al., 2013; Troy and Bosco, 2016). Tahamtan et al. (2018) reported an association between CB2 variant Q63R and a high risk of hospitalization in children with acute respiratory tract infection. Further, children with the QQ genotype were more vulnerable to severe acute respiratory tract infection. The increased risk of developing severe acute respiratory tract infection secondary to RSV infection is more than 2-fold higher in children who carry the Q allele. In Balb/c mice, JWH133 significantly reduced the influx of bronchoalveolar lavage (BAL) cells and abolished leukocyte migration into the lungs in RSV.

Moreover, CB2 stimulation by JWH133 resulted in a significant reduction in the levels of IFN-γ and MIP-1α and increased IL-10 levels in the BAL of mice while mitigating lung pathology. JWH133 also inhibited the accumulation of immune cells in the peribronchial and perivascular spaces of the lung after RSV infection. Therefore, CB2R is the primary signaling pathway for endocannabinoid-mediated immune modulation and might play a pivotal role in regulating immune homeostasis and maintaining the extent of the immunological response via a negative regulatory mechanism.

JWH133 in Wound Repair, Healing, and Differentiation

Several studies have demonstrated that cannabinoids promote wound healing by enhancing cellular migration, resulting in the preservation of vascular integrity (Zhang et al., 2010); corneal wound healing (Yang et al., 2010); and epithelial wound closure in colonic tissues (Wright et al., 2005). CB2Rs have demonstrated wound healing effects in various models (Li et al., 2016; Wang et al., 2016). Ruhl et al. (2020) found that incubation with JWH133 elevated the population of human adipose tissue mesenchymal stromal cells (atMSC), which release many cytokines and growth factors that control cell differentiation, angiogenesis, and the immune response to mediate the repair of damaged tissue (Yancopoulos et al., 2000; Pakyari et al., 2013). JWH133 enhanced VEGF, TGF-β1, and HGF secretion, which then enhanced the regenerative activity of atMSCs. Thus, CB2R agonists could be a promising target for increasing the regenerative potential of atMSCs.

Schmuhl et al. (2014) showed that JWH133 stimulated the migration of mesenchymal stem cells, which mediate wound healing. This effect was suppressed by AM-630 and the p42/44 MAPK activation antagonist PD98059, indicating that CB2R stimulation by JWH133 induces p42/44 MAPK. Furthermore, JWH133 mitigated TGF-β1-mediated production of fibronectin, collagen I and III, and expression of MMP-1 and MMP-3 in cultured human Tenon's fibroblasts (Guan et al., 2017). JWH133 also attenuated TGF-β1-mediated matrix contraction and remodeling in a dose-dependent manner, in conjunction with a remarkable suppression of activated MAPKs, such as ERK1/2, p38, and JNK as well as extracellular matrix synthesis and the contractility of human Tenon's fibroblasts *in vitro*. Therefore, pharmacological stimulation of CB2R could protect against scar formation in wound healing after glaucoma filtration surgery. Murataeva et al. (2019) showed that CB2R activation by JWH133 induced a chemorepulsive effect in cultured corneal epithelial cells (CECs), but did not change CEC growth. CB2R activation also induced *p*-ERK expression and cAMP production, the latter being due to Gs-coupling. Additionally, wound closure was delayed in CB2R-knockout mice and the presence of CB2R blockade by SR144528. Thus, CB2R receptor activation could support wound healing, possibly by chemorepulsion.

The physiological balance between self-renewal and differentiation is necessary for hematopoietic stem cell function and hematopoiesis. CB2Rs localize to human and murine hematopoietic stem and progenitor cells (HSPCs), and

JWH133 activation induces colony formation and HSPC recruitment *in vitro* and accelerates colony formation of bone marrow cells via ERK, PI3-kinase, and Gαi-Rac1 signaling (Jiang et al. 2011). However, granulocyte colony-stimulating factor-stimulated migration of HSPCs was significantly attenuated by AM630 and was absent in $CB2^{-/-}$ mice. These findings implicate the cannabinoid system in hematopoiesis and suggest that CB2 activation mediates repopulation and migration of HSPCs, indicating its clinical value in bone marrow transplantation.

JWH133 augmented oligodendrocyte progenitor cell differentiation, as demonstrated by the increased expression of stage-specific antigens and myelin basic protein, and this effect was reversed by AM630 (Gomez et al., 2011). Enhanced oligodendrocyte differentiation was owing to the JWH133-stimulated CB2R activation of *p*-AKT and mTOR signaling. Therefore, CB2R stimulation could profoundly affect oligodendrocytes and consequently affect brain repair. In a rat model of skeletal muscle contusion, CB2R activation by JWH133 significantly reduced the fibrotic area and inhibited the expression of collagen type I/III as well as amplified the number of multinucleated regenerating myofibers in the injured area (Yu et al., 2015). These results were directly attributed to the reduced expression of TGF-β1, fibronectin-EIIIA, and α-SMA; decreased production of myofibroblasts; and concurrent upregulation of MMP-1/2 by JWH133. Therefore, CB2R activation inhibited fibrotic formation and improved muscle regeneration, suggesting a therapeutic value in patients with skeletal muscle injuries and disorders. JWH133 stimulation of CB2R attenuated the infiltration of M1 macrophages and enhanced M2 populations in a mouse model of incised skin wound healing (Du et al. 2018). JWH133 also downregulated the expression of the M1-related cytokines IL-6, IL-12, CD86, and iNOS and upregulated the expression of the M2-related cytokines IL-4, IL-10, CD206, and Arg-1. Inhibition of the inflammatory process by CBR2 activation might lead to the development of novel therapies for cutaneous inflammation.

CONCLUSION

JWH133 is a synthetic cannabinoid with seemingly limitless therapeutic potential for different pathological conditions, primarily owing to its CB2R specificity, which in addition to making this synthetic ligand devoid of psychoactive effects, determines its major biological activities. The available studies reviewed here suggest that JWH133 inhibits inflammation, oxidative stress, and apoptosis, among other effects, resulting in the mitigation of various pathologies. JWH133 is considered a suitable CB2R agonist for preclinical target validation, based on the following features: 1) selective agonism on CB2R over CB1R in both humans and mice, 2) well-balanced stimulation of signaling transduction on human CB2R, 3) negligeable number of off-target activities at its effective doses, 4) reasonable pharmacokinetics properities and 5) deficiency of cannabimimetic pharmacological effects *in vivo* suggestive of CB1R activity. The bulk of our knowledge about the polypharmacological effects of JWH133 in *in vitro* and *in vivo* models is derived from the aforementioned studies. Much of this work displayed potent antioxidant, anti-inflammatory, and antiapoptotic activities, confirmed *in vitro* and *in vivo* mechanisms of JWH133 actions and could allow a successful transfer of preclinical data to the patient's bedside. However, further investigations in animals are needed to delineate the pharmacokinetic properties as well as safety and toxicity of JWH133 before large scale human studies are conducted. Such investigations may recognize more clinically suitable routes of administration, establish the extent of drug stability and metabolism while providing evidences about potential adverse effects of JWH133. As the U.S. Drug Enforcement Administration criminalizes any extract "containing one or more cannabinoids," JWH133 is a scheduled substance in the U.S. This is despite the low addictive potential relative to its sister compounds such as JWH-018, as JWH133 is highly selective for the non-psychoactive CB2R and thus lacks significant psychoactive effects.

AUTHOR CONTRIBUTIONS

SO conceptualized the hypotheses. HH performed the literature search, drafted tables and wrote first draft. NJ draw the schemes and the artwork. CS, HH, NJ, SG and SO significantly contributed to the editing of the manuscript. All authors read, edited, and approved the manuscript.

FUNDING

The authors are thankful to the open access publication support from UAE University.

ACKNOWLEDGMENTS

The authors are grateful to the United Arab Emirates University for the award of the graduate (PhD) research fellowship to HH and research grant support to SO from Zayed Bin Sultan Center for Health Sciences (21R075). The authors also sincerely acknowledge the research fund support from ADEK award for Research Excellence (AARE18-194).

REFERENCES

Aghazadeh Tabrizi, M., Baraldi, P. G., Borea, P. A., and Varani, K. (2016). Medicinal Chemistry, Pharmacology, and Potential Therapeutic Benefits of Cannabinoid CB2 Receptor Agonists. *Chem. Rev.* 116, 519–560. doi:10.1021/acs.chemrev.5b00411

Agirregoitia, E., Ibarra-Lecue, I., Totorikaguena, L., Mendoza, R., Expósito, A., Matorras, R., et al. (2015). Dynamics of Expression and Localization of the Cannabinoid System in Granulosa Cells during Oocyte Nuclear Maturation. *Fertil. Sterility* 104, 753–760. doi:10.1016/j.fertnstert.2015.06.013

Aguado, T., Carracedo, A., Julien, B., Velasco, G., Milman, G., Mechoulam, R., et al. (2007). Cannabinoids Induce Glioma Stem-like Cell Differentiation and Inhibit Gliomagenesis. *J. Biol. Chem.* 282, 6854–6862. doi:10.1074/jbc.m608900200

Akhmetshina, A., Dees, C., Busch, N., Beer, J., Sarter, K., Zwerina, J., et al. (2009). The Cannabinoid Receptor CB2 Exerts Antifibrotic Effects in Experimental Dermal Fibrosis. *Arthritis Rheum.* 60, 1129–1136. doi:10.1002/art.24395

Ambrose, T., and Simmons, A. (2019). Cannabis, Cannabinoids, and the Endocannabinoid System-Is There Therapeutic Potential for Inflammatory Bowel Disease?. *J. Crohns Colitis* 13, 525–535. doi:10.1093/ecco-jcc/jjy185

Amin, M. R., and Ali, D. W. (2019). Pharmacology of Medical Cannabis. *Adv. Exp. Med. Biol.* 1162, 151–165. doi:10.1007/978-3-030-21737-2_8

An, D., Peigneur, S., Hendrickx, L. A., and Tytgat, J. (2020). Targeting Cannabinoid Receptors: Current Status and Prospects of Natural Products. *Int. J. Mol. Sci.* 21. doi:10.3390/ijms21145064

Anderson, M. E. (2016). Update on Survival in Osteosarcoma. *Orthop. Clin. North America* 47, 283–292. doi:10.1016/j.ocl.2015.08.022

Angermayr, B., Mejias, M., Gracia-Sancho, J., Garcia-Pagan, J. C., Bosch, J., and Fernandez, M. (2006). Heme Oxygenase Attenuates Oxidative Stress and Inflammation, and Increases VEGF Expression in portal Hypertensive Rats. *J. Hepatol.* 44, 1033–1039. doi:10.1016/j.jhep.2005.09.021

Arévalo-Martín, A., Vela, J. M., Molina-Holgado, E., Borrell, J., and Guaza, C. (2003). Therapeutic Action of Cannabinoids in a Murine Model of Multiple Sclerosis. *J. Neurosci.* 23, 2511–2516.

Axford, J., Butt, A., Heron, C., Hammond, J., Morgan, J., Alavi, A., et al. (2010). Prevalence of Anxiety and Depression in Osteoarthritis: Use of the Hospital Anxiety and Depression Scale as a Screening Tool. *Clin. Rheumatol.* 29, 1277–1283. doi:10.1007/s10067-010-1547-7

Bachelder, R. E., Yoon, S.-O., Franci, C., De Herreros, A. G., and Mercurio, A. M. (2005). Glycogen Synthase Kinase-3 Is an Endogenous Inhibitor of Snail Transcription. *J. Cel Biol* 168, 29–33. doi:10.1083/jcb.200409067

Badal, S., Smith, K. N., and Rajnarayanan, R. (2017). Analysis of Natural Product Regulation of Cannabinoid Receptors in the Treatment of Human Disease. *Pharmacol. Ther.* 180, 24–48. doi:10.1016/j.pharmthera.2017.06.003

Baldassano, S., Serio, R., and Mule', F. (2008). Cannabinoid CB1 Receptor Activation Modulates Spontaneous Contractile Activity in Mouse Ileal Longitudinal Muscle. *Eur. J. Pharmacol.* 582, 132–138. doi:10.1016/j.ejphar.2007.12.016

Barutta, F., Piscitelli, F., Pinach, S., Bruno, G., Gambino, R., Rastaldi, M. P., et al. (2011). Protective Role of Cannabinoid Receptor Type 2 in a Mouse Model of Diabetic Nephropathy. *Diabetes* 60, 2386–2396. doi:10.2337/db10-1809

Baselga, J., and Swain, S. M. (2009). Novel Anticancer Targets: Revisiting ERBB2 and Discovering ERBB3. *Nat. Rev. Cancer* 9, 463–475. doi:10.1038/nrc2656

Basha, R. H., and Sankaranarayanan, C. (2014). β-Caryophyllene, a Natural Sesquiterpene, Modulates Carbohydrate Metabolism in Streptozotocin-Induced Diabetic Rats. *Acta Histochem.* 116, 1469–1479. doi:10.1016/j.acthis.2014.10.001

Bátkai, S., Osei-Hyiaman, D., Pan, H., El-Assal, O., Rajesh, M., Mukhopadhyay, P., et al. (2007). Cannabinoid-2 Receptor Mediates protection against Hepatic Ischemia/reperfusion Injury. *FASEB j.* 21, 1788–1800. doi:10.1096/fj.06-7451com

Bellini, G., Torella, M., Manzo, I., Tortora, C., Luongo, L., Punzo, F., et al. (2017). Pkcβii-mediated Cross-Talk of TRPV1/CB2 Modulates the Glucocorticoid-Induced Osteoclast Overactivity. *Pharmacol. Res.* 115, 267–274. doi:10.1016/j.phrs.2016.11.039

Bi, G. H., Galaj, E., He, Y., and Xi, Z. X. (2020). Cannabidiol Inhibits Sucrose Self-Administration by CB1 and CB2 Receptor Mechanisms in Rodents. *Addict. Biol.* 25, e12783. doi:10.1111/adb.12783

Bifulco, M., Malfitano, A. M., Pisanti, S., and Laezza, C. (2008). Endocannabinoids in Endocrine and Related Tumours. *Endocr. Relat. Cancer* 15, 391–408. doi:10.1677/erc-07-0258

Blázquez, C., Carracedo, A., Barrado, L., José Real, P., Luis Fernández-Luna, J., Velasco, G., et al. (2006). Cannabinoid Receptors as Novel Targets for the Treatment of Melanoma. *FASEB j.* 20, 2633–2635. doi:10.1096/fj.06-6638fje

Blázquez, C., Casanova, M. L., Planas, A., Gómez Del Pulgar, T., Villanueva, C., Fernández-Aceñero, M. J., et al. (2003). Inhibition of Tumor Angiogenesis by Cannabinoids. *Faseb j* 17, 529–531. doi:10.1096/fj.02-0795fje

Blázquez, C., Salazar, M., Carracedo, A., Lorente, M., Egia, A., González-Feria, L., et al. (2008). Cannabinoids Inhibit Glioma Cell Invasion by Down-Regulating Matrix Metalloproteinase-2 Expression. *Cancer Res.* 68, 1945–1952. doi:10.1158/0008-5472.can-07-5176

Borchers, A. T., Chang, C., Gershwin, M. E., and Gershwin, L. J. (2013). Respiratory Syncytial Virus-A Comprehensive Review. *Clinic Rev. Allerg Immunol.* 45, 331–379. doi:10.1007/s12016-013-8368-9

Bouaboula, M., Poinot-Chazel, C., Marchand, J., Canat, X., Bourrié, B., Rinaldi-Carmona, M., et al. (1996). Signaling Pathway Associated with Stimulation of CB2 Peripheral Cannabinoid Receptor. Involvement of Both Mitogen-Activated Protein Kinase and Induction of Krox-24 Expression. *Eur. J. Biochem.* 237, 704–711. doi:10.1111/j.1432-1033.1996.0704p.x

Bozkurt, T. E., Kaya, Y., Durlu-Kandilci, N. T., Onder, S., and Sahin-Erdemli, I. (2016). The Effect of Cannabinoids on Dinitrofluorobenzene-Induced Experimental Asthma in Mice. *Respir. Physiol. Neurobiol.* 231, 7–13. doi:10.1016/j.resp.2016.05.012

Burston, J. J., Sagar, D. R., Shao, P., Bai, M., King, E., Brailsford, L., et al. (2013). Cannabinoid CB2 Receptors Regulate central Sensitization and Pain Responses Associated with Osteoarthritis of the Knee Joint. *PLoS One* 8, e80440. doi:10.1371/journal.pone.0080440

Cabañero, D., Ramírez-López, A., Drews, E., Schmöle, A., Otte, D. M., Wawrzczak-Bargiela, A., et al. (2020). Protective Role of Neuronal and Lymphoid Cannabinoid CB(2) Receptors in Neuropathic Pain. *eLife* 9, e55582. doi:10.7554/elife.55582

Caffarel, M. M., Andradas, C., Mira, E., Pérez-Gómez, E., Cerutti, C., Moreno-Bueno, G., et al. (2010). Cannabinoids Reduce ErbB2-Driven Breast Cancer Progression through Akt Inhibition. *Mol. Cancer* 9, 196. doi:10.1186/1476-4598-9-196

Çakır, M., Tekin, S., Doğanyiğit, Z., Çakan, P., and Kaymak, E. (2019a). The Protective Effect of Cannabinoid Type 2 Receptor Activation on Renal Ischemia-Reperfusion Injury. *Mol. Cel Biochem.* 462, 123–132.

Çakır, M., Tekin, S., Doğanyiğit, Z., Erden, Y., Soytürk, M., Çiğremiş, Y., et al. (2019b). Cannabinoid Type 2 Receptor Agonist JWH-133, Attenuates Okadaic Acid Induced Spatial Memory Impairment and Neurodegeneration in Rats. *Life Sci.* 217, 25–33.

Çakır, M., Tekin, S., Okan, A., Çakan, P., and Doğanyiğit, Z. (2020). The Ameliorating Effect of Cannabinoid Type 2 Receptor Activation on Brain, Lung, Liver and Heart Damage in Cecal Ligation and Puncture-Induced Sepsis Model in Rats. *Int. Immunopharmacol.* 78, 105978.

Cao, Q., Yang, F., and Wang, H. (2020). CB2R Induces a Protective Response against Epileptic Seizures through ERK and P38 Signaling Pathways. *Int. J. Neurosci.*, 1–10. doi:10.1080/00207454.2020.1796661

Caraceni, P., Viola, A., Piscitelli, F., Giannone, F., Berzigotti, A., Cescon, M., et al. (2010). Circulating and Hepatic Endocannabinoids and Endocannabinoid-Related Molecules in Patients with Cirrhosis. *Liver Int.* 30, 816–825. doi:10.1111/j.1478-3231.2009.02137.x

Carbone, F., Mach, F., Vuilleumier, N., and Montecucco, F. (2014). Cannabinoid Receptor Type 2 Activation in Atherosclerosis and Acute Cardiovascular Diseases. *Cmc* 21, 4046–4058. doi:10.2174/0929867321666140915141332

Carracedo, A., Gironella, M., Lorente, M., Garcia, S., Guzmán, M., Velasco, G., et al. (2006). Cannabinoids Induce Apoptosis of Pancreatic Tumor Cells via Endoplasmic Reticulum Stress-Related Genes. *Cancer Res.* 66, 6748–6755. doi:10.1158/0008-5472.can-06-0169

Casanova, M. L., Blázquez, C., Martínez-Palacio, J., Villanueva, C., Fernández-Aceñero, M. J., Huffman, J. W., et al. (2003). Inhibition of Skin Tumor Growth and Angiogenesis *In Vivo* by Activation of Cannabinoid Receptors. *J. Clin. Invest.* 111, 43–50. doi:10.1172/jci200316116

Caspi, R. R. (2003). Experimental Autoimmune Uveoretinitis in the Rat and Mouse. *Curr. Protoc. Immunol.* 53, 15–1615 16.20. doi:10.1002/0471142735.im1506s5311

Choi, H.-N., Jeong, S.-M., Huh, G. H., and Kim, J.-I. (2015). Quercetin Ameliorates Insulin Sensitivity and Liver Steatosis Partly by Increasing Adiponectin Expression in Ob/ob Mice. *Food Sci. Biotechnol.* 24, 273–279. doi:10.1007/s10068-015-0036-9

Chung, H., Fierro, A., and Pessoa-Mahana, C. D. (2019). Cannabidiol Binding and Negative Allosteric Modulation at the Cannabinoid Type 1 Receptor in the Presence of delta-9-tetrahydrocannabinol: An In Silico Study. *PloS one* 14–e0220025. doi:10.1371/journal.pone.0220025

Cianchi, F., Papucci, L., Schiavone, N., Lulli, M., Magnelli, L., Vinci, M. C., et al. (2008). Cannabinoid Receptor Activation Induces Apoptosis through Tumor Necrosis Factor α-Mediated Ceramide De Novo Synthesis in Colon Cancer Cells. *Clin. Cancer Res.* 14, 7691–7700. doi:10.1158/1078-0432.ccr-08-0799

Citti, C., Linciano, P., Russo, F., Luongo, L., Iannotta, M., Maione, S., et al. (2019). A Novel Phytocannabinoid Isolated from Cannabis Sativa L. With an *In Vivo* Cannabimimetic Activity Higher Than Δ(9)-tetrahydrocannabinol: Δ(9)-Tetrahydrocannabiphorol. *Sci. Rep.* 9, 20335. doi:10.1038/s41598-019-56785-1

Colonna, M., Trinchieri, G., and Liu, Y.-J. (2004). Plasmacytoid Dendritic Cells in Immunity. *Nat. Immunol.* 5, 1219–1226. doi:10.1038/ni1141

Correa, F., Mestre, L., Docagne, F., and Guaza, C. (2005). Activation of Cannabinoid CB2 Receptor Negatively Regulates IL-12p40 Production in Murine Macrophages: Role of IL-10 and ERK1/2 Kinase Signaling. *Br. J. Pharmacol.* 145, 441–448. doi:10.1038/sj.bjp.0706215

Cristino, L., Bisogno, T., and Di Marzo, V. (2020). Cannabinoids and the Expanded Endocannabinoid System in Neurological Disorders. *Nat. Rev. Neurol.* 16, 9–29. doi:10.1038/s41582-019-0284-z

Cui, Y.-Y., D'agostino, B., Risse, P.-A., Marrocco, G., Naline, E., Zhang, Y., et al. (2007). Cannabinoid CB2 Receptor Activation Prevents Bronchoconstriction and Airway Oedema in a Model of Gastro-Oesophageal Reflux. *Eur. J. Pharmacol.* 573, 206–213. doi:10.1016/j.ejphar.2007.06.040

De Domenico, E., Todaro, F., Rossi, G., Dolci, S., Geremia, R., Rossi, P., et al. (2017). Overactive Type 2 Cannabinoid Receptor Induces Meiosis in Fetal Gonads and Impairs Ovarian reserve. *Cell Death Dis.* 8, e3085. doi:10.1038/cddis.2017.496

Defer, N., Wan, J., Souktani, R., Escoubet, B., Perier, M., Caramelle, P., et al. (2009). The Cannabinoid Receptor Type 2 Promotes Cardiac Myocyte and Fibroblast Survival and Protects against Ischemia/reperfusion-Induced Cardiomyopathy. *FASEB j.* 23, 2120–2130. doi:10.1096/fj.09-129478

Den Hengst, W. A., Gielis, J. F., Lin, J. Y., Van Schil, P. E., De Windt, L. J., and Moens, A. L. (2010). Lung Ischemia-Reperfusion Injury: a Molecular and Clinical View on a Complex Pathophysiological Process. *Am. J. Physiology-Heart Circulatory Physiol.* 299, H1283–H1299. doi:10.1152/ajpheart.00251.2010

Denaës, T., Lodder, J., Chobert, M.-N., Ruiz, I., Pawlotsky, J.-M., Lotersztajn, S., et al. (2016). The Cannabinoid Receptor 2 Protects against Alcoholic Liver Disease via a Macrophage Autophagy-dependent Pathway. *Scientific Rep.* 6, 28806. doi:10.1038/srep28806

Depetrocellis, L., Marini, P., Matias, I., Moriello, A., Starowicz, K., Cristino, L., et al. (2007). Mechanisms for the Coupling of Cannabinoid Receptors to Intracellular Calcium Mobilization in Rat Insulinoma β-cells. *Exp. Cel Res.* 313, 2993–3004. doi:10.1016/j.yexcr.2007.05.012

Deveaux, V., Cadoudal, T., Ichigotani, Y., Teixeira-Clerc, F., Louvet, A., Manin, S., et al. (2009). Cannabinoid CB2 Receptor Potentiates Obesity-Associated Inflammation, Insulin Resistance and Hepatic Steatosis. *PLoS One* 4, e5844. doi:10.1371/journal.pone.0005844

Di Giacomo, D., De Domenico, E., Sette, C., Geremia, R., and Grimaldi, P. (2016). Type 2 Cannabinoid Receptor Contributes to the Physiological Regulation of Spermatogenesis. *FASEB j.* 30, 1453–1463. doi:10.1096/fj.15-279034

Dibba, P., Li, A. A., Cholankeril, G., Iqbal, U., Gadiparthi, C., Khan, M. A., et al. (2018). The Role of Cannabinoids in the Setting of Cirrhosis. *Medicines (Basel)*, 5. doi:10.3390/medicines5020052

Dinis-Oliveira, R. J., Duarte, J. A., Sánchez-Navarro, A., Remião, F., Bastos, M. L., and Carvalho, F. (2008). Paraquat Poisonings: Mechanisms of Lung Toxicity, Clinical Features, and Treatment. *Crit. Rev. Toxicol.* 38, 13–71. doi:10.1080/10408440701669959

Du, Y., Ren, P., Wang, Q., Jiang, S. K., Zhang, M., Li, J. Y., et al. (2018). Cannabinoid 2 Receptor Attenuates Inflammation during Skin Wound Healing by Inhibiting M1 Macrophages rather Than Activating M2 Macrophages. *J. Inflamm. (Lond)* 15, 25. doi:10.1186/s12950-018-0201-z

Duncan, M., Mouihate, A., Mackie, K., Keenan, C. M., Buckley, N. E., Davison, J. S., et al. (2008). Cannabinoid CB2 Receptors in the Enteric Nervous System Modulate Gastrointestinal Contractility in Lipopolysaccharide-Treated Rats. *Am. J. Physiology-Gastrointestinal Liver Physiol.* 295, G78–g87. doi:10.1152/ajpgi.90285.2008

Elbaz, M., Ahirwar, D., Ravi, J., Nasser, M. W., and Ganju, R. K. (2017). Novel Role of Cannabinoid Receptor 2 in Inhibiting EGF/EGFR and IGF-I/IGF-IR Pathways in Breast Cancer. *Oncotarget* 8, 29668–29678. doi:10.18632/oncotarget.9408

Elmes, S. J. R., Winyard, L. A., Medhurst, S. J., Clayton, N. M., Wilson, A. W., Kendall, D. A., et al. (2005). Activation of CB1 and CB2 Receptors Attenuates the Induction and Maintenance of Inflammatory Pain in the Rat. *Pain* 118, 327–335. doi:10.1016/j.pain.2005.09.005

Engeli, S. (2012). Central and Peripheral Cannabinoid Receptors as Therapeutic Targets in the Control of Food Intake and Body Weight. *Handb Exp. Pharmacol.*, 357–381. doi:10.1007/978-3-642-24716-3_17

Ernst, J., Grabiec, U., Greither, T., Fischer, B., and Dehghani, F. (2016). The Endocannabinoid System in the Human Granulosa Cell Line KGN. *Mol. Cell Endocrinol.* 423, 67–76. doi:10.1016/j.mce.2016.01.006

Farid, N. R., Shi, Y., and Zou, M. (1994). Molecular Basis of Thyroid Cancer*. *Endocr. Rev.* 15, 202–232. doi:10.1210/edrv-15-2-202

Fechtner, S., Singh, A. K., and Ahmed, S. (2019). Role of Cannabinoid Receptor 2 in Mediating Interleukin-1β-Induced Inflammation in Rheumatoid Arthritis Synovial Fibroblasts. *Clin. Exp. Rheumatol.* 37, 1026–1035.

Feizi, A., Jafari, M.-R., Hamedivafa, F., Tabrizian, P., and Djahanguiri, B. (2008). The Preventive Effect of Cannabinoids on Reperfusion-Induced Ischemia of Mouse Kidney. *Exp. Toxicologic Pathol.* 60, 405–410. doi:10.1016/j.etp.2008.04.006

Fraga-Silva, R. A., Costa-Fraga, F. P., Montecucco, F., Faye, Y., Savergnini, S. Q., Lenglet, S., et al. (2013). Treatment with CB2 Agonist JWH-133 Reduces Histological Features Associated with Erectile Dysfunction in Hypercholesterolemic Mice. *Clin. Dev. Immunol.* 2013, 263846. doi:10.1155/2013/263846

Frei, R. B., Luschnig, P., Parzmair, G. P., Peinhaupt, M., Schranz, S., Fauland, A., et al. (2016). Cannabinoid Receptor 2 Augments Eosinophil Responsiveness and Aggravates Allergen-Induced Pulmonary Inflammation in Mice. *Allergy* 71, 944–956. doi:10.1111/all.12858

Fu, Q., Zheng, Y., Dong, X., Wang, L., and Jiang, C. G. (2017). Activation of Cannabinoid Receptor Type 2 by JWH133 Alleviates Bleomycin-Induced Pulmonary Fibrosis in Mice. *Oncotarget* 8, 103486–103498. doi:10.18632/oncotarget.21975

Fukuda, H., Abe, T., and Yoshihara, S. (2010). The Cannabinoid Receptor Agonist WIN 55,212-2 Inhibits Antigen-Induced Plasma Extravasation in guinea Pig Airways. *Int. Arch. Allergy Immunol.* 152, 295–300. doi:10.1159/000283042

Fukuda, S., Kohsaka, H., Takayasu, A., Yokoyama, W., Miyabe, C., Miyabe, Y., et al. (2014). Cannabinoid Receptor 2 as a Potential Therapeutic Target in Rheumatoid Arthritis. *BMC Musculoskelet. Disord.* 15, 275. doi:10.1186/1471-2474-15-275

Fulmer, M. L., and Thewke, D. P. (2018). The Endocannabinoid System and Heart Disease: The Role of Cannabinoid Receptor Type 2. *Chddt* 18, 34–51. doi:10.2174/1871529x18666180206161457

Gao, B., and Bataller, R. (2011). Alcoholic Liver Disease: Pathogenesis and New Therapeutic Targets. *Gastroenterology* 141, 1572–1585. doi:10.1053/j.gastro.2011.09.002

Gertsch, J., Leonti, M., Raduner, S., Racz, I., Chen, J.-Z., Xie, X.-Q., et al. (2008). Beta-caryophyllene Is a Dietary Cannabinoid. *Pnas* 105, 9099–9104. doi:10.1073/pnas.0803601105

Ghonim, A. E., Ligresti, A., Rabbito, A., Mahmoud, A. M., Di Marzo, V., Osman, N. A., et al. (2019). Structure-activity Relationships of Thiazole and Benzothiazole Derivatives as Selective Cannabinoid CB2 Agonists with *In Vivo* Anti-inflammatory Properties. *Eur. J. Med. Chem.* 180, 154–170. doi:10.1016/j.ejmech.2019.07.002

Gomez, O., Sanchez-Rodriguez, A., Le, M., Sanchez-Caro, C., Molina-Holgado, F., and Molina-Holgado, E. (2011). Cannabinoid Receptor Agonists Modulate Oligodendrocyte Differentiation by Activating PI3K/Akt and the Mammalian Target of Rapamycin (mTOR) Pathways. *Br. J. Pharmacol.* 163, 1520–1532. doi:10.1111/j.1476-5381.2011.01414.x

Grassin-Delyle, S., Naline, E., Buenestado, A., Faisy, C., Alvarez, J.-C., Salvator, H., et al. (2014). Cannabinoids Inhibit Cholinergic Contraction in Human Airways

through Prejunctional CB1receptors. *Br. J. Pharmacol.* 171, 2767–2777. doi:10.1111/bph.12597

Gratzke, C., Streng, T., Stief, C. G., Downs, T. R., Alroy, I., Rosenbaum, J. S., et al. (2010). Effects of Cannabinor, a Novel Selective Cannabinoid 2 Receptor Agonist, on Bladder Function in Normal Rats. *Eur. Urol.* 57, 1093–1100. doi:10.1016/j.eururo.2010.02.027

Grimaldi, P., Orlando, P., Di Siena, S., Lolicato, F., Petrosino, S., Bisogno, T., et al. (2009). The Endocannabinoid System and Pivotal Role of the CB2 Receptor in Mouse Spermatogenesis. *Proc. Natl. Acad. Sci.* 106, 11131–11136. doi:10.1073/pnas.0812789106

Groot Kormelink, T., Thio, M., Blokhuis, B. R., Nijkamp, F. P., and Redegeld, F. A. (2009). Atopic and Non-atopic Allergic Disorders: Current Insights into the Possible Involvement of Free Immunoglobulin Light Chains. *Clin. Exp. Allergy* 39, 33–42. doi:10.1111/j.1365-2222.2008.03135.x

Gruden, G., Barutta, F., Kunos, G., and Pacher, P. (2016). Role of the Endocannabinoid System in Diabetes and Diabetic Complications. *Br. J. Pharmacol.* 173, 1116–1127. doi:10.1111/bph.13226

Guan, T., Zhao, G., Duan, H., Liu, Y., and Zhao, F. (2017). Activation of Type 2 Cannabinoid Receptor (CB2R) by Selective Agonists Regulates the Deposition and Remodelling of the Extracellular Matrix. *Biomed. Pharmacother.* 95, 1704–1709. doi:10.1016/j.biopha.2017.09.085

Gui, H., Liu, X., Liu, L.-R., Su, D.-F., and Dai, S.-M. (2015). Activation of Cannabinoid Receptor 2 Attenuates Synovitis and Joint Distruction in Collagen-Induced Arthritis. *Immunobiology* 220, 817–822. doi:10.1016/j.imbio.2014.12.012

Guillot, A., Hamdaoui, N., Bizy, A., Zoltani, K., Souktani, R., Zafrani, E.-S., et al. (2014). Cannabinoid Receptor 2 Counteracts Interleukin-17-Induced Immune and Fibrogenic Responses in Mouse Liver. *Hepatology* 59, 296–306. doi:10.1002/hep.26598

Gutsche, M., Rosen, G. D., and Swigris, J. J. (2012). Connective Tissue Disease-Associated Interstitial Lung Disease: A Review. *Curr. Respir. Care Rep.* 1, 224–232. doi:10.1007/s13665-012-0028-7

Guzmán, M. (2003). Cannabinoids: Potential Anticancer Agents. *Nat. Rev. Cancer* 3, 745–755. doi:10.1038/nrc1188

Haase, J., Weyer, U., Immig, K., Klöting, N., Blüher, M., Eilers, J., et al. (2014). Local Proliferation of Macrophages in Adipose Tissue during Obesity-Induced Inflammation. *Diabetologia* 57, 562–571. doi:10.1007/s00125-013-3139-y

Hansson, G. K., and Libby, P. (2006). The Immune Response in Atherosclerosis: a Double-Edged Sword. *Nat. Rev. Immunol.* 6, 508–519. doi:10.1038/nri1882

Hanuš, L., Breuer, A., Tchilibon, S., Shiloah, S., Goldenberg, D., Horowitz, M., et al. (1999). HU-308: A Specific Agonist for CB_2, a Peripheral Cannabinoid Receptor, Proc Natl Acad Sci. 96–14228–14233. 10.1073/pnas.96.25.14228

Hao, M.-x., Jiang, L.-s., Fang, N.-y., Pu, J., Hu, L.-h., Shen, L.-H., et al. (2010). The Cannabinoid WIN55,212-2 Protects against Oxidized LDL-Induced Inflammatory Response in Murine Macrophages. *J. Lipid Res.* 51, 2181–2190. doi:10.1194/jlr.m001511

Haskó, J., Fazakas, C., Molnár, J., Nyúl-Tóth, Á., Herman, H., Hermenean, A., et al. (2014). CB2 Receptor Activation Inhibits Melanoma Cell Transmigration through the Blood-Brain Barrier. *Ijms* 15, 8063–8074. doi:10.3390/ijms15058063

He, Q., Xiao, F., Yuan, Q., Zhang, J., Zhan, J., and Zhang, Z. (2019). Cannabinoid Receptor 2: a Potential Novel Therapeutic Target for Sepsis?. *Acta Clinica Belgica* 74, 70–74. doi:10.1080/17843286.2018.1461754

Henriquez, J. E., Crawford, R. B., and Kaminski, N. E. (2019). Suppression of CpG-ODN-Mediated IFNα and TNFα Response in Human Plasmacytoid Dendritic Cells (pDC) by Cannabinoid Receptor 2 (CB2)-specific Agonists. *Toxicol. Appl. Pharmacol.* 369, 82–89. doi:10.1016/j.taap.2019.02.013

Hervás, E. S. (2017). Synthetic Cannabinoids: Characteristics, Use and Clinical Implications. *Arch. Psychiatry Psychotherapy* 19, 42–48.

Howlett, A. C. (2005). Cannabinoid Receptor Signaling. *Handb Exp. Pharmacol.*, 53–79. doi:10.1007/3-540-26573-2_2

Howlett, A. C., and Abood, M. E. (2017). CB 1 and CB 2 Receptor Pharmacology. *Adv. Pharmacol.* 80, 169–206. doi:10.1016/bs.apha.2017.03.007

Hsu, C.-y., Mcculloch, C. E., Fan, D., Ordoñez, J. D., Chertow, G. M., and Go, A. S. (2007). Community-based Incidence of Acute Renal Failure. *Kidney Int.* 72, 208–212. doi:10.1038/sj.ki.5002297

Huang, H.-C., Wang, S.-S., Hsin, I.-F., Chang, C.-C., Lee, F.-Y., Lin, H.-C., et al. (2012). Cannabinoid Receptor 2 Agonist Ameliorates Mesenteric Angiogenesis and Portosystemic Collaterals in Cirrhotic Rats. *Hepatology* 56, 248–258. doi:10.1002/hep.25625

Huang, W., Xiong, Y., Chen, Y., Cheng, Y., and Wang, R. (2020). NOX2 Is Involved in CB2-Mediated protection against Lung Ischemia-Reperfusion Injury in Mice. *Int. J. Clin. Exp. Pathol.* 13, 277–285.

Huffman, J. W., Liddle, J., Yu, S., Aung, M. M., Abood, M. E., Wiley, J. L., et al. (1999). 3-(1′,1′-Dimethylbutyl)-1-deoxy-Δ 8 -THC and Related Compounds: Synthesis of Selective Ligands for the CB 2 Receptor. *Bioorg. Med. Chem.* 7, 2905–2914. doi:10.1016/s0968-0896(99)00219-9

Huffman, J. W., Yu, S., Showalter, V., Abood, M. E., Wiley, J. L., Compton, D. R., et al. (1996). Synthesis and Pharmacology of a Very Potent Cannabinoid Lacking a Phenolic Hydroxyl with High Affinity for the CB2 Receptor. *J. Med. Chem.* 39, 3875–3877. doi:10.1021/jm960394y

Hytti, M., Andjelic, S., Josifovska, N., Piippo, N., Korhonen, E., Hawlina, M., et al. (2017). CB(2) Receptor Activation Causes an ERK1/2-dependent Inflammatory Response in Human RPE Cells. *Scientific Rep.* 7, 16169. doi:10.1038/s41598-017-16524-w

Idris, A. I., Sophocleous, A., Landao-Bassonga, E., Van't Hof, R. J., and Ralston, S. H. (2008). Regulation of Bone Mass, Osteoclast Function, and Ovariectomy-Induced Bone Loss by the Type 2 Cannabinoid Receptor. *Endocrinology* 149, 5619–5626. doi:10.1210/en.2008-0150

Idris, A. I. (2012). The Promise and Dilemma of Cannabinoid Therapy: Lessons from Animal Studies of Bone Disease. *BoneKEy Rep.* 1, 224. doi:10.1038/bonekey.2012.224

Innocenzi, E., De Domenico, E., Ciccarone, F., Zampieri, M., Rossi, G., Cicconi, R., et al. (2019). Paternal Activation of CB2 Cannabinoid Receptor Impairs Placental and Embryonic Growth via an Epigenetic Mechanism. *Scientific Rep.* 9, 17034. doi:10.1038/s41598-019-53579-3

Ishiguro, H., Carpio, O., Horiuchi, Y., Shu, A., Higuchi, S., Schanz, N., et al. (2010). A Nonsynonymous Polymorphism in Cannabinoid CB2 Receptor Gene Is Associated with Eating Disorders in Humans and Food Intake Is Modified in Mice by its Ligands. *Synapse* 64, 92–96. doi:10.1002/syn.20714

Ivy, D., Palese, F., Vozella, V., Fotio, Y., Yalcin, A., Ramirez, G., et al. (2020). Cannabinoid CB2 Receptors Mediate the Anxiolytic-like Effects of Monoacylglycerol Lipase Inhibition in a Rat Model of Predator-Induced Fear. *Neuropsychopharmacol.* 45, 1330–1338. doi:10.1038/s41386-020-0696-x

Jemal, A., Center, M. M., Ward, E., and Thun, M. J. (2009a). Cancer Occurrence. *Methods Mol. Biol. (Clifton, N.J.)* 471, 3–29. doi:10.1007/978-1-59745-416-2_1

Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., and Thun, M. J. (2009b). Cancer Statistics, 2009. *CA: A Cancer J. Clinicians* 59, 225–249. doi:10.3322/caac.20006

Jenkin, K. A., Mcainch, A. J., Grinfeld, E., and Hryciw, D. H. (2010). Role for Cannabinoid Receptors in Human Proximal Tubular Hypertrophy. *Cell Physiol. Biochem.* 26, 879–886. doi:10.1159/000323997

Jenkin, K. A., O'keefe, L., Simcocks, A. C., Briffa, J. F., Mathai, M. L., Mcainch, A. J., et al. (2016). Renal Effects of Chronic Pharmacological Manipulation of CB2receptors in Rats with Diet-Induced Obesity. *Br. J. Pharmacol.* 173, 1128–1142. doi:10.1111/bph.13056

Jia, Y., Deng, H., Qin, Q., and Ma, Z. (2020). JWH133 Inhibits MPP+-induced Inflammatory Response and Iron Influx in Astrocytes. *Neurosci. Lett.* 720, 134779. doi:10.1016/j.neulet.2020.134779

Jiang, L., Chen, Y., Huang, X., Yuan, A., Shao, Q., Pu, J., et al. (2016). Selective Activation of CB2 Receptor Improves Efferocytosis in Cultured Macrophages. *Life Sci.* 161, 10–18. doi:10.1016/j.lfs.2016.07.013

Jiang, S., Alberich-Jorda, M., Zagozdzon, R., Parmar, K., Fu, Y., Mauch, P., et al. (2011). Cannabinoid Receptor 2 and its Agonists Mediate Hematopoiesis and Hematopoietic Stem and Progenitor Cell Mobilization. *Blood* 117, 827–838. doi:10.1182/blood-2010-01-265082

Jing, N., Fang, B., Li, Z., and Tian, A. (2020). Exogenous Activation of Cannabinoid-2 Receptor Modulates TLR4/MMP9 Expression in a Spinal Cord Ischemia Reperfusion Rat Model. *J. Neuroinflammation* 17, 101. doi:10.1186/s12974-020-01784-7

Jonsson, K.-O., Persson, E., and Fowler, C. J. (2006). The Cannabinoid CB2 Receptor Selective Agonist JWH133 Reduces Mast Cell Oedema in Response to Compound 48/80 *In Vivo* but Not the Release of β-hexosaminidase from Skin Slices *In Vitro*. *Life Sci.* 78, 598–606. doi:10.1016/j.lfs.2005.05.059

Jordan, C. J., and Xi, Z.-X. (2019). Progress in Brain Cannabinoid CB2 Receptor Research: From Genes to Behavior. *Neurosci. Biobehavioral Rev.* 98, 208–220. doi:10.1016/j.neubiorev.2018.12.026

Joseph, J., Niggemann, B., Zaenker, K. S., and Entschladen, F. (2004). Anandamide Is an Endogenous Inhibitor for the Migration of Tumor Cells and T Lymphocytes. *Cancer Immunol. Immunother.* 53, 723–728. doi:10.1007/s00262-004-0509-9

Juan-Picó, P., Fuentes, E., Javier Bermúdez-Silva, F., Javier Díaz-Molina, F., Ripoll, C., Rodríguez de Fonseca, F., et al. (2006). Cannabinoid Receptors Regulate Ca2+ Signals and Insulin Secretion in Pancreatic β-cell. *Cell Calcium* 39, 155–162. doi:10.1016/j.ceca.2005.10.005

Karsak, M., Gaffal, E., Date, R., Wang-Eckhardt, L., Rehnelt, J., Petrosino, S., et al. (2007). Attenuation of Allergic Contact Dermatitis through the Endocannabinoid System. *Science* 316, 1494–1497. doi:10.1126/science.1142265

Kaur, J. (2014). A Comprehensive Review on Metabolic Syndrome. *Cardiol. Res. Pract.* 2014, 943162. doi:10.1155/2014/943162

Khan, A. M., Mydra, H., and Nevarez, A. (2017). Clinical Practice Updates in the Management of Immune Thrombocytopenia. *P T : a peer-reviewed J. formulary Manag.* 42, 756–763.

Killilea, M., Kerr, D. M., Mallard, B. M., Roche, M., and Wheatley, A. M. (2020). Exacerbated LPS/GalN-Induced Liver Injury in the Stress-Sensitive Wistar Kyoto Rat Is Associated with Changes in the Endocannabinoid System. *Molecules* 25, 3834. doi:10.3390/molecules25173834

Kimball, E. S., Schneider, C. R., Wallace, N. H., and Hornby, P. J. (2006). Agonists of Cannabinoid Receptor 1 and 2 Inhibit Experimental Colitis Induced by Oil of Mustard and by Dextran Sulfate Sodium. *Am. J. Physiology-Gastrointestinal Liver Physiol.* 291, G364–G371. doi:10.1152/ajpgi.00407.2005

Kimball, E. S., Wallace, N. H., Schneider, C. R., D'andrea, M. R., and Hornby, P. J. (2010). Small Intestinal Cannabinoid Receptor Changes Following a Single Colonic Insult with Oil of Mustard in Mice. *Front. Pharmacol.* 1, 132. doi:10.3389/fphar.2010.00132

Kruk-Slomka, M., Michalak, A., and Biala, G. (2015). Antidepressant-like Effects of the Cannabinoid Receptor Ligands in the Forced Swimming Test in Mice: Mechanism of Action and Possible Interactions with Cholinergic System. *Behav. Brain Res.* 284, 24–36. doi:10.1016/j.bbr.2015.01.051

Kuo, Y. L., and Lai, C. J. (2008). Ovalbumin Sensitizes Vagal Pulmonary C-Fiber Afferents in Brown Norway Rats. *J. Appl. Physiol.* 105, 611–620. doi:10.1152/japplphysiol.01099.2007

La Porta, C., Bura, S. A., Llorente-Onaindia, J., Pastor, A., Navarrete, F., García-Gutiérrez, M. S., et al. (2015). Role of the Endocannabinoid System in the Emotional Manifestations of Osteoarthritis Pain. *Pain* 156, 2001–2012. doi:10.1097/j.pain.0000000000000260

Lagneux, C., and Lamontagne, D. (2001). Involvement of Cannabinoids in the Cardioprotection Induced by Lipopolysaccharide. *Br. J. Pharmacol.* 132, 793–796. doi:10.1038/sj.bjp.0703902

Leber, K., Perron, V. D., and Sinni-Mckeehen, B. (1999). Common Skin Cancers in the United States: a Practical Guide for Diagnosis and Treatment. *Nurse Pract. Forum* 10, 106–112.

Leggett, J. J., Johnston, B. T., Mills, M., Gamble, J., and Heaney, L. G. (2005). Prevalence of Gastroesophageal Reflux in Difficult Asthma. *Chest* 127, 1227–1231. doi:10.1016/s0012-3692(15)34471-8

Lépicier, P., Bibeau-Poirier, A., Lagneux, C., Servant, M. J., and Lamontagne, D. (2006). Signaling Pathways Involved in the Cardioprotective Effects of Cannabinoids. *J. Pharmacol. Sci.* 102, 155–166. doi:10.1254/jphs.crj06011x

Leroy, E. C., and Medsger, T. A., Jr. (2001). Criteria for the Classification of Early Systemic Sclerosis. *J. Rheumatol.* 28, 1573–1576.

Li, L., Dong, X., Tu, C., Li, X., Peng, Z., Zhou, Y., et al. (2019a). Opposite Effects of Cannabinoid CB 1 and CB 2 Receptors on Antipsychotic Clozapine-Induced Cardiotoxicity. *Br. J. Pharmacol.* 176, 890–905. doi:10.1111/bph.14591

Li, Q., Guo, H.-c., Maslov, L. N., Qiao, X.-w., Zhou, J.-j., and Zhang, Y. (2014). Mitochondrial Permeability Transition Pore Plays a Role in the Cardioprotection of CB2 Receptor against Ischemia-Reperfusion Injury. *Can. J. Physiol. Pharmacol.* 92, 205–214. doi:10.1139/cjpp-2013-0293

Li, Q., Wang, F., Zhang, Y.-M., Zhou, J.-J., and Zhang, Y. (2013a). Activation of Cannabinoid Type 2 Receptor by JWH133 Protects Heart against Ischemia/reperfusion-Induced Apoptosis. *Cel. Physiol. Biochem.* 31, 693–702. doi:10.1159/000350088

Li, S.-S., Wang, L.-L., Liu, M., Jiang, S.-K., Zhang, M., Tian, Z.-L., et al. (2016). Cannabinoid CB2 Receptors Are Involved in the Regulation of Fibrogenesis during Skin Wound Repair in Mice. *Mol. Med. Rep.* 13, 3441–3450. doi:10.3892/mmr.2016.4961

Li, X., Peng, Z., Zhou, Y., Wang, J., Lin, X., Dong, X., et al. (2019b). Quetiapine Induces Myocardial Necroptotic Cell Death through Bidirectional Regulation of Cannabinoid Receptors. *Toxicol. Lett.* 313, 77–90. doi:10.1016/j.toxlet.2019.06.005

Li, y.-y., Li, y.-n., Ni, j.-b., Chen, c.-j., Lv, S., Chai, s.-y., et al. (2010). Involvement of Cannabinoid-1 and Cannabinoid-2 Receptors in Septic Ileus. *Neurogastroenterol Motil.* 22, 350–e88. doi:10.1111/j.1365-2982.2009.01419.x

Li, Y., Sun, Y., Zhang, Z., Feng, X., Meng, H., Li, S., et al. (2013b). Cannabinoid Receptors 1 and 2 Are Associated with Bladder Dysfunction in an Experimental Diabetic Rat Model. *BJU Int.* 112, E143–E150. doi:10.1111/bju.12172

Libby, P. (2002). Inflammation in Atherosclerosis. *Nature* 420, 868–874. doi:10.1038/nature01323

Lin, L., Yihao, T., Zhou, F., Yin, N., Qiang, T., Haowen, Z., et al. (2017). Inflammatory Regulation by Driving Microglial M2 Polarization: Neuroprotective Effects of Cannabinoid Receptor-2 Activation in Intracerebral Hemorrhage. *Front. Immunol.* 8, 112. doi:10.3389/fimmu.2017.00112

Liu, Q., Wu, Z., Liu, Y., Chen, L., Zhao, H., Guo, H., et al. (2020a). Cannabinoid Receptor 2 Activation Decreases Severity of Cyclophosphamide-Induced Cystitis via Regulating Autophagy. *Neurourology and Urodynamics* 39, 158–169. doi:10.1002/nau.24205

Liu, X., Zhang, D., Dong, X., Zhu, R., Ye, Y., Li, L., et al. (2020b). Pharmacological Activation of CB2 Receptor Protects against Ethanol-Induced Myocardial Injury Related to RIP1/RIP3/MLKL-Mediated Necroptosis. *Mol. Cel Biochem* 474, 1–14. doi:10.1007/s11010-020-03828-1

Liu, Z., Wang, Y., Zhao, H., Zheng, Q., Xiao, L., and Zhao, M. (2014). CB2 Receptor Activation Ameliorates the Proinflammatory Activity in Acute Lung Injury Induced by Paraquat. *Biomed. Res. Int.* 2014, 971750. doi:10.1155/2014/971750

Loftus, E. V., Jr. (2004). Clinical Epidemiology of Inflammatory Bowel Disease: Incidence, Prevalence, and Environmental Influences. *Gastroenterology* 126, 1504–1517. doi:10.1053/j.gastro.2004.01.063

Lotersztajn, S., Julien, B., Teixeira-Clerc, F., Grenard, P., and Mallat, A. (2005). Hepatic Fibrosis: Molecular Mechanisms and Drug Targets. *Annu. Rev. Pharmacol. Toxicol.* 45, 605–628. doi:10.1146/annurev.pharmtox.45.120403.095906

Louvet, A., Teixeira-Clerc, F., Chobert, M.-N., Deveaux, V., Pavoine, C., Zimmer, A., et al. (2011). Cannabinoid CB2 Receptors Protect against Alcoholic Liver Disease by Regulating Kupffer Cell Polarization in Mice. *Hepatology* 54, 1217–1226. doi:10.1002/hep.24524

Lu, Y., Akinwumi, B. C., Shao, Z., and Anderson, H. D. (2014). Ligand Activation of Cannabinoid Receptors Attenuates Hypertrophy of Neonatal Rat Cardiomyocytes. *J. Cardiovasc. Pharmacol.* 64, 420–430. doi:10.1097/fjc.0000000000000134

Luca, T., Di Benedetto, G., Scuderi, M. R., Palumbo, M., Clementi, S., Bernardini, R., et al. (2009). The CB1/CB2 Receptor Agonist WIN-55,212-2 Reduces Viability of Human Kaposi's Sarcoma Cells *In Vitro*. *Eur. J. Pharmacol.* 616, 16–21. doi:10.1016/j.ejphar.2009.06.004

Luchicchi, A., and Pistis, M. (2012). Anandamide and 2-arachidonoylglycerol: Pharmacological Properties, Functional Features, and Emerging Specificities of the Two Major Endocannabinoids. *Mol. Neurobiol.* 46, 374–392. doi:10.1007/s12035-012-8299-0

Maccarrone, M., Barboni, B., Paradisi, A., Bernabò, N., Gasperi, V., Pistilli, M. G., et al. (2005). Characterization of the Endocannabinoid System in Boar Spermatozoa and Implications for Sperm Capacitation and Acrosome Reaction. *J. Cel. Sci.* 118, 4393–4404. doi:10.1242/jcs.02536

Maggo, S., and Ashton, J. C. (2018). Effect of Cannabinoid Receptor Agonists on Isolated Rat Atria. *J. Cardiovasc. Pharmacol.* 72, 191–194. doi:10.1097/fjc.0000000000000613

Maher, E. A., Furnari, F. B., Bachoo, R. M., Rowitch, D. H., Louis, D. N., Cavenee, W. K., et al. (2001). Malignant Glioma: Genetics and Biology of a Grave Matter. *Genes Dev.* 15, 1311–1333. doi:10.1101/gad.891601

Malfait, A. M., Gallily, R., Sumariwalla, P. F., Malik, A. S., Andreakos, E., Mechoulam, R., et al. (2000). The Nonpsychoactive Cannabis Constituent Cannabidiol Is an Oral Anti-arthritic Therapeutic in Murine Collagen-Induced Arthritis. *Proc. Natl. Acad. Sci.* 97, 9561–9566. doi:10.1073/pnas.160105897

Martínez-Martínez, E., Martín-Ruiz, A., Martín, P., Calvo, V., Provencio, M., and García, J. M. (2016). CB2 Cannabinoid Receptor Activation Promotes colon Cancer Progression via AKT/GSK3β Signaling Pathway. *Oncotarget* 7, 68781–68791. doi:10.18632/oncotarget.11968

Mathison, R., Ho, W., Pittman, Q. J., Davison, J. S., and Sharkey, K. A. (2004). Effects of Cannabinoid Receptor-2 Activation on Accelerated Gastrointestinal Transit in Lipopolysaccharide-Treated Rats. *Br. J. Pharmacol.* 142, 1247–1254. doi:10.1038/sj.bjp.0705889

Mcdonnell, C., Leánez, S., and Pol, O. (2017). The Inhibitory Effects of Cobalt Protoporphyrin IX and Cannabinoid 2 Receptor Agonists in Type 2 Diabetic Mice. *Int. J. Mol. Sci.* 18. doi:10.3390/ijms18112268

Mcdougall, J. J., Yu, V., and Thomson, J. (2008). *In Vivo* effects of CB2 Receptor-Selective Cannabinoids on the Vasculature of normal and Arthritic Rat Knee Joints. *Br. J. Pharmacol.* 153, 358–366. doi:10.1038/sj.bjp.0707565

Mcinnes, I. B., and Schett, G. (2017). Pathogenetic Insights from the Treatment of Rheumatoid Arthritis. *The Lancet* 389, 2328–2337. doi:10.1016/s0140-6736(17)31472-1

Mechoulam, R., Lander, N., University, A., and Zahalka, J. (1990). Synthesis of the Individual, Pharmacologically Distinct, Enantiomers of a Tetrahydrocannabinol Derivative. *Tetrahedron: Asymmetry* 1, 315–318. doi:10.1016/s0957-4166(00)86322-3

Mendez-Sanchez, N., Zamora-Valdes, D., Pichardo-Bahena, R., Barredo-Prieto, B., Ponciano-Rodriguez, G., Bermejo-Martínez, L., et al. (2007). Endocannabinoid Receptor CB2 in Nonalcoholic Fatty Liver Disease. *Liver Int.* 27, 215–219. doi:10.1111/j.1478-3231.2006.01401.x

Michler, T., Storr, M., Kramer, J., Ochs, S., Malo, A., Reu, S., et al. (2013). Activation of Cannabinoid Receptor 2 Reduces Inflammation in Acute Experimental Pancreatitis via Intra-acinar Activation of P38 and MK2-dependent Mechanisms. *Am. J. Physiology-Gastrointestinal Liver Physiol.* 304, G181–G192. doi:10.1152/ajpgi.00133.2012

Molica, F., Matter, C. M., Burger, F., Pelli, G., Lenglet, S., Zimmer, A., et al. (2012). Cannabinoid Receptor CB2 Protects against Balloon-Induced Neointima Formation. *Am. J. Physiology-Heart Circulatory Physiol.* 302, H1064–H1074. doi:10.1152/ajpheart.00444.2011

Montecucco, F., Di Marzo, V., Da Silva, R. F., Vuilleumier, N., Capettini, L., Lenglet, S., et al. (2012). The Activation of the Cannabinoid Receptor Type 2 Reduces Neutrophilic Protease-Mediated Vulnerability in Atherosclerotic Plaques. *Eur. Heart J.* 33, 846–856. doi:10.1093/eurheartj/ehr449

Montecucco, F., Lenglet, S., Braunersreuther, V., Burger, F., Pelli, G., Bertolotto, M., et al. (2009). CB2 Cannabinoid Receptor Activation Is Cardioprotective in a Mouse Model of Ischemia/reperfusion. *J. Mol. Cell Cardiol.* 46, 612–620. doi:10.1016/j.yjmcc.2008.12.014

Moradi, H., Oveisi, F., Khanifar, E., Moreno-Sanz, G., Vaziri, N. D., and Piomelli, D. (2016). Increased Renal 2-Arachidonoylglycerol Level Is Associated with Improved Renal Function in a Mouse Model of Acute Kidney Injury. *Cannabis cannabinoid Res.* 1, 218–228. doi:10.1089/can.2016.0013

Morales, P., Hernandez-Folgado, L., Goya, P., and Jagerovic, N. (2016). Cannabinoid Receptor 2 (CB2) Agonists and Antagonists: a Patent Update. *Expert Opin. Ther. Patents* 26, 843–856. doi:10.1080/13543776.2016.1193157

Morales, P., Hurst, D. P., and Reggio, P. H. (2017). Molecular Targets of the Phytocannabinoids: A Complex Picture. *Prog. Chem. Org. Nat. Prod.* 103, 103–131. doi:10.1007/978-3-319-45541-9_4

Moriarty, O., Mcguire, B. E., and Finn, D. P. (2011). The Effect of Pain on Cognitive Function: a Review of Clinical and Preclinical Research. *Prog. Neurobiol.* 93, 385–404. doi:10.1016/j.pneurobio.2011.01.002

Mugnaini, C., Rabbito, A., Brizzi, A., Palombi, N., Petrosino, S., Verde, R., et al. (2019). Synthesis of Novel 2-(1-adamantanylcarboxamido)thiophene Derivatives. Selective Cannabinoid Type 2 (CB2) Receptor Agonists as Potential Agents for the Treatment of Skin Inflammatory Disease. *Eur. J. Med. Chem.* 161, 239–251. doi:10.1016/j.ejmech.2018.09.070

Mulè, F., Amato, A., Baldassano, S., and Serio, R. (2007). Evidence for a Modulatory Role of Cannabinoids on the Excitatory NANC Neurotransmission in Mouse colon. *Pharmacol. Res.* 56, 132–139. doi:10.1016/j.phrs.2007.04.019

Muñoz-Luque, J., Ros, J., Fernández-Varo, G., Tugues, S., Morales-Ruiz, M., Alvarez, C. E., et al. (2008). Regression of Fibrosis after Chronic Stimulation of Cannabinoid CB2 Receptor in Cirrhotic Rats. *J. Pharmacol. Exp. Ther.* 324, 475–483. doi:10.1124/jpet.107.131896

Munro, S., Thomas, K. L., and Abu-Shaar, M. (1993). Molecular Characterization of a Peripheral Receptor for Cannabinoids. *Nature* 365, 61–65. doi:10.1038/365061a0

Munson, A. E., Harris, L. S., Friedman, M. A., Dewey, W. L., and Carchman, R. A. (1975). Antineoplastic Activity of Cannabinoids2. *J. Natl. Cancer Inst.* 55, 597–602. doi:10.1093/jnci/55.3.597

Murataeva, N., Miller, S., Dhopeshwarkar, A., Leishman, E., Daily, L., Taylor, X., et al. (2019). Cannabinoid CB2R Receptors Are Upregulated with Corneal Injury and Regulate the Course of Corneal Wound Healing. *Exp. Eye Res.* 182, 74–84. doi:10.1016/j.exer.2019.03.011

Norooznezhad, A. H., and Norooznezhad, F. (2017). Cannabinoids: Possible Agents for Treatment of Psoriasis via Suppression of Angiogenesis and Inflammation. *Hypotheses* 99, 15–18. doi:10.1016/j.mehy.2016.12.003

Pacher, P., Bátkai, S., and Kunos, G. (2006). The Endocannabinoid System as an Emerging Target of Pharmacotherapy. *Pharmacol. Rev.* 58, 389–462. doi:10.1124/pr.58.3.2

Pacher, P., and Haskó, G. (2008). Endocannabinoids and Cannabinoid Receptors in Ischaemia-Reperfusion Injury and Preconditioning. *Br. J. Pharmacol.* 153, 252–262. doi:10.1038/sj.bjp.0707582

Pacher, P., and Mechoulam, R. (2011). Is Lipid Signaling through Cannabinoid 2 Receptors Part of a Protective System?. *Prog. lipid Res.* 50, 193–211. doi:10.1016/j.plipres.2011.01.001

Pacher, P., Steffens, S., Haskó, G., Schindler, T. H., and Kunos, G. (2018). Cardiovascular Effects of Marijuana and Synthetic Cannabinoids: the Good, the Bad, and the Ugly. *Nat. Rev. Cardiol.* 15, 151–166. doi:10.1038/nrcardio.2017.130

Pagano, E., Orlando, P., Finizio, S., Rossi, A., Buono, L., Iannotti, F. A., et al. (2017). Role of the Endocannabinoid System in the Control of Mouse Myometrium Contractility during the Menstrual Cycle. *Biochem. Pharmacol.* 124, 83–93. doi:10.1016/j.bcp.2016.11.023

Pakyari, M., Farrokhi, A., Maharlooei, M. K., and Ghahary, A. (2013). Critical Role of Transforming Growth Factor Beta in Different Phases of Wound Healing. *Adv. Wound Care* 2, 215–224. doi:10.1089/wound.2012.0406

Patel, H. J., Birrell, M. A., Crispino, N., Hele, D. J., Venkatesan, P., Barnes, P. J., et al. (2003). Inhibition of guinea-pig and Human Sensory Nerve Activity and the Cough Reflex in guinea-pigs by Cannabinoid (CB2) Receptor Activation. *Br. J. Pharmacol.* 140, 261–268. doi:10.1038/sj.bjp.0705435

Patel, R. P., Moellering, D., Murphy-Ullrich, J., Jo, H., Beckman, J. S., and Darley-Usmar, V. M. (2000). Cell Signaling by Reactive Nitrogen and Oxygen Species in Atherosclerosis. *Free Radic. Biol. Med.* 28, 1780–1794. doi:10.1016/s0891-5849(00)00235-5

Pertwee, R. G., Howlett, A. C., Abood, M. E., Alexander, S. P. H., Di Marzo, V., Elphick, M. R., et al. (2010). International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid Receptors and Their Ligands: Beyond CB1and CB2. *Pharmacol. Rev.* 62, 588–631. doi:10.1124/pr.110.003004

Pertwee, R. G. (1997). Pharmacology of Cannabinoid CB1 and CB2 Receptors. *Pharmacol. Ther.* 74, 129–180. doi:10.1016/s0163-7258(97)82001-3

Pertwee, R. G. (1999). Pharmacology of Cannabinoid Receptor Ligands. *Curr. Med. Chem.* 6, 635–664. doi:10.1159/000057150

Pertwee, R. G. (2008). The Diverse CB1 and CB2 Receptor Pharmacology of Three Plant Cannabinoids: Δ9 -tetrahydrocannabinol, Cannabidiol and Δ9 -tetrahydrocannabivarin. *Br. J. Pharmacol.* 153, 199–215. doi:10.1038/sj.bjp.0707442

Pini, A., Mannaioni, G., Pellegrini-Giampietro, D., Beatrice Passani, M., Mastroianni, R., Bani, D., et al. (2012). The Role of Cannabinoids in Inflammatory Modulation of Allergic Respiratory Disorders, Inflammatory Pain and Ischemic Stroke. *Cdt* 13, 984–993. doi:10.2174/138945012800675786

Pisani, P., Renna, M. D., Conversano, F., Casciaro, E., Di Paola, M., Quarta, E., et al. (2016). Major Osteoporotic Fragility Fractures: Risk Factor Updates and Societal Impact. *Wjo* 7, 171–181. doi:10.5312/wjo.v7.i3.171

Preet, A., Qamri, Z., Nasser, M. W., Prasad, A., Shilo, K., Zou, X., et al. (2011). Cannabinoid Receptors, CB1 and CB2, as Novel Targets for Inhibition of Non-small Cell Lung Cancer Growth and Metastasis, *Cancer Prev. Res.* 4. Philadelphia, Pa: Cancer prevention research, 65–75. doi:10.1158/1940-6207.capr-10-0181

Pressly, J. D., Mustafa, S. M., Adibi, A. H., Alghamdi, S., Pandey, P., Roy, K. K., et al. (2018). Selective Cannabinoid 2 Receptor Stimulation Reduces Tubular

Epithelial Cell Damage after Renal Ischemia-Reperfusion Injury. *J. Pharmacol. Exp. Ther.* 364, 287–299. doi:10.1124/jpet.117.245522

Puig-Junoy, J., and Ruiz Zamora, A. (2015). Socio-economic Costs of Osteoarthritis: a Systematic Review of Cost-Of-Illness Studies. *Semin. Arthritis Rheum.* 44, 531–541. doi:10.1016/j.semarthrit.2014.10.012

Punzo, F., Manzo, I., Tortora, C., Pota, E., Angelo, V. D., Bellini, G., et al. (2018a). Effects of CB2 and TRPV1 Receptors' Stimulation in Pediatric Acute T-Lymphoblastic Leukemia. *Oncotarget* 9, 21244–21258. doi:10.18632/oncotarget.25052

Punzo, F., Tortora, C., Di Pinto, D., Manzo, I., Bellini, G., Casale, F., et al. (2017). Anti-proliferative, Pro-apoptotic and Anti-invasive Effect of EC/EV System in Human Osteosarcoma. 8, 54459–54471. doi:10.18632/oncotarget.17089

Punzo, F., Tortora, C., Di Pinto, D., Pota, E., Argenziano, M., Di Paola, A., et al. (2018b). Bortezomib and Endocannabinoid/endovanilloid System: a Synergism in Osteosarcoma. *Pharmacol. Res.* 137, 25–33. doi:10.1016/j.phrs.2018.09.017

Puxeddu, I., Piliponsky, A. M., Bachelet, I., and Levi-Schaffer, F. (2003). Mast Cells in Allergy and beyond. *Int. J. Biochem. Cel Biol.* 35, 1601–1607. doi:10.1016/s1357-2725(03)00208-5

Qamri, Z., Preet, A., Nasser, M. W., Bass, C. E., Leone, G., Barsky, S. H., et al. (2009). Synthetic Cannabinoid Receptor Agonists Inhibit Tumor Growth and Metastasis of Breast Cancer. *Mol. Cancer Ther.* 8, 3117–3129. doi:10.1158/1535-7163.mct-09-0448

Qian, H., Zhao, Y., Peng, Y., Han, C., Li, S., Huo, N., et al. (2010). Activation of Cannabinoid Receptor CB2 Regulates Osteogenic and Osteoclastogenic Gene Expression in Human Periodontal Ligament Cells. *J. Periodontal Res.* 45, 504–511. doi:10.1111/j.1600-0765.2009.01265.x

Rajesh, M., Mukhopadhyay, P., Bátkai, S., Haskó, G., Liaudet, L., Huffman, J. W., et al. (2007). CB2-receptor Stimulation Attenuates TNF-α-Induced Human Endothelial Cell Activation, Transendothelial Migration of Monocytes, and Monocyte-Endothelial Adhesion. *Am. J. Physiology-Heart Circulatory Physiol.* 293, H2210–H2218. doi:10.1152/ajpheart.00688.2007

Rajesh, M., Mukhopadhyay, P., Haskó, G., Huffman, J. W., Mackie, K., and Pacher, P. (2008). CB2 Cannabinoid Receptor Agonists Attenuate TNF-α-Induced Human Vascular Smooth Muscle Cell Proliferation and Migration. *Br. J. Pharmacol.* 153, 347–357. doi:10.1038/sj.bjp.0707569

Ramer, R., Fischer, S., Haustein, M., Manda, K., and Hinz, B. (2014). Cannabinoids Inhibit Angiogenic Capacities of Endothelial Cells via Release of Tissue Inhibitor of Matrix Metalloproteinases-1 from Lung Cancer Cells. *Biochem. Pharmacol.* 91, 202–216. doi:10.1016/j.bcp.2014.06.017

Reifart, J., Rentsch, M., Mende, K., Coletti, R., Sobocan, M., Thasler, W. E., et al. (2015). Modulating CD4+ T Cell Migration in the Postischemic Liver. *Transplantation* 99, 41–47. doi:10.1097/tp.0000000000000461

Rivera, P., Vargas, A., Pastor, A., Boronat, A., López-Gambero, A. J., Sánchez-Marín, L., et al. (2020). Differential Hepatoprotective Role of the Cannabinoid CB 1 and CB 2 Receptors in Paracetamol-Induced Liver Injury. *Br. J. Pharmacol.* 177, 3309–3326. doi:10.1111/bph.15051

Romero-Zerbo, S. Y., Garcia-Gutierrez, M. S., Suárez, J., Rivera, P., Ruz-Maldonado, I., Vida, M., et al. (2012). Overexpression of Cannabinoid CB2 Receptor in the Brain Induces Hyperglycaemia and a Lean Phenotype in Adult Mice. *J. Neuroendocrinol.* 24, 1106–1119. doi:10.1111/j.1365-2826.2012.02325.x

Rossi, F., Bellini, G., Luongo, L., Manzo, I., Tolone, S., Tortora, C., et al. (2016). Cannabinoid Receptor 2 as Antiobesity Target: Inflammation, Fat Storage, and Browning Modulation. *J. Clin. Endocrinol. Metab.* 101, 3469–3478. doi:10.1210/jc.2015-4381

Rossi, F., Tortora, C., Palumbo, G., Punzo, F., Argenziano, M., Casale, M., et al. (2019a). CB2 Receptor Stimulation and Dexamethasone Restore the Anti-inflammatory and Immune-Regulatory Properties of Mesenchymal Stromal Cells of Children with Immune Thrombocytopenia. *Int. J. Mol. Sci.* 20. doi:10.3390/ijms20051049

Rossi, F., Tortora, C., Punzo, F., Bellini, G., Argenziano, M., Di Paola, A., et al. (2019b). The Endocannabinoid/Endovanilloid System in Bone: From Osteoporosis to Osteosarcoma. *Ijms* 20, 1919. doi:10.3390/ijms20081919

Ruhl, T., Karthaus, N., Kim, B.-S., and Beier, J. P. (2020). The Endocannabinoid Receptors CB1 and CB2 Affect the Regenerative Potential of Adipose Tissue MSCs. *Exp. Cel Res.* 389, 111881. doi:10.1016/j.yexcr.2020.111881

Sánchez, A. J., González-Pérez, P., Galve-Roperh, I., and García-Merino, A. (2006). R-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)-pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphtalenylmethanone (WIN-2) Ameliorates Experimental Autoimmune Encephalomyelitis and Induces Encephalitogenic T Cell Apoptosis: Partial Involvement of the CB2 Receptor. *Biochem. Pharmacol.* 72, 1697–1706. doi:10.1016/j.bcp.2006.08.018

Sánchez, C., De Ceballos, M. L., Gomez Del Pulgar, T., Rueda, D., Corbacho, C., Velasco, G., et al. (2001). Inhibition of Glioma Growth *In Vivo* by Selective Activation of the CB(2) Cannabinoid Receptor. *Cancer Res.* 61, 5784–5789.

Schmid, P. C., Paria, B. C., Krebsbach, R. J., Schmid, H. H. O., and Dey, S. K. (1997). Changes in Anandamide Levels in Mouse Uterus Are Associated with Uterine Receptivity for Embryo Implantation. *Proc. Natl. Acad. Sci.* 94, 4188–4192. doi:10.1073/pnas.94.8.4188

Schmitz, K., Mangels, N., Häussler, A., Ferreirós, N., Fleming, I., and Tegeder, I. (2016). Pro-inflammatory Obesity in Aged Cannabinoid-2 Receptor-Deficient Mice. *Int. J. Obes.* 40, 366–379. doi:10.1038/ijo.2015.169

Schmuhl, E., Ramer, R., Salamon, A., Peters, K., and Hinz, B. (2014). Increase of Mesenchymal Stem Cell Migration by Cannabidiol via Activation of P42/44 MAPK. *Biochem. Pharmacol.* 87, 489–501. doi:10.1016/j.bcp.2013.11.016

Schuel, H., and Burkman, L. J. (2005). A Tale of Two Cells: Endocannabinoid-Signaling Regulates Functions of Neurons and Sperm. *Biol. Reprod.* 73, 1078–1086. doi:10.1095/biolreprod.105.043273

Seitz, H. M., Camenisch, T. D., Lemke, G., Earp, H. S., and Matsushima, G. K. (2007). Macrophages and Dendritic Cells Use Different Axl/Mertk/Tyro3 Receptors in Clearance of Apoptotic Cells. *J. Immunol.* 178, 5635–5642. doi:10.4049/jimmunol.178.9.5635

Servettaz, A., Kavian, N., Nicco, C., Deveaux, V., Chéreau, C., Wang, A., et al. (2010). Targeting the Cannabinoid Pathway Limits the Development of Fibrosis and Autoimmunity in a Mouse Model of Systemic Sclerosis. *Am. J. Pathol.* 177, 187–196. doi:10.2353/ajpath.2010.090763

Shang, Y., and Tang, Y. (2017). The central Cannabinoid Receptor Type-2 (CB2) and Chronic Pain. *Int. J. Neurosci.* 127, 812–823. doi:10.1080/00207454.2016.1257992

Sheng, W. S., Chauhan, P., Hu, S., Prasad, S., and Lokensgard, J. R. (2019). Antiallodynic Effects of Cannabinoid Receptor 2 (CB2R) Agonists on Retrovirus Infection-Induced Neuropathic Pain. *Pain Res. Manag.* 2019. 1260353. doi:10.1155/2019/1260353

Shi, Y., Zou, M., Baitei, E. Y., Alzahrani, A. S., Parhar, R. S., Al-Makhalafi, Z., et al. (2008). Cannabinoid 2 Receptor Induction by IL-12 and its Potential as a Therapeutic Target for the Treatment of Anaplastic Thyroid Carcinoma. *Cancer Gene Ther.* 15, 101–107. doi:10.1038/sj.cgt.7701101

Singh, U. P., Singh, N. P., Singh, B., Price, R. L., Nagarkatti, M., and Nagarkatti, P. S. (2012). Cannabinoid Receptor-2 (CB2) Agonist Ameliorates Colitis in IL-$10^{-/-}$ Mice by Attenuating the Activation of T Cells and Promoting Their Apoptosis. *Toxicol. Appl. Pharmacol.* 258, 256–267. doi:10.1016/j.taap.2011.11.005

Soethoudt, M., Grether, U., Fingerle, J., Grim, T. W., Fezza, F., De Petrocellis, L., et al. (2017). Cannabinoid CB(2) Receptor Ligand Profiling Reveals Biased Signalling and Off-Target Activity. *Nat. Commun.* 8, 13958. doi:10.1038/ncomms13958

Sophocleous, A., Landao-Bassonga, E., van't Hof, R. J., Idris, A. I., and Ralston, S. H. (2011). The Type 2 Cannabinoid Receptor Regulates Bone Mass and Ovariectomy-Induced Bone Loss by Affecting Osteoblast Differentiation and Bone Formation. *Endocrinology* 152, 2141–2149. doi:10.1210/en.2010-0930

Sophocleous, A., Marino, S., Logan, J. G., Mollat, P., Ralston, S. H., and Idris, A. I. (2015). Bone Cell-Autonomous Contribution of Type 2 Cannabinoid Receptor to Breast Cancer-Induced Osteolysis. *J. Biol. Chem.* 290, 22049–22060. doi:10.1074/jbc.m115.649608

Staiano, R. I., Loffredo, S., Borriello, F., Iannotti, F. A., Piscitelli, F., Orlando, P., et al. (2016). Human Lung-Resident Macrophages Express CB1 and CB2 Receptors Whose Activation Inhibits the Release of Angiogenic and Lymphangiogenic Factors. *J. Leukoc. Biol.* 99, 531–540. doi:10.1189/jlb.3hi1214-584r

Steffens, S., and Pacher, P. (2012). Targeting Cannabinoid Receptor CB2 in Cardiovascular Disorders: Promises and Controversies. *Br. J. Pharmacol.* 167, 313–323. doi:10.1111/j.1476-5381.2012.02042.x

Steffens, S., Veillard, N. R., Arnaud, C., Pelli, G., Burger, F., Staub, C., et al. (2005). Low Dose Oral Cannabinoid Therapy Reduces Progression of Atherosclerosis in Mice. *Nature* 434, 782–786. doi:10.1038/nature03389

Steib, C. J., Gmelin, L., Pfeiler, S., Schewe, J., Brand, S., Göke, B., et al. (2013). Functional Relevance of the Cannabinoid Receptor 2 - Heme Oxygenase Pathway: a Novel Target for the Attenuation of portal Hypertension. *Life Sci.* 93, 543–551. doi:10.1016/j.lfs.2013.08.018

Storr, M. A., Keenan, C. M., Zhang, H., Patel, K. D., Makriyannis, A., and Sharkey, K. A. (2009). Activation of the Cannabinoid 2 Receptor (CB2) Protects against Experimental Colitis. *Inflamm. Bowel Dis.* 15, 1678–1685. doi:10.1002/ibd.20960

Suárez-Pinilla, P., López-Gil, J., and Crespo-Facorro, B. (2014). Immune System: a Possible Nexus between Cannabinoids and Psychosis. *Brain Behav. Immun.* 40, 269–282. doi:10.1016/j.bbi.2014.01.018

Sun, X., and Dey, S. K. (2012). Endocannabinoid Signaling in Female Reproduction. *ACS Chem. Neurosci.* 3, 349–355. doi:10.1021/cn300014e

Tahamtan, A., Samieipoor, Y., Nayeri, F. S., Rahbarimanesh, A. A., Izadi, A., Rashidi-Nezhad, A., et al. (2018). Effects of Cannabinoid Receptor Type 2 in Respiratory Syncytial Virus Infection in Human Subjects and Mice. *Virulence* 9, 217–230. doi:10.1080/21505594.2017.1389369

Tanai, E., and Frantz, S. (2015). Pathophysiology of Heart Failure. *Compr. Physiol.* 6, 187–214. doi:10.1002/cphy.c140055

Tartakover Matalon, S., Ringel, Y., Konikoff, F., Drucker, L., Pery, S., and Naftali, T. (2020). Cannabinoid Receptor 2 Agonist Promotes Parameters Implicated in Mucosal Healing in Patients with Inflammatory Bowel Disease. *United Eur. Gastroenterol. j.* 8, 271–283. doi:10.1177/2050640619889773

Tashkin, D. P., Shapiro, B. J., and Frank, I. M. (1974). Acute Effects of Smoked Marijuana and Oral delta9-tetrahydrocannabinol on Specific Airway Conductance in Asthmatic Subjects. *Am. Rev. Respir. Dis.* 109, 420–428. doi:10.1164/arrd.1974.109.4.420

Tashkin, D. P., Shapiro, B. J., Lee, Y. E., and Harper, C. E. (1975). Effects of Smoked Marijuana in Experimentally Induced Asthma. *Am. Rev. Respir. Dis.* 112, 377–386. doi:10.1164/arrd.1975.112.3.377

Teixeira-Clerc, F., Belot, M.-P., Manin, S., Deveaux, V., Cadoudal, T., Chobert, M.-N., et al. (2010). Beneficial Paracrine Effects of Cannabinoid Receptor 2 on Liver Injury and Regeneration. *Hepatology* 52, 1046–1059. doi:10.1002/hep.23779

Terwilliger, T., and Abdul-Hay, M. (2017). Acute Lymphoblastic Leukemia: a Comprehensive Review and 2017 Update. *Blood Cancer J.* 7, e577. doi:10.1038/bcj.2017.53

Tham, M., Yilmaz, O., Alaverdashvili, M., Kelly, M. E. M., Denovan-Wright, E. M., and Laprairie, R. B. (2019). Allosteric and Orthosteric Pharmacology of Cannabidiol and Cannabidiol-Dimethylheptyl at the Type 1 and Type 2 Cannabinoid Receptors. *Br. J. Pharmacol.* 176, 1455–1469. doi:10.1111/bph.14440

Tomar, S., E. Zumbrun, E., Nagarkatti, M., and Nagarkatti, P. S. (2015). Protective Role of Cannabinoid Receptor 2 Activation in Galactosamine/lipopolysaccharide-Induced Acute Liver Failure through Regulation of Macrophage Polarization and microRNAs. *J. Pharmacol. Exp. Ther.* 353, 369–379. doi:10.1124/jpet.114.220368

Troy, N. M., and Bosco, A. (2016). Respiratory Viral Infections and Host Responses; Insights from Genomics. *Respir. Res.* 17, 156. doi:10.1186/s12931-016-0474-9

Turcotte, C., Blanchet, M.-R., Laviolette, M., and Flamand, N. (2016). The CB2 Receptor and its Role as a Regulator of Inflammation. *Cell. Mol. Life Sci.* 73, 4449–4470. doi:10.1007/s00018-016-2300-4

Turner, S. E., Williams, C. M., Iversen, L., and Whalley, B. J. (2017). Molecular Pharmacology of Phytocannabinoids. *Prog. Chem. Org. Nat. Prod.* 103, 61–101. doi:10.1007/978-3-319-45541-9_3

Unal, E., Anderson, B., Helber, A., and Marks, J. H. (2020). Cannabinoids: A Guide for Use in the World of Gastrointestinal Disease. *J. Clin. Gastroenterol.* 54. doi:10.1097/mcg.0000000000001287

Valenzano, K. J., Tafesse, L., Lee, G., Harrison, J. E., Boulet, J. M., Gottshall, S. L., 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. doi:10.1016/j.neuropharm.2004.12.008

Van Gaal, L. F., Mertens, I. L., and De Block, C. E. (2006). Mechanisms Linking Obesity with Cardiovascular Disease. *Nature* 444, 875–880. doi:10.1038/nature05487

Verty, A. N., Stefanidis, A., Mcainch, A. J., Hryciw, D. H., and Oldfield, B. (2015). Anti-Obesity Effect of the CB2 Receptor Agonist JWH-015 in Diet-Induced Obese Mice. *PLoS One* 10, e0140592. doi:10.1371/journal.pone.0140592

Vidinský, B., Gál, P., Pilátová, M., Vidová, Z., Solár, P., Varinská, L., et al. (2012). Anti-proliferative and Anti-angiogenic Effects of CB2R Agonist (JWH-133) in Non-small Lung Cancer Cells (A549) and Human Umbilical Vein Endothelial Cells: an *In Vitro* Investigation. *Folia Biol. (Praha)* 58, 75–80.

Wang, L.-L., Zhao, R., Li, J.-Y., Li, S.-S., Liu, M., Wang, M., et al. (2016). Pharmacological Activation of Cannabinoid 2 Receptor Attenuates Inflammation, Fibrogenesis, and Promotes Re-epithelialization during Skin Wound Healing. *Eur. J. Pharmacol.* 786, 128–136. doi:10.1016/j.ejphar.2016.06.006

Watkins, B. A., and Kim, J. (2014). The Endocannabinoid System: Directing Eating Behavior and Macronutrient Metabolism. *Front. Psychol.* 5, 1506. doi:10.3389/fpsyg.2014.01506

Wawryk-Gawda, E., Chłapek, K., Zarobkiewicz, M. K., Lis-Sochocka, M., Chylińska-Wrzos, P., Boguszewska-Czubara, A., et al. (2018). CB2R Agonist Prevents Nicotine Induced Lung Fibrosis. *Exp. Lung Res.* 44, 344–351. doi:10.1080/01902148.2018.1543368

Whyte, L., Ford, L., Ridge, S., Cameron, G., Rogers, M., and Ross, R. (2012). Cannabinoids and Bone: Endocannabinoids Modulate Human Osteoclast Function *In Vitro*. *Br. J. Pharmacol.* 165, 2584–2597. doi:10.1111/j.1476-5381.2011.01519.x

Wilhelm, I., Molnár, J., Fazakas, C., Haskó, J., and Krizbai, I. (2013). Role of the Blood-Brain Barrier in the Formation of Brain Metastases. *Ijms* 14, 1383–1411. doi:10.3390/ijms14011383

Willecke, F., Zeschky, K., Ortiz Rodriguez, A., Colberg, C., Auwärter, V., Kneisel, S., et al. (2011). Cannabinoid Receptor 2 Signaling Does Not Modulate Atherogenesis in Mice. *loS one* 6, e19405. doi:10.1371/journal.pone.0019405

Wright, K., Rooney, N., Feeney, M., Tate, J., Robertson, D., Welham, M., et al. (2005). Differential Expression of Cannabinoid Receptors in the Human colon: Cannabinoids Promote Epithelial Wound Healing. *Gastroenterology* 129, 437–453. doi:10.1053/j.gastro.2005.05.026

Wu, H. M., Kim, T. H., Kim, A., Koo, J. H., Joo, M. S., and Kim, S. G. (2019). Liver X Receptor α-Induced Cannabinoid Receptor 2 Inhibits Ubiquitin-specific Peptidase 4 through miR-27b, Protecting Hepatocytes from TGF-β. *Hepatol. Commun.* 3, 1373–1387. doi:10.1002/hep4.1415

Wu, Q., Ma, Y., Liu, Y., Wang, N., Zhao, X., and Wen, D. (2020). CB2R Agonist JWH-133 Attenuates Chronic Inflammation by Restraining M1 Macrophage Polarization via Nrf2/HO-1 Pathway in Diet-Induced Obese Mice. *Life Sci.* 260, 118424. doi:10.1016/j.lfs.2020.118424

Xia, K.-K., Shen, J.-X., Huang, Z.-B., Song, H.-M., Gao, M., Chen, D.-J., et al. (2019). Heterogeneity of Cannabinoid Ligand-Induced Modulations in Intracellular Ca2+ Signals of Mouse Pancreatic Acinar Cells *In Vitro*. *Acta Pharmacol. Sin*. 40, 410–417. doi:10.1038/s41401-018-0074-y

Xu, A., Yang, Y., Shao, Y., Wu, M., and Sun, Y. (2020). Activation of Cannabinoid Receptor Type 2-induced Osteogenic Differentiation Involves Autophagy Induction and P62-Mediated Nrf2 Deactivation. *ell Commun. Signaling* 18, 9. doi:10.1186/s12964-020-0512-6

Xu, H., Cheng, C. L., Chen, M., Manivannan, A., Cabay, L., Pertwee, R. G., et al. (2007). Anti-inflammatory Property of the Cannabinoid Receptor-2-Selective Agonist JWH-133 in a Rodent Model of Autoimmune Uveoretinitis. *J. Leukoc. Biol.* 82, 532–541. doi:10.1189/jlb.0307159

Yancopoulos, G. D., Davis, S., Gale, N. W., Rudge, J. S., Wiegand, S. J., and Holash, J. (2000). Vascular-specific Growth Factors and Blood Vessel Formation. *Nature* 407, 242–248. doi:10.1038/35025215

Yang, H., Wang, Z., Capó-Aponte, J. E., Zhang, F., Pan, Z., and Reinach, P. S. (2010). Epidermal Growth Factor Receptor Transactivation by the Cannabinoid Receptor (CB1) and Transient Receptor Potential Vanilloid 1 (TRPV1) Induces Differential Responses in Corneal Epithelial Cells. *Exp. Eye Res.* 91, 462–471. doi:10.1016/j.exer.2010.06.022

Yang, Y.-Y., Hsieh, S.-L., Lee, P.-C., Yeh, Y.-C., Lee, K.-C., Hsieh, Y.-C., et al. (2014). Long-term Cannabinoid Type 2 Receptor Agonist Therapy Decreases Bacterial Translocation in Rats with Cirrhosis and Ascites. *J. Hepatol.* 61, 1004–1013. doi:10.1016/j.jhep.2014.05.049

Yeh, C.-M., Ruan, T., Lin, Y.-J., and Hsu, T.-H. (2016). Activation of Cannabinoid CB1 Receptors Suppresses the ROS-Induced Hypersensitivity of Rat Vagal Lung C-Fiber Afferents. *Pulm. Pharmacol. Ther.* 40, 22–29. doi:10.1016/j.pupt.2016.06.002

Yellon, D. M., and Hausenloy, D. J. (2007). Myocardial Reperfusion Injury. *N. Engl. J. Med.* 357, 1121–1135. doi:10.1056/nejmra071667

Yoshihara, S., Morimoto, H., Yamada, Y., Abe, T., and Arisaka, O. (2004). Cannabinoid Receptor Agonists Inhibit Sensory Nerve Activation in guinea Pig Airways. *Am. J. Respir. Crit. Care Med.* 170, 941–946. doi:10.1164/rccm.200306-775oc

Yu, T., Wang, X., Zhao, R., Zheng, J., Li, L., Ma, W., et al. (2015). Beneficial Effects of Cannabinoid Receptor Type 2 (CB2R) in Injured Skeletal Muscle post-contusion. *Histol. Histopathol.* 30, 737–49. doi:10.14670/HH-30.737

Yu, W., Jin, G., Zhang, J., and Wei, W. (2019). Selective Activation of Cannabinoid Receptor 2 Attenuates Myocardial Infarction via Suppressing NLRP3 Inflammasome. *Inflammation* 42, 904–914. doi:10.1007/s10753-018-0945-x

Zeng, J., Li, X., Cheng, Y., Ke, B., and Wang, R. (2019). Activation of Cannabinoid Receptor Type 2 Reduces Lung Ischemia Reperfusion Injury through PI3K/Akt Pathway. *Int. J. Clin. Exp. Pathol.* 12, 4096–4105.

Zhan, Y., Wang, Z., Yang, P., Wang, T., Xia, L., Zhou, M., et al. (2014). Adenosine 5′-monophosphate Ameliorates D-Galactosamine/lipopolysaccharide-Induced Liver Injury through an Adenosine Receptor-independent Mechanism in Mice. *Cel. Death Dis.* 5, e985. doi:10.1038/cddis.2013.516

Zhang, X., Maor, Y., Wang, J., Kunos, G., and Groopman, J. (2010). Endocannabinoid-like N-Arachidonoyl Serine Is a Novel Pro-angiogenic Mediator. *Br. J. Pharmacol.* 160, 1583–1594. doi:10.1111/j.1476-5381.2010.00841.x

Zhao, J., Wang, M., Liu, W., Ma, Z., and Wu, J. (2020). Activation of Cannabinoid Receptor 2 Protects Rat Hippocampal Neurons against Aβ-Induced Neuronal Toxicity. *Neurosci. Lett.* 735, 135207. doi:10.1016/j.neulet.2020.135207

Zhao, Y., Yuan, Z., Liu, Y., Xue, J., Tian, Y., Liu, W., et al. (2010). Activation of Cannabinoid CB2 Receptor Ameliorates Atherosclerosis Associated with Suppression of Adhesion Molecules. *J. Cardiovasc. Pharmacol.* 55, 292–298. doi:10.1097/fjc.0b013e3181d2644d

Zheng, W., Liu, C., Lei, M., Han, Y., Zhou, X., Li, C., et al. (2019). Evaluation of Common Variants in the CNR2 Gene and its Interaction with Abdominal Obesity for Osteoporosis Susceptibility in Chinese post-menopausal Females. *Bone Jt. Res.* 8, 544–549. doi:10.1302/2046-3758.811.bjr-2018-0284.r1

Zhu, M., Yu, B., Bai, J., Wang, X., Guo, X., Liu, Y., et al. (2019). Cannabinoid Receptor 2 Agonist Prevents Local and Systemic Inflammatory Bone Destruction in Rheumatoid Arthritis. *J. Bone Miner. Res.* 34, 739–751. doi:10.1002/jbmr.3637

Zoja, C., Locatelli, M., Corna, D., Villa, S., Rottoli, D., Nava, V., et al. (2016). Therapy with a Selective Cannabinoid Receptor Type 2 Agonist Limits Albuminuria and Renal Injury in Mice with Type 2 Diabetic Nephropathy. *Nephron* 132, 59–69. doi:10.1159/000442679

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.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 09 September 2021
doi: 10.3389/fphar.2021.722476

β-caryophyllene, an FDA-Approved Food Additive, Inhibits Methamphetamine-Taking and Methamphetamine-Seeking Behaviors Possibly *via* CB2 and Non-CB2 Receptor Mechanisms

*Xiang-Hu He[1,2], Ewa Galaj[1], Guo-Hua Bi[1], Yi He[1], Briana Hempel[1], Yan-Lin Wang[2], Eliot L. Gardner[1] and Zheng-Xiong Xi[1]**

[1]Molecular Targets and Medications Discovery Branch, National Institute on Drug Abuse, Intramural Research Program, Baltimore, MD, United States, [2]Department of Anesthesiology, Zhongnan Hospital of Wuhan University, Hubei, China

OPEN ACCESS

Edited by:
Cecilia Hillard, Medical College of Wisconsin, United States

Reviewed by:
África Flores, Pompeu Fabra University, Spain
Julian Romero, Universidad Francisco de Vitoria, Spain

***Correspondence:**
Zheng-Xiong Xi
zxi@intra.nida.nih.gov

Specialty section:
This article was submitted to Experimental Pharmacology and Drug Discovery, a section of the journal Frontiers in Pharmacology

Received: *08 June 2021*
Accepted: *23 August 2021*
Published: *09 September 2021*

Citation:
He X-H, Galaj E, Bi G-H, He Y, Hempel B, Wang Y-L, Gardner EL and Xi Z-X (2021) β-caryophyllene, an FDA-Approved Food Additive, Inhibits Methamphetamine-Taking and Methamphetamine-Seeking Behaviors Possibly via CB2 and Non-CB2 Receptor Mechanisms. Front. Pharmacol. 12:722476. doi: 10.3389/fphar.2021.722476

Recent research indicates that brain cannabinoid CB2 receptors are involved in drug reward and addiction. However, it is unclear whether β-caryophyllene (BCP), a natural product with a CB2 receptor agonist profile, has therapeutic effects on methamphetamine (METH) abuse and dependence. In this study, we used animal models of self-administration, electrical brain-stimulation reward (BSR) and *in vivo* microdialysis to explore the effects of BCP on METH-taking and METH-seeking behavior. We found that systemic administration of BCP dose-dependently inhibited METH self-administration under both fixed-ratio and progressive-ratio reinforcement schedules in rats, indicating that BCP reduces METH reward, METH intake, and incentive motivation to seek and take METH. The attenuating effects of BCP were partially blocked by AM 630, a selective CB2 receptor antagonist. Genetic deletion of CB2 receptors in CB2-knockout (CB2-KO) mice also blocked low dose BCP-induced reduction in METH self-administration, suggesting possible involvement of a CB2 receptor mechanism. However, at high doses, BCP produced a reduction in METH self-administration in CB2-KO mice in a manner similar as in WT mice, suggesting that non-CB2 receptor mechanisms underlie high dose BCP-produced effects. In addition, BCP dose-dependently attenuated METH-enhanced electrical BSR and inhibited METH-primed and cue-induced reinstatement of drug-seeking in rats. *In vivo* microdialysis assays indicated that BCP alone did not produce a significant reduction in extracellular dopamine (DA) in the nucleus accumbens (NAc), while BCP pretreatment significantly reduced METH-induced increases in extracellular NAc DA in a dose-dependent manner, suggesting a DA-dependent mechanism involved in BCP action. Together, the present findings suggest that BCP might be a promising therapeutic candidate for the treatment of METH use disorder.

Keywords: β-caryophyllene, dopamine, CB2 receptor, methamphetamine, self-administration, reinstatement

INTRODUCTION

Methamphetamine (METH) is one of the most addictive psychostimulants. Following cannabis, it is the second most widely abused illicit drug worldwide–possibly due to its widespread availability and relatively low costs (Brensilver et al., 2013; Panenka et al., 2013; Rawson, 2013). METH abuse produces serious social and public health problems worldwide (Vearrier et al., 2012; Courtney and Ray, 2014). A number of therapeutic ligands such as methylphenidate (Miles et al., 2013), modafinil (Shearer et al., 2009), topiramate (Johnson et al., 2007), aripiprazole (Newton et al., 2008) and sertraline (Zorick et al., 2011) have been evaluated in clinical trials for the treatment of METH use disorder (Ling et al., 2006). However, to date, no effective medications have been approved by the United States Food and Drug Administration (FDA) for the treatment of METH addiction (Brackins et al., 2011; Rawson, 2013).

Accumulating evidence indicates that the endocannabinoid system in the brain is involved in the rewarding effects of drugs of abuse (Covey et al., 2015; Zlebnik and Cheer, 2016; Galaj and Xi, 2019; Jordan et al., 2020). The endocannabinoid system consists of cannabinoid receptors, endogenous ligands and enzymes (Di Marzo, 2009; Galaj and Xi, 2020). To date, both CB1 and CB2 receptors have been cloned and identified as G-protein-coupled receptors (Svízenská et al., 2008). Early studies have mainly focused on brain CB1 receptors, because CB1 receptors are highly expressed in the central nervous system (Wilson and Nicoll, 2002; Wilson and Nicoll, 2002; Iversen, 2003). Indeed, numerous studies have demonstrated that CB1 receptors play a vital role in drug reward and addiction. Some cannabinoid CB1 receptor antagonists have been tested against the effects of cocaine (Gobira et al., 2019), heroin (Solinas et al., 2003; Navarro et al., 2004), METH (Vinklerová et al., 2002; Schindler et al., 2010; Rodriguez et al., 2011), and nicotine (Shoaib, 2008) in animal models. However, clinical trials with rimonabant, a selective CB1 receptor antagonist or inverse agonist, failed due to severe unwanted side-effects such as depression and suicidal tendency (Le Foll et al., 2009).

In addition to the CB1 receptor, recent studies indicate that CB2 receptors are also expressed in brain regions related to drug abuse and addiction (Gong et al., 2006; Svízenská et al., 2008; Zhang et al., 2014, 2015). CB2 receptors have been found to modulate cocaine self-administration (Aracil-Fernández et al., 2012; Galaj et al., 2020a; Jordan et al., 2020) and cocaine- or nicotine-induced conditioned place preference (Ignatowska-Jankowska et al., 2013). Our previous study found that JWH 133, a selective CB2 receptor agonist, dose-dependently inhibits intravenous cocaine self-administration and this effect is blocked by AM630, a selective CB2 receptor antagonist, and is absent in CB2-KO mice (Xi et al., 2011). These findings suggest that brain CB2 receptors might be a new target in medication development for the treatment of substance use disorders.

(E)-β-caryophyllene (BCP) is a common constituent of essential oils in numerous spice and food plants and a major component in the cannabis sativa plant (Mediavilla and Steinemann, 1997; Sharma et al., 2016). Due to its distinctive flavor and an excellent safety profile, BCP has been approved by the FDA as a "generally recognized as safe" food or cosmetic additive (CFR - Code of Federal Regulations Title 21, 2020). BCP was first synthesized in 1964 (Corey et al., 1964) and later identified as a selective agonist of CB_2 receptors (K_i = 155 nM) with ~60-fold selectivity for CB2 over CB1 receptor (Ki > 10 μM) (Gertsch et al., 2008). BCP has been shown to exhibit its anti-inflammatory, antioxidant, antiviral, and analgesic effects (Cho et al., 2007; Gertsch et al., 2008; Katsuyama et al., 2013; Chicca et al., 2014; Guo et al., 2014; Klauke et al., 2014; Fidyt et al., 2016). Recently, BCP has been found to confer protection against various diseases, including cerebral ischemic injury (Chang et al., 2013), anxiety and depressive disorders (Bahi et al., 2014), alcohol use disorder (Al Mansouri et al., 2014), nicotine dependence (He et al., 2020) and cocaine abuse (Galaj et al., 2021). However, it is unknown whether BCP is also effective against METH reward, intake, and relapse.

Therefore, in the present study, we investigated: 1) whether BCP treatment can inhibit METH self-administration under both fixed-ratio 2 (FR2) and progressive-ratio (PR) schedules of reinforcement in rats; 2) whether deletion of CB2 receptors in CB2-knockout (CB2-KO) mice prevents BCP action on METH self-administration; 3) whether BCP can block METH action on electrical brain-stimulation reward in rats; 4) whether BCP can reduce METH- or cue-induced reinstatement of drug seeking; and 5) whether a dopamine-dependent mechanism is involved in BCP's potential therapeutic effects against METH-taking and METH-seeking behavior, as assessed by *in vivo* microdialysis.

MATERIALS AND METHODS

Animals

Male Long–Evans rats (Charles River Laboratories, Raleigh, NC) were used in all experiments. Wild-type (WT) and CB2-KO mice with C57BL/6J genetic backgrounds were used only in METH self-administration experiment to determine whether a CB2 receptor-dependent mechanism underlies BCP action. WT and CB2-KO mice (Buckley et al., 2000) were bred within the Transgenic Animal Breeding Facility of the National Institute on Drug Abuse (NIDA). All animals were housed individually in a climate-controlled animal room on a reversed light–dark cycle with free access to food and water. All experimental procedures were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* of the United States National Research Council and were approved by the NIDA Animal Care and Use Committee.

Drugs and Chemicals

Methamphetamine HCl (METH) was provided by the Research Pharmacy of the National Institute on Drug Abuse Intramural Research Program and dissolved in sterile 0.9% physiological saline. BCP was obtained from MilliporeSigma (Burlington, MA, United States) and dissolved in 5% Kolliphor EL (i.e., Cremophor) (BASF Pharma, Ludwigshafen, Germany). The BCP doses were chosen from our previous reports (Galaj and Xi, 2020; He et al., 2020). AM630 was purchased from Tocris Division of Bio-Techne (Minneapolis, MN, United States) and dissolved in saline; the doses of AM630 (3, 10 mg/kg) were chosen based on our previous experiments (Galaj and Xi, 2020).

Surgery

Under standard aseptic surgical techniques, all animals were prepared for experimentation by surgical catheterization of the right external jugular vein as described by Xi et al. (Xi et al., 2011; Galaj et al., 2020b). After all animals were anesthetized by an intraperitoneal injection of sodium pentobarbital (65 mg/kg, i.p.), a microrenathane catheter (Braintree Scientific Inc., Braintree, MA, United States) was inserted into the right jugular vein. After being sutured into place, the catheter was passed subcutaneously to the top of the skull and exited into a connector (a modified 24 g cannula; Plastics One, Roanoke, VA, United States), then mounted to the skull with jeweler's screws and dental acrylic. To prevent clogging, the catheters were flushed daily with a gentamicin-heparin-saline solution (30 IU/ml heparin) (ICN Biochemicals, Cleveland, OH, United States).

Apparatus

The intravenous self-administration experiments were conducted in operant chambers (32 × 25 × 33 cm) from MED Associates Inc. (Georgia, VT, United States). Each chamber contained two levers: one active and one inactive, located 6.5 cm above the floor. A cue light and a speaker were located 12 cm above the active lever. The house light was turned on during each 3 h test session. To facilitate acquisition and maintenance of drug self-administration behavior, each drug infusion was paired with a conditioned cue light and a cue sound (tone). Each press of the active lever activated the infusion pump; presses of the inactive lever were counted but had no consequences.

General Procedure

After recovery from surgery, animals were placed into standard operant chambers for drug self-administration under a fixed ratio 1 (FR1) reinforcement schedule. Each session lasted 3 h during which active lever presses produced delivery of i.v. METH (0.05 mg/kg/infusion) in a volume of 0.08 ml over 4.6 s. During the 4.6 s infusion time, additional responses on the active lever were recorded but did not lead to additional infusions. Inactive lever presses were counted but had no consequence. After a stable pattern of self-administration was established, rats were then randomly assigned to one of the following three groups: 1) METH self-administration under an FR2 schedule of reinforcement in rats; 2) METH self-administration under a PR schedule of reinforcement in rats; 3) METH self-administration under a FR2 schedule of reinforcement followed by extinction (or forced abstinence) and reinstatement tests. As described previously (Xi et al., 2008, 2011; Zhang et al., 2014), in all experiments, BCP was given (i.p.) 30 min prior to testing. The CB2 antagonist AM 630 was administrated (i.p.) 30 min prior to the injection of BCP.

Experiment 1. Methamphetamine Self-Administration Under a Fixed-Ratio 2 Reinforcement Schedule in Rats

After transition from a FR1 to FR2 schedule of reinforcement, drug self-administration training continued with METH (0.05 mg/kg/infusion). The following criteria were used to assess whether stable drug-maintained responding was established: less than 10% variability in intra-session responding and less than 10% variability in the number of active lever presses for at least three consecutive days. To prevent drug overdose, each animal was limited to a maximum of 50 infusions per self-administration session. After stable rates of responding were established, each subject randomly received one of four doses of BCP (10, 25, 50, 100 mg/kg, i.p.), or vehicle (equal volume of 5% Kolliphor solution) 30 min prior to the test session. For subjects that received pretreatment with the CB2 antagonist AM630, the antagonist (3 or 10 mg/kg, i.p.) was administrated 30 min prior to BCP. Animals then received an additional 5–7 days of self-administration of METH alone until a baseline response rate was reestablished prior to being tested with another dose of BCP. The order of testing with different doses of BCP or AM 630 was counterbalanced.

Experiment 2. Methamphetamine Self-Administration Under a Progressive-Ratio Reinforcement Schedule in Rats

After stable METH self-administration under a FR2 schedule of reinforcement was established, an additional group of rats were switched to METH self-administration (0.05 mg/kg/infusion) under a progressive-ratio (PR) schedule, during which the lever-pressing work requirement needed to receive a single i.v. METH infusion was progressively raised within each test session [see details in (Richardson and Roberts, 1996)] according to the following PR series: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328, 402, 492, and 603 until a break point was reached. The break point was defined as the maximum number of lever presses completed for the last METH infusion prior to a 1-h period during which no infusions were obtained. Animals self-administered METH daily under the PR reinforcement conditions until day-to-day variability in break points fell within 1-2 ratio increments for three consecutive days. After a stable break point was established, subjects were assigned to different subgroups to determine the effects of BCP (10, 25, 50 mg/kg, i.p.) or vehicle (equal volume of 5% Kolliphor solution) on PR break point for METH self-administration. To evaluate the mechanism by which BCP produces its effects on METH self-administration, AM630, a CB2 antagonist (3 mg/kg) was administrated 30 min prior to the injection of BCP. Since it is relatively difficult to re-establish a stable break point level after each drug test, we used a between-subjects design rather than a within-subjects design to determine the dose–response effects of BCP and BCP plus AM 630 on break point for METH.

Experiment 3: Locomotor Activity in Rats

Three groups of rats were used to observe the effects of BCP on spontaneous locomotor activity. On the test day, rats were initially placed in locomotor detection chambers (Accuscan, Columbus, OH, United States) for a 30-min habituation period, and then each rat was administered one of the two doses of BCP (25, 50 mg/kg, i.p.) or vehicle (5% Kolliphor solution). The habituation was chosen because animal locomotor activity within the initial 30 min in locomotor chamber is high and variable, and

therefore, we chose to observe the locomotor effects of BCP after basal level of locomotion stabilized. After the BCP injection, rats were placed back into the locomotor chambers for 2 h to record possible alterations in locomotion. Total distance was used to evaluate the effects of BCP on locomotion.

Experiment 4. Methamphetamine Self-Administration in Wild-Type and CB2-Knockout Mice

To further examine possible involvement of a CB2 receptor mechanism in BCP's action, we used CB2-KO mice as controls (n = 8) and their WT littermates (n = 9) in a self-administration paradigm. Briefly, animals were trained to self-administer METH (0.05 mg/kg, i.v.) under an FR1 schedule of reinforcement during daily 3-h sessions for approximately 2–3 weeks. Responding on the active lever activated the syringe pump–producing an i.v. infusion of METH (0.015 ml) and presentation of the light cue above the active lever and the tone cue. Responses on the inactive lever were counted but had no consequences. During the 4.2-s infusion period, additional responses on the active lever were recorded but did not lead to additional infusions. Animals were tested with BCP (0, 25, 50, 100 mg/kg i.p., 30 min prior to the test session) after stable METH self-administration was achieved, defined as 1) at least 20 METH infusions during the 3-h session, 2) less than 20% variability in daily METH infusions across two consecutive days, and 3) an active/inactive lever press ratio exceeding 2:1. Mice then received an additional 5–7 days of METH self-administration between BCP tests until stable self-administration was re-established as described above. The order of BCP doses was counterbalanced.

Experiment 5: Methamphetamine-Induced Reinstatement of Drug Seeking in Rats

After stable METH self-administration training, a third group of rats was exposed to extinction conditions, during which METH was replaced by saline, and the METH-associated cue light and tone were turned off. Daily extinction sessions continued until lever pressing was <10 per 3 h session for three consecutive days. Then, rats were divided into three BCP dose groups. On the reinstatement test day, each group received either vehicle (5% Kolliphor solution) or one of the BCP doses (25, 50 mg/kg). Thirty min later, rats were given a priming injection of METH (1 mg/kg, i.p.) and immediately tested in a reinstatement test. During the reinstatement test, which lasted 3 h, lever-pressing responses did not lead to either METH infusions or presentation of the conditioned cues. METH-induced lever-pressing responses were recorded. This priming dose of METH was found to produce robust reinstatement of METH seeking in our previous studies (Higley et al., 2011).

Experiment 6: Cue-Induced Methamphetamine Seeking in Rats

Additional groups of rats were used to assess the effects of BCP pretreatment on contextual cue-induced METH-seeking behavior. This "incubation of craving" model was chosen because it mimics relapse in humans after forced abstinence (Altshuler et al., 2021). In addition, we have found over many years of experience that contextual cue-induced drug seeking is more robust than classical cue-induced reinstatement responding, and therefore, it is a more sensitive measure of cue-induced changes in drug-seeking behavior. After stable METH self-administration was achieved under a FR2 schedule of reinforcement, rats underwent forced abstinence in their home cages. After 21 days of withdrawal from METH self-administration, rats were divided into four experimental groups; each group received either vehicle (5% Kolliphor solution) or one of the three doses of BCP (25, 50, 100 mg/kg). 30 min after the injection on the test day, the rats were re-placed into the same self-administration chambers. Contextual cue-induced drug seeking was conducted under conditions identical to that of self-administration, except that responses on the active lever (under a FR2 schedule) resulted in contingent presentation of the cues without METH availability (no infusions). Responses on the inactive lever were recorded but had no programmed consequences. Each reinstatement test lasted for 3 h.

Experiment 7: Electrical Brain Stimulation Reward in Rats

We then assessed the effects of BCP on METH-enhanced electrical brain-stimulation reward (BSR). The procedures of electrical BSR were the same as we reported recently (Spiller et al., 2019). Briefly, lever pressing for electrical BSR was reinforced by a stimulation current at different frequencies from 141 to 25 Hz in a decreasing series of 16 discrete 0.05 log steps. At each pulse frequency, there were two 30-s trials, each followed by lever retraction for 5 s. A response rate for each frequency was defined as the mean number of lever responses during two 30-s trials. The BSR threshold (θ_0) was defined as the minimum frequency at which an animal responded for stimulation, calculated using the Gompertz sigmoidal model (Coulombe and Miliaressis, 1987). In addition, Ymax was measured as maximum number of lever presses. The testing phase began once stable BSR responding was achieved (<20% variation in θ_0 over three consecutive days). On the test day rats received systemic injection of BCP (0, 50, or 100 mg/kg) 30 min prior to METH injection (2 mg/kg) and later were allowed to lever-press for brain-stimulation. After each test, a new baseline θ_0 was established and rats were re-tested with a different dose of BCP in the presence of METH treatment. The BCP effects on BSR were also evaluated in the absence of METH.

Experiment 8: *In vivo* Brain Microdialysis in Rats

Microdialysis experiments were performed in six additional groups of rats to evaluate the effects of vehicle (5% Kolliphor solution) or BCP (25, 50 mg/kg) alone on basal levels of extracellular DA or BCP pretreatment on METH-enhanced NAc dopamine (DA). Microdialysis protocols and probe construction were as reported previously (Xi et al., 2006). Guide cannulae (20 gauge; Plastics One, Roanoke,VA) were surgically implanted into the NAc (anteroposterior, +1.6 mm;

mediolateral, ±1.8 mm; dorsoventral,—4.3 mm, angled 6° from vertical) using standard surgical and stereotaxic techniques. Microdialysis probes were inserted into the NAc 12 h before the experiment to minimize damage-induced neurotransmitter release. During the experiment, microdialysis buffer was perfused through the probe (2.0 ml/min) for at least 2 h before sampling started. Samples were collected every 20 min into 10 µl of 0.5 M perchloric acid to prevent neurotransmitter degradation. After 1 h baseline collection, one of the two doses of BCP (25, 50 mg/kg, i.p.) or vehicle (5% Kolliphor solution) were administered 40 min prior to METH administration. All samples were frozen at 80°C until analyzed. After microdialysis experiments were completed, rats were anesthetized with a high dose of pentobarbital (>100 mg/kg i.p.) and perfused transcardially with 0.9% saline followed by 10% formalin. Brains were removed and placed in 10% formalin for histological verification of microdialysis probe locations in rat brain.

Microdialysate DA was measured by high performance liquid chromatography (HPLC) with an ESA (ESA Biosciences, Chelmsford, MA) electrochemical (EC) detection system as described previously (Xi et al., 2006), upgraded by a Coulochem III EC detector. Areas under the curve (AUC) for DA were measured and quantified with external standard curves. The minimum detection limit for DA was 1–10 fmol.

Data Analysis

All data are presented as means ± SEM. Separate one-way analyses of variance (ANOVAs) were used to analyze the effects of BCP on drug self-administration, methamphetamine or cue-induced reinstatement, NAc DA and locomotion. A two-way ANOVA with time as the repeated measure was used to analyze the effects of BCP on METH self-administration in WT and CB2-KO mice and on NAc DA. The Student–Newman–Keuls post-hoc test or Tukey's honestly significant difference (HSD) test was used for multiple group comparisons. The statistical significance was set at a probability level of $p < 0.05$ for all tests.

RESULTS

β-Caryophyllene Attenuates Methamphetamine Self-Administration Under a Fixed-Ratio 2 Schedule of Reinforcement

Figure 1Ashows the effects of BCP on METH self-administration under a FR2 reinforcement schedule. Treatment with BCP dose-dependently inhibited METH self-administration. A two-way ANOVA with repeated measurements over BCP doses revealed a statistically significant infusion *vs* inactive lever response main effect ($F_{1, 11} = 126.92$, $p < 0.001$) and, most relevantly, a significant interaction effect between BCP dose and infusion vs inactive lever responding ($F_{4,42} = 6.41$, $p < 0.001$). Post-hoc tests revealed a statistically significant reduction in METH self-administration after 25 mg/kg ($q = 5.26$, $p < 0.001$), 50 mg/kg ($q = 5.99$, $p < 0.001$) or 100 mg/kg ($q = 7.17$, $p < 0.001$), but not after 10 mg/kg ($q = 2.54$, p = NS) BCP, when compared to the vehicle group.

To explore a potential role of CB2 receptors in BCP's action on METH self-administration, we administered the CB2 receptor antagonist AM630 (3 mg/kg, i.p.) 30 min prior to BCP treatment. As shown in **Figure 1B**, pretreatment with AM630 blocked the inhibitory effects of 25 mg/kg BCP on METH self-administration ($F_{2, 27} = 4.57$, $p < 0.05$). Post-hoc tests revealed a statistically significant reduction in METH infusions after 25 mg/kg ($q = 4.27$, $p < 0.05$), but not after 25 mg/kg BCP plus 3 mg/kg AM630 ($q = 1.81$, $p > 0.05$), as compared to the vehicle control group.

Similarly, pretreatment with AM 630 (10 mg/kg, i.p.) also reversed the inhibitory effects of 100 mg/kg BCP on METH self-administration (**Figure 1C**, $F_{2, 28} = 8.84$, $p < 0.001$). Post-hoc tests revealed a statistically significant reduction in METH infusions after 100 mg/kg ($q = 5.88$, $p < 0.001$), but not after 100 mg/kg BCP plus 10 mg/kg AM630 ($q = 1.74$, $p > 0.05$), when compared to the vehicle treatment group.

β-Caryophyllene Reduces Progressive-Ratio Break-point Level for Methamphetamine Self-Administration

Figure 1Dshows that treatment with BCP (10, 25, 50 mg/kg, i.p.) dose-dependently shifted the PR break-point for METH self-administration downward ($F_{3, 35} = 14.93$, $p < 0.001$). Post-hoc between group comparisons revealed a significant reduction in break-point for METH self-administration after 10 mg/kg ($q = 6.69$, $p < 0.001$), 25 mg/kg ($q = 7.74$, $p < 0.001$) or 50 mg/kg ($q = 8.32$, $p < 0.001$) BCP treatment, as compared to the vehicle treatment group.

As shown in **Figure 1E**, pretreatment with AM630 blocked the effects of BCP (25 mg/kg) on the PR break-point for METH self-administration ($F_{3, 27} = 4.76$, $p < 0.01$). Post-hoc tests revealed a statistically significant reduction in break-point after 25 mg/kg BCP ($q = 4.91$, $p < 0.01$), but not after 25 mg/kg BCP plus 3 mg/kg AM630 ($q = 1.68$, $p > 0.05$) or 3 mg/kg AM630 alone ($q = 0.14$, $p > 0.05$), when compared with the vehicle treatment group.

To determine whether the reduction in METH self-administration was due to BCP-induced sedation or locomotor impairment, we evaluated the effect of BCP on open field locomotion in rats. **Figure 1F** shows that BCP, at the same doses, failed to alter open-field locomotion. A two-way ANOVA with BCP treatment and time as repeated-measures factors revealed a statistically significant main effect of time ($F_{17,255} = 54.716$, $p < 0.05$) but no main effect of BCP treatment ($F_{3,45} = 0.929$; $p < 0.43$) or time × treatment interaction ($F_{51,765} = 1.00$; $p = 0.465$).

β-Caryophyllene Reduces Methamphetamine Self-Administration in Wild-Type Mice and at a High Dose in CB2-Knockout Mice

To further assess the potential involvement of CB2 receptors in the inhibitory effects of BCP on METH self-administration, we used transgenic mice lacking CB2 receptors. WT and CB2-KO mice were trained to self-administer METH under FR1 reinforcement. Systemic administration of BCP dose-dependently inhibited METH self-administration in WT mice and CB2-KO mice (**Figure 2**). A two-

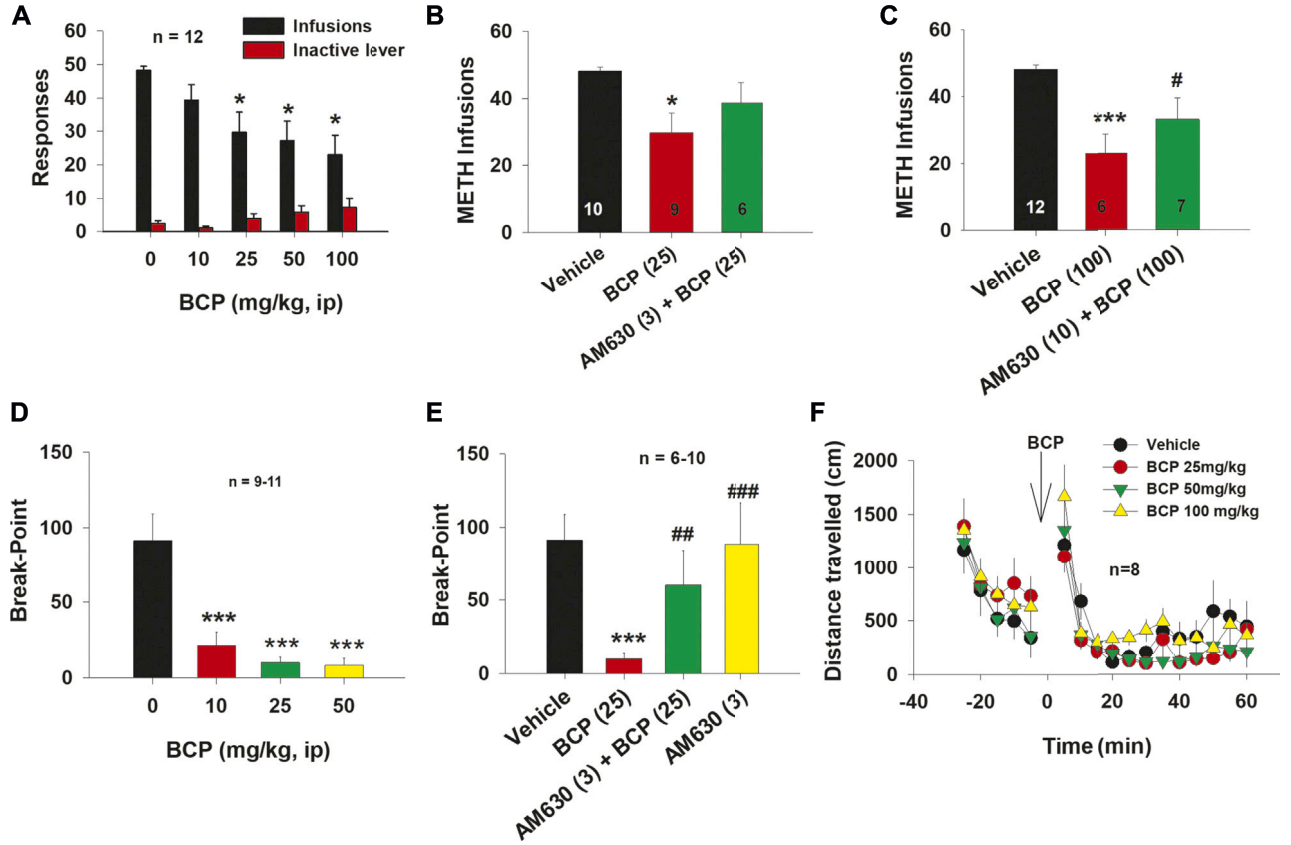


FIGURE 1 | The effect of BCP on METH self-administration under FR2 and progressive-ratio reinforcement schedules in rats. **(A)**: Administration of BCP dose-dependently decreased the number of METH self-infusions. **(B):** Pretreatment with AM630 (3 mg/kg) blocked BCP (25 mg/kg)-induced reduction in METH self-administration. **(C)**: Pretreatment with AM630 (10 mg/kg) also blocked the attenuating effects of BCP (100 mg/kg) on METH self-administration. **(D)**: BCP dose-dependently reduced the break-point level for METH self-administration under PR reinforcement conditions. **(E)**: Pretreatment with AM630 (3 mg/kg) blocked BCP's action under PR reinforcement conditions (25 mg/kg), while AM630 (3 mg/kg) alone failed to alter the break-point for METH self-administration. **(F)**: BCP, at 25, 50 and 100 mg/kg, did not alter open-field locomotor activity in rats. Data are presented as means ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$, when compared to the vehicle group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$, compared to "BCP (25)" or "BCP (100)" groups.

way ANOVA with repeated measurements for BCP doses revealed a significant strain (WT vs CB2-KO) main effect ($F_{1, 15} = 13.29$, $p < 0.01$) and BCP dose main effect ($F_{3, 45} = 15.78$, $p < 0.001$), but without strain × BCP interaction ($F_{3, 45} = 2.17$, $p > 0.05$). Post-hoc individual group comparisons revealed a significant reduction in infusions for METH self-administration in WT mice after 25 mg/kg ($q = 3.00$, $p < 0.05$), 50 mg/kg ($q = 6.00$, $p < 0.001$) or 100 mg/kg ($q = 7.54$, $p < 0.001$) BCP, as compared to the vehicle treatment group. Similar post-hoc tests revealed a significant reduction in infusions for METH self-administration in CB2-KO mice after administration of 100 mg/kg ($q = 5.60$, $p < 0.01$), but not after 25 mg/kg ($q = 0.18$, $p > 0.05$) or 50 mg/kg ($q = 0.85$, $p > 0.05$) of BCP, when compared to the vehicle treatment group.

β-Caryophyllene Reduces Methamphetamine-Enhanced Brain-Stimulation Reward

Next, we used the highly sensitive BSR paradigm to shed further light upon the effects of BCP on METH reward. **Figure 3A** shows the general experimental procedures, in which electrical stimulation was targeted at the medial forebrain bundle at the level for the lateral hypothalamus. **Figure 3B** shows representative rate-frequency functions for BSR, indicating the BSR stimulation threshold θ0, M50, Ymax, and the effects of METH on BSR in the presence or absence of BCP. METH (0.2 mg/kg, i.p.) significantly decreased the BSR threshold θ_0 value (i.e., shifted the curve to the left) without affecting asymptotic rates of responding (i.e., no change in Ymax level), indicating that lower stimulation intensity (Hz) was required to produce BSR in the presence of METH, suggesting that METH and rewarding brain stimulation produce an additive or synergistic effect (i.e., that METH enhances BSR). **Figure 3C** shows that pretreatment with BCP dose-dependently decreased METH-enhanced BSR, as indicated by an increase in BSR stimulation threshold θ_0 values ($F_{2,22} = 5.018$; $p < 0.05$). Treatment with BCP did not significantly alter the M50 value (**Figure 3D**, $F_{2,22} = 3.024$; $p > 0.05$) or the Ymax level (**Figure 3E**, $F_{2,22} = 3.299$; $p > 0.05$). The latter finding concerning Ymax suggests a lack of motoric impairment after BCP and METH administration.

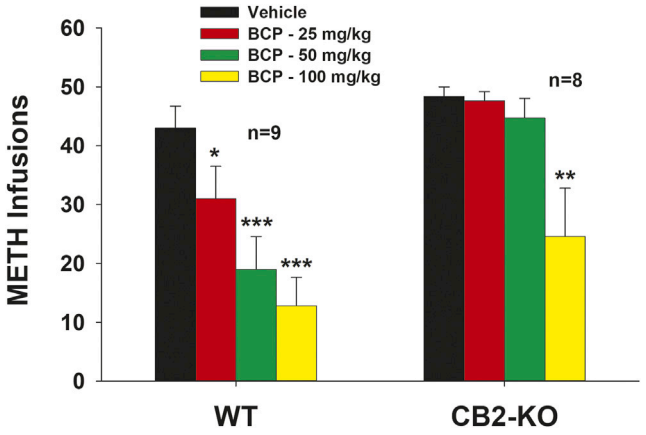


FIGURE 2 | The effects of BCP on METH self-administration under a fixed-ratio 1 (FR1) reinforcement schedule in WT and CB2-KO mice. BCP dose-dependently decreased METH self-administration in WT mice, while only at the very high dose of 100 mg/kg, BCP inhibited METH self-administration in CB2-KO mice. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, when compared to the vehicle group.

β-Caryophyllene Reduces Methamphetamine-Primed Reinstatement of Drug Seeking

Figure 4 illustrates the total numbers of active and inactive lever presses observed during the last session of METH self-administration, the last session of extinction, and the reinstatement test session in the three different dose groups for BCP (vehicle, 25, 50 mg/kg). A single, non-contingent METH priming injection (1 mg/kg) produced robust reinstatement of extinguished operant responding (i.e., active lever presses) in rats with a history of METH self-administration. Treatment with BCP produced a significant reduction in METH-induced reinstatement of drug-seeking behavior (**Figure 4A**, active lever responding: $F_{2,30} = 3.96$, $p < 0.05$). Post-hoc tests revealed a significant reduction in METH seeking after 25 mg/kg ($q = 3.95$, $p < 0.05$) or 50 mg/kg ($q = 3.79$, $p < 0.05$) BCP, when compared to the vehicle control group. There were no significant differences in inactive lever responding across BCP dose groups (**Figure 4B**).

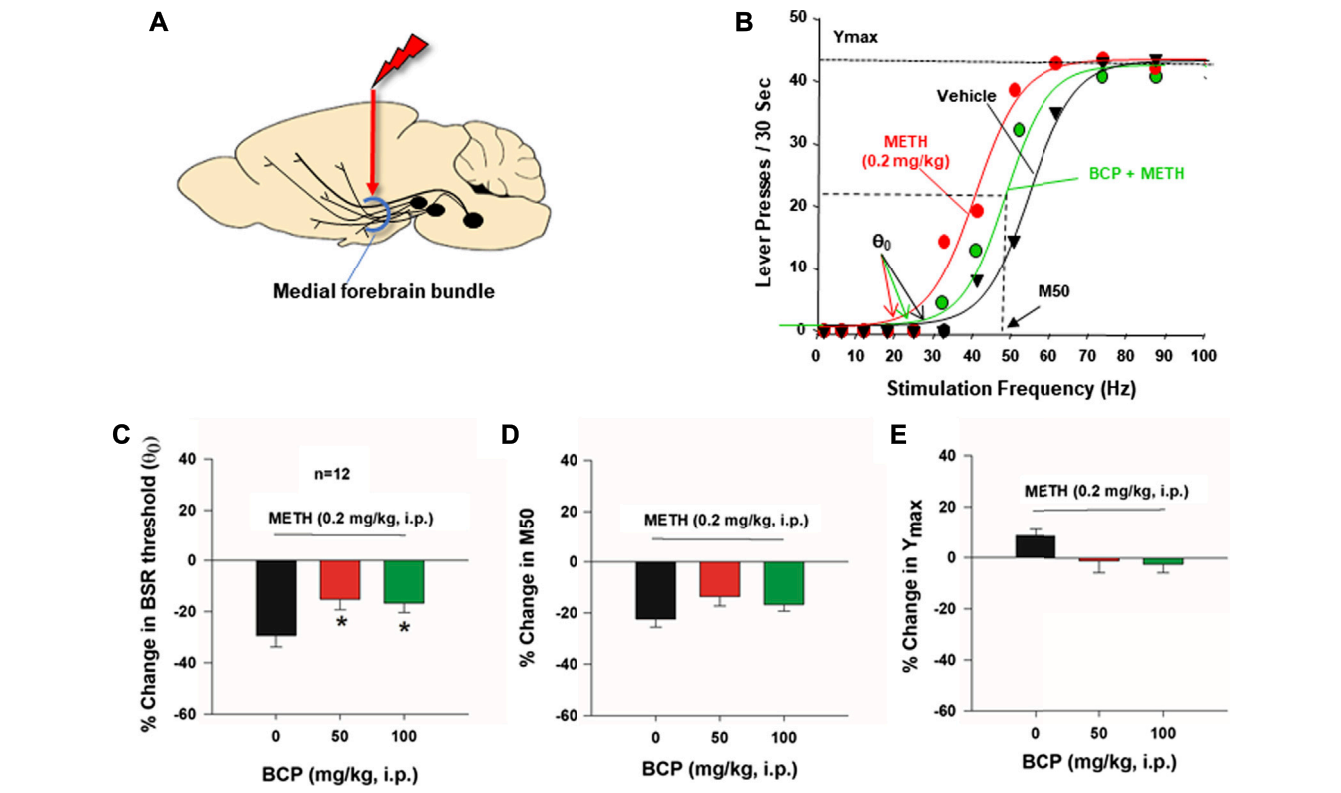


FIGURE 3 | The effects of BCP on METH-enhanced electrical brain-stimulation reward (BSR) in rats ($n = 12$). **(A)**: A diagram showing that electrical stimulation of the medial forebrain bundle at the hypothalamus produces BSR. **(B)**: Representative stimulation–response curves, indicating that METH treatment shifted the stimulation-response curve to the left and decreased the BSR stimulation threshold (θ_0 value) but not M50; **(C)**: Averaged % changes in BSR stimulation threshold (θ_0 value), indicating that BCP pretreatment significantly attenuated METH-induced reduction in the θ_0 value. **(D)**: BCP did not produce a significant reduction in M50. **(E)**: METH and BCP did not produce a significant change in Ymax. *$p < 0.05$, compared to the vehicle group.

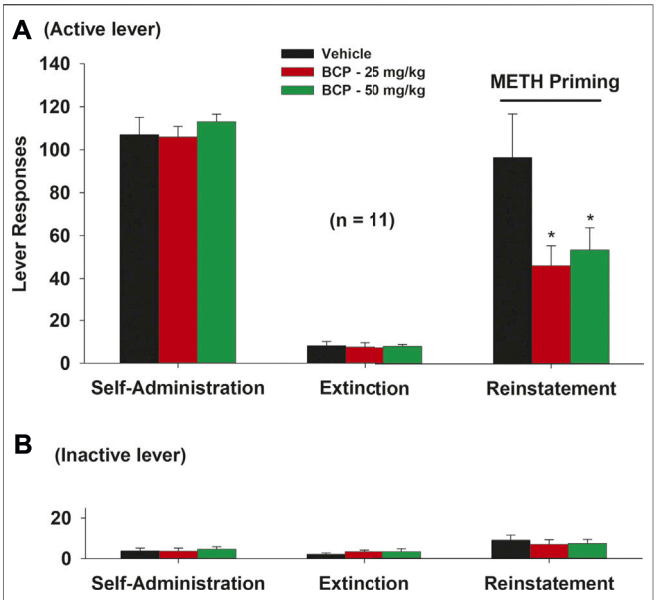


FIGURE 4 | The effects of BCP pretreatment on METH-induced reinstatement of drug seeking in rats. **(A):** Active lever presses during the last session of METH self-administration, last extinction session, and reinstatement test, illustrating that BCP (25, 50 mg/kg, i.p., 30 min prior to test) significantly reduced METH priming-induced reinstatement. **(B)**: BCP, at the same dose, had no effect on inactive lever presses during reinstatement testing. Data are presented as means ± SEM. *$p < 0.05$, when compared to the vehicle group.

β-Caryophyllene Attenuates Cue-Induced Methamphetamine Seeking

We also observed the effects of BCP treatment on cue-induced drug seeking in rats after 3 weeks of withdrawal from METH self-administration (e.g., in a forced abstinence craving model). We found that BCP dose-dependently attenuated METH-associated cue-induced drug seeking (**Figure 5**). A one-way ANOVA of the cue-triggered response data revealed a significant BCP treatment main effect (**Figure 5A**: $F_{3,36} = 11.78$, $p < 0.001$) on active lever presses. Post-hoc tests revealed that 25 mg/kg ($q = 3.17$, $p < 0.05$), 50 mg/kg ($q = 4.53$, $p < 0.01$) or 100 mg/kg ($q = 8.29$, $p < 0.001$) of BCP significantly reduced active lever responding, when compared to the vehicle control group. There were no significant differences in inactive lever responding across different BCP dose groups (**Figure 5B**).

β-Caryophyllene Attenuates Methamphetamine-Enhanced Dopamine in the Nucleus Accumbens

Finally, we examined whether a DA-dependent mechanism might underlie BCP actions against METH by using *in vivo* brain microdialysis. **Figure 6A** shows that BCP alone, at the doses of 25 or 50 mg/kg, produced no statistically significant effect on extracellular DA in the NAc. A two-way ANOVA with time as the repeated-measures factor revealed a significant main effect of time ($F_{11, 165} = 2.09$, $p < 0.05$), but did not reveal a BCP treatment main effect ($F_{2, 15} = 1.02$, $p > 0.05$) or a BCP × time interaction ($F_{22, 165} = 1.14$, $p > 0.05$), suggesting that BCP alone did not significantly alter NAc DA release. **Figure 6B** shows that METH (1 mg/kg) in the vehicle pretreatment group caused a rapid and significant increase in extracellular DA level in drug-naive rats, which lasted 2–3 h with a peak effect at 1 h after the injection. Treatment with 50 mg/kg, but not 25 mg/kg, of BCP significantly attenuated the METH-induced increase in extracellular DA. Two-way ANOVAs with time as the repeated-measures factor and BCP dose as the between-subjects factor revealed a significant main effect of time ($F_{12,300} = 46.176$, $p < 0.0001$) and BCP treatment × time interaction ($F_{24,300} = 2.189$, $p < 0.0001$), but no main effect of BCP dose ($F_{2,25} = 1.599$; $p = 0.22$). Post-hoc (Tukey) tests for multiple group comparisons indicated that METH-induced enhancement of extracellular DA was significantly reduced by 50 mg/kg, but not by 25 mg/kg, of BCP, when compared to Veh + METH groups (**Figure 6B**).

DISCUSSION

In the present study, we found that systemic administration of the natural CB2R agonist BCP (Gertsch et al., 2008) dose-dependently inhibited intravenous METH self-administration, METH-enhanced brain-stimulation reward, and METH- or cue-induced drug-seeking in rats. Importantly, the inhibitory effects of BCP on METH self-administration were attenuated by the cannabinoid CB2 receptor antagonist AM630, and genetic deletion of CB2 receptors also blocked low dose (25, 50 mg/kg)

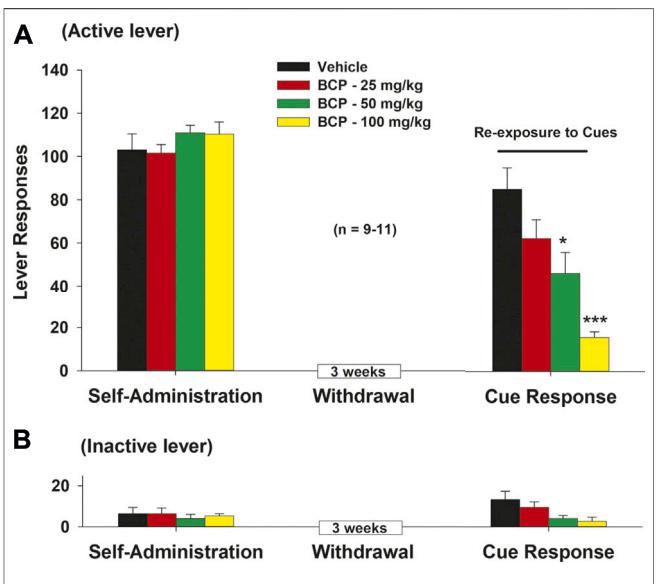


FIGURE 5 | The effects of BCP on METH-associated cue-induced drug seeking in rats after forced abstinence. **(A)**: Systemic administration of BCP (25, 50, 100 mg/kg, i.p., 30 min prior to test) dose-dependently inhibited cue-triggered drug-seeking in rats after 3 weeks of withdrawal from METH self-administration. **(B)**: BCP, at the same doses, failed to alter inactive lever responses during cue exposure test. Data are presented as means ± SEM. *$p < 0.05$, ***$p < 0.001$, when compared to the vehicle group.

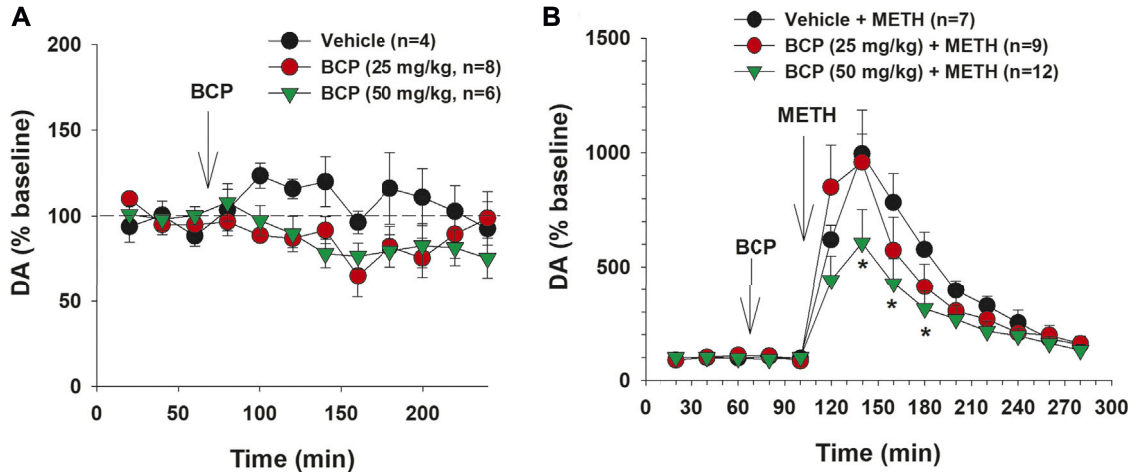


FIGURE 6 | The effects of BCP and METH on extracellular DA in the nucleus accumbens (NAc). **(A)**: BCP, at 25 and 50 mg/kg, failed to produce a significant reduction in extracellular NAc DA. **(B)**: Pretreatment with BCP dose-dependently attenuated METH-induced enhancement of extracellular NAc DA. Data are presented as means ± SEM. *$p < 0.05$, when compared to the vehicle pretreatment group.

BCP-induced reduction in METH self-administration, suggesting the possible involvement of CB2 receptor mechanisms. Notably, BCP, at a high dose (100 mg/kg), also inhibited METH self-administration in CB2-KO mice, suggesting that non-CB2 receptor mechanisms are involved in high dose BCP-mediated effects. This is consistent with our previous reports that systemic administration of BCP, at high doses (50, 100 mg/kg), also inhibits cocaine or nicotine self-administration in CB2-KO mice (He et al., 2020; Galaj et al., 2021), suggesting that BCP's selectivity as a CB2 receptor agonist depends on the BCP dose, and at high doses, it also binds to other (non-CB2) receptors. Furthermore, BCP alone did not produce a significant decrease in extracellular NAc DA, while pretreatment with BCP dose-dependently attenuated METH-induced increase in extracellular DA, suggesting that a DA-dependent mechanism at least in part underlies BCP's actions against METH.

We and others have previously reported the presence of functional CB2 receptors in the brain, especially in reward-related areas such as the ventral tegmental area (VTA) and the NAc (Gong et al., 2006; Zhang et al., 2014, 2019, 2021a, 2021b; Foster et al., 2016; Jordan and Xi, 2019), suggesting the potential involvement of CB2 receptors in drug abuse. This hypothesis is supported by a number of studies indicating that CB2R agonists or inverse agonists (JWH133, O-1966, Xie2-64, BCP) significantly inhibit cocaine self-administration, cocaine-induced conditioned place preference (CPP), cocaine-induced hyperlocomotion and locomotor sensitization (Xi et al., 2011; Aracil-Fernández et al., 2012; Ignatowska-Jankowska et al., 2013; Zhang et al., 2015; Delis et al., 2017; Jordan et al., 2020; Galaj et al., 2021). Congruently, overexpression of CB2 receptor in the brain also produces anti-cocaine effects (Aracil-Fernández et al., 2012). In addition, BCP, at low doses (10, 25 mg/kg) significantly decreased the break-point for METH self-administration under PR reinforcement, suggesting that BCP has the ability to attenuate animals' motivation for the drug. The reduction in METH self-administration is unlikely due to non-specific sedative effects or locomotor impairment, because BCP, at the same doses, did not alter basal or cocaine-enhanced locomotor activity (Galaj et al., 2019). The present anti-METH findings are congruent with previous reports that BCP attenuates intravenous cocaine or nicotine self-administration and oral alcohol consumption in rats and mice (Al Mansouri et al., 2014; He et al., 2020; Galaj et al., 2021). They are also congruent with recent reports that a CB2 receptor mechanism mediates the analgesic, anxiolytic and anti-depressant effects of BCP (Bahi et al., 2014; Klauke et al., 2014; Youssef et al., 2019).

We note that the effective doses of BCP that inhibit self-administration of nicotine, cocaine and METH are different. Lower doses (25, 50 mg/kg) of BCP are able to inhibit nicotine (He et al., 2020) or METH self-administration, while a higher dose (100 mg/kg) of BCP is required to inhibit cocaine self-administration, which is not blocked by deletion of the CB2 receptor in CB2-KO mice (Galaj et al., 2020a). This may be related to the reinforcing strength or the doses of drugs of abuse used in those studies. The facts that nicotine is a weak reinforcer compared to cocaine and that the METH dose (0.05 mg/kg/infusion) used in our self-administration experiments is 10-fold lower than the cocaine dose (0.5 mg/kg/infusion) may well explain why BCP, at lower doses, is able to inhibit nicotine or METH, but not cocaine, self-administration, and why genetic deletion of the CB2 receptor in CB2-KO mice is able to prevent low dose, but not high dose, BCP-induced attenuation of drug self-administration, given that BCP at high doses binds to non-CB2 off-targets (Galaj et al., 2020b, but see; Finlay et al., 2020; Santiago et al., 2019).

The precise non-CB2 receptor mechanisms that may be involved remain unclear. We previously reported that genetic deletion and/or pharmacological blockade of the CB1, GRP55, mu opioid, and toll-like receptor 4 (TLR4) failed to alter BCP's

action on cocaine self-administration, suggesting that these receptors are not involved in BCP's action against cocaine (Galaj et al., 2021). Unexpectedly, we found that peroxisome proliferator-activated receptor-α (PPARα) or PPARγ antagonists dose-dependently attenuated BCP's action against cocaine self-administration (Galaj et al., 2020a), suggesting that these two receptors may be also involved in BCP's action against METH. Clearly, more studies are required to test this hypothesis.

It is not fully understood how BCP produces inhibitory effects on METH-taking and METH-seeking behaviors. It is widely believed that the brain CB2 receptor is mainly or exclusively expressed in microglia, not in neurons, and can be upregulated in activated microglia during neuroinflammation (Atwood and Mackie, 2010; López et al., 2018). However, this view is not supported by our findings that neither CB2-immunostaining nor CB2 mRNA was detected in microglia in either normal healthy subjects (Zhang et al., 2014, 2017, 2019) or in mice after acute administration of lipopolysaccharide, an endotoxin that causes severe neuroinflammation and microglia activation (Zhang et al., 2014) or chronic administration of cocaine (Zhang et al., 2017; 2021a). In contrast, we demonstrated clear CB2 receptor expression in multiple phenotypes of neurons, including VTA DA neurons (Zhang et al., 2014, 2017, 2019; Humburg et al., 2021), red nucleus glutamate neurons (Zhang et al., 2021b), and striatal GABA neurons (Zhang et al., 2021a; see a comprehensive review by; Jordan and Xi, 2019). Furthermore, chronic cocaine administration significantly up-regulates CB2 receptor expression in VTA DA neurons and NAc D1 receptor-expressing medium-spiny neurons, not in microglia (Zhang et al., 2014; 2021a). Consistent with these findings, genetic deletion of CB2 receptors from lymphocytes, mainly from monocytes (the precursors of microglial cells), failed to alter JWH133 self-administration (self-medication) to relieve neuropathic pain (Cabañero et al., 2020). In contrast, genetic deletion of CB2 receptor from neurons (syn-Cre X CB2-floxed) significantly altered JWH133 self-administration (Cabañero et al., 2020), suggesting that neuronal CB2 receptor mechanisms underlie the analgesic effects of CB2 receptor activation. However, other work using targeted expression of fluorescent proteins in CB2-reporter mice failed to detect CB2 receptor expression in neurons (Schmöle et al., 2015; López et al., 2018), suggesting that more work is required to further address the role of neuronal *versus* microglial CB2 in BCP action.

It is well documented that drug abuse and addiction are closely associated with an increase in extracellular DA in the NAc (Di Chiara and Imperato, 1988; Ranaldi et al., 1999; Le Foll and Goldberg, 2005; Galaj et al., 2019). With respect to the present topic, METH's highly addictive properties have been attributed to its effect on DA release. METH is a substrate for the dopamine transporter (DAT) and the vesicular monoamine transporter 2 (VMAT2). METH is first taken into the cytoplasm via the DAT and then enters vesicles via the VMAT2. Each molecule of METH that undergoes vesicular entry causes two protons to be extruded, which diminishes vesicular H^+ concentration. The pH gradient is the main driving force for vesicular loading and retention of DA. In the absence of this pH gradient, DA is rapidly accumulated in the cytoplasm, which reverses the functional direction of the DAT and releases DA into the extracellular space (Elkashef et al., 2008; Freyberg et al., 2016). As noted above, a series of studies have shown that CB2 receptor genes and receptors are expressed in midbrain DA neurons and negatively modulate DA neuronal activity mainly by activation of M-type K^+ channels (Xi et al., 2011; Zhang et al., 2014, 2017; Foster et al., 2016; Ma et al., 2019). Thus, a working hypothesis is that BCP may initially bind to CB2 receptors on midbrain DA neurons and decrease DA neuronal activity or excitability, which may then decrease NAc DA response to METH and subsequent DA-dependent behavior (**Figure 7**).

To test this hypothesis, we used *in vivo* brain microdialysis to measure extracellular DA in the NAc. We found that systemic administration of METH (1 mg/kg) caused a robust (10-fold) increase in extracellular DA levels in the NAc immediately after administration, which lasted for about 2 h. Pretreatment with BCP, at the same doses that inhibited METH self-administration and reinstatement responding, produced a dose-dependent reduction in METH-enhanced DA release, suggesting that DA-dependent mechanisms may in part underlie BCP's action against METH (**Figure 7**).

Notably, BCP alone, at 25 and 50 mg/kg, did not produce a significant alteration in extracellular DA in the NAc, suggesting that it is not rewarding or aversive by itself. This is supported by previous findings that BCP failed to maintain self-administration after substitution for cocaine in rats previously self-administering cocaine (Galaj et al., 2020a) nor produced CPP or conditioned place aversion in mice (Al Mansouri et al., 2014). However, it is slightly different from our previous report that BCP, at higher doses (50, 100 mg/kg), dose-dependently inhibit brain-stimulation reward maintained by either electrical stimulation of the medial forebrain bundle at the lateral hypothalamic level in rats or by optical stimulation of midbrain DA neurons in DAT-Cre mice (He et al., 2020), suggesting that high doses of BCP may be required to produce a significant reduction in NAc DA release. We have previously reported that JWH133, a highly selective CB2 receptor agonist, dose-dependently inhibits cocaine self-administration and decreases NAc DA release, but itself does not produce conditioned place aversion (Xi et al., 2011), suggesting that a reduction in NAc DA release may not necessarily lead to dysphoric or aversive effects. Similarly, aversive stimuli may also increase DA release and individual groups of DA cells make a unique contribution to the processing of reward and aversion (Weele et al., 2019; Verharen et al., 2020), suggesting that multiple neural mechanisms may underlie drug aversion and that BCP's potentially therapeutic anti-METH effects are unlikely to be mediated by its aversive effects.

In conclusion, BCP is a major component in the essential oils of cannabis and other spice and food plants (Sharma et al., 2016; Galaj and Xi, 2019). In the present study, we demonstrate that systemic administration of BCP is highly effective in attenuating METH-taking and METH-seeking in rodents

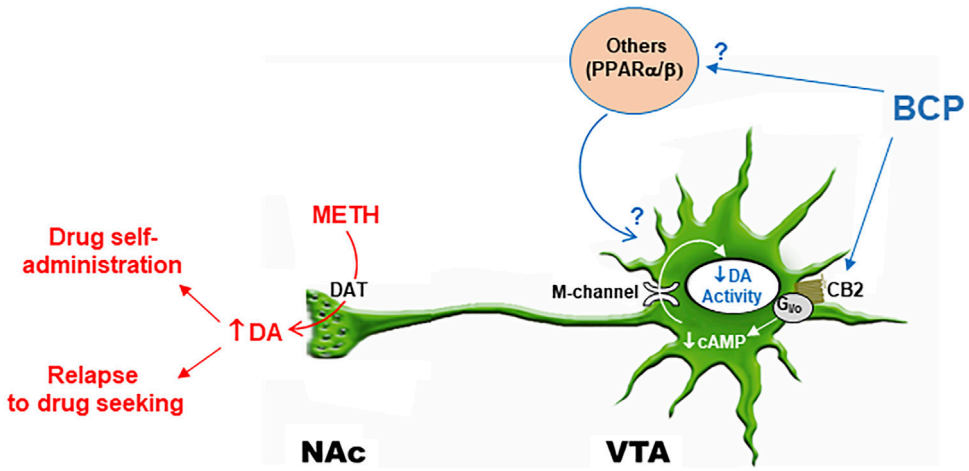


FIGURE 7 | Schematic diagram illustrating possible interaction of BCP and METH in the mesolimbic DA system. METH promotes DA release from presynaptic DA terminals in the NAc and other projection regions via the membrane DA transporter (DAT) and intracellular type 2 vesicular monoamine transports (VMAT2, not shown). BCP binds to CB2 receptors on DA neurons and inhibits DA neuron activity by activation of M-type K^+ channel via a Gi/o-cAMP-PKA signal pathway (Zhang et al., 2014; Ma et al., 2019), which subsequently attenuates NAc DA response to METH and DA-dependent addiction-related behavior. In addition, BCP may also bind to other non-CB2 receptors such as PPARα/β, which indirectly modulates DA neuron activity.

via both CB2- and non-CB2-dependent mechanisms. Given that BCP is an FDA-approved food additive with good oral bioavailability, favorable pharmacokinetics, and low toxicity, BCP deserves further research as a promising repurposed drug in translational studies for the treatment of METH use disorder.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by NIDA ACUC Committee.

AUTHOR CONTRIBUTIONS

Y-LW, ELG, Z-XX designed the experiments. XHH, G-HB, YH conducted the experiments. X-HH, EG, BH and Z-XX performed data analyses. X-HH, EG and Z-XX wrote the manuscript. BH and ELG revised the manuscript. All authors have approved the final version of this article.

REFERENCES

Al Mansouri, S., Ojha, S., Al Maamari, E., Al Ameri, M., Nurulain, S. M., and Bahi, A. (2014). The Cannabinoid Receptor 2 Agonist, β-caryophyllene, Reduced Voluntary Alcohol Intake and Attenuated Ethanol-Induced Place Preference and Sensitivity in Mice. *Pharmacol. Biochem. Behav.* 124, 260–268. doi:10.1016/j.pbb.2014.06.025

Altshuler, R. D., Lin, H., and Li, X. (2020). Neural Mechanisms Underlying Incubation of Methamphetamine Craving: A Mini-Review. *Pharmacol. Biochem. Behav.* 199, 173058. doi:10.1016/j.pbb.2020.173058

Aracil-Fernández, A., Trigo, J. M., García-Gutiérrez, M. S., Ortega-Álvaro, A., Ternianov, A., Navarro, D., et al. (2012). Decreased Cocaine Motor Sensitization and Self-Administration in Mice Overexpressing Cannabinoid CB_2 Receptors. *Neuropsychopharmacology* 37, 1749–1763. doi:10.1038/npp.2012.22

Atwood, B. K., and Mackie, K. (2010). CB2: a Cannabinoid Receptor with an Identity Crisis. *Br. J. Pharmacol.* 160, 467–479. doi:10.1111/j.1476-5381.2010.00729.x

Bahi, A., Al Mansouri, S., Al Memari, E., Al Ameri, M., Nurulain, S. M., and Ojha, S. (2014). β-Caryophyllene, a CB2 Receptor Agonist Produces Multiple Behavioral Changes Relevant to Anxiety and Depression in Mice. *Physiol. Behav.* 135, 119–124. doi:10.1016/j.physbeh.2014.06.003

Brackins, T., Brahm, N. C., and Kissack, J. C. (2011). Treatments for Methamphetamine Abuse: a Literature Review for the Clinician. *J. Pharm. Pract.* 24, 541–550. doi:10.1177/0897190011426557

Brensilver, M., Heinzerling, K. G., and Shoptaw, S. (2013). Pharmacotherapy of Amphetamine-type Stimulant Dependence: an Update. *Drug Alcohol. Rev.* 32, 449–460. doi:10.1111/dar.12048

Buckley, N. E., McCoy, K. L., Mezey, E., Bonner, T., Zimmer, A., Felder, C. C., et al. (2000). Immunomodulation by Cannabinoids Is Absent in Mice Deficient for the Cannabinoid CB(2) Receptor. *Eur. J. Pharmacol.* 396, 141–149. doi:10.1016/s0014-2999(00)00211-9

Cabañero, D., Ramírez-López, A., Drews, E., Schmöle, A., Otte, D. M., Wawrzczak-Bargiela, A., et al. (2020). Protective Role of Neuronal and Lymphoid Cannabinoid CB2 Receptors in Neuropathic Pain. *Elife* 9, e55582. doi:10.7554/eLife.55582

CFR - Code of Federal Regulations Title 21 (2020). Volume 3. 21CFR172.515. Available at: https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=172.515&SearchTerm=caryophyllene (Accessed April 1, 2020)

Chang, H. J., Kim, J. M., Lee, J. C., Kim, W. K., and Chun, H. S. (2013). Protective Effect of β-caryophyllene, a Natural Bicyclic Sesquiterpene, against Cerebral Ischemic Injury. *J. Med. Food* 16, 471–480. doi:10.1089/jmf.2012.2283

Chicca, A., Caprioglio, D., Minassi, A., Petrucci, V., Appendino, G., Taglialatela-Scafati, O., et al. (2014). Functionalization of β-caryophyllene Generates Novel

Polypharmacology in the Endocannabinoid System. *ACS Chem. Biol.* 9, 1499–1507. doi:10.1021/cb500177c

Cho, J. Y., Chang, H. J., Lee, S. K., Kim, H. J., Hwang, J. K., and Chun, H. S. (2007). Amelioration of Dextran Sulfate Sodium-Induced Colitis in Mice by Oral Administration of Beta-Caryophyllene, a Sesquiterpene. *Life Sci.* 80, 932–939. doi:10.1016/j.lfs.2006.11.038

Corey, E. J., Mitra, R. B., and Uda, H. (1964). Total Synthesis of D,l-Caryophyllene and D,l-Isocaryophyllene. *J. Am. Chem. Soc.* 86, 485–492. doi:10.1021/ja01057a040

Coulombe, D., and Miliaressis, E. (1987). Fitting Intracranial Self-Stimulation Data with Growth Models. *Behav. Neurosci.* 101, 209–214. doi:10.1037//0735-7044.101.2.209

Courtney, K. E., and Ray, L. A. (2014). Methamphetamine: an Update on Epidemiology, Pharmacology, Clinical Phenomenology, and Treatment Literature. *Drug Alcohol Depend* 143, 11–21. doi:10.1016/j.drugalcdep.2014.08.003

Covey, D. P., Wenzel, J. M., and Cheer, J. F. (2015). Cannabinoid Modulation of Drug Reward and the Implications of Marijuana Legalization. *Brain Res.* 1628, 233–243. doi:10.1016/j.brainres.2014.11.034

Delis, F., Polissidis, A., Poulia, N., Justinova, Z., Nomikos, G. G., Goldberg, S. R., et al. (2017). Attenuation of Cocaine-Induced Conditioned Place Preference and Motor Activity via Cannabinoid CB2 Receptor Agonism and CB1 Receptor Antagonism in Rats. *Int. J. Neuropsychopharmacol.* 20, 269–278. doi:10.1093/ijnp/pyw102

Di Chiara, G., and Imperato, A. (1988). Drugs Abused by Humans Preferentially Increase Synaptic Dopamine Concentrations in the Mesolimbic System of Freely Moving Rats. *Proc. Natl. Acad. Sci. U S A.* 85, 5274–5278. doi:10.1073/pnas.85.14.5274

Di Marzo, V. (2009). The Endocannabinoid System: its General Strategy of Action, Tools for its Pharmacological Manipulation and Potential Therapeutic Exploitation. *Pharmacol. Res.* 60, 77–84. doi:10.1016/j.phrs.2009.02.010

Elkashef, A., Vocci, F., Hanson, G., White, J., Wickes, W., and Tiihonen, J. (2008). Pharmacotherapy of Methamphetamine Addiction: an Update. *Subst. Abus* 29, 31–49. doi:10.1080/08897070802218554

Fidyt, K., Fiedorowicz, A., Strządała, L., and Szumny, A. (2016). β-Caryophyllene and β-caryophyllene Oxide-Natural Compounds of Anticancer and Analgesic Properties. *Cancer Med.* 5, 3007–3017. doi:10.1002/cam4.816

Finlay, D. B., Sircombe, K. J., Nimick, M., Jones, C., and Glass, M. (2020). Terpenoids from Cannabis Do Not Mediate an Entourage Effect by Acting at Cannabinoid Receptors. *Front. Pharmacol.* 11, 359. doi:10.3389/fphar.2020.00359

Foster, D. J., Wilson, J. M., Remke, D. H., Mahmood, M. S., Uddin, M. J., Wess, J., et al. (2016). Antipsychotic-like Effects of M4 Positive Allosteric Modulators Are Mediated by CB2 Receptor-dependent Inhibition of Dopamine Release. *Neuron* 91, 1244–1252. doi:10.1016/j.neuron.2016.08.017

Freyberg, Z., Sonders, M. S., Aguilar, J. I., Hiranita, T., Karam, C. S., Flores, J., et al. (2016). Mechanisms of Amphetamine Action Illuminated through Optical Monitoring of Dopamine Synaptic Vesicles in Drosophila Brain. *Nat. Commun.* 7, 10652. doi:10.1038/ncomms10652

Galaj, E., Bi, G. H., Moore, A., Chen, K., He, Y., Gardner, E., et al. (2021). Beta-caryophyllene Inhibits Cocaine Addiction-Related Behavior by Activation of PPARα and PPARγ: Repurposing a FDA-Approved Food Additive for Cocaine Use Disorder. *Neuropsychopharmacology* 46, 860–870. doi:10.1038/s41386-020-00885-4

Galaj, E., Bi, G. H., Yang, H. J., and Xi, Z. X. (2020a). Cannabidiol Attenuates the Rewarding Effects of Cocaine in Rats by CB2, 5-HT1A and TRPV1 Receptor Mechanisms. *Neuropharmacology* 167, 107740. doi:10.1016/j.neuropharm.2019.107740

Galaj, E., Han, X., Shen, H., Jordan, C. J., He, Y., Humburg, B., et al. (2020b). Dissecting the Role of GABA Neurons in the VTA versus SNr in Opioid Reward. *J. Neurosci.* 40, 8853–8869. doi:10.1523/JNEUROSCI.0988-20.2020

Galaj, E., and Xi, Z. X. (2020). Possible Receptor Mechanisms Underlying Cannabidiol Effects on Addictive-like Behaviors in Experimental Animals. *Int. J. Mol. Sci.* 22. doi:10.3390/ijms22010134

Galaj, E., and Xi, Z. X. (2019). Potential of Cannabinoid Receptor Ligands as Treatment for Substance Use Disorders. *CNS Drugs* 33, 1001–1030. doi:10.1007/s40263-019-00664-w

Galaj, E., Bi, G.-H., Yang, H.-J., and Xi, Z.-X. (2019). Cannabidiol Attenuates the Rewarding Effects of Cocaine by CB2, 5-TH1A and TRPV1 Receptor Mechanisms. *Neuropharmacology*

Gertsch, J., Leonti, M., Raduner, S., Racz, I., Chen, J. Z., Xie, X. Q., et al. (2008). Beta-caryophyllene Is a Dietary Cannabinoid. *Proc. Natl. Acad. Sci. U S A.* 105, 9099–9104. doi:10.1073/pnas.0803601105

Gobira, P. H., Oliveira, A. C., Gomes, J. S., da Silveira, V. T., Asth, L., Bastos, J. R., et al. (2019). Opposing Roles of CB1 and CB2 Cannabinoid Receptors in the Stimulant and Rewarding Effects of Cocaine. *Br. J. Pharmacol.* 176, 1541–1551. doi:10.1111/bph.14473

Gong, J. P., Onaivi, E. S., Ishiguro, H., Liu, Q. R., Tagliaferro, P. A., Brusco, A., et al. (2006). Cannabinoid CB2 Receptors: Immunohistochemical Localization in Rat Brain. *Brain Res.* 1071, 10–23. doi:10.1016/j.brainres.2005.11.035

Guo, K., Mou, X., Huang, J., Xiong, N., and Li, H. (2014). Trans-caryophyllene Suppresses Hypoxia-Induced Neuroinflammatory Responses by Inhibiting NF-Kb Activation in Microglia. *J. Mol. Neurosci.* 54, 41–48. doi:10.1007/s12031-014-0243-5

He, Y., Galaj, E., Bi, G. H., Wang, X. F., Gardner, E., and Xi, Z. X. (2020). β-Caryophyllene, a Dietary Terpenoid, Inhibits Nicotine Taking and Nicotine Seeking in Rodents. *Br. J. Pharmacol.* 177, 2058–2072. doi:10.1111/bph.14969

Humburg, B. A., Jordan, C. J., Zhang, H. Y., Shen, H., Han, X., Bi, G. H., et al. (2021). Optogenetic Brain-stimulation Reward: A New Procedure to Re-evaluate the Rewarding versus Aversive Effects of Cannabinoids in Dopamine transporter-Cre Mice. *Addict. Biol.* 26, e13005. doi:10.1111/adb.13005

Ignatowska-Jankowska, B. M., Muldoon, P. P., Lichtman, A. H., and Damaj, M. I. (2013). The Cannabinoid CB2 Receptor Is Necessary for Nicotine-Conditioned Place Preference, but Not Other Behavioral Effects of Nicotine in Mice. *Psychopharmacology (Berl)* 229, 591–601. doi:10.1007/s00213-013-3117-6

Iversen, L. (2003). Cannabis and the Brain. *Brain* 126, 1252–1270. doi:10.1093/brain/awg143

Johnson, B. A., Roache, J. D., Ait-Daoud, N., Wells, L. T., Wallace, C. L., Dawes, M. A., et al. (2007). Effects of Topiramate on Methamphetamine-Induced Changes in Attentional and Perceptual-Motor Skills of Cognition in Recently Abstinent Methamphetamine-dependent Individuals. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 31, 123–130. doi:10.1016/j.pnpbp.2006.08.002

Jordan, C. J., Feng, Z. W., Galaj, E., Bi, G. H., Xue, Y., Liang, Y., et al. (2020). Xie2-64, a Novel CB2 Receptor Inverse Agonist, Reduces Cocaine Abuse-Related Behaviors in Rodents. *Neuropharmacology* 176, 108241. doi:10.1016/j.neuropharm.2020.108241

Jordan, C. J., and Xi, Z. X. (2019). Progress in Brain Cannabinoid CB2 Receptor Research: From Genes to Behavior. *Neurosci. Biobehav Rev.* 98, 208–220. doi:10.1016/j.neubiorev.2018.12.026

Katsuyama, S., Mizoguchi, H., Kuwahata, H., Komatsu, T., Nagaoka, K., Nakamura, H., et al. (2013). Involvement of Peripheral Cannabinoid and Opioid Receptors in β-caryophyllene-induced Antinociception. *Eur. J. Pain* 17, 664–675. doi:10.1002/j.1532-2149.2012.00242.x

Klauke, A. L., Racz, I., Pradier, B., Markert, A., Zimmer, A. M., Gertsch, J., et al. (2014). The Cannabinoid CB_2 Receptor-Selective Phytocannabinoid Beta-Caryophyllene Exerts Analgesic Effects in Mouse Models of Inflammatory and Neuropathic Pain. *Eur. Neuropsychopharmacol.* 24, 608–620. doi:10.1016/j.euroneuro.2013.10.008

Le Foll, B., and Goldberg, S. R. (2005). Cannabinoid CB1 Receptor Antagonists as Promising New Medications for Drug Dependence. *J. Pharmacol. Exp. Ther.* 312, 875–883. doi:10.1124/jpet.104.077974

Le Foll, B., Gorelick, D. A., and Goldberg, S. R. (2009). The Future of Endocannabinoid-Oriented Clinical Research after CB1 Antagonists. *Psychopharmacology (Berl)* 205, 171–174. doi:10.1007/s00213-009-1506-7

Ling, W., Rawson, R., Shoptaw, S., and Ling, W. (2006). Management of Methamphetamine Abuse and Dependence. *Curr. Psychiatry Rep.* 8, 345–354. doi:10.1007/s11920-006-0035-x

López, A., Aparicio, N., Pazos, M. R., Grande, M. T., Barreda-Manso, M. A., Benito-Cuesta, I., et al. (20182018). Cannabinoid CB2 Receptors in the Mouse Brain: Relevance for Alzheimer's Disease. *J.* Neuroinflammation 15 (1), 158. doi:10.1186/s12974-018-1174-9

Ma, Z., Gao, F., Larsen, B., Gao, M., Luo, Z., Chen, D., et al. (2019). Mechanisms of Cannabinoid CB2 Receptor-Mediated Reduction of Dopamine Neuronal Excitability in Mouse Ventral Tegmental Area. *EBioMedicine* 42, 225–237. doi:10.1016/j.ebiom.2019.03.040

Mediavilla, V., and Steinemann, S. (1997). Essential Oil of Cannabis Sativa L. Strains. *J. Intl Hemp Assoc.*, 82–84.

Miles, S. W., Sheridan, J., Russell, B., Kydd, R., Wheeler, A., Walters, C., et al. (2013). Extended-release Methylphenidate for Treatment of Amphetamine/methamphetamine Dependence: a Randomized, Double-Blind, Placebo-Controlled Trial. *Addiction* 108, 1279–1286. doi:10.1111/add.12109

Navarro, M., Carrera, M. R., Del Arco, I., Trigo, J. M., Koob, G. F., and Rodríguez de Fonseca, F. (2004). Cannabinoid Receptor Antagonist Reduces Heroin Self-Administration Only in Dependent Rats. *Eur. J. Pharmacol.* 501, 235–237. doi:10.1016/j.ejphar.2004.08.022

Newton, T. F., Reid, M. S., De La Garza, R., Mahoney, J. J., Abad, A., Condos, R., et al. (2008). Evaluation of Subjective Effects of Aripiprazole and Methamphetamine in Methamphetamine-dependent Volunteers. *Int. J. Neuropsychopharmacol.* 11, 1037–1045. doi:10.1017/S1461145708009097

Panenka, W. J., Procyshyn, R. M., Lecomte, T., MacEwan, G. W., Flynn, S. W., Honer, W. G., et al. (2013). Methamphetamine Use: a Comprehensive Review of Molecular, Preclinical and Clinical Findings. *Drug Alcohol Depend* 129, 167–179. doi:10.1016/j.drugalcdep.2012.11.016

Ranaldi, R., Pocock, D., Zereik, R., and Wise, R. A. (1999). Dopamine Fluctuations in the Nucleus Accumbens during Maintenance, Extinction, and Reinstatement of Intravenous D-Amphetamine Self-Administration. *J. Neurosci.* 19, 4102–4109. doi:10.1523/jneurosci.19-10-04102.1999

Rawson, R. A. (2013). Current Research on the Epidemiology, Medical and Psychiatric Effects, and Treatment of Methamphetamine Use. *J. Food Drug Anal.* 21, S77–S81. doi:10.1016/j.jfda.2013.09.039

Richardson, N. R., and Roberts, D. C. (1996). Progressive Ratio Schedules in Drug Self-Administration Studies in Rats: a Method to Evaluate Reinforcing Efficacy. *J. Neurosci. Methods* 66, 1–11. doi:10.1016/0165-0270(95)00153-0

Rodriguez, J. S., Boctor, S. Y., Flores, L. C., Phelix, C. F., and Martinez, J. L. (2011). Local Pretreatment with the Cannabinoid CB1 Receptor Antagonist AM251 Attenuates Methamphetamine Intra-accumbens Self-Administration. *Neurosci. Lett.* 489, 187–191. doi:10.1016/j.neulet.2010.12.013

Santiago, M., Sachdev, S., Arnold, J. C., McGregor, I. S., and Connor, M. (2019). Absence of Entourage: Terpenoids Commonly Found in Cannabis Sativa Do Not Modulate the Functional Activity of Δ9-THC at Human CB1 and CB2 Receptors. *Cannabis Cannabinoid Res.* 4, 165–176. doi:10.1089/can.2019.0016

Schindler, C. W., Panlilio, L. V., Gilman, J. P., Justinova, Z., Vemuri, V. K., Makriyannis, A., et al. (2010). Effects of Cannabinoid Receptor Antagonists on Maintenance and Reinstatement of Methamphetamine Self-Administration in Rhesus Monkeys. *Eur. J. Pharmacol.* 633, 44–49. doi:10.1016/j.ejphar.2010.02.005

Schmöle, A. C., Lundt, R., Gennequin, B., Schrage, H., Beins, E., Krämer, A., et al. (2015). Expression Analysis of CB2-GFP BAC Transgenic Mice. *PLoS One* 10, e0138986. doi:10.1371/journal.pone.0138986

Sharma, C., Al Kaabi, J. M., Nurulain, S. M., Goyal, S. N., Kamal, M. A., and Ojha, S. (2016). Polypharmacological Properties and Therapeutic Potential of β-Caryophyllene: A Dietary Phytocannabinoid of Pharmaceutical Promise. *Curr. Pharm. Des.* 22, 3237–3264. doi:10.2174/1381612822666160311115226

Shearer, J., Darke, S., Rodgers, C., Slade, T., van Beek, I., Lewis, J., et al. (2009). A Double-Blind, Placebo-Controlled Trial of Modafinil (200 Mg/day) for Methamphetamine Dependence. *Addiction* 104, 224–233. doi:10.1111/j.1360-0443.2008.02437.x

Shoaib, M. (2008). The Cannabinoid Antagonist AM251 Attenuates Nicotine Self-Administration and Nicotine-Seeking Behaviour in Rats. *Neuropharmacology* 54, 438–444. doi:10.1016/j.neuropharm.2007.10.011

Solinas, M., Panlilio, L. V., Antoniou, K., Pappas, L. A., and Goldberg, S. R. (2003). The Cannabinoid CB1 Antagonist N-Piperidinyl-5-(4-Chlorophenyl)-1-(2,4-Dichlorophenyl) -4-Methylpyrazole-3-Carboxamide (SR-141716A) Differentially Alters the Reinforcing Effects of Heroin under Continuous Reinforcement, Fixed Ratio, and Progressive Ratio Schedules of Drug Self-Administration in Rats. *J. Pharmacol. Exp. Ther.* 306, 93–102. doi:10.1124/jpet.102.047928

Svízenská, I., Dubový, P., and Sulcová, A. (2008). Cannabinoid Receptors 1 and 2 (CB1 and CB2), Their Distribution, Ligands and Functional Involvement in Nervous System Structures-Aa Short Review. *Pharmacol. Biochem. Behav.* 90, 501–511. doi:10.1016/j.pbb.2008.05.010

Vearrier, D., Greenberg, M. I., Miller, S. N., Okaneku, J. T., and Haggerty, D. A. (2012). Methamphetamine: History, Pathophysiology, Adverse Health Effects, Current Trends, and Hazards Associated with the Clandestine Manufacture of Methamphetamine. *Dis. Mon* 58, 38–89. doi:10.1016/j.disamonth.2011.09.004

Verharen, J. P. H., Zhu, Y., and Lammel, S. (2020). Aversion Hot Spots in the Dopamine System. *Curr. Opin. Neurobiol.* 64, 46–52. doi:10.1016/j.conb.2020.02.002

Vinklerová, J., Nováková, J., and Sulcová, A. (2002). Inhibition of Methamphetamine Self-Administration in Rats by Cannabinoid Receptor Antagonist AM 251. *J. Psychopharmacol.* 16, 139–143. doi:10.1177/026988110201600204

Weele, C. M. V., Siciliano, C. A., and Tye, K. M. (2019). Dopamine Tunes Prefrontal Outputs to Orchestrate Aversive Processing. *Brain Res.* 1713, 16–31. doi:10.1016/j.brainres.2018.11.044

Wilson, R. I., and Nicoll, R. A. (2002). Endocannabinoid Signaling in the Brain. *Science* 296, 678–682. doi:10.1126/science.1063545

Xi, Z. X., Gilbert, J. G., Peng, X. Q., Pak, A. C., Li, X., and Gardner, E. L. (2006). Cannabinoid CB1 Receptor Antagonist AM251 Inhibits Cocaine-Primed Relapse in Rats: Role of Glutamate in the Nucleus Accumbens. *J. Neurosci.* 26, 8531–8536. doi:10.1523/JNEUROSCI.0726-06.2006

Xi, Z. X., Peng, X. Q., Li, X., Song, R., Zhang, H. Y., Liu, Q. R., et al. (2011). Brain Cannabinoid CB_2 Receptors Modulate Cocaine's Actions in Mice. *Nat. Neurosci.* 14, 1160–1166. doi:10.1038/nn.2874

Xi, Z. X., Spiller, K., Pak, A. C., Gilbert, J., Dillon, C., Li, X., et al. (2008). Cannabinoid CB1 Receptor Antagonists Attenuate Cocaine's Rewarding Effects: Experiments with Self-Administration and Brain-Stimulation Reward in Rats. *Neuropsychopharmacology* 33, 1735–1745. doi:10.1038/sj.npp.1301552

Youssef, D. A., El-Fayoumi, H. M., and Mahmoud, M. F. (2019). Beta-caryophyllene Alleviates Diet-Induced Neurobehavioral Changes in Rats: The Role of CB2 and PPAR-γ Receptors. *Biomed. Pharmacother.* 110, 145–154. doi:10.1016/j.biopha.2018.11.039

Zhang, H.-Y., De Biase, L., Chandra, R., Shen, H., Liu, Q.-R., Gardner, E., et al. (2021a2021). Repeated Cocaine Administration Upregulates CB2 Receptor Expression in Striatal Medium-Spiny Neurons that Express Dopamine D1 Receptors in Mice. *Acta Pharmacol. Sin.* doi:10.1038/s41401-021-00712-6

Zhang, H. Y., Bi, G. H., Li, X., Li, J., Qu, H., Zhang, S. J., et al. (2015). Species Differences in Cannabinoid Receptor 2 and Receptor Responses to Cocaine Self-Administration in Mice and Rats. *Neuropsychopharmacology* 40, 1037–1051. doi:10.1038/npp.2014.297

Zhang, H. Y., Gao, M., Liu, Q. R., Bi, G. H., Li, X., Yang, H. J., et al. (2014). Cannabinoid CB2 Receptors Modulate Midbrain Dopamine Neuronal Activity and Dopamine-Related Behavior in Mice. *Proc. Natl. Acad. Sci. U S A.* 111, E5007–E5015. doi:10.1073/pnas.1413210111

Zhang, H. Y., Gao, M., Shen, H., Bi, G. H., Yang, H. J., Liu, Q. R., et al. (2017). Expression of Functional Cannabinoid CB2 Receptor in VTA Dopamine Neurons in Rats. *Addict. Biol.* 22, 752–765. doi:10.1111/adb.12367

Zhang, H. Y., Shen, H., Gao, M., Ma, Z., Hempel, B. J., Bi, G. H., et al. (2021b). Cannabinoid CB2 Receptors Are Expressed in Glutamate Neurons in the Red Nucleus and Functionally Modulate Motor Behavior in Mice. *Neuropharmacology* 189, 108538. doi:10.1016/j.neuropharm.2021.108538

Zhang, H. Y., Shen, H., Jordan, C. J., Liu, Q. R., Gardner, E. L., Bonci, A., et al. (2019). CB2 Receptor Antibody Signal Specificity: Correlations with the Use of Partial CB2-Knockout Mice and Anti-rat CB2 Receptor Antibodies. *Acta Pharmacol. Sin.* 40, 398–409. doi:10.1038/s41401-018-0037-3

Zlebnik, N. E., and Cheer, J. F. (2016). Beyond the CB1 Receptor: Is Cannabidiol the Answer for Disorders of Motivation?. *Annu. Rev. Neurosci.* 39, 1–17. doi:10.1146/annurev-neuro-070815-014038

Zorick, T., Sugar, C. A., Hellemann, G., Shoptaw, S., and London, E. D. (2011). Poor Response to Sertraline in Methamphetamine Dependence Is Associated with Sustained Craving for Methamphetamine. *Drug Alcohol Depend* 118, 500–503. doi:10.1016/j.drugalcdep.2011.04.015

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

REVIEW
published: 18 January 2022
doi: 10.3389/fphar.2021.809965

Anti-Inflammatory and Pro-Autophagy Effects of the Cannabinoid Receptor CB2R: Possibility of Modulation in Type 1 Diabetes

Qing-Rong Liu[1], Kanikkai Raja Aseer[1], Qin Yao[1], Xiaoming Zhong[2], Paritosh Ghosh[1], Jennifer F. O'Connell[1] and Josephine M. Egan[1]**

[1]Laboratory of Clinical Investigation, National Institute on Aging, NIH, Baltimore, MD, United States, [2]Ben May Department for Cancer Research, The University of Chicago, Chicago, IL, United States

OPEN ACCESS

Edited by:
Pal Pacher,
Laboratory of Cardiovascular Physiology and Tissue Injury (NIH), United States

Reviewed by:
Nicholas V. DiPatrizio,
University of California, Riverside, United States
Barbara Kaplan,
Mississippi State University, United States

***Correspondence:**
Qing-Rong Liu
qliu@mail.nih.gov
Josephine M. Egan
eganj@grc.nia.nih.gov

Specialty section:
This article was submitted to Experimental Pharmacology and Drug Discovery, a section of the journal Frontiers in Pharmacology

Received: 05 November 2021
Accepted: 21 December 2021
Published: 18 January 2022

Citation:
Liu Q-R, Aseer KR, Yao Q, Zhong X, Ghosh P, O'Connell JF and Egan JM (2022) Anti-Inflammatory and Pro-Autophagy Effects of the Cannabinoid Receptor CB2R: Possibility of Modulation in Type 1 Diabetes. Front. Pharmacol. 12:809965. doi: 10.3389/fphar.2021.809965

Type 1 diabetes mellitus (T1DM) is an autoimmune disease resulting from loss of insulin-secreting β-cells in islets of Langerhans. The loss of β-cells is initiated when self-tolerance to β-cell-derived contents breaks down, which leads to T cell-mediated β-cell damage and, ultimately, β-cell apoptosis. Many investigations have demonstrated the positive effects of antagonizing cannabinoid receptor 1 (CB1R) in metabolic diseases such as fatty liver disease, obesity, and diabetes mellitus, but the role of cannabinoid receptor 2 (CB2R) in such diseases is relatively unknown. Activation of CB2R is known for its immunosuppressive roles in multiple sclerosis, rheumatoid arthritis, Crohn's, celiac, and lupus diseases, and since autoimmune diseases can share common environmental and genetic factors, we propose CB2R specific agonists may also serve as disease modifiers in diabetes mellitus. The *CNR2* gene, which encodes CB2R protein, is the result of a gene duplication of *CNR1*, which encodes CB1R protein. This ortholog evolved rapidly after transitioning from invertebrates to vertebrate hundreds of million years ago. Human specific *CNR2* isoforms are induced by inflammation in pancreatic islets, and a *CNR2* nonsynonymous SNP (Q63R) is associated with autoimmune diseases. We collected evidence from the literature and from our own studies demonstrating that CB2R is involved in regulating the inflammasome and especially release of the cytokine interleukin 1B (IL-1β). Furthermore, CB2R activation controls intracellular autophagy and may regulate secretion of extracellular vesicles from adipocytes that participate in recycling of lipid droplets, dysregulation of which induces chronic inflammation and obesity. CB2R activation may play a similar role in islets of Langerhans. Here, we will discuss future strategies to unravel what roles, if any, CB2R modifiers potentially play in T1DM.

Keywords: type 1 diabetes mellitus, cannabinoid receptor, autoimmunity, autophagy, inflammation, immunetolerance, lysosome

INTRODUCTION

Overview of Type 1 Diabetes Mellitus and its Etiology

Based on the 2020 CDC's National Diabetes Statistics Report, the number of people in the United States suffering from type 1 diabetes mellitus (T1DM) increased from 1.25 to 1.6 million between 2017 and 2020 (https://www.cdc.gov/diabetes/data/statistics-report/index.html). In addition to the increasing incidence, the peak age at diagnoses has shifted to an even younger age group (Ilonen et al., 2019). Overall, the highest incidence of T1DM is in Northern European countries and the island of Sardinia, while lower incidences are reported from India and China (Patterson et al., 2019). The underlying mechanism of pancreatic β-cell failure involves a strong genetic predisposition and transgenerational epigenome reprogramming (King and Skinner, 2020), but genetics alone is unlikely to account for such an increase: pollutants (e.g., microplastics) (Campanale et al., 2020), obesogenic diets causing increased stress on β-cells (Polsky and Ellis, 2015), infection during pregnancy (Group, 2007), sedentary lifestyle (Maja Cigrovski Berkovic et al., 2017), and microbiota shift (Knip and Siljander, 2016) also seem to be playing their parts.

T1DM shows significant geographic, ethnic, age, and gender differences, with the incidence peaking between 4 and 19 years of age, then leveling off, and once again gradually increasing after the fifth decade of life (Rogers et al., 2017), indicating defective central thymic and peripheral tolerance (Littman and Rudensky, 2010; Zucchelli et al., 2005). Self-tolerance is induced in the primary lymphoid organs (thymus and bone marrow), and in spleen and lymph nodes, where self-reactive T cells are deleted, thereby guaranteeing, in normal physiology, that self-reactive T cells do not get into the circulation (Theofilopoulos et al., 2017). β-cells in islets of Langerhans exposed to viral infections (such as enteroviruses, Coxsackie B), an array of cytokines (IL-1β, TNF-α, IFN-γ), injury by toxins, and stress (such as increased ROS production, ER stress, post-translational modifications) conditions may present auto/neoantigenic peptides (β-Ag) on major histocompatibility complex molecules I (MHC-I) to the cell surface, thereby attracting cytotoxic $CD8^+$T cells (Eizirik et al., 2009). $CD8^+$T-lymphocytes, which recognize MHC-I peptide complexes, dominate the pro-inflammatory milieu of islet infiltration (insulitis) and are thought to be major effectors of β-cell death (Carré and Mallone, 2021). The processes in β-cell that produce MHC-I restricted antigens are poorly understood in T1DM. Autophagy (Atg) may, however, intersect with the intracellular MHC-I presentation by lessening the amount of neoantigens that are formed (**Figure 1**). Pancreatic β-cells are vulnerable because insulin transcription accounts for 40% of the transcriptome whereas genes involved in cellular protection such as those for chaperones, autophagy, ubiquitin, proteosome, protection from reactive oxygen species, and ER unfolded protein responses are expressed at lower levels than in other islet cell types (Benner et al., 2014; Segerstolpe et al., 2016; Diedisheim et al., 2018). Genetic susceptibility, environmental triggering, autoantibody appearance are the pre-disposing events to β-cell damage. Reduced insulin secretion and dysglycemia occur when T cells and macrophages infiltrate into the islets and

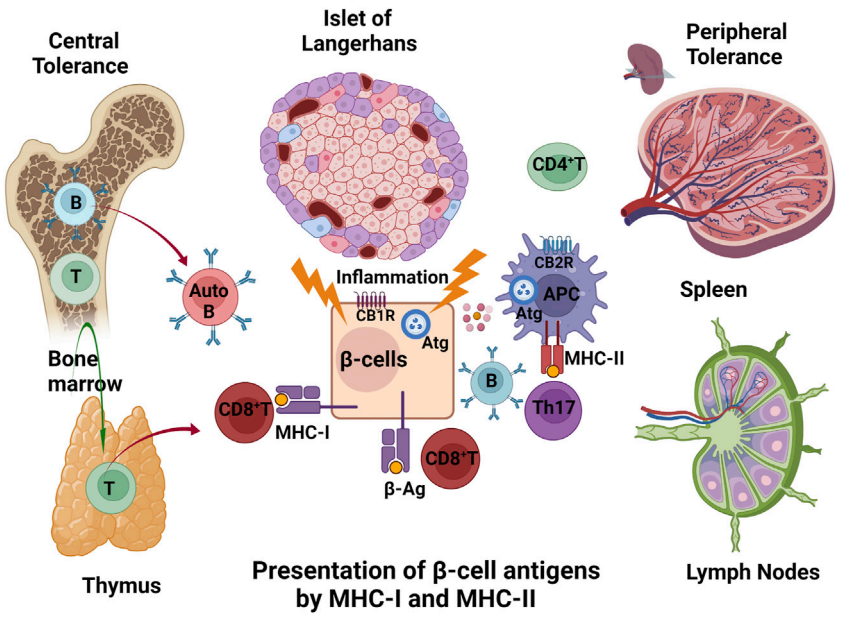


FIGURE 1 | Central and peripheral breakdown of immune tolerance in T1DM: Auto-reactive cytotoxic T ($CD8^+$T) and B (Auto B) cells escape from primary lymphoid (bone marrow and thymus) and secondary lymphoid organs (spleen and lymph nodes). The β-cells exposed to viral infections, an array of cytokines (IL-1β, TNF-α, IFN-γ), injury by toxins, and stress (such as increased ROS production, ER stress, post-translational modifications) conditions may present their auto/neoantigenic peptides (β-Ag) on MHC-I complexes to the cell surface, thereby attracting cytotoxic $CD8^+$T cells. Autoreactive antigens that are endocytosed by antigen presenting cells (APC) activate $CD4^+$Th17 T and B cells. CB1R in β-cells and CB2R in APC cells are players in the outcome to β-cells, possibly through actions on autophagy (Atg).

gradually destroy β-cells. Finally insulin-dependent diabetes occurs when approximately 80% of the β-cells are destroyed: this is the pathological sequence of events (Eisenbarth, 1986; Insel et al., 2015; Ilonen et al., 2019). Susceptible HLA (human leukocyte antigen) DR/DQ alleles and detection of at least two autoantibodies specifically targeting β-cells are pre-diagnostic markers for T1DM (Michels et al., 2015). A humanized anti-CD3 monoclonal antibody (Teplizumab) is currently a FDA approved drug to delay occurrence of T1DM symptoms by slowing down destruction of β-cells (Herold et al., 2019). Certain natural and synthetic cannabinoids are known for their potent immunosuppressive and anti-inflammatory properties that are effective against several autoimmune diseases (Rieder et al., 2010); however, little research is carried out for early intervention with cannabinoids on T1DM risk cohorts.

Overview of the Endocannabinoid System in relation to Islets of Langerhans

Cannabinoids are endogenously produced, lipid-derived mediators of multiple organ functions-hence the name endocannabinoids (eCBs) (Pacher et al., 2020; Lu and Mackie, 2021). The most studied eCBs are anandamide (N-arachidonoylethanolamide, AEA) and 2-arachidonoyl-sn-glycerol (2-AG), both of which are synthesized in β-cells in islets upon cellular depolarization. The whole eCB system also consists of the enzymes involved in the synthesis and degradation of the eCBs and the eCB receptors (CBRs) (Joshi and Onaivi, 2019), of which there are primarily two such receptors, CB1R and CB2R. Both are class-A G-protein-coupled receptors that function through Gi/o/q proteins and the β-arrestin signaling pathway (Aseer and Egan, 2021). In general, CB1R is highly expressed in the central nervous system while CB2R is mostly found in immune cells. However, of pertinence to this review, CBRs are also present in cells of the islets of Langerhans. There are five cell types in islets, called α-, β-, δ-, ε-, and PP-cells. These cells produce glucagon, insulin, somatostatin, ghrelin, and pancreatic polypeptide, respectively. In general, more than 50% of the islet cells are β-cells, while α-cells are the next most common cell type. Using FACS sorted mouse and human β-cells it was found that CB1R mRNA levels in mouse β-cells (GSE54973) are more than 10-fold higher than in human β-cells (GSE103383) (Benner et al., 2014; Diedisheim et al., 2018). CB1R but not CB2R mRNA was found in human β-cells by single cell sequencing also (GSE81608) (Benner et al., 2014; Xin et al., 2016). Human CB2R transcripts were found in α-, δ-, and ε-cells more than are CB1R transcripts while in PP-cells both transcripts were reported to be equally expressed (Xin et al., 2016). Low basal expression of CNS-enriched CB1R is also present in myocytes, adipocytes and hepatocytes (González-Mariscal et al., 2016), while leukocyte-enriched CB2R is found in adipocytes, neurons and microglia (Karaliota et al., 2009; Liu et al., 2020a).

Exogenous cannabinoids are also available in marijuana plants; Δ9-tetrahydrocannabinol (Δ9–THC), cannabidiol (CBD), and (−)-β-caryophyllene (BCP). Δ9–THC is a ligand for both CB1R and CB2R, while BCP is a selective CB2R ligand. All the molecular target receptors of CBD are unknown but CBD is thought to be involved in enhancing serotonin 5-HT_{1A} receptor and transient receptor potential cation channel (TRPV1) activity (Pacher et al., 2020; Lu and Mackie, 2021). Inverse agonists (antagonism) of CB1R were developed 20 years ago as treatments for obesity: however, rimonabant that did come into use for that purpose was quickly withdrawn because of severe adverse psychiatric effects (Sam et al., 2011). Further development of CB1R antagonists and inverse agonists by pharmaceutical companies was then halted. Unrelated to CNS effects, we have shown that, in regards to β-cells, peripheral inhibition of CB1R leads to: improved insulin secretion in response to glucose; enhanced responses to incretins; increased intracellular cAMP levels; resistance to inflammation from high fat diets; and protects against apoptosis due to toxins and high fat diets (Gonzalez-Mariscal et al., 2018). CB2R has a yin-yang relationship with CB1R structurally and functionally (Shao et al., 2016; Li et al., 2019) in the context of cell types. While activation of CB2R has general anti-inflammation effects (Basu and Dittel, 2011; Wu et al., 2018), cell type specific CB1R deletion in β-cells, myocytes, and hepatocytes has anti-inflammatory effects in mice (Gonzalez-Mariscal et al., 2018; González-Mariscal et al., 2019; Kim et al., 2020). CB2R enriched in various cell types of the immune system (Fernández-Ruiz et al., 2007; Hu et al., 2020) appears to result in little or no adverse CNS effects, unlike CB1R, when activated (Buckley et al., 2000; Turcotte et al., 2016). CB2R activation in the immune system is also thought to be anti-inflammatory and pro-tolerance and therefore may aid in preventing autoimmune-mediated self-destruction (Eisenstein and Meissler, 2015). There are rich sources of natural and synthetic CB2R selective agonists that potentially could be investigated for intervention at the pre-symptomatic phase of T1DM. Hemp seeds (Pellati et al., 2018), cloves (Siani et al., 2013), black pepper (Geddo et al., 2019), and manacá (Galdino et al., 2012) with high content of β-caryophyllene are widely consumed in India, China, and Brazil (Patterson et al., 2019). A synthetic cannabidiol quinone derivative (THP-101), a CBD analog with CB2R agonist properties, added another potential remedy for autoimmune diseases (Navarrete et al., 2018). We will now analyze the literature with regards to the possible molecular mechanisms whereby regulating activity of CB2R might have therapeutic potential in the spectrum of T1DM with emphasis on molecular evolution, immune tolerance, anti-inflammation, autophagy, and extracellular vesicles secretion.

CB2R and Evolution

No *CNR1*/*CNR2* orthologs are present in protostome invertebrates even though specific enzymes necessary for eCB synthesis and breakdown are present, as are vanilloid-type ion channels that could serve as eCB receptors (Elphick, 2012). A single *CNR1*/*CNR2* ortholog is present in genomes of deuterostome chordates such as the sea squirt *Ciona intestinalis* (ciCBR, 423 AA) and lancelet *Branchiostoma floridae* (bfCBR, 410 AA), expressed in branchial pharynx, heart, cerebral ganglion, testis, ovaries, and gut (Elphick, 2007; Elphick et al., 2003; Matias et al., 2005). The primitive chordate

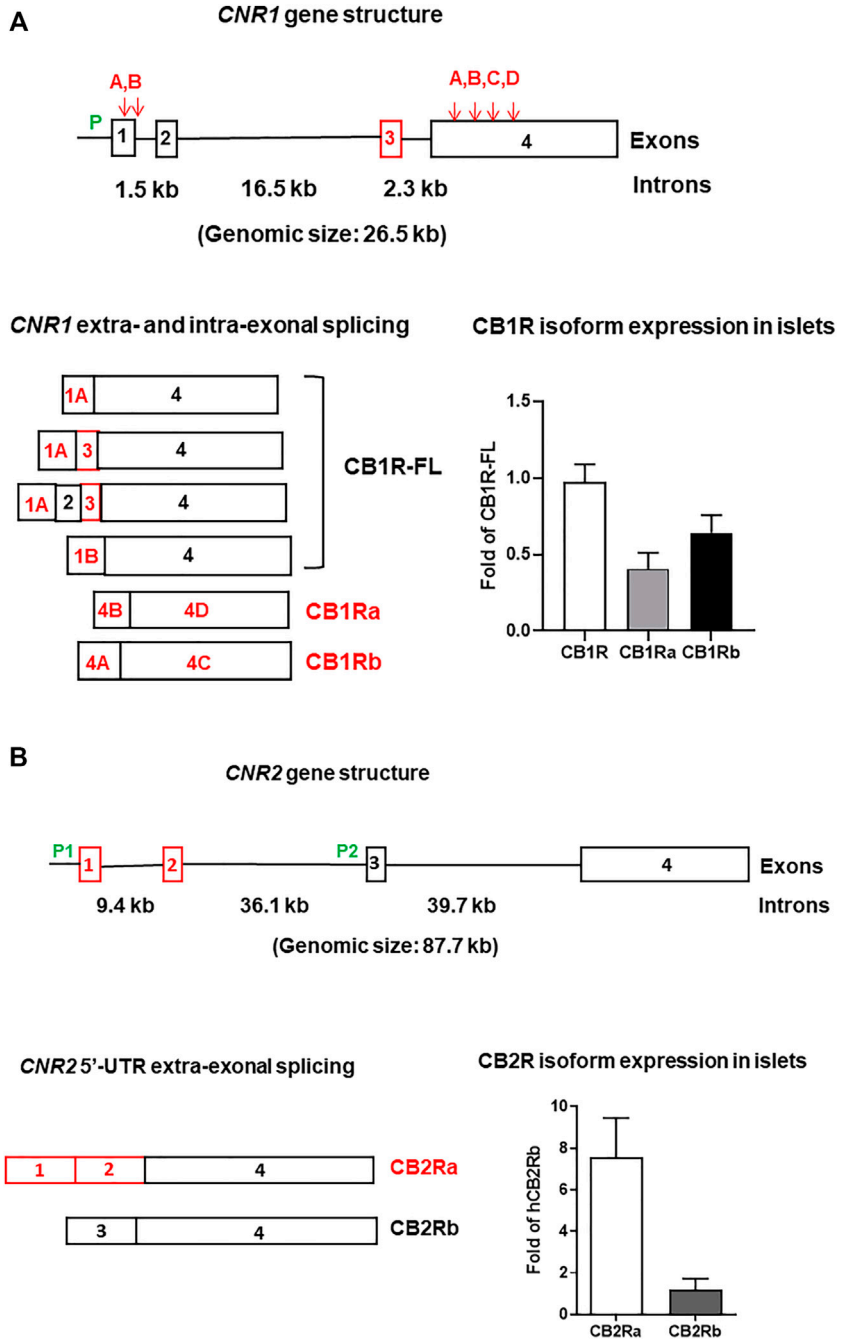


FIGURE 2 | Human *CNR1* **(A)** and *CNR2* **(B)** gene structures, their alternatively spliced isoforms, and expression in pancreatic islets. P (green lettering) represents promoters. Exons are open boxes and introns solid lines. The exon numbers are inside the open boxes and intron sizes are marked in kb (kilobase). Downward arrows and capital letters are at the intra-exonal splicing sites. Red letterings, boxes, and arrows represent human specific isoforms, exons, and splicing sites, respectively. The reference of CB1R isoform islet expression (n = 6) is full-length CB1R (CB1R-FL) and the reference of CB2R isoform islet expression (n = 3) is CB2Rb (unpublished data from Diabetes Section, LCI/NIA/NIH).

CNR gene has only one promoter without upstream exons encoding different 5′UTRs (McPartland et al., 2006) as is observed in *CNR1* and *CNR2* of mammalian species (Zhang et al., 2015; Liu et al., 2019). Human CB1R (472 AA) is enriched in neurons and is more homologous to chordate ciCBR and bfCBR (Elphick, 2007). CB2R (360 AA) is enriched in the immune system (Liu et al., 2020a) and *CNR2* likely arose due to vertebrate genome duplication about 500 million years ago (Elphick, 2002) when the adaptive immune system and major histocompatibility complex class I (MHC-I)

and class II (MHC-II) are reported to have first appeared in jawed fish (Flajnik and Kasahara, 2010; Wu et al., 2021). During mammalian evolution, human gene exonization (Li et al., 2018) and splicing isoform evolution (Zhang et al., 2017a) contributed to multiple upstream exons with a single promoter in *CNR1* and two promoters in *CNR2* (González-Mariscal et al., 2016; Liu et al., 2009) to diversify eCB signaling in a specific cell type context (Marti-Solano et al., 2020) and the genomic size of *CNR2* is more than 3-fold larger than that of *CNR1* (**Figures 2A,B**). Human *CNR1* has one promoter and four exons that are spliced into six variants including two human-specific N-terminal amino acid (AA) altered isoforms (González-Mariscal et al., 2016), while *CNR2* gene has two separate promoters and four exons that are spliced into CBR2a (human-specific) and CBR2b isoforms, encoding the same peptide sequences (Liu et al., 2019). *CNR1* contains human-specific exon-3 and intra-exonal splice sites of exon-1 and coding exon-4, creating altered N-terminal AA isoforms of CB1Ra and CB1Rb (González-Mariscal et al., 2016). *CNR2* contains human-specific exon-1 and -2 encoding isoform CB2Ra that is under control of human-specific promoter-1, whereas the promoter-2 controls expression of generic exon-3 and -4 encoding CB2Rb isoform that is preferentially expressed in immune system (Liu et al., 2009). The human-specific evolution of eCB system could explain that THC is rewarding to humans but not rodents (Zhang et al., 2015; Han et al., 2017). Although CB1R is predominantly expressed in mammalian brain, we observed low basal expression of CB1R in many peripheral tissues, and interestingly the liver of humans has a predominant N-terminal intra-exonal spliced isoform (CB1Rb), expression of which is increased by obesity (González-Mariscal et al., 2016). Global CB1R knockout mice, though fertile, have detrimental phenotypes of increased morbidity and weight loss, agitation, and early death (Zimmer et al., 1999). In contrast, global CB2R knockout mice, while also fertile, appear healthy unless challenged with endotoxins (Kapellos et al., 2017) and high fat-sugar diet (Agudo et al., 2010), implying that manipulation of CB2R might not have severe adverse CNS side effects.

CB2R and Immunity

As stated above, CB2R is predominantly expressed in the immune system with a rank order of B-cells (B-lymphocytes) > granulocytes > dendritic cells > macrophages > $CD8^+$Tcells > natural killer T-cells > $CD4^+$T-cells > natural killer cells (Galiègue et al., 1995; López et al., 2018). CB2R expression is highly inducible during inflammatory processes and its activation polarizes macrophages from a classical pro-inflammatory (M1) state to an alternative anti-inflammatory (M2) state (Braun et al., 2018). For example, there is a 40-fold increase in CB2R expression by the 5th day in mouse right brain cortex when the right middle cerebral artery is occluded for 30 min and causes right cortical ischemia. This gradually subsides to the basal level by the 10th day to levels similar to those of the left non-ischemic cortex (Yu et al., 2015). Activation of CB2R by GP1a (a CB2R agonist) reduced HLA DQ expression by 10-fold in an ipsilateral mouse brain hemisphere that was stereotactically injected with HIV-1 infected human monocyte-derived-macrophages in comparison with the non-injected contralateral hemisphere (Gorantla et al., 2010). Both CB2Ra and CB2Rb isoforms are activated by inflammation and psychiatric stress (Zhang et al., 2015). Activation of CB2R resulted in decreases in cell surface expression of MHC-II molecules and the pro-inflammatory cytokines IL-1β and IL-12p40 (Mestre et al., 2005). Although CB2R is enriched in the immune system, we observed CB2R expression in microglia, as might be expected, and neurons in different mouse brain regions (Liu et al., 2020a). Interestingly, we found that CB2Ra but not CB2Rb is expressed in human testis (Liu et al., 2009) and Nielson et al. reported that CB2Ra is involved in germ cell maturation and is localized in the cytoplasm of late spermatocytes and round spermatids but not early spermatocytes (Nielsen et al., 2019). We found that the CB2Ra transcript levels are about 8-fold higher than that of CB2Rb in human islets (**Figure 2B**), indicating that the upstream promoter is more active in cell types outside of immune system (Zhang et al., 2017b). The expression of CB2R in non-immune system implies that CB2R is not only involved in MHC class II (MHC-II) immune cell response (Gorantla et al., 2010) but also in pan MHC class I (MHC-I) cells that present oncogenic and invading intracellular virus antigens to cell surface (Karmaus et al., 2013). Whether CB2R plays a role in immune tolerance in T1DM is currently not reported.

CB2R and Autoimmune Diseases

T1DM shares genetic and phenotypic comorbidity with other autoimmune diseases and CB2R activation can ameliorate symptoms of multiple sclerosis (Annunziata et al., 2017), thyroiditis autoimmune diseases (Alcigir et al., 2017), celiac disease (Tortora et al., 2020), Crohn's disease (Leinwand et al., 2017), and rheumatoid arthritis (Gui et al., 2014). Several human leukocyte antigen (HLA) gene polymorphisms of MHC class I and II (Noble and Valdes, 2011), insulin gene short VNTR (variable number tandem repeat) (Bennett et al., 1995), and a nonsynonymous *CNR2* SNP (Q63R) are risk alleles that co-segregate with several autoimmune diseases (Rossi et al., 2012; Mahmoud Gouda and Mohamed Kamel, 2013; Bellini et al., 2015; Ismail and Khawaja, 2018; Strisciuglio et al., 2018). However, we could not find any study of CB2R and its level of activation in T1DM in the literature. Damage to β-cells in T1DM patients is initiated by recruiting circulating T cells and macrophages that migrate across vascular endothelium cells by orchestrated multimodal movements; tethering, rolling, arrest, firm adhesion, and migration that are mediated by selectins, integrins, and cytoskeleton molecules (Alon and Shulman, 2011). Activation of CB2R has been shown to reduce formation of leukocyte lamellipodia by downregulation of integrins (ITGA4 and ITGB2) and small GTPases (RAC1 and RHOA) that promote adhesion and cytoskeleton dynamics, respectively, necessary for trans-endothelium migration (Rom et al., 2013). Activation of CB2R has also been shown to protect from tissue damage by controlling recruitment of $CD34^+$ myeloid progenitor cells and neutrophils, reducing infiltration $CD4^+$ T-lymphocyte subset of T helper 17 (Th17) cells (Cencioni et al., 2010), suppressing $CD8^+$T

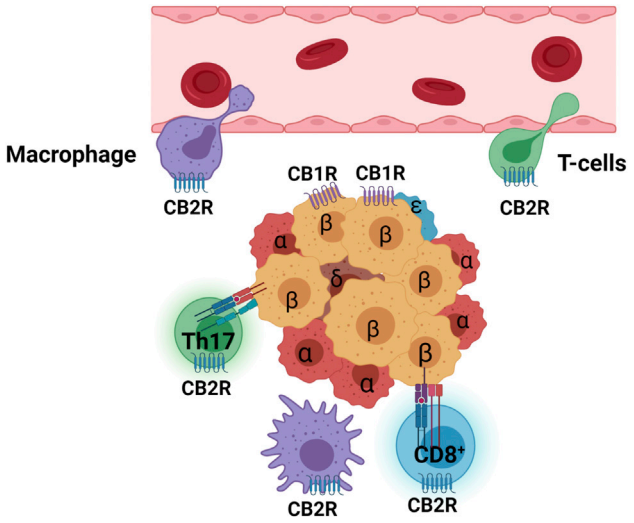


FIGURE 3 | Activated CB2R suppresses macrophage (purple) and T cell (green) infiltration from endothelium barrier of blood vessel and inhibits M1 macrophages (purple ameba shape), self-reactive HMC-I $CD8^+$T cells (blue with halo), and $CD4^+$Th17 cells (green with halo). Islet cell types are marked with Greek letters (α, β, δ, ε). Purple serpentine represents CB1R in β-cells and blue serpentine CB2R in immune cells.

lymphocytes (Joseph et al., 2004), and regulating macrophage function by altering expression of pro- and anti-inflammatory cytokines and their receptors (Palazuelos et al., 2008; Kapellos et al., 2017; Kapellos et al., 2019) (**Figure 3**).

CB2R and Immune Tolerance

The intracellular autoantigens of β-cells are processed by ubiquitin–proteasome system into small peptides of 8–11 AAs that are transported into the endoplasmic reticulum (ER) by TAP (transporter associated with antigen processing), and then bind to MHC-I with assistance of chaperone, and further translocated to plasma membrane *via* Golgi apparatus (Strehl et al., 2005). Cytotoxic $CD8^+$T cells with specific T cell receptors (TCRs) for the autogenic peptides are activated and exert apoptotic effects on β-cells (Gupta et al., 2006). CB2R is expressed in thymus (Schatz et al., 1997) where autoimmune regulator AIRE (mutated in APS-1, autoimmune polyendocrine syndrome type 1) stimulates ectopic expression of intracellular T1DM autoantigens (e.g., insulin and GAD65) in medullary thymus epithelium cells (mTECs) in which the endogenous peptides are presented to the cell surface by MHC-I (Alexandropoulos et al., 2015). Insulin gene (*INS*) with long-VNTR alleles promotes higher expression of insulin in mTECs that present more insulin peptides to educate $CD8^+$T cells not to be self-reactive (Fan et al., 2009; Mathis and Benoist, 2009; Levi and Polychronakos, 2013). Proteasome processing of T1DM autoantigens for MHC-I presentation requires unfolding of protein monomers and is not capable of unfolding oligomeric insulin. In that case, autophagosomes and lysosomes are involved in the autoantigen presentation in mTECs (Yedidi et al., 2017; Øynebråten, 2020). CB2R expression is 6-fold higher than CB1R in mTECs (GSE89892) within the thymus (Guha et al., 2017) and is upregulated in activated T cells where very little or no CB1R is found (Schatz et al., 1997; Coopman et al., 2007), and therefore the pro-autophagy function of CB2R may prevent insulin from being mispresented. Clonal selection of immunosuppressive regulatory T cells ($CD4^+FOXP3^+$Treg cells) prevents self-reactivating T cells from exiting into the circulation (Kraj and Ignatowicz, 2018). Deletion of $FOXP3^+$Treg accelerates onset of T1DM (Mariño et al., 2009) and infusion of $FOXP3^+$Treg cells delays the onset of T1DM in young NOD mice (Spence et al., 2018). CB2R expression is preferentially induced in $FOXP3^+$Treg-cells and the agonist GP1a enhances $FOXP3^+$Treg immunosuppressive function in Crohn's disease (Leinwand et al., 2017). Pancreatic β-cells do not express MHC-II because it is restricted to professional antigen presenting cells (APCs), such as CB2R enriched macrophages, dendritic and B cells (Roche and Furuta, 2015). The β-cells secrete and present the autoantigens that are endocytosed by APCs and fused with lysosomes, and further processed by endosome-lysosome pathway (Lundberg and McDevitt, 1992) to peptides of 12–25 AAs (Wu et al., 2021) that bind to MHC-II-Ii (Invariant chain) complex in ER and translocate *via* Golgi apparatus to endolysosomes in which Ii is cleaved by cathepsin L and the remaining CLIP (class II-associated invariant chain peptide) prevents autoantigen presentation to APCs that regulate $CD4^+$T cell differentiation (Jurewicz and Stern, 2019). During inflammation, the activated APCs present more β-cell-derived neo- and autoantigen peptides that stimulate pathological Th17 cytotoxic cell expansion in lymph nodes and in circulation (Honkanen et al., 2010). Peripherally, in the secondary lymphoid organs (lymph nodes, spleen, tonsils, and mucous membranes), pathologic autoreactive $CD4^+$T helper cells ($Th17/Th1^+$) cause breakdown of peripheral tolerance and

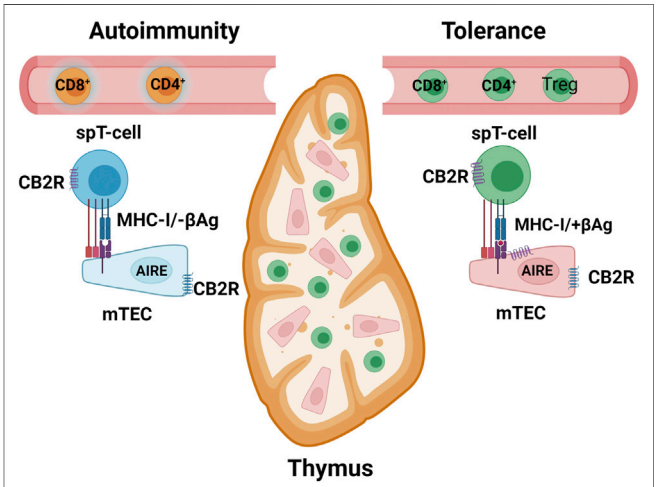


FIGURE 4 | MHC-I β-cell restricted antigen (+βAg) or no antigen (–βAg) presentation in medullary epithelium cells (mTEC) inside thymus. AIRE, autoimmune regulator for ectopic expression of β-cell-specific genes. spT cells, single-positive $CD8^+$ or $CD4^+$ T cells. Yellow represents self-reactive and green self-tolerance T cells. Purple serpentine represents CB1R in β-cells and blue serpentine CB2R in immune cells.

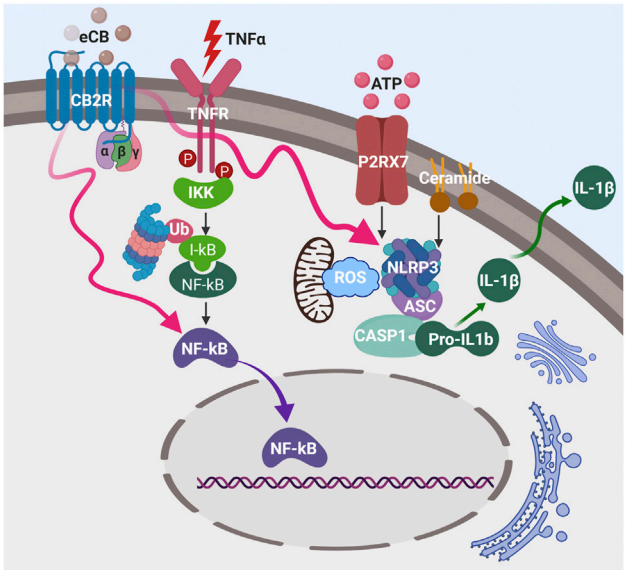


FIGURE 5 | Activation of plasma membrane CB2R pathway inhibits NLRP3 inflammasome complex and NFκB activation (red wavy arrows) during inflammation that is initiated by TNFα, ATP, and ceramide, thereby reduces IL-1β secretion.

inflammation (Cencioni et al., 2010; Fava et al., 2016). The CB2R synthetic agonist JWH015 reduces IL-17, TNF-α, and IFN-γ secreted by Th17 cells, and suppresses anti-CD3/anti-CD28 induced $CD4^+$ and $CD8^+$T cell proliferation by reducing T cell growth factor IL-2 (Cencioni et al., 2010) (**Figure 4**). Whether CB2R is involved in breakdown of central and peripheral immune tolerance in T1DM is unknown.

CB2R and Inflammation

Inflammatory components of the innate immune system such as toll-like receptors (TLRs), NLRP3 (NLR family pyrin domain containing 3) inflammasome, and IL-1β contribute to the etiology of T1DM and their activation recruits inflammatory T cells and macrophages into islets where they are cytotoxic to β-cells (Grishman et al., 2012). CB2R is prominently upregulated by inflammation and a selective synthetic CB2R agonist, JWH-133, inhibits the TLR4/NF-κB signaling pathway, reduces infiltration of immune cells across endothelium, thereby mitigating against immune-mediated tissue damage (Yu et al., 2015; Chen et al., 2019; Jing et al., 2020). Another selective synthetic CB2R agonist, HU-308, inhibits NLRP3 inflammasome expression and activation, leading to reduction of IL-1β secretion from macrophages and microglia in a mouse model of dextran sulphate sodium (DSS)-induced colitis and experimental autoimmune encephalomyelitis (EAE) (Shao et al., 2014; Ke et al., 2016). The naturally occurring CB2R selective agonist, β-caryophyllene, inhibits hypoxia-induced cytotoxicity by decreasing proinflammatory cytokine secretion of IL-1β, TNF-α, and IL-6 in a murine microglia cell line, BV2 (Guo et al., 2014). A selective CB2R agonist, AM124, used in a rat model of complete Freund's adjuvant (CFA)-induced inflammatory dermatitis decreases the expression of IL-1β, IL-6 and TNF-α (Nascimento et al., 2012; Su et al., 2012). It has been shown that, under stress damaged mitochondria release mtDNA into the cytosol and enhance production of reactive oxygen species (ROS) in an inflammasome-dependent manner in both macrophages and $Th17^+$T cells (Nakahira et al., 2011; Kaufmann et al., 2019). Since the inflammasome is exquisitely sensitive to nucleic acid and ROS, the activated inflammasome produces the proinflammatory cytokines, IL-1β and IL-18, resulting in vicious inflammatory cycle (Nakahira et al., 2011). Since CB2R agonists stimulate calcium release from lysosomes that tether and transfer calcium to mitochondria to reduce NLRP3 inflammasome activation (Peng et al., 2020) and ROS production they may be possible therapeutic agents to mitigate inflammation induction (**Figure 5**). In sum, although CB2R activation reduces the proinflammatory cytokines in certain disease models, there is no published research on the possibility of CB2R activation being protective of β-cell destruction due to proinflammatory cytokine-induced cytotoxicity during onset of T1DM.

CB2R and Autophagy

Malfunction of intracellular membrane trafficking is involved in autoantigen presentations by MHC-I and -II and autophagy compensates compromised protease activities of ubiquitin-proteasome system in antigen presentation and impaired autophagy has been documented in T1DM (Valecka et al., 2018; Muralidharan et al., 2021). Autophagy is classified as macro-, micro-autophagy, and chaperone-mediated autophagy that share intracellular proteolytic pathway and membrane trafficking machinery as MHC antigen presentation pathways and are potentially able to cross-present β-cell-derived autoantigens (Valecka et al., 2018; Germic et al., 2019). Autophagy is an evolutionarily conserved mechanism that helps all cells degrade and recycle biological materials under a range of situations, including ER stress. Specifically, macroautophagy (hereafter referred to as autophagy) involves the transport of cargo contained in double-membraned autophagosomes to the lysosome (Parzych and Klionsky, 2014). Hyperglycemia and the buildup of ROS, as well as endoplasmic reticulum (ER) stress, are known to disturb β-cell homeostasis (Gerber and Rutter, 2017; Newsholme et al., 2019). Furthermore, excessive ROS can damage proteins and organelles, making it more difficult for the cell to activate its adaptive stress response systems. Endogenous activities that help to pacify these cellular stressors and restore homeostasis are thus crucial for β-cell survival. In this context, the role of autophagy in maintaining β-cell homeostasis and increasing cell survival has been examined (Marasco and Linnemann, 2018; Vivot et al., 2020).

CB2R, as described above, is mainly located in the cells of the immune system and participates in the modulation of immune responses (Basu and Dittel, 2011). Moreover, CB2R stimulation has been shown to promote autophagy in various cellular and animal models. Notably, JWH-133 reduced the expression of lipopolysaccharide (LPS)-induced inflammatory genes in autophagy related protein 5 (ATG5)-sufficient macrophages but not in ATG5-deficient cells, and JWH-133 treatment also

protected mice from alcohol-induced liver inflammation and steatosis but was not protective in mice lacking ATG5 in myeloid cells (Denaës et al., 2016). As a result, activation of CB2R in macrophages protects against alcohol-induced steatosis through an autophagy-dependent route (Denaës et al., 2016). Selective activation of CB2R with HU308 had a cardio-protective effect against diabetic cardiomyopathy and protected the cardiomyocytes by promoting autophagy *via* the AMPK-mTOR-p70S6K signaling pathway when maintained under the stress of high glucose (Wu et al., 2018). In addition, autophagy induction and p62-mediated Nrf2 deactivation are linked to CB2R activation-induced osteoblastic differentiation *in vitro* (Xu et al., 2020). The synthetic CB2R agonist AM1241 protects rats from cardiac ischemia-reperfusion injury by triggering autophagy through activation of the Pink1/Parkin pathway (Liu et al., 2021). HU-308 (a CB2R agonist) promotes autophagy, inhibits the NLRP3 inflammasome, and protects mice from autoimmune encephalomyelitis (Shao et al., 2014). JWH133 orchestrates neuronal autophagy in the hippocampus of developing rats with status epilepticus through modulating the mTOR signaling pathway (Wu et al., 2020a). Taken together, these studies imply that activating the CB2R promotes autophagy *in vitro* and *in vivo*. It is therefore reasonable to propose that CB2R plays a critical role in autophagy processes and consequently may protect from the autoimmunity of T1DM by this mechanism.

Antigen-presenting cells such as dendritic cells (DCs) that lack the core autophagy machinery that enables ATG8 (autophagy related protein 8) lipidation, for example, have increased surface MHC-I expression, which is linked to hyper-reactive $CD8^+$T cell responses (Hubbard-Lucey et al., 2014). Blocked internalization and degradation of MHC-I molecules, which involves recruitment of MHC-I molecules *via* (probably membrane coupled) LC3B (microtubule-associated proteins 1A/1B light chain 3B), are among the mechanisms underlying loss of components in the autophagy machinery in MHC-I restricted antigen presentation in DCs (Loi et al., 2016). Interestingly, CB2R has been reported to regulate autophagy in non-pancreatic cells. Nevertheless, there have been no investigations on CB2R-mediated autophagy in pancreatic islets or β-cells, as there have been for antigen processing, immune cell differentiation, and macrophage migration in the context of airway immunomodulation (Carayon et al., 1998; McCoy et al., 1999). Because CB2R activation is known to increase autophagy in other cellular/tissue contexts, CB2R agonists could be a viable treatment option to control $CD8^+$T cell response and MHC-I antigen presentation leading to stress conditions in pancreatic islets during T1DM initiation and progression. Hence, future research into the novel role of CB2R in T1DM and its complications, particularly in pancreatic islets and its immune cell infiltration, would be worthwhile.

CB1R might regulate MHC-I in β-cells and CB2R regulate MHC-II in immune cells since CB1R and not CB2R is found in β-cells (Benner et al., 2014). Autophagy is highly dynamic, ATP-dependent, and maintains photostatic homeostasis in β-cell when proteosome machinery is compromised and could not properly present antigenic peptide through MHC-I in β-cell (Broca et al., 2014). Targeting autophagy pathways regulated by cannabinoids for prevention of T1DM is a pathway worth investigating as a way to prevent presentation of auto- and neo-antigens to APCs (Fierabracci, 2014). Intracellular CB1R and CB2R also play important roles in metabolism and immunity (Brailoiu et al., 2014; Brailoiu et al., 2011). Activation of mitochondria CB1R dysregulates astrocyte glucose metabolism and promotes glycolysis in activated T cells (Jimenez-Blasco et al., 2020). The activation also modulates inflammation by reducing microglia oxygen consumption (Beji et al., 2020) and reduces mitophagy (Kataoka et al., 2020). Rimonabant was found to protect liver ischemia-induced inflammation through increasing autophagic flux, as illustrated by upregulation of proteins in the autophagy pathway, p62 (SQSTM1), Beclin-1 and LC3B-I to LC3B-II conversion (Rezq et al., 2021). On the other hand, CB2R is localized intracellularly at endolysosomes and microinjection of 2-AG into bone sarcoma U2OS cells induced faster and higher amplitude Ca^{2+} release from intracellular calcium pools (Brailoiu et al., 2014) than cytoplasmic CB2R activation. Calcineurin is then activated by calcium and dephosphorylates transcription factor EB (TFEB opposing mTORC1 kinase) (Medina et al., 2015). Dephosphorylation of autophagy Top-Chef TFEB (Cuervo, 2011; Settembre et al., 2011) causes its activation and translocation to the nucleus. The nuclear TFEB subsequently promotes lysosome biogenesis and exocytosis, and upregulates genes involved in autophagy (Settembre et al., 2011), implying links between CB2R and downstream effects on enhancing autophagy. Indeed, increased expression of CB2R is associated with enhanced autophagic flux as shown by enhanced LC3B-I to LC3B-II conversion, upregulation of Beclin-1, and increased p62 degradation in hFOB 1.19 cells derived from osteoblasts (Xu et al., 2020). Furthermore, mice treated with HU308 had some protection from diabetic cardiomyopathy and reduced ischemic myocardial infarction size through similar increases in autophagic flux (Wu et al., 2018; Xu et al., 2020). We propose that CB2R activation causes Ca^{2+} release from endolysosomes through the lysosomal calcium efflux channel MCOLN1 (transient receptor potential mucolipin 1) that not only causes dephosphorylation of TFEB and results in its nuclear translocation, but also increases lysosome contact sites with mitochondria and aids in actively transfer of Ca^{2+} into mitochondria, resulting in reducing their production of ROS, and increasing energy supply for lysosome biogenesis (Peng et al., 2020). Calcium influx and efflux regulate immune cell activation that is intertwined with autophagy (Jia et al., 2013). CB2R's influence in autophagy may be that it participates in the delicate intracellular calcium homeostasis that regulate neo- and auto-antigen presentation in APC cells (**Figure 6**). The yin-yang relationship of CB1R and CB2R actions in islets illustrates the potential therapeutic of Δ^9-tetrahydrocannabivarin (THCV), a dual antagonist/agonist for CB1R and CB2R respectively, for treating T1DM that may improve pancreatic β-cell function (Abioye et al., 2020), possibly by promoting autophagy through antagonism of CB1R

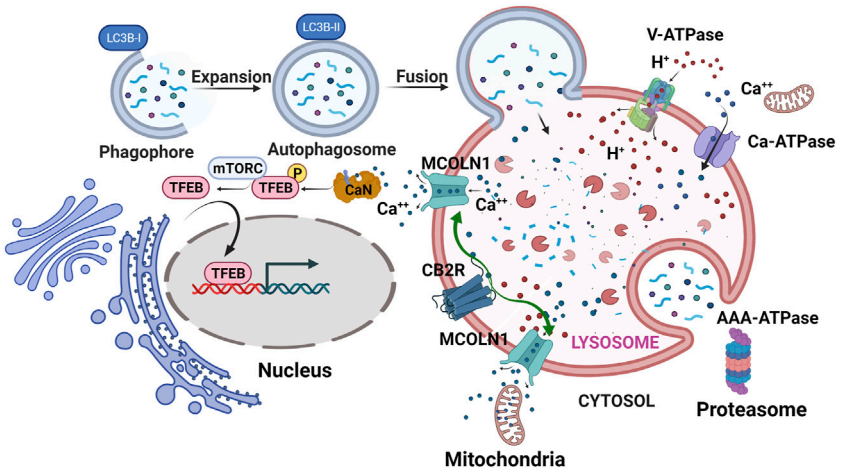


FIGURE 6 | CB2R pro-autophagy effect. Lysosomal CB2R activation results in release of intra-lysosomal Ca^{2+} through MCOLN1 (Mucolipin TRP Cation Channel 1). The released Ca^{2+} tethers mitochondria to lysosomes and some Ca^{2+} ions are transferred to the mitochondria where less ROS is then produced. Calcineurin (CaN) is also activated that then dephosphorylates TFEB causing its translocation to nucleus, downstream of which autophagosomes and lysosomes are generated. Green arrows represent CB2R stimulation of MCOLN1 to release Ca^{2+} ions that enter mitochondria and activate calcineurin (CaN) for TFEB nuclear translocation. V-ATPase, vacuolar-type ATPase; Ca-ATPase, Calcium ATPase; AAA-ATPase, ATPases Associated with diverse cellular activities.

within β-cells and agonism of CB2R in APC cells (Jadoon et al., 2016).

CB2R and Obesity

Obesity increases risk for T1DM, especially in children (Polsky and Ellis, 2015). Adipose tissues from obese individuals contain enlarged adipocytes that secrete inflammatory cytokines such as IL-6, soluble IL6R, TNF-α and MCP-1 into circulation and thereby induce infiltration of macrophages (Lauterbach and Wunderlich, 2017). Obesity associated chronic inflammation causes insulin resistance in muscle, liver, heart, and the endothelial layer of blood vessels by stimulation of Ser/Thr phosphorylation of IRS1 proteins, and by inhibiting insulin receptor signaling both directly and indirectly through action of JNK and IKK-β (Chia and Egan, 2020). eCBs are components of the paracrine and endocrine pathways that regulate appetite/satiety and fatty acid metabolism through central and peripheral actions (Lynes et al., 2019; Behl et al., 2021). Low levels of CBRs are present in mature adipocytes and in primary cultures of rat adipocytes, and under obese conditions, their CB1R expression increases while CB2R decreases (Karaliota et al., 2009). Functions of dually and singly expressed CB1R and CB2R depends on the cell context and the low basal expression of CB1R in peripheral tissues and CB2R in brain regions exert cell type specific amplifiable actions similar to Pascal's leverage (Kim et al., 2020; Liu et al., 2017; Xi et al., 2011), e.g., activation of CB2R induces hyperpolarization of hippocampal and cortical neurons (Stempel et al., 2016; Stumpf et al., 2018). The dynamic ranges of CB1R and CB2R mRNA levels from CNS to peripheral tissues are among the highest of the GPCR superfamily (Liu et al., 2020a) and CB2R is more inducible than is CB1R in the setting of obesity-related inflammation (Yu et al., 2015; Wu et al., 2020b). Pharmacological and genetic inhibition of total-body CB1R results in significant weight loss (Sam et al., 2011; Zimmer et al., 1999). We also found that ablation of CB1R in β-cells, myocytes, and hepatocytes lessens inflammation and improves metabolism in those tissues, especially when animals are placed on high-fat, high-sugar diets (Gonzalez-Mariscal et al., 2018; González-Mariscal et al., 2019; Kim et al., 2020). Peripherally restricted CB1R inverse agonists (Cinar et al., 2020) and CB1R blocking antibodies show promising anti-obesity effects and are under early-stage clinical development (Dao and François, 2021). On the other hand, CB2R germline knockout mice are reported to have increased food intake and total body fat content, especially as they age (Agudo et al., 2010; Alshaarawy et al., 2019). Activation of CB2R promotes β-oxidation (Zheng et al., 2013) and reduces body fat in diet-induced obesity by inhibiting pro-inflammatory M1 macrophage polarization and inducing M2 macrophages to secrete anti-inflammatory cytokines (Wu et al., 2020b). Recently LC3B dependent extracellular vesicle (EV) loading/secretion (LDELS) of lipid droplets was found to be dependent on LC3B-II conjugation to lysosomes, lipidation by ATG7 (autophagy related protein 7), and ceramide synthesis, as distinct from classical autophagy (Leidal and Debnath, 2021; Leidal et al., 2020). We propose that LC3B activation by CB2R is not only involved in intracellular membrane trafficking but also in intercellular signaling in the regulation of EV loading and secretion by lysosomal exocytosis, exosome release, and secretory autophagy (Leidal and Debnath, 2021; Liu et al., 2020b; Buratta et al., 2020). Secreted materials range from cytokines, lipids, and granules to virus particles. Secretory autophagy has been implicated in multiple diseases including cancer and neurodegeneration (New and Thomas, 2019). Pancreatic β-cells secrete insulin-containing EVs into the islet milieu that are recognized by the infiltrating dendritic cells and macrophages in NOD (non-obese diabetic) mice (Ferris et al., 2016), a mouse model of T1DM, resulting in the activation of APCs, which in turn with the help of MHC-II, are responsible for presenting insulin B-chain peptide and its fragments to reactive $CD4^+$T cells (Vomund et al., 2015). Anti-inflammatory CB2R

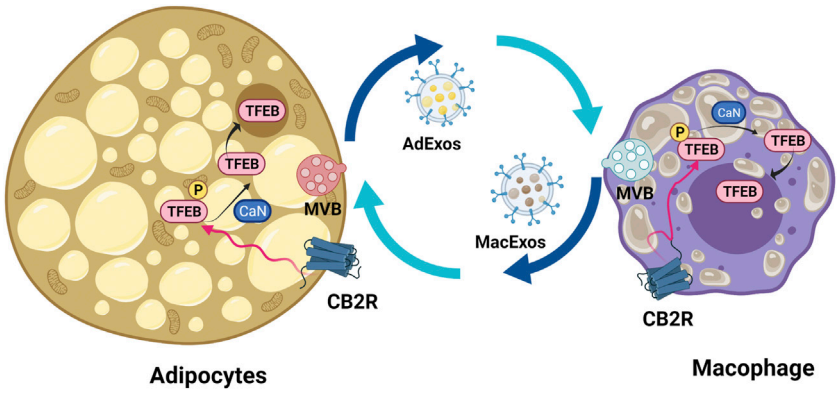


FIGURE 7 | Lipid droplet-loaded extracellular vesicle recycling between adipocyte and adipose tissue macrophage. Activation of CB2R results in TFEB translocation to nucleus and activation of autophagy and lysosome biogenesis, thereby improving the lipid cycle between adipocytes and macrophages. ApExos represents adipocyte exosomes and MacExos macrophage exosomes. The adipocyte multivesicular body (MVB) is represented with pink color and macrophage light blue color.

restrains M1 macrophage activation in the lean state (Wu et al., 2020b; Xu et al., 2013), however, it seems to lose this ability in obese states. Obese adipocytes are depleted of TFEB (Trivedi et al., 2016) and secrete more lipid-filled exosome-sized vesicles (AdExos) that are taken up by adipose tissue macrophages (ATMs) for triacylglyceride hydrolysis that then returns to adipocytes through macrophage presenting exosome-sized vesicles (MacExos) (Flaherty et al., 2019). The accumulation of lipofuscin in ATMs causes a switch from a lean M2 "alternatively activated" state to an obese M1 "classically activated" state generating a F4/80^{+}CD11c^{+}CD45hi dendritic cell subpopulation (Lumeng et al., 2007). CB2R activation reduces Iba1^{+} M1 population and increases the M2 population that might exert protective effects against the vicious lipid cycle between obese adipocytes and ATMs (Zarruk et al., 2012) and activation of TFEB due to dephosphorylation by calcineurin then activates autophagy-based hydrolysis of lipid droplets and protects against obesity-induced insulin resistance (Kim et al., 2021) (**Figure 7**). Global CB2R knockout mice have an obese phenotype; however, whether this is due to dendritic and macrophage CB2R deficiency is not yet known. In order to eventually answer this, we have created *Cnr2*-floxed mice that can be crossed with CX3CR1-Cre and CD11C-Cre mice to generate M1/M2-macrophages and F4/80^{+}CD11c^{+} obesity-associated dendritic cell specific conditional CB2 knockout mice so that we can study macrophage activation and intercellular extracellular vesicle signaling and trafficking between adipocytes, β-cells and APCs (Liu et al., 2020a).

CONCLUSION

The yin-yang relationship of CB1R and CB2R in pancreatic islets involves signaling *via* plasma membrane downstream signaling pathways and intra- and inter-cellular membranal trafficking. We propose that modulation of cannabinoid receptors will ameliorate T1DM by modulation of the mTORC/TFEB/calcineurin axis (Chiocco et al., 2010; Pan et al., 2020) and promotion of lysosome biogenesis that is a hub for T1DM tolerance, autophagy, and extracellular vesicle signaling. There is presently no cannabinoid therapeutic that increases the robustness of β-cells that can withstand the genetic lottery lost by people with pre-symptomatic and symptomatic T1DM. Cost effective, naturally occurring CB2R selective agonists widely used in traditional medicines and diets in Asia and South America for early intervention of diabetes are worthy of study in this regard. Additionally, next generation, selective, peripherally restricted synthetic cannabinoids that work by intervening in both CB1R and CB2R signaling are in the pipeline.

AUTHOR CONTRIBUTIONS

Q-RL and JE conceptualized the idea, did CB1R and CB2R isoform islet expressions, pathway drawing, and wrote the manuscript. KA, QY, PG, and JO'C contribute parts of CB2R roles in autophagy, inflammation, immune tolerance, and obesity.

ACKNOWLEDGMENTS

Q-RL, KA, QY, PG, JO'C, and JE are supported by the Intramural Research Program (IRP) of the National Institute on Aging, NIH; XZ is supported by Ben May Department for Cancer Research, The University of Chicago. Pathway drawings are created using BioRender.com.

REFERENCES

Abioye, A., Ayodele, O., Marinkovic, A., Patidar, R., Akinwekomi, A., and Sanyaolu, A. (2020). Δ9-Tetrahydrocannabivarin (THCV): a Commentary on Potential Therapeutic Benefit for the Management of Obesity and Diabetes. *J. Cannabis Res.* 2, 6. doi:10.1186/s42238-020-0016-7

Agudo, J., Martin, M., Roca, C., Molas, M., Bura, A. S., Zimmer, A., et al. (2010). Deficiency of CB2 Cannabinoid Receptor in Mice Improves Insulin Sensitivity but Increases Food Intake and Obesity with Age. *Diabetologia* 53, 2629–2640. doi:10.1007/s00125-010-1894-6

Alcigir, M. E., Dogan, H. O., Atalay Vural, S., and Yilmaz, F. M. (2017). Neuroprotective Activity of Cannabinoid Receptor-2 against Oxidative Stress and Apoptosis in Rat Pups Having Experimentally-Induced Congenital Hypothyroidism. *Dev. Neurobiol.* 77, 1334–1347. doi:10.1002/dneu.22516

Alexandropoulos, K., Bonito, A. J., Weinstein, E. G., and Herbin, O. (2015). Medullary Thymic Epithelial Cells and central Tolerance in Autoimmune Hepatitis Development: Novel Perspective from a New Mouse Model. *Int. J. Mol. Sci.* 16, 1980–2000. doi:10.3390/ijms16011980

Alon, R., and Shulman, Z. (2011). Chemokine Triggered Integrin Activation and Actin Remodeling Events Guiding Lymphocyte Migration across Vascular Barriers. *Exp. Cel Res* 317, 632–641. doi:10.1016/j.yexcr.2010.12.007

Alshaarawy, O., Kurjan, E., Truong, N., and Olson, L. K. (2019). Diet-Induced Obesity in Cannabinoid-2 Receptor Knockout Mice and Cannabinoid Receptor 1/2 Double-Knockout Mice. *Obesity (Silver Spring)* 27, 454–461. doi:10.1002/oby.22403

Annunziata, P., Cioni, C., Mugnaini, C., and Corelli, F. (2017). Potent Immunomodulatory Activity of a Highly Selective Cannabinoid CB2 Agonist on Immune Cells from Healthy Subjects and Patients with Multiple Sclerosis. *J. Neuroimmunol* 303, 66–74. doi:10.1016/j.jneuroim.2016.12.009

Aseer, K. R., and Egan, J. M. (2021). An Autonomous Cannabinoid System in Islets of Langerhans. *Front. Endocrinol. (Lausanne)* 12, 699661. doi:10.3389/fendo.2021.699661

Basu, S., and Dittel, B. N. (2011). Unraveling the Complexities of Cannabinoid Receptor 2 (CB2) Immune Regulation in Health and Disease. *Immunol. Res.* 51, 26–38. doi:10.1007/s12026-011-8210-5

Behl, T., Chadha, S., Sachdeva, M., Sehgal, A., Kumar, A., Venkatachalam, T, et al. (2021). Understanding the Possible Role of Endocannabinoid System in Obesity. *Prostaglandins Other Lipid Mediat* 152, 106520. doi:10.1016/j.prostaglandins.2020.106520

Beji, C., Loucif, H., Telittchenko, R., Olagnier, D., Dagenais-Lussier, X., and van Grevenynghe, J. (2020). Cannabinoid-Induced Immunomodulation during Viral Infections: A Focus on Mitochondria. *Viruses* 12, 875. doi:10.3390/v12080875

Bellini, G., Olivieri, A. N., Grandone, A., Alessio, M., Gicchino, M. F., Nobili, B., et al. (2015). Association between Cannabinoid Receptor Type 2 Q63R Variant and Oligo/polyarticular Juvenile Idiopathic Arthritis. *Scand. J. Rheumatol.* 44, 284–287. doi:10.3109/03009742.2015.1020863

Benner, C., van der Meulen, T., Cacéres, E., Tigyi, K., Donaldson, C. J., and Huising, M. O. (2014). The Transcriptional Landscape of Mouse Beta Cells Compared to Human Beta Cells Reveals Notable Species Differences in Long Non-coding RNA and Protein-Coding Gene Expression. *BMC Genomics* 15, 620. doi:10.1186/1471-2164-15-620

Bennett, S. T., Lucassen, A. M., Gough, S. C., Powell, E. E., Undlien, D. E., Pritchard, L. E., et al. (1995). Susceptibility to Human Type 1 Diabetes at IDDM2 Is Determined by Tandem Repeat Variation at the Insulin Gene Minisatellite Locus. *Nat. Genet.* 9, 284–292. doi:10.1038/ng0395-284

Brailoiu, G. C., Deliu, E., Marcu, J., Hoffman, N. E., Console-Bram, L., Zhao, P., et al. (2014). Differential Activation of Intracellular versus Plasmalemmal CB2 Cannabinoid Receptors. *Biochemistry* 53, 4990–4999. doi:10.1021/bi500632a

Brailoiu, G. C., Oprea, T. I., Zhao, P., Abood, M. E., and Brailoiu, E. (2011). Intracellular Cannabinoid Type 1 (CB1) Receptors Are Activated by Anandamide. *J. Biol. Chem.* 286, 29166–29174. doi:10.1074/jbc.M110.217463

Braun, M., Khan, Z. T., Khan, M. B., Kumar, M., Ward, A., Achyut, B. R., et al. (2018). Selective Activation of Cannabinoid Receptor-2 Reduces Neuroinflammation after Traumatic Brain Injury via Alternative Macrophage Polarization. *Brain Behav. Immun.* 68, 224–237. doi:10.1016/j.bbi.2017.10.021

Broca, C., Varin, E., Armanet, M., Tourrel-Cuzin, C., Bosco, D., Dalle, S., et al. (2014). Proteasome Dysfunction Mediates High Glucose-Induced Apoptosis in Rodent Beta Cells and Human Islets. *PLoS One* 9, e92066. doi:10.1371/journal.pone.0092066

Buckley, N. E., McCoy, K. L., Mezey, E., Bonner, T., Zimmer, A., Felder, C. C., et al. (2000). Immunomodulation by Cannabinoids Is Absent in Mice Deficient for the Cannabinoid CB(2) Receptor. *Eur. J. Pharmacol.* 396, 141–149. doi:10.1016/s0014-2999(00)00211-9

Buratta, S., Tancini, B., Sagini, K., Delo, F., Chiaradia, E., Urbanelli, L., et al. (2020). Lysosomal Exocytosis, Exosome Release and Secretory Autophagy: The Autophagic- and Endo-Lysosomal Systems Go Extracellular. *Int. J. Mol. Sci.* 21. doi:10.3390/ijms21072576

Campanale, C., Massarelli, C., Savino, I., Locaputo, V., and Uricchio, V. F. (2020). A Detailed Review Study on Potential Effects of Microplastics and Additives of Concern on Human Health. *Int. J. Environ. Res. Public Health* 17. doi:10.3390/ijerph17041212

Carayon, P., Marchand, J., Dussossoy, D., Derocq, J. M., Jbilo, O., Bord, A., et al. (1998). Modulation and Functional Involvement of CB2 Peripheral Cannabinoid Receptors during B-Cell Differentiation. *Blood* 92, 3605–3615. doi:10.1182/blood.v92.10.3605.422k05_3605_3615

Carré, A., and Mallone, R. (2021). Making Insulin and Staying Out of Autoimmune Trouble: The Beta-Cell Conundrum. *Front. Immunol.* 12, 639682. doi:10.3389/fimmu.2021.639682

Cencioni, M. T., Chiurchiù, V., Catanzaro, G., Borsellino, G., Bernardi, G., Battistini, L., et al. (2010). Anandamide Suppresses Proliferation and Cytokine Release from Primary Human T-Lymphocytes Mainly via CB2 Receptors. *PLoS One* 5, e8688. doi:10.1371/journal.pone.0008688

Chen, T., Xiong, Y., Long, M., Zheng, D., Ke, H., Xie, J., et al. (2019). Electro-acupuncture Pretreatment at Zusanli (ST36) Acupoint Attenuates Lipopolysaccharide-Induced Inflammation in Rats by Inhibiting Ca2+ Influx Associated with Cannabinoid CB2 Receptors. *Inflammation* 42, 211–220. doi:10.1007/s10753-018-0885-5

Chia, C., and Egan, J. (2020). Incretins in Obesity and Diabetes. *Ann. N. Y Acad. Sci.* 1461, 104–126. doi:10.1111/nyas.14211

Chiocco, M. J., Zhu, X., Walther, D., Pletnikova, O., Troncoso, J. C., Uhl, G. R., et al. (2010). Fine Mapping of Calcineurin (PPP3CA) Gene Reveals Novel Alternative Splicing Patterns, Association of 5'UTR Trinucleotide Repeat with Addiction Vulnerability, and Differential Isoform Expression in Alzheimer's Disease. *Subst. Use Misuse* 45, 1809–1826. doi:10.3109/10826084.2010.482449

Cinar, R., Iyer, M. R., and Kunos, G. (2020). The Therapeutic Potential of Second and Third Generation CB1R Antagonists. *Pharmacol. Ther.* 208, 107477. doi:10.1016/j.pharmthera.2020.107477

Coopman, K., Smith, L. D., Wright, K. L., and Ward, S. G. (2007). Temporal Variation in CB2R Levels Following T Lymphocyte Activation: Evidence that Cannabinoids Modulate CXCL12-Induced Chemotaxis. *Int. Immunopharmacol* 7, 360–371. doi:10.1016/j.intimp.2006.11.008

Cuervo, A. M. (2011). Cell Biology. Autophagy's Top Chef. *Science* 332, 1392–1393. doi:10.1126/science.1208607

Dao, M., and François, H. (2021). Cannabinoid Receptor 1 Inhibition in Chronic Kidney Disease: A New Therapeutic Toolbox. *Front. Endocrinol. (Lausanne)* 12, 720734. doi:10.3389/fendo.2021.720734

Denaës, T., Lodder, J., Chobert, M. N., Ruiz, I., Pawlotsky, J. M., Lotersztajn, S., et al. (2016). The Cannabinoid Receptor 2 Protects against Alcoholic Liver Disease via a Macrophage Autophagy-dependent Pathway. *Sci. Rep.* 6, 28806. doi:10.1038/srep28806

Diedisheim, M., Oshima, M., Albagli, O., Huldt, C. W., Ahlstedt, I., Clausen, M., et al. (2018). Modeling Human Pancreatic Beta Cell Dedifferentiation. *Mol. Metab.* 10, 74–86. doi:10.1016/j.molmet.2018.02.002

Eisenbarth, G. S. (1986). Type I Diabetes Mellitus. A Chronic Autoimmune Disease. *N. Engl. J. Med.* 314, 1360–1368. doi:10.1056/NEJM198605223142106

Eisenstein, T. K., and Meissler, J. J. (2015). Effects of Cannabinoids on T-Cell Function and Resistance to Infection. *J. Neuroimmune Pharmacol.* 10, 204–216. doi:10.1007/s11481-015-9603-3

Eizirik, D. L., Colli, M. L., and Ortis, F. (2009). The Role of Inflammation in Insulitis and Beta-Cell Loss in Type 1 Diabetes. *Nat. Rev. Endocrinol.* 5, 219–226. doi:10.1038/nrendo.2009.21

Elphick, M. R. (2007). BfCBR: a Cannabinoid Receptor Ortholog in the Cephalochordate Branchiostoma floridae (Amphioxus). *Gene* 399, 65–71. doi:10.1016/j.gene.2007.04.025

Elphick, M. R. (2002). Evolution of Cannabinoid Receptors in Vertebrates: Identification of a CB(2) Gene in the Puffer Fish Fugu Rubripes. *Biol. Bull.* 202, 104–107. doi:10.2307/1543648

Elphick, M. R., Satou, Y., and Satoh, N. (2003). The Invertebrate Ancestry of Endocannabinoid Signalling: an Orthologue of Vertebrate Cannabinoid Receptors in the Urochordate Ciona intestinalis. *Gene* 302, 95–101. doi:10.1016/s0378-1119(02)01094-6

Elphick, M. R. (2012). The Evolution and Comparative Neurobiology of Endocannabinoid Signalling. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 367, 3201–3215. doi:10.1098/rstb.2011.0394

Fan, Y., Rudert, W. A., Grupillo, M., He, J., Sisino, G., and Trucco, M. (2009). Thymus-specific Deletion of Insulin Induces Autoimmune Diabetes. *EMBO J.* 28, 2812–2824. doi:10.1038/emboj.2009.212

Fava, A., Cimbro, R., Wigley, F. M., Liu, Q. R., Rosen, A., and Boin, F. (2016). Frequency of Circulating Topoisomerase-I-specific CD4 T Cells Predicts Presence and Progression of Interstitial Lung Disease in Scleroderma. *Arthritis Res. Ther.* 18, 99. doi:10.1186/s13075-016-0993-2

Fernández-Ruiz, J., Romero, J., Velasco, G., Tolón, R. M., Ramos, J. A., and Guzmán, M. (2007). Cannabinoid CB2 Receptor: a New Target for Controlling Neural Cell Survival? *Trends Pharmacol. Sci.* 28, 39–45. doi:10.1016/j.tips.2006.11.001

Ferris, S. T., Carrero, J. A., and Unanue, E. R. (2016). Antigen Presentation Events during the Initiation of Autoimmune Diabetes in the NOD Mouse. *J. Autoimmun.* 71, 19–25. doi:10.1016/j.jaut.2016.03.007

Fierabracci, A. (2014). The Putative Role of Proteolytic Pathways in the Pathogenesis of Type 1 Diabetes Mellitus: the 'autophagy' Hypothesis. *Med. Hypotheses* 82, 553–557. doi:10.1016/j.mehy.2014.02.010

Flaherty, S. E., 3rd, Grijalva, A., Xu, X., Ables, E., Nomani, A., and Ferrante, A. W., Jr. (2019). A Lipase-independent Pathway of Lipid Release and Immune Modulation by Adipocytes. *Science* 363, 989–993. doi:10.1126/science.aaw2586

Flajnik, M. F., and Kasahara, M. (2010). Origin and Evolution of the Adaptive Immune System: Genetic Events and Selective Pressures. *Nat. Rev. Genet.* 11, 47–59. doi:10.1038/nrg2703

Galdino, P. M., Nascimento, M. V., Florentino, I. F., Lino, R. C., Fajemiroye, J. O., Chaibub, B. A., et al. (2012). The Anxiolytic-like Effect of an Essential Oil Derived from Spiranthera Odoratissima A. St. Hil. Leaves and its Major Component, β-caryophyllene, in Male Mice. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 38, 276–284. doi:10.1016/j.pnpbp.2012.04.012

Galiègue, S., Mary, S., Marchand, J., Dussossoy, D., Carrière, D., Carayon, P., et al. (1995). Expression of central and Peripheral Cannabinoid Receptors in Human Immune Tissues and Leukocyte Subpopulations. *Eur. J. Biochem.* 232, 54–61. doi:10.1111/j.1432-1033.1995.tb20780.x

Geddo, F., Scandiffio, R., Antoniotti, S., Cottone, E., Querio, G., Maffei, M. E., et al. (2019). PipeNig®-FL, a Fluid Extract of Black Pepper (Piper Nigrum L.) with a High Standardized Content of Trans-β-caryophyllene, Reduces Lipid Accumulation in 3T3-L1 Preadipocytes and Improves Glucose Uptake in C2C12 Myotubes. *Nutrients* 11, 2788. doi:10.3390/nu11112788

Gerber, P. A., and Rutter, G. A. (2017). The Role of Oxidative Stress and Hypoxia in Pancreatic Beta-Cell Dysfunction in Diabetes Mellitus. *Antioxid. Redox Signal.* 26, 501–518. doi:10.1089/ars.2016.6755

Germic, N., Frangez, Z., Yousefi, S., and Simon, H. U. (2019). Regulation of the Innate Immune System by Autophagy: Monocytes, Macrophages, Dendritic Cells and Antigen Presentation. *Cell Death Differ* 26, 715–727. doi:10.1038/s41418-019-0297-6

González-Mariscal, I., Krzysik-Walker, S. M., Doyle, M. E., Liu, Q. R., Cimbro, R., Santa-Cruz Calvo, S., et al. (2016). Human CB1 Receptor Isoforms, Present in Hepatocytes and β-cells, Are Involved in Regulating Metabolism. *Sci. Rep.* 6, 33302. doi:10.1038/srep33302

González-Mariscal, I., Montoro, R. A., O'Connell, J. F., Kim, Y., Gonzalez-Freire, M., Liu, Q. R., et al. (2019). Muscle Cannabinoid 1 Receptor Regulates Il-6 and Myostatin Expression, Governing Physical Performance and Whole-Body Metabolism. *FASEB J.* 33, 5850–5863. doi:10.1096/fj.201801145R

Gonzalez-Mariscal, I., Montoro, R. A., Doyle, M. E., Liu, Q. R., Rouse, M., O'Connell, J. F., et al. (2018). Absence of Cannabinoid 1 Receptor in Beta Cells Protects against High-Fat/high-Sugar Diet-Induced Beta Cell Dysfunction and Inflammation in Murine Islets. *Diabetologia* 61, 1470. doi:10.1007/s00125-018-4576-4

Gorantla, S., Makarov, E., Roy, D., Finke-Dwyer, J., Murrin, L. C., Gendelman, H. E., et al. (2010). Immunoregulation of a CB2 Receptor Agonist in a Murine Model of neuroAIDS. *J. Neuroimmune Pharmacol.* 5, 456–468. doi:10.1007/s11481-010-9225-8

Grishman, E. K., White, P. C., and Savani, R. C. (2012). Toll-like Receptors, the NLRP3 Inflammasome, and Interleukin-1β in the Development and Progression of Type 1 Diabetes. *Pediatr. Res.* 71, 626–632. doi:10.1038/pr.2012.24

Group, T. S. (2007). The Environmental Determinants of Diabetes in the Young (TEDDY) Study: Study Design. *Pediatr. Diabetes* 8, 286–298. doi:10.1111/j.1399-5448.2007.00269.x

Guha, M., Saare, M., Maslovskaja, J., Kisand, K., Liiv, I., Haljasorg, U., et al. (2017). DNA Breaks and Chromatin Structural Changes Enhance the Transcription of Autoimmune Regulator Target Genes. *J. Biol. Chem.* 292, 6542–6554. doi:10.1074/jbc.M116.764704

Gui, H., Liu, X., Wang, Z. W., He, D. Y., Su, D. F., and Dai, S. M. (2014). Expression of Cannabinoid Receptor 2 and its Inhibitory Effects on Synovial Fibroblasts in Rheumatoid Arthritis. *Rheumatology (Oxford)* 53, 802–809. doi:10.1093/rheumatology/ket447

Guo, K., Mou, X., Huang, J., Xiong, N., and Li, H. (2014). Trans-caryophyllene Suppresses Hypoxia-Induced Neuroinflammatory Responses by Inhibiting NF-Kb Activation in Microglia. *J. Mol. Neurosci.* 54, 41–48. doi:10.1007/s12031-014-0243-5

Gupta, M., Graham, J., McNeeny, B., Zarghami, M., Landin-Olsson, M., Hagopian, W. A., et al. (2006). MHC Class I Chain-Related Gene-A Is Associated with IA2 and IAA but Not GAD in Swedish Type 1 Diabetes Mellitus. *Ann. N. Y Acad. Sci.* 1079, 229–239. doi:10.1196/annals.1375.036

Han, X., He, Y., Bi, G. H., Zhang, H. Y., Song, R., Liu, Q. R., et al. (2017). CB1 Receptor Activation on VgluT2-Expressing Glutamatergic Neurons Underlies Δ9-Tetrahydrocannabinol (Δ9-Thc)-Induced Aversive Effects in Mice. *Sci. Rep.* 7, 12315. doi:10.1038/s41598-017-12399-z

Herold, K. C., Bundy, B. N., Long, S. A., Bluestone, J. A., DiMeglio, L. A., Dufort, M. J., et al. (2019). An Anti-CD3 Antibody, Teplizumab, in Relatives at Risk for Type 1 Diabetes. *N. Engl. J. Med.* 381, 603–613. doi:10.1056/NEJMoa1902226

Honkanen, J., Nieminen, J. K., Gao, R., Luopajarvi, K., Salo, H. M., Ilonen, J., et al. (2010). IL-17 Immunity in Human Type 1 Diabetes. *J. Immunol.* 185, 1959–1967. doi:10.4049/jimmunol.1000788

Hu, Y., Ranganathan, M., Shu, C., Liang, X., Ganesh, S., Osafo-Addo, A., et al. (2020). Single-cell Transcriptome Mapping Identifies Common and Cell-type Specific Genes Affected by Acute Delta9-tetrahydrocannabinol in Humans. *Sci. Rep.* 10, 3450. doi:10.1038/s41598-020-59827-1

Hubbard-Lucey, V. M., Shono, Y., Maurer, K., West, M. L., Singer, N. V., Ziegler, C. G., et al. (2014). Autophagy Gene Atg16L1 Prevents Lethal T Cell Alloreactivity Mediated by Dendritic Cells. *Immunity* 41, 579–591. doi:10.1016/j.immuni.2014.09.011

Ilonen, J., Lempainen, J., and Veijola, R. (2019). The Heterogeneous Pathogenesis of Type 1 Diabetes Mellitus. *Nat. Rev. Endocrinol.* 15, 635–650. doi:10.1038/s41574-019-0254-y

Insel, R. A., Dunne, J. L., Atkinson, M. A., Chiang, J. L., Dabelea, D., Gottlieb, P. A., et al. (2015). Staging Presymptomatic Type 1 Diabetes: a Scientific Statement of JDRF, the Endocrine Society, and the American Diabetes Association. *Diabetes Care* 38, 1964–1974. doi:10.2337/dc15-1419

Ismail, M., and Khawaja, G. (2018). Study of Cannabinoid Receptor 2 Q63R Gene Polymorphism in Lebanese Patients with Rheumatoid Arthritis. *Clin. Rheumatol.* 37, 2933–2938. doi:10.1007/s10067-018-4217-9

Jadoon, K. A., Ratcliffe, S. H., Barrett, D. A., Thomas, E. L., Stott, C., Bell, J. D., et al. (2016). Efficacy and Safety of Cannabidiol and Tetrahydrocannabivarin on Glycemic and Lipid Parameters in Patients with Type 2 Diabetes: A Randomized, Double-Blind, Placebo-Controlled, Parallel Group Pilot Study. *Diabetes Care* 39, 1777–1786. doi:10.2337/dc16-0650

Jia, W., He, M., McLeod, I., and He, Y. (2013). Autophagy, a Novel Pathway to Regulate Calcium Mobilization in T Lymphocytes. *Front. Immunol.* 4, 179. doi:10.3389/fimmu.2013.00179

Jimenez-Blasco, D., Busquets-Garcia, A., Hebert-Chatelain, E., Serrat, R., Vicente-Gutierrez, C., Ioannidou, C., et al. (2020). Glucose Metabolism Links Astroglial Mitochondria to Cannabinoid Effects. *Nature* 583, 603–608. doi:10.1038/s41586-020-2470-y

Jing, N., Fang, B., Li, Z., and Tian, A. (2020). Exogenous Activation of Cannabinoid-2 Receptor Modulates TLR4/MMP9 Expression in a Spinal Cord Ischemia Reperfusion Rat Model. *J. Neuroinflammation* 17, 101. doi:10.1186/s12974-020-01784-7

Joseph, J., Niggemann, B., Zaenker, K. S., and Entschladen, F. (2004). Anandamide Is an Endogenous Inhibitor for the Migration of Tumor Cells and T Lymphocytes. *Cancer Immunol. Immunother.* 53, 723–728. doi:10.1007/s00262-004-0509-9

Joshi, N., and Onaivi, E. S. (2019). Endocannabinoid System Components: Overview and Tissue Distribution. *Adv. Exp. Med. Biol.* 1162, 1–12. doi:10.1007/978-3-030-21737-2_1

Jurewicz, M. M., and Stern, L. J. (2019). Class II MHC Antigen Processing in Immune Tolerance and Inflammation. *Immunogenetics* 71, 171–187. doi:10.1007/s00251-018-1095-x

Kapellos, T. S., Recio, C., Greaves, D. R., and Iqbal, A. J. (2017). Cannabinoid Receptor 2 Modulates Neutrophil Recruitment in a Murine Model of Endotoxemia. *Mediators Inflamm.* 2017, 4315412. doi:10.1155/2017/4315412

Kapellos, T. S., Taylor, L., Feuerborn, A., Valaris, S., Hussain, M. T., Rainger, G. E., et al. (2019). Cannabinoid Receptor 2 Deficiency Exacerbates Inflammation and Neutrophil Recruitment. *FASEB J.* 33, 6154–6167. doi:10.1096/fj.201802524R

Karaliota, S., Siafaka-Kapadai, A., Gontinou, C., Psarra, K., and Mavri-Vavayanni, M. (2009). Anandamide Increases the Differentiation of Rat Adipocytes and Causes PPARgamma and CB1 Receptor Upregulation. *Obesity (Silver Spring)* 17, 1830–1838. doi:10.1038/oby.2009.177

Karmaus, P. W., Chen, W., Crawford, R., Kaplan, B. L., and Kaminski, N. E. (2013). Δ9-tetrahydrocannabinol Impairs the Inflammatory Response to Influenza Infection: Role of Antigen-Presenting Cells and the Cannabinoid Receptors 1 and 2. *Toxicol. Sci.* 131, 419–433. doi:10.1093/toxsci/kfs315

Kataoka, K., Bilkei-Gorzo, A., Nozaki, C., Togo, A., Nakamura, K., Ohta, K., et al. (2020). Age-dependent Alteration in Mitochondrial Dynamics and Autophagy in Hippocampal Neuron of Cannabinoid CB1 Receptor-Deficient Mice. *Brain Res. Bull.* 160, 40–49. doi:10.1016/j.brainresbull.2020.03.014

Kaufmann, U., Kahlfuss, S., Yang, J., Ivanova, E., Koralov, S. B., and Feske, S. (2019). Calcium Signaling Controls Pathogenic Th17 Cell-Mediated Inflammation by Regulating Mitochondrial Function. *Cell Metab* 29, 1104–e6. doi:10.1016/j.cmet.2019.01.019

Ke, P., Shao, B. Z., Xu, Z. Q., Wei, W., Han, B. Z., Chen, X. W., et al. (2016). Activation of Cannabinoid Receptor 2 Ameliorates DSS-Induced Colitis through Inhibiting NLRP3 Inflammasome in Macrophages. *PLoS One* 11, e0155076. doi:10.1371/journal.pone.0155076

Kim, J., Kim, S. H., Kang, H., Lee, S., Park, S. Y., Cho, Y., et al. (2021). TFEB-GDF15 axis Protects against Obesity and Insulin Resistance as a Lysosomal Stress Response. *Nat. Metab.* 3, 410–427. doi:10.1038/s42255-021-00368-w

Kim, Y., Gautam, S., Aseer, K. R., Kim, J., Chandrasekaran, P., Mazucanti, C. H., et al. (2020). Hepatocyte Cannabinoid 1 Receptor Nullification Alleviates Toxin-Induced Liver Damage via NF-Kb Signaling. *Cell Death Dis* 11, 1044. doi:10.1038/s41419-020-03261-8

King, S. E., and Skinner, M. K. (2020). Epigenetic Transgenerational Inheritance of Obesity Susceptibility. *Trends Endocrinol. Metab.* 31, 478–494. doi:10.1016/j.tem.2020.02.009

Knip, M., and Siljander, H. (2016). The Role of the Intestinal Microbiota in Type 1 Diabetes Mellitus. *Nat. Rev. Endocrinol.* 12, 154–167. doi:10.1038/nrendo.2015.218

Kraj, P., and Ignatowicz, L. (2018). The Mechanisms Shaping the Repertoire of CD4+ Foxp3+ Regulatory T Cells. *Immunology* 153, 290–296. doi:10.1111/imm.12859

Lauterbach, M. A., and Wunderlich, F. T. (2017). Macrophage Function in Obesity-Induced Inflammation and Insulin Resistance. *Pflugers Arch.* 469, 385–396. doi:10.1007/s00424-017-1955-5

Leidal, A. M., and Debnath, J. (2021). Emerging Roles for the Autophagy Machinery in Extracellular Vesicle Biogenesis and Secretion. *FASEB Bioadv* 3, 377–386. doi:10.1096/fba.2020-00138

Leidal, A. M., Huang, H. H., Marsh, T., Solvik, T., Zhang, D., Ye, J., et al. (2020). The LC3-Conjugation Machinery Specifies the Loading of RNA-Binding Proteins into Extracellular Vesicles. *Nat. Cel Biol* 22, 187–199. doi:10.1038/s41556-019-0450-y

Leinwand, K. L., Jones, A. A., Huang, R. H., Jedlicka, P., Kao, D. J., de Zoeten, E. F., et al. (2017). Cannabinoid Receptor-2 Ameliorates Inflammation in Murine Model of Crohn's Disease. *J. Crohns Colitis* 11, 1369–1380. doi:10.1093/ecco-jcc/jjx096

Levi, D., and Polychronakos, C. (2013). Expression Profile of a Clonal Insulin-Expressing Epithelial Cell in the Thymus. *Mol. Immunol.* 56, 804–810. doi:10.1016/j.molimm.2013.07.015

Li, X., Hua, T., Vemuri, K., Ho, J. H., Wu, Y., Wu, L., et al. (2019). Crystal Structure of the Human Cannabinoid Receptor CB2. *Cell* 176, 459–e13. doi:10.1016/j.cell.2018.12.011

Li, Y., Li, C., Li, S., Peng, Q., An, N. A., He, A., et al. (2018). Human Exonization through Differential Nucleosome Occupancy. *Proc. Natl. Acad. Sci. U S A.* 115, 8817–8822. doi:10.1073/pnas.1802561115

Littman, D. R., and Rudensky, A. Y. (2010). Th17 and Regulatory T Cells in Mediating and Restraining Inflammation. *Cell* 140, 845–858. doi:10.1016/j.cell.2010.02.021

Liu, Q., Wu, Z., Liu, Y., Chen, L., Zhao, H., Guo, H., et al. (2020). Cannabinoid Receptor 2 Activation Decreases Severity of Cyclophosphamide-Induced Cystitis via Regulating Autophagy. *Neurourol Urodyn* 39, 158–169. doi:10.1002/nau.24205

Liu, Q. R., Canseco-Alba, A., Zhang, H. Y., Tagliaferro, P., Chung, M., Dennis, E., et al. (2017). Cannabinoid Type 2 Receptors in Dopamine Neurons Inhibits Psychomotor Behaviors, Alters Anxiety, Depression and Alcohol Preference. *Sci. Rep.* 7, 17410. doi:10.1038/s41598-017-17796-y

Liu, Q. R., Canseco-Alba, A., Liang, Y., Ishiguro, H., and Onaivi, E. S. (2020). Low Basal CB2R in Dopamine Neurons and Microglia Influences Cannabinoid Tetrad Effects. *Int. J. Mol. Sci.* 21. doi:10.3390/ijms21249763

Liu, Q. R., Huang, N. S., Qu, H., O'Connell, J. F., Gonzalez-Mariscal, I., Santa-Cruz-Calvo, S., et al. (2019). Identification of Novel Mouse and Rat CB1R Isoforms and In Silico Modeling of Human CB1R for Peripheral Cannabinoid Therapeutics. *Acta Pharmacol. Sin* 40, 387–397. doi:10.1038/s41401-018-0152-1

Liu, Q. R., Pan, C. H., Hishimoto, A., Li, C. Y., Xi, Z. X., Llorente-Berzal, A., et al. (2009). Species Differences in Cannabinoid Receptor 2 (CNR2 Gene): Identification of Novel Human and Rodent CB2 Isoforms, Differential Tissue Expression and Regulation by Cannabinoid Receptor Ligands. *Genes Brain Behav.* 8, 519–530. doi:10.1111/j.1601-183X.2009.00498.x

Liu, W., Chen, C., Gu, X., Zhang, L., Mao, X., Chen, Z., et al. (2021). AM1241 Alleviates Myocardial Ischemia-Reperfusion Injury in Rats by Enhancing Pink1/Parkin-Mediated Autophagy. *Life Sci.* 272, 119228. doi:10.1016/j.lfs.2021.119228

Loi, M., Müller, A., Steinbach, K., Niven, J., Barreira da Silva, R., Paul, P., et al. (2016). Macroautophagy Proteins Control MHC Class I Levels on Dendritic Cells and Shape Anti-viral CD8(+) T Cell Responses. *Cell Rep* 15, 1076–1087. doi:10.1016/j.celrep.2016.04.002

López, A., Aparicio, N., Pazos, M. R., Grande, M. T., Barreda-Manso, M. A., Benito-Cuesta, I., et al. (2018). Cannabinoid CB2 Receptors in the Mouse Brain: Relevance for Alzheimer's Disease. *J. Neuroinflammation* 15, 158. doi:10.1186/s12974-018-1174-9

Lu, H. C., and Mackie, K. (2021). Review of the Endocannabinoid System. *Biol. Psychiatry Cogn. Neurosci. Neuroimaging* 6, 607–615. doi:10.1016/j.bpsc.2020.07.016

Lumeng, C. N., Bodzin, J. L., and Saltiel, A. R. (2007). Obesity Induces a Phenotypic Switch in Adipose Tissue Macrophage Polarization. *J. Clin. Invest.* 117, 175–184. doi:10.1172/JCI29881

Lundberg, A. S., and McDevitt, H. O. (1992). Evolution of Major Histocompatibility Complex Class II Allelic Diversity: Direct Descent in Mice and Humans. *Proc. Natl. Acad. Sci. U S A.* 89, 6545–6549. doi:10.1073/pnas.89.14.6545

Lynes, M., Kodani, S., and Tseng, Y. (2019). Lipokines and Thermogenesis. *Endocrinology* 160, 2314–2325. doi:10.1210/en.2019-00337

Mahmoud Gouda, H., and Mohamed Kamel, N. R. (2013). Cannabinoid CB2 Receptor Gene (CNR2) Polymorphism Is Associated with Chronic Childhood Immune Thrombocytopenia in Egypt. *Blood Coagul. Fibrinolysis* 24, 247–251. doi:10.1097/MBC.0b013e32835aba1d

Maja Cigrovski Berkovic, M. C., Bilic-Curcic, I., Gradiser, M., Herman-Mahecic, D., Cigrovski, V., and Ivandic, M. (2017). Are We Compensating for the Lack of Physical Activity in Our Diabetic Patients with Treatment Intensification? *Sports (Basel)* 5, 58. doi:10.3390/sports5030058

Marasco, M. R., and Linnemann, A. K. (2018). β-Cell Autophagy in Diabetes Pathogenesis. *Endocrinology* 159, 2127–2141. doi:10.1210/en.2017-03273

Mariño, E., Villanueva, J., Walters, S., Liuwantara, D., Mackay, F., and Grey, S. T. (2009). CD4(+)CD25(+) T-Cells Control Autoimmunity in the Absence of B-Cells. *Diabetes* 58, 1568–1577. doi:10.2337/db08-1504

Marti-Solano, M., Crilly, S. E., Malinverni, D., Munk, C., Harris, M., Pearce, A., et al. (2020). Combinatorial Expression of GPCR Isoforms Affects Signalling and Drug Responses. *Nature* 587, 650–656. doi:10.1038/s41586-020-2888-2

Mathis, D., and Benoist, C. (2009). Aire. *Annu. Rev. Immunol.* 27, 287–312. doi:10.1146/annurev.immunol.25.022106.141532

Matias, I., McPartland, J. M., and Di Marzo, V. (2005). Occurrence and Possible Biological Role of the Endocannabinoid System in the Sea Squirt Ciona intestinalis. *J. Neurochem.* 93, 1141–1156. doi:10.1111/j.1471-4159.2005.03103.x

McCoy, K. L., Matveyeva, M., Carlisle, S. J., and Cabral, G. A. (1999). Cannabinoid Inhibition of the Processing of Intact Lysozyme by Macrophages: Evidence for CB2 Receptor Participation. *J. Pharmacol. Exp. Ther.* 289, 1620–1625.

McPartland, J. M., Matias, I., Di Marzo, V., and Glass, M. (2006). Evolutionary Origins of the Endocannabinoid System. *Gene* 370, 64–74. doi:10.1016/j.gene.2005.11.004

Medina, D. L., Di Paola, S., Peluso, I., Armani, A., De Stefani, D., Venditti, R., et al. (2015). Lysosomal Calcium Signalling Regulates Autophagy through Calcineurin and TFEB. *Nat. Cel Biol* 17, 288–299. doi:10.1038/ncb3114

Mestre, L., Correa, F., Arévalo-Martín, A., Molina-Holgado, E., Valenti, M., Ortar, G., et al. (2005). Pharmacological Modulation of the Endocannabinoid System in a Viral Model of Multiple Sclerosis. *J. Neurochem.* 92, 1327–1339. doi:10.1111/j.1471-4159.2004.02979.x

Michels, A., Zhang, L., Khadra, A., Kushner, J. A., Redondo, M. J., and Pietropaolo, M. (2015). Prediction and Prevention of Type 1 Diabetes: Update on success of Prediction and Struggles at Prevention. *Pediatr. Diabetes* 16, 465–484. doi:10.1111/pedi.12299

Muralidharan, C., Conteh, A. M., Marasco, M. R., Crowder, J. J., Kuipers, J., de Boer, P., et al. (2021). Pancreatic Beta Cell Autophagy Is Impaired in Type 1 Diabetes. *Diabetologia* 64, 865–877. doi:10.1007/s00125-021-05387-6

Nakahira, K., Haspel, J. A., Rathinam, V. A., Lee, S. J., Dolinay, T., Lam, H. C., et al. (2011). Autophagy Proteins Regulate Innate Immune Responses by Inhibiting the Release of Mitochondrial DNA Mediated by the NALP3 Inflammasome. *Nat. Immunol.* 12, 222–230. doi:10.1038/ni.1980

Nascimento, M. V., Galdino, P. M., Florentino, I. F., de Brito, A. F., Vanderlinde, F. A., de Paula, J. R., et al. (2012). Anti-inflammatory Effect of Spiranthera Odoratissima A. St.-Hil. Leaves Involves Reduction of TNF-α. *Nat. Prod. Res.* 26, 2274–2279. doi:10.1080/14786419.2011.653973

Navarrete, C., Carrillo-Salinas, F., Palomares, B., Mecha, M., Jiménez-Jiménez, C., Mestre, L., et al. (2018). Hypoxia Mimetic Activity of VCE-004.8, a Cannabidiol Quinone Derivative: Implications for Multiple Sclerosis Therapy. *J. Neuroinflammation* 15, 64. doi:10.1186/s12974-018-1103-y

New, J., and Thomas, S. M. (2019). Autophagy-dependent Secretion: Mechanism, Factors Secreted, and Disease Implications. *Autophagy* 15, 1682–1693. doi:10.1080/15548627.2019.1596479

Newsholme, P., Keane, K. N., Carlessi, R., and Cruzat, V. (2019). Oxidative Stress Pathways in Pancreatic β-cells and Insulin-Sensitive Cells and Tissues: Importance to Cell Metabolism, Function, and Dysfunction. *Am. J. Physiol. Cel Physiol* 317, C420–C433. doi:10.1152/ajpcell.00141.2019

Nielsen, J. E., Rolland, A. D., Rajpert-De Meyts, E., Janfelt, C., Jørgensen, A., Winge, S. B., et al. (2019). Characterisation and Localisation of the Endocannabinoid System Components in the Adult Human Testis. *Sci. Rep.* 9, 12866. doi:10.1038/s41598-019-49177-y

Noble, J. A., and Valdes, A. M. (2011). Genetics of the HLA Region in the Prediction of Type 1 Diabetes. *Curr. Diab Rep.* 11, 533–542. doi:10.1007/s11892-011-0223-x

Øynebråten, I. (2020). Involvement of Autophagy in MHC Class I Antigen Presentation. *Scand. J. Immunol.* 92, e12978. doi:10.1111/sji.12978

Pacher, P., Kogan, N. M., and Mechoulam, R. (2020). Beyond THC and Endocannabinoids. *Annu. Rev. Pharmacol. Toxicol.* 60, 637–659. doi:10.1146/annurev-pharmtox-010818-021441

Palazuelos, J., Davoust, N., Julien, B., Hatterer, E., Aguado, T., Mechoulam, R., et al. (2008). The CB(2) Cannabinoid Receptor Controls Myeloid Progenitor Trafficking: Involvement in the Pathogenesis of an Animal Model of Multiple Sclerosis. *J. Biol. Chem.* 283, 13320–13329. doi:10.1074/jbc.M707960200

Pan, B., Li, J., Parajuli, N., Tian, Z., Wu, P., Lewno, M. T., et al. (2020). The Calcineurin-TFEB-P62 Pathway Mediates the Activation of Cardiac Macroautophagy by Proteasomal Malfunction. *Circ. Res.* 127, 502–518. doi:10.1161/CIRCRESAHA.119.316007

Parzych, K. R., and Klionsky, D. J. (2014). An Overview of Autophagy: Morphology, Mechanism, and Regulation. *Antioxid. Redox Signal.* 20, 460–473. doi:10.1089/ars.2013.5371

Patterson, C. C., Karuranga, S., Salpea, P., Saeedi, P., Dahlquist, G., Soltesz, G., et al. (2019). Worldwide Estimates of Incidence, Prevalence and Mortality of Type 1 Diabetes in Children and Adolescents: Results from the International Diabetes Federation Diabetes Atlas, 9th Edition. *Diabetes Res. Clin. Pract.* 157, 107842. doi:10.1016/j.diabres.2019.107842

Pellati, F., Brighenti, V., Sperlea, J., Marchetti, L., Bertelli, D., and Benvenuti, S. (2018). New Methods for the Comprehensive Analysis of Bioactive Compounds in Cannabis Sativa L. (Hemp). *Molecules* 23, 2639. doi:10.3390/molecules23102639

Peng, W., Wong, Y. C., and Krainc, D. (2020). Mitochondria-lysosome Contacts Regulate Mitochondrial Ca2+ Dynamics via Lysosomal TRPML1. *Proc. Natl. Acad. Sci. U S A.* 117, 19266–19275. doi:10.1073/pnas.2003236117

Polsky, S., and Ellis, S. L. (2015). Obesity, Insulin Resistance, and Type 1 Diabetes Mellitus. *Curr. Opin. Endocrinol. Diabetes Obes.* 22, 277–282. doi:10.1097/MED.0000000000000170

Rezq, S., Hassan, R., and Mahmoud, M. F. (2021). Rimonabant Ameliorates Hepatic Ischemia/reperfusion Injury in Rats: Involvement of Autophagy via Modulating ERK- and PI3K/AKT-mTOR Pathways. *Int. Immunopharmacol* 100, 108140. doi:10.1016/j.intimp.2021.108140

Rieder, S. A., Chauhan, A., Singh, U., Nagarkatti, M., and Nagarkatti, P. (2010). Cannabinoid-induced Apoptosis in Immune Cells as a Pathway to Immunosuppression. *Immunobiology* 215, 598–605. doi:10.1016/j.imbio.2009.04.001

Roche, P. A., and Furuta, K. (2015). The Ins and Outs of MHC Class II-Mediated Antigen Processing and Presentation. *Nat. Rev. Immunol.* 15, 203–216. doi:10.1038/nri3818

Rogers, M. A. M., Kim, C., Banerjee, T., and Lee, J. M. (2017). Fluctuations in the Incidence of Type 1 Diabetes in the United States from 2001 to 2015: a Longitudinal Study. *BMC Med.* 15, 199. doi:10.1186/s12916-017-0958-6

Rom, S., Zuluaga-Ramirez, V., Dykstra, H., Reichenbach, N. L., Pacher, P., and Persidsky, Y. (2013). Selective Activation of Cannabinoid Receptor 2 in Leukocytes Suppresses Their Engagement of the Brain Endothelium and Protects the Blood-Brain Barrier. *Am. J. Pathol.* 183, 1548–1558. doi:10.1016/j.ajpath.2013.07.033

Rossi, F., Bellini, G., Tolone, C., Luongo, L., Mancusi, S., Papparella, A., et al. (2012). The Cannabinoid Receptor Type 2 Q63R Variant Increases the Risk of Celiac Disease: Implication for a Novel Molecular Biomarker and Future Therapeutic Intervention. *Pharmacol. Res.* 66, 88–94. doi:10.1016/j.phrs.2012.03.011

Sam, A. H., Salem, V., and Ghatei, M. A. (2011). Rimonabant: From RIO to Ban. *J. Obes.* 2011, 432607. doi:10.1155/2011/432607

Schatz, A. R., Lee, M., Condie, R. B., Pulaski, J. T., and Kaminski, N. E. (1997). Cannabinoid Receptors CB1 and CB2: a Characterization of Expression and Adenylate Cyclase Modulation within the Immune System. *Toxicol. Appl. Pharmacol.* 142, 278–287. doi:10.1006/taap.1996.8034

Segerstolpe, Å., Palasantza, A., Eliasson, P., Andersson, E. M., Andréasson, A. C., Sun, X., et al. (2016). Single-Cell Transcriptome Profiling of Human Pancreatic Islets in Health and Type 2 Diabetes. *Cel Metab* 24, 593–607. doi:10.1016/j.cmet.2016.08.020

Settembre, C., Di Malta, C., Polito, V. A., Garcia Arencibia, M., Vetrini, F., Erdin, S., et al. (2011). TFEB Links Autophagy to Lysosomal Biogenesis. *Science* 332, 1429–1433. doi:10.1126/science.1204592

Shao, B. Z., Wei, W., Ke, P., Xu, Z. Q., Zhou, J. X., and Liu, C. (2014). Activating Cannabinoid Receptor 2 Alleviates Pathogenesis of Experimental Autoimmune Encephalomyelitis via Activation of Autophagy and Inhibiting NLRP3 Inflammasome. *CNS Neurosci. Ther.* 20, 1021–1028. doi:10.1111/cns.12349

Shao, Z., Yin, J., Chapman, K., Grzemska, M., Clark, L., Wang, J., et al. (2016). High-resolution crystal Structure of the Human CB1 Cannabinoid Receptor. *Nature* 540, 602. doi:10.1038/nature20613

Siani, A. C., Souza, M. C., Henriques, M. G., and Ramos, M. F. (2013). Anti-inflammatory Activity of Essential Oils from Syzygium Cumini and Psidium Guajava. *Pharm. Biol.* 51, 881–887. doi:10.3109/13880209.2013.768675

Spence, A., Purtha, W., Tam, J., Dong, S., Kim, Y., Ju, C. H., et al. (2018). Revealing the Specificity of Regulatory T Cells in Murine Autoimmune Diabetes. *Proc. Natl. Acad. Sci. U S A.* 115, 5265–5270. doi:10.1073/pnas.1715590115

Stempel, A. V., Stumpf, A., Zhang, H. Y., Özdoğan, T., Pannasch, U., Theis, A. K., et al. (2016). Cannabinoid Type 2 Receptors Mediate a Cell Type-specific Plasticity in the Hippocampus. *Neuron* 90, 795–809. doi:10.1016/j.neuron.2016.03.034

Strehl, B., Seifert, U., Krüger, E., Heink, S., Kuckelkorn, U., and Kloetzel, P. M. (2005). Interferon-gamma, the Functional Plasticity of the Ubiquitin-Proteasome System, and MHC Class I Antigen Processing. *Immunol. Rev.* 207, 19–30. doi:10.1111/j.0105-2896.2005.00308.x

Strisciuglio, C., Bellini, G., Miele, E., Martinelli, M., Cenni, S., Tortora, C., et al. (2018). Cannabinoid Receptor 2 Functional Variant Contributes to the Risk for Pediatric Inflammatory Bowel Disease. *J. Clin. Gastroenterol.* 52, e37–e43. doi:10.1097/MCG.0000000000000755

Stumpf, A., Parthier, D., Sammons, R. P., Stempel, A. V., Breustedt, J., Rost, B. R., et al. (2018). Cannabinoid Type 2 Receptors Mediate a Cell Type-specific Self-Inhibition in Cortical Neurons. *Neuropharmacology* 139, 217–225. doi:10.1016/j.neuropharm.2018.07.020

Su, T. F., Zhao, Y. Q., Zhang, L. H., Peng, M., Wu, C. H., Pei, L., et al. (2012). Electroacupuncture Reduces the Expression of Proinflammatory Cytokines in Inflamed Skin Tissues through Activation of Cannabinoid CB2 Receptors. *Eur. J. Pain* 16, 624–635. doi:10.1002/j.1532-2149.2011.00055.x

Theofilopoulos, A. N., Kono, D. H., and Baccala, R. (2017). The Multiple Pathways to Autoimmunity. *Nat. Immunol.* 18, 716–724. doi:10.1038/ni.3731

Tortora, C., Punzo, F., Argenziano, M., Di Paola, A., Tolone, C., Strisciuglio, C., et al. (2020). The Role of Cannabinoid Receptor Type 2 in the Bone Loss Associated with Pediatric Celiac Disease. *J. Pediatr. Gastroenterol. Nutr.* 71, 633–640. doi:10.1097/MPG.0000000000002863

Trivedi, P. C., Bartlett, J. J., Perez, L. J., Brunt, K. R., Legare, J. F., Hassan, A., et al. (2016). Glucolipotoxicity Diminishes Cardiomyocyte TFEB and Inhibits Lysosomal Autophagy during Obesity and Diabetes. *Biochim. Biophys. Acta* 1861, 1893–1910. doi:10.1016/j.bbalip.2016.09.004

Turcotte, C., Blanchet, M. R., Laviolette, M., and Flamand, N. (2016). The CB2 Receptor and its Role as a Regulator of Inflammation. *Cell Mol Life Sci* 73, 4449–4470. doi:10.1007/s00018-016-2300-4

Valecka, J., Almeida, C. R., Su, B., Pierre, P., and Gatti, E. (2018). Autophagy and MHC-Restricted Antigen Presentation. *Mol. Immunol.* 99, 163–170. doi:10.1016/j.molimm.2018.05.009

Vivot, K., Pasquier, A., Goginashvili, A., and Ricci, R. (2020). Breaking Bad and Breaking Good: β-Cell Autophagy Pathways in Diabetes. *J. Mol. Biol.* 432, 1494–1513. doi:10.1016/j.jmb.2019.07.030

Vomund, A. N., Zinselmeyer, B. H., Hughes, J., Calderon, B., Valderrama, C., Ferris, S. T., et al. (2015). Beta Cells Transfer Vesicles Containing Insulin to Phagocytes for Presentation to T Cells. *Proc. Natl. Acad. Sci. U S A.* 112, E5496–E5502. doi:10.1073/pnas.1515954112

Wu, A., Hu, P., Lin, J., Xia, W., and Zhang, R. (2018). Activating Cannabinoid Receptor 2 Protects against Diabetic Cardiomyopathy through Autophagy Induction. *Front. Pharmacol.* 9, 1292. doi:10.3389/fphar.2018.01292

Wu, Q., Ma, Y., Liu, Y., Wang, N., Zhao, X., and Wen, D. (2020). CB2R Agonist JWH-133 Attenuates Chronic Inflammation by Restraining M1 Macrophage Polarization via Nrf2/HO-1 Pathway in Diet-Induced Obese Mice. *Life Sci.* 260, 118424. doi:10.1016/j.lfs.2020.118424

Wu, Q., Zhang, M., Liu, X., Zhang, J., and Wang, H. (2020). CB2R Orchestrates Neuronal Autophagy through Regulation of the mTOR Signaling Pathway in the hippocampus of Developing Rats with Status Epilepticus. *Int. J. Mol. Med.* 45, 475–484. doi:10.3892/ijmm.2019.4439

Wu, Y., Zhang, N., Hashimoto, K., Xia, C., and Dijkstra, J. M. (2021). Structural Comparison between MHC Classes I and II; in Evolution, a Class-II-like Molecule Probably Came First. *Front. Immunol.* 12, 621153. doi:10.3389/fimmu.2021.621153

Xi, Z. X., Peng, X. Q., Li, X., Song, R., Zhang, H. Y., Liu, Q. R., et al. (2011). Brain Cannabinoid CB_2 Receptors Modulate Cocaine's Actions in Mice. *Nat. Neurosci.* 14, 1160–1166. doi:10.1038/nn.2874

Xin, Y., Kim, J., Okamoto, H., Ni, M., Wei, Y., Adler, C., et al. (2016). RNA Sequencing of Single Human Islet Cells Reveals Type 2 Diabetes Genes. *Cel Metab* 24, 608–615. doi:10.1016/j.cmet.2016.08.018

Xu, A., Yang, Y., Shao, Y., Wu, M., and Sun, Y. (2020). Activation of Cannabinoid Receptor Type 2-induced Osteogenic Differentiation Involves Autophagy Induction and P62-Mediated Nrf2 Deactivation. *Cell Commun Signal* 18, 9. doi:10.1186/s12964-020-0512-6

Xu, X., Grijalva, A., Skowronski, A., van Eijk, M., Serlie, M. J., and Ferrante, A. W., Jr. (2013). Obesity Activates a Program of Lysosomal-dependent Lipid Metabolism in Adipose Tissue Macrophages Independently of Classic Activation. *Cel Metab* 18, 816–830. doi:10.1016/j.cmet.2013.11.001

Yedidi, R. S., Wendler, P., and Enenkel, C. (2017). AAA-ATPases in Protein Degradation. *Front. Mol. Biosci.* 4, 42. doi:10.3389/fmolb.2017.00042

Yu, S. J., Reiner, D., Shen, H., Wu, K. J., Liu, Q. R., and Wang, Y. (2015). Time-Dependent Protection of CB2 Receptor Agonist in Stroke. *PLoS One* 10, e0132487. doi:10.1371/journal.pone.0132487

Zarruk, J. G., Fernández-López, D., García-Yébenes, I., García-Gutiérrez, M. S., Vivancos, J., Nombela, F., et al. (2012). Cannabinoid Type 2 Receptor Activation Downregulates Stroke-Induced Classic and Alternative Brain Macrophage/microglial Activation Concomitant to Neuroprotection. *Stroke* 43, 211–219. doi:10.1161/STROKEAHA.111.631044

Zhang, H. Y., Bi, G. H., Li, X., Li, J., Qu, H., Zhang, S. J., et al. (2015). Species Differences in Cannabinoid Receptor 2 and Receptor Responses to Cocaine Self-Administration in Mice and Rats. *Neuropsychopharmacology* 40, 1037–1051. doi:10.1038/npp.2014.297

Zhang, H. Y., Gao, M., Shen, H., Bi, G. H., Yang, H. J., Liu, Q. R., et al. (2017). Expression of Functional Cannabinoid CB2 Receptor in VTA Dopamine Neurons in Rats. *Addict. Biol.* 22, 752–765. doi:10.1111/adb.12367

Zhang, S. J., Wang, C., Yan, S., Fu, A., Luan, X., Li, Y., et al. (2017). Isoform Evolution in Primates through Independent Combination of Alternative RNA Processing Events. *Mol. Biol. Evol.* 34, 2453–2468. doi:10.1093/molbev/msx212

Zheng, X., Sun, T., and Wang, X. (2013). Activation of Type 2 Cannabinoid Receptors (CB2R) Promotes Fatty Acid Oxidation through the SIRT1/PGC-1α Pathway. *Biochem. Biophys. Res. Commun.* 436, 377–381. doi:10.1016/j.bbrc.2013.05.108

Zimmer, A., Zimmer, A. M., Hohmann, A. G., Herkenham, M., and Bonner, T. I. (1999). Increased Mortality, Hypoactivity, and Hypoalgesia in Cannabinoid CB1 Receptor Knockout Mice. *Proc. Natl. Acad. Sci. U S A.* 96, 5780–5785. doi:10.1073/pnas.96.10.5780

Zucchelli, S., Holler, P., Yamagata, T., Roy, M., Benoist, C., and Mathis, D. (2005). Defective central Tolerance Induction in NOD Mice: Genomics and Genetics. *Immunity* 22, 385–396. doi:10.1016/j.immuni.2005.01.015

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 21 January 2022
doi: 10.3389/fphar.2021.805758

Spontaneous Activity of CB_2 Receptors Attenuates Stress-Induced Behavioral and Neuroplastic Deficits in Male Mice

Melissa A. Ribeiro[1†], *Rafael P. Aguiar*[2†], *Franciele F. Scarante*[2], *Eduardo J. Fusse*[3], *Rubia M. W. de Oliveira*[2], *Francisco S. Guimaraes*[1] *and Alline C Campos*[4*]

[1]Department of Pharmacology and Therapeutics, State University of Maringá, Maringá, Brazil, [2]Department of Pharmacology-Ribeirão Preto Medical School, University of São Paulo, São Paulo, Brazil, [3]Mental Health Graduate Program- Ribeirão Preto Medical School, University of São Paulo, São Paulo, Brazil, [4]Pharmacology, University of São Paulo, São Paulo, Brazil

OPEN ACCESS

Edited by:
Cecilia Hillard,
Medical College of Wisconsin,
United States

Reviewed by:
Russell D Romeo,
Columbia University, United States
Sebastiano Alfio Torrisi,
University of Catania, Italy

***Correspondence:**
Alline C Campos
allinecampos@usp.br

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Experimental Pharmacology and Drug Discovery, a section of the journal Frontiers in Pharmacology

Received: 30 October 2021
Accepted: 31 December 2021
Published: 21 January 2022

Citation:
Ribeiro MA, Aguiar RP, Scarante FF, Fusse EJ, de Oliveira RMW, Guimaraes FS and Campos AC (2022) Spontaneous Activity of CB_2 Receptors Attenuates Stress-Induced Behavioral and Neuroplastic Deficits in Male Mice. Front. Pharmacol. 12:805758. doi: 10.3389/fphar.2021.805758

The monoaminergic theory of depression/anxiety disorders cannot fully explain the behavioral and neuroplastic changes observed after ADs chronic treatment. Endocannabinoid system, which comprises CB2 receptors, has been associated with the chronic effects of these drugs, especially in stressed mice. CB2-KO mice display more vulnerability to stressful stimuli. In the present study, we hypothesized that the behavioral and neuroplastic effects observed after repeated treatment with the AD escitalopram (Esc) in chronically stressed mice depend on CB2 receptor signaling. Male mice submitted to chronic unpredictable stress (CUS) paradigm (21 days) were treated daily with AM630 (0.01; 0.03 or 0.3 mg/kg, i.p) a CB2 receptor antagonist/inverse agonist. At e 19th day of the CUS protocol, mice were submitted to Open field test and Tail-suspension test to evaluate antidepressant-like behavior. At the end of the stress protocol, mice were submitted to Novel Suppressed Feeding test (day 22nd) to evaluate anxiety-like behavior. In a second series of experiments, male mice treated with Esc (10 mg/kg, daily, 21 days) in the presence or not of AM630 (0.30 mg/kg) were submitted to the same round of behavioral tests in the same conditions as performed in the dose-response curve protocol. Animals were then euthanized under deep anesthesia, and their brains/hippocampi removed for immunohistochemistry (Doublecortin-DCX) or Western Blot assay. Our results demonstrated that chronic treatment with AM630, a CB2 antagonist/inverse agonist, induces anxiolytic-like effects in stressed mice. Moreover, chronic reduction of CB2 receptor endogenous activity by AM630 attenuated the neuroplastic (potentiating stress-induced decreased expression of pro-BDNF, but enhanced pmTOR and DAGL expression in the hippocampus reduced in stressed mice), the antidepressant- but not the anxiolytic-like effects of Esc. AM630 alone or in combination with Esc decreased the expression of DCX + cell in both the subgranular and granular layers of the dentate gyrus (DG), indicating a general reduction of DCX + neuroblasts and a decrease in their migration through the DG layers. We suggest that the antidepressant-like behavior and the pro-neurogenic effect, but not the anxiolytic like behavior, promoted by Esc in stressed mice are, at least in part, mediated by CB2 receptors.

Keywords: chronic stress, CB2 inverse agonist, escitalopram, hippocampus, neuroplasticity

INTRODUCTION

The incomplete knowledge about the mechanisms involved in the regulation of emotional states and stress coping represents a limiting factor for the efficacy of antidepressants (ADs) and the monoaminergic hypothesis of mood and anxiety disorders (Delgado, 2000). The discovery of new intracellular pathways, neurochemical elements, neurobiological basis and neuroplastic events involved in the control of emotional states has changed the understanding of the clinical and therapeutical aspects of these mental disorders, open new possibilities for the development of new and better therapeutic targets (Delgado, 2000; Dale et al., 2015; Kopschina Feltes et al., 2017).

In the last 2 decades, the endocannabinoid system (ECB), its receptors CB_1 and CB_2), and endogenous ligands (endocannabinoids) raised as one the major neuromodulator system controlling the fine tune of neurotransmitters (GABA, glutamate, monoamines) (Hájos et al., 2001; Wotjak, 2005; Mechoulam & Parker, 2013). As one of the most expressed G coupled receptors expressed in the brain, CB1 and CB2 receptors are current seeing as promising future targets and a missing link in the etiology of stress-related disorders, including their participation in the pharmacological effects of the current antidepressant (Hill et al., 2006; Poleszak et al., 2020)

After its initial description in 1995, CB_2 was thought to be expressed mainly in peripheral cells of the immune system (e.g., lymphocytes and macrophages) (Ashton et al., 2006; Onaivi, 2006) and in the brain, restricted to pathological and neurodegenerative conditions such as gliomas (Sánchez et al., 2001); Alzheimer's disease (Benito et al., 2003), Multiple Sclerosis and Amyotrophic Lateral Sclerosis (Yiangou et al., 2006). Nowadays, the expression of CB_2 receptors in healthy brain cells remains controversial, and the current knowledge suggest that CB_2 gene and protein are expressed in microglial cells (Carlisle et al., 2002; Klegeris et al., 2003; Maresz et al., 2005) and in different brain regions, such as the striatum and hypothalamus of rats (Gong et al., 2006; Onaivi, 2006; Onaivi et al., 2008) and in the cingulate cortex, amygdala, hippocampus, hypothalamus, substantia nigra, dorsal and medial raphe of mice (Gong et al., 2006; Onaivi, 2006; Onaivi et al., 2008; García-Gutiérrez et al., 2010)

These pieces of evidence suggest the distribution of CB_2 receptors in the CNS in brain areas responsible for emotional behavior and stress coping. In a pioneering study investigating a possible reported a decrease in the density of these receptors in the mice midbrain, striatum and hippocampus after stress exposure (Onaivi et al., 2008). Additionally, CB_2 receptor knockout mice (CB_2-KO) display more vulnerability to stressful stimuli in the Tail Suspension Test (TST), light-dark box and elevated plus maze test (Ortega-Alvaro et al., 2011).

Pharmacological manipulations of CB2, however, showed conflicting results. Acute and chronic treatments with the CB_2 receptor antagonist/inverse agonist, AM630, promote antidepressant-like effects in both the forced swimming test and chronic mild stress model (García-Gutiérrez et al., 2010). On the other hand, the study published by Kruk-Slomka and collaborators (2015) suggested that acute doses of CB_2 receptor agonist, JWH 133, or the CB_2 receptor antagonist/inverse agonist, AM630, evoked antidepressant-like effect in the FST in mice. Interestingly, the antidepressant-like effects induced by acute injection of oleamide and JWH 133, were attenuated by a single administration of non-effective dose of AM630, suggesting a complex involvement of CB_2 receptors in the antidepressant-related responses (Kruk-Slomka et al., 2015).

In addition to control emotional states and stress coping in rodents, CB_1 and CB_2 receptors are implicated in the regulation of adult hippocampal neurogenesis, a complex process that seem to be positively regulated and somehow necessary for the effects of antidepressant drugs (Malberg and Duman, 2003; Santarelli et al., 2003, Aguado et al., 2007, Palazuelos et al., 2012, Campos et al., 2013).

Antidepressants and cannabinoids receptors seem to share more that similar behavioral and pro-neurogenic mechanisms. Series of good studies conducted by Canadian groups, suggested that some behavioral and neuroplastic effects of antidepressants, involve CB_1 activation (Hill et al., 2015). However, little is known about the involvement of CB_2 receptors in the pharmacological and pro-neurogenic actions of antidepressants.

Additionally, to the classic monoaminergic theories of mood and anxiety disorders, cannabinoid receptors, specially CB_2 due its primary expression in microglia cells, are current linked to the neuroimmune hypothesis of stress related disorders (Lisboa et al., 2016). It have been demonstrated that both CB_2 receptors (Ashton and Glass, 2007; Benito et al., 2008) and antidepressants (Tynan et al., 2012; Kopschina Feltes et al., 2017) can decrease the pro-inflammatory environment of the brain. Therefore, in the present study we tested the hypothesis that CB_2 receptor activity contribute negatively to the anti-stress effects of the antidepressant escitalopram (focused on its the behavioral and pro-neurogenic actions) in male mice.

MATERIAL AND METHODS

Animals

90 adult male C57BL6 (8–10 weeks old at the beginning of the protocols) were provided by the colony of the Central Animal Facility of the University of São Paulo, Ribeirão Preto Campus. Mice were allowed to acclimatize for at least 2 weeks in our local animal facility (Department of Pharmacology) before the

TABLE 1 | Detailed list of daily stressor used in the 3 week chronic unprectible stress protocol.

1st Week	2nd Week	3rd Week
forced swimming	forced swimming	wet sawdust
sawdust removal	light/dark cycle reversal	inclined box
restraint stress	food deprivation	forced swimming
light/dark cycle reversal	wet sawdust	sawdust removal
wet sawdust	sawdust removal	restraint stress
inclined box	restraint stress	light/dark cycle reversal
food deprivation	inclined box	food deprivation

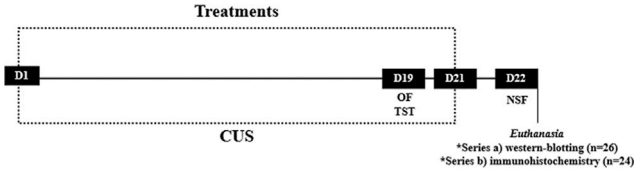


FIGURE 1 | Timeline of our experimental designs. On the 19th day of Chronic Unpredictable Stress (CUS) and treatment, mice were submitted to Open Field (OF) and Tail Suspension Test (TST). 24 h following the CUS protocol, mice were submitted to Novelty Supressed Feeding (NSF). After the d of the protocol, brains were processed for WB and immunohistochemistry.

beginning of the experiments. They were housed in separated cages with 4–6 mice per cage and kept in a quiet room with controlled temperature and humidity, in a 12:12 h light/dark cycle (lights on at 6:30 am) and free access to food and water, except for short periods during the stress protocols when the daily stressor required for food deprivation (see in **Table 1**). Animals were randomly separated into stressed or non-stressed groups and arbitrarily assigned for pharmacological treatments. Stress procedures and the behavioral tasks were carried out in separate quiet rooms. The Ethical Committee of Animal Experimentation of the Ribeirão Preto Medical School (FMRP)- USP approved the experimental protocols according to the Brazilian laws and the ARRIVE Guide (CEUA/FMRP 032/2015-1, 01/2019).

Drugs

Esc (SSRI; Prati & Donaduzzi Cia. Ltda, PR, Brazil) was dissolved in saline 0.9% (w/v) and AM630 (CB_2 antagonist/inverse agonist; Tocris Bioscience, Bristol, United Kingdom) was dissolved in Tween 20 2% + DMSO 0.2% (v/v). Esc (10 mg/kg) dose was based in Seo et al. (2017). AM630 (dose was determined based on a dose-response curve (0.01, 0.03 and 0.30 mg/kg) performed in the present work. All solutions were freshly prepared unde sterile conditions and injected in a volume of 10 ml/kg intraperitoneally (i.p.).

Experimental Design

Firstly, a dose response curve was performed in order to choose the AM630 dose. Male mice submitted to chronic unpredictable stress (CUS) were treated with AM630 at the doses of 0.01 mg/kg; 0.03 mg/kg or 0.3 mg/kg (i.p.). Independent groups of animals (groups: non-stress/Veh, CUS/Veh, CUS/AM630 (0.01 mg/kg), CUS/AM630 (0.03 mg/kg) and CUS/AM630 (0.3 mg/kg); n = 8/group) were submitted to the CUS paradigm for 21 days. In the 19th of the CUS protocol and treatment, mice were submitted to Open Field (OF) to evaluate locomotor activity followed by the Tail Suspension Test (TST) to evaluate antidepressant-like behavior. 24 h after the last stress episode and drug treatment, mice were submitted to the Novel Suppressed Feeding test (NSF) to evaluate anxiety-like behavior. Then, an independent subset of experiments was conducted to evaluate the behavioral and neuroplastic effects of chronic CB_2 spontaneous activity/antagonism (AM630, 0.3 mg/kg i.p.) prior to antidepressant treatment (Esc, 10 mg/kg, i.p). Independent groups of animals (groups: non-stress/Veh + Veh (n = 10), CUS/Veh + Veh (n = 10), CUS/Veh + Esc (n = 9), CUS/AM630 + Veh (n = 10) and CUS/AM630 + Esc (n = 10)) were submitted to the CUS paradigm for 21 days similarly to the experiments of the dose-response curve. Animals were then euthanized, and brains removed for immunohistochemistry assay or the hippocampi dissected for WB assays. Experimental procedures of both sets of experiments followed the scheme described in the **Figure 1**.

Chronic Stress Protocol and Behavioral Tests

Chronic Unpredictable Stress

Chronic and unpredictable stress exposure is an established key factor for the development of several psychological disorders. Unpredictable stressors have greater negative impact than predictable ones, perhaps due to temporal uncertainty (Willner and Mitchell, 2002). The Chronic Unpredictable Stress (CUS) paradigm was developed aiming to maximize unpredictability, in that the animals are exposed to the stressors in seemingly random order. During the light period of the cycle, mice were submitted to a modified CUS paradigm (Campos et al., 2013) during 21 consecutive days. Randomly assigned, different mild stressors were used and apply daily, one per day: forced swimming during 15 min; restraint stress for 2 h; sawdust removal for 24 h; exposure to wet sawdust for 24 h; food deprivation for 24 h, light/dark cycle reversal for 24 h and inclined box overnight. The daily stressor order performed is shown in **Table 1**. During all the procedures, all efforts were made to minimize animal suffering.

Open Field

The OF test is broadly employed to evaluate locomotion and exploration (Gould et al., 2009). The circular OF was made of acrylic (transparent- 50 cm high wall, and 40 cm of diameter) and had white acrylic floor. On the day of the test, each mouse was gently removed from its home cage and put immediately in the center of the apparatus. Mice were allowed to freely explore the arena during 10 min. All trials were recorded and analyzed automatically (in a live mode) by the software AnyMaze (Stoelting, Germany). The total distance traveled, in meters, was recorded as a measure of basal locomotor activity.

Tail-Suspension Test

The TST is a classical test performed to evaluate passive and active coping behavior. In the present study, it was modified from the version validated in mice by Steru et al. (1985). On the day of the experiments, all mice were transported from the holding facility to the testing room. Then, they were left there undisturbed for at least 3 h. Each mouse was individually suspended by the tail to a horizontal ring-stand bar (35 cm of distance from floor) using adhesive tape (2 cm of distance

from the tip of tail). As the test session progressed, mice demonstrated several escape-oriented movements interspersed with bouts of immobility of increasing length. The test session was recorded during 6 min, and the total immobility time was measured by an experienced experimenter blind to the groups.

Novelty Suppressed Feeding Test

The NSF test is another classical test to evaluate anxiety-like behaviors. It was performed in a 10 min test session, as previously described by Campos and colleagues (2013). 24 h prior the test, all animals were food-deprived. The apparatus consisted of a square acrylic box (40 × 40 × 30 cm) covered by 2 cm of sawdust. On the day of the test, a single regular chow pellet was placed in a white platform located in the center of the arena. Each animal was carefully placed in one of the corners of the apparatus, and the latency to start ingest food in the new environment was recorded. The stopwatch was immediately stopped when the mouse bit the chow, using its forepaws sitting on its haunches. After the test, all animals were returned to their home cages, and the amount of food consumed in 5 min was measured, as a test control of basal hungry.

Tissue Preparation

In the last set of experiments, after the last behavioral test, mice were quickly euthanized under deep anesthesia (Ketamine/Xylazine;100/8 mg/kg: 0.1 ml, i.p - Syntec, Brazil) and the hippocampi were rapidly dissected. The samples were lysed in a tissue buffer containing 50 mM Tris (pH 7.6) and complete proteinase inhibitor (1:10 dilution of stock; Sigma-Aldrich, St. Louis, MO, United States). After homogenization and centrifugation twice (12000 rpm, 10 min, 4°C) the supernatant was individually collected and properly stored at −80°C until the beginning of the Western blot analysis. In another subgroup of mice, brain tissues were prepared for immunohistochemistry procedure. Mice were perfused transcardially (under deep anesthesia) with PBS, followed by tissue fixation with 4% paraformaldehyde solution. Brains were removed, cryoprotected for 72 h in a 30% sucrose solution and cut in 30 µm slices through the hippocampus (bregma from -1.46 to – 3.08 mm; Franklin and Paxinos, 2008) using a cryostat (Leica, Wetzlar, Germany).

Western Blot

The protein concentrations in the stored supernatant from the hippocampi were determined using the Bradford method. Total proteins (20 µg/20ml) were electrophoresed (NuPAGE, Invitrogen, MA, United States) and transferred into a nitrocellulose membrane (Amersham Potran, LittleChalfont, United Kingdom). Membranes were blocked in 10% non-fat milk (Bio-Rad) (dissolved in Tris- saline- buffer +0.5% of Tween20 -TBSt) for 2 h. After blocking, membranes were rinsed quickly with TBSt to remove the excess of blocking solution and then incubated with the primary antibody at 4°C overnight at the following dilutions: anti-BDNF (1:2,500; Santa Cruz Biotechnology, Santa Cruz, CA, United States), anti-mTOR (1:2000; QED Bioscience, San Diego, United States), anti-pmTOR (1:2,500; Santa Cruz Biotechnology, Santa Cruz, United States) and anti-DAGL (1:2000; QED Bioscience, San Diego, United States). After a washing step with TBS, membranes were incubated for 2 h with donkey anti-mouse IgG (1:2,000; Amersham, Little Chalfont, United Kingdom). The reactive bands were detected using an enhanced chemiluminescence reagent (ECLPrime®, Amersham, Little Chalfont, United Kingdom) and visualized using ChemiDoc Imaging Systems (GE ImageQuant LAS, United States). Intensities of specific bands were quantified using Image Studio Lite (LI-COR, NE, United States) and normalized to anti-α-tubulin (1: 20,000; Sigma-Aldrich, MI, United States) protein levels. Data were presented as % of the non-stressed-Veh group (control).

Doublecortin Immunohistochemistry

The sections containing the hippocampal formation received three washes in TBS. Slices underwent an antigen retrieval step in citrate buffer (10 mM Citric Acid, 0.05% Tween 20, pH 6.0) for 30 min at 30°C and were left in the bench for cooling down at room temperature. Then, slices were incubated in a blocking solution (BSA 1% + 0.25% Triton 100X in TBS) for 2 h. The slices were incubated overnight with the primary antibody (goat anti-DCX- Santa Cruz Biotechnology, 1:200, United States) and incubated with the secondary antibody for 1 h (1:1,000 Vectastin anti-goat biotinylated). An additional step of incubation with A + B complex for 1 hour (1:1,000ABC Elite-Vectastin kit, Vector Labs—Burlingame, United States) was performed followed by the color development using 3,3′-Diaminobenzidine (DAB 0.2 mg/ml- 10 min, Sigma-Aldrich, Missouri, United States). Slices containing hippocampi were mounted on glass slides/coverslips with Permount (DPX-Fisher Scientific, Loughborough, United Kingdom) as mounting media.

Doublecortin Analysis

Six to eight slices containing dorsal parts of the hippocampus (series of hippocampal sections located between 1.3 and 2.5 mm posterior to bregma) were analyzed for each experimental animal. DCX + cells were counted in a 40x objective of a light microscope (Olympus BX60, Germany) by an experimenter blinded for the treatments and conditions. Cells were considered positive for DCX only if the cell body was stained, and cells were located in the subgranular or granular zone of the dentate gyrus. The total number of cells was normalized to the dentate gyrus area determined with 10x objective. The number of positive cells was estimated by calculating the total hippocampal volume as determined by the sum of the areas of the sampled sections multiplied by the distances between them (series of hippocampal sections located between 1.3 and 2.5 mm posterior to bregma) (Campos et al., 2013; Campos et al, 2014). Positive cells located at a distance of at least 1 cell body in the granular layer of the dentate gyrus were considered to be in the migration phase.

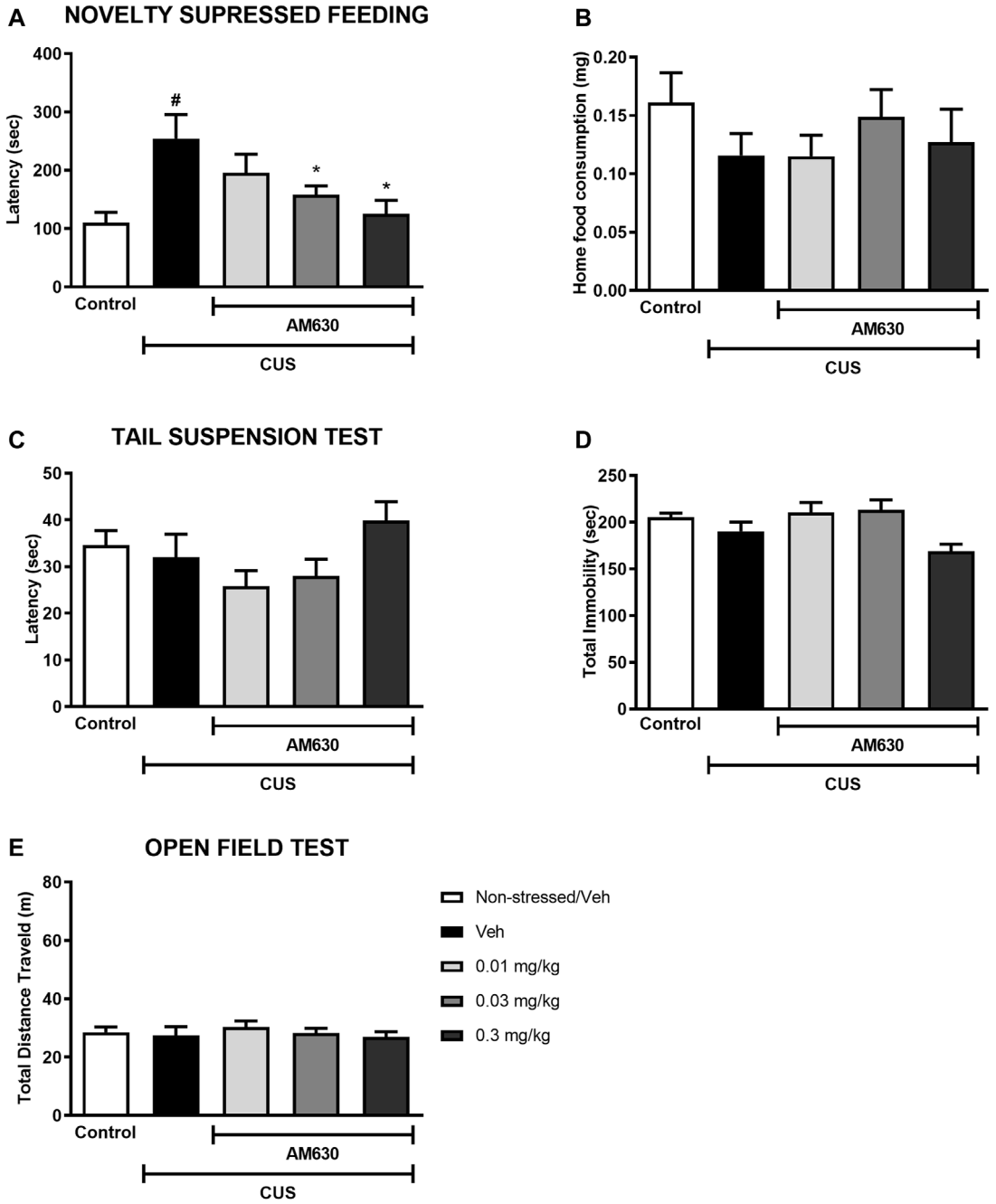


FIGURE 2 | The CB_2 receptor inverse agonist AM630 induces an anxiolytic-like effect in stressed mice after chronic treatment. Latency to feed in the NSF test in seconds **(A)**, total food consumption in the home-cage **(B)**, first immobility episode time in the TST in seconds **(C)**, total immobility time in the TST in seconds **(D)**, and the total distance traveled, in meters **(E)** by animals submitted to CUS protocol and treated with Vehicle or AM630 (0.01 mg/kg; 0.03 mg/kg or 0.3 mg/kg). *N* = 8/group. Data represented as Mean ± SEM; (#) represents $p < 0.05$ relative to the non-stressed control group (*t*-Student test); (*) indicated $p < 0.05$ relative to the CUS group treated with Vehicle (One-way ANOVA followed by Duncan).

Statistical Analysis

Statistical analysis was performed following the principles previously published by our group using the SPSS software (version 16.0, IBM, United States) (Fernandes et al., 2021). Data were analyzed by Levene's test and met the assumption of homogeneity of variances ($p > 0.05$), then analyzed by One-way ANOVA

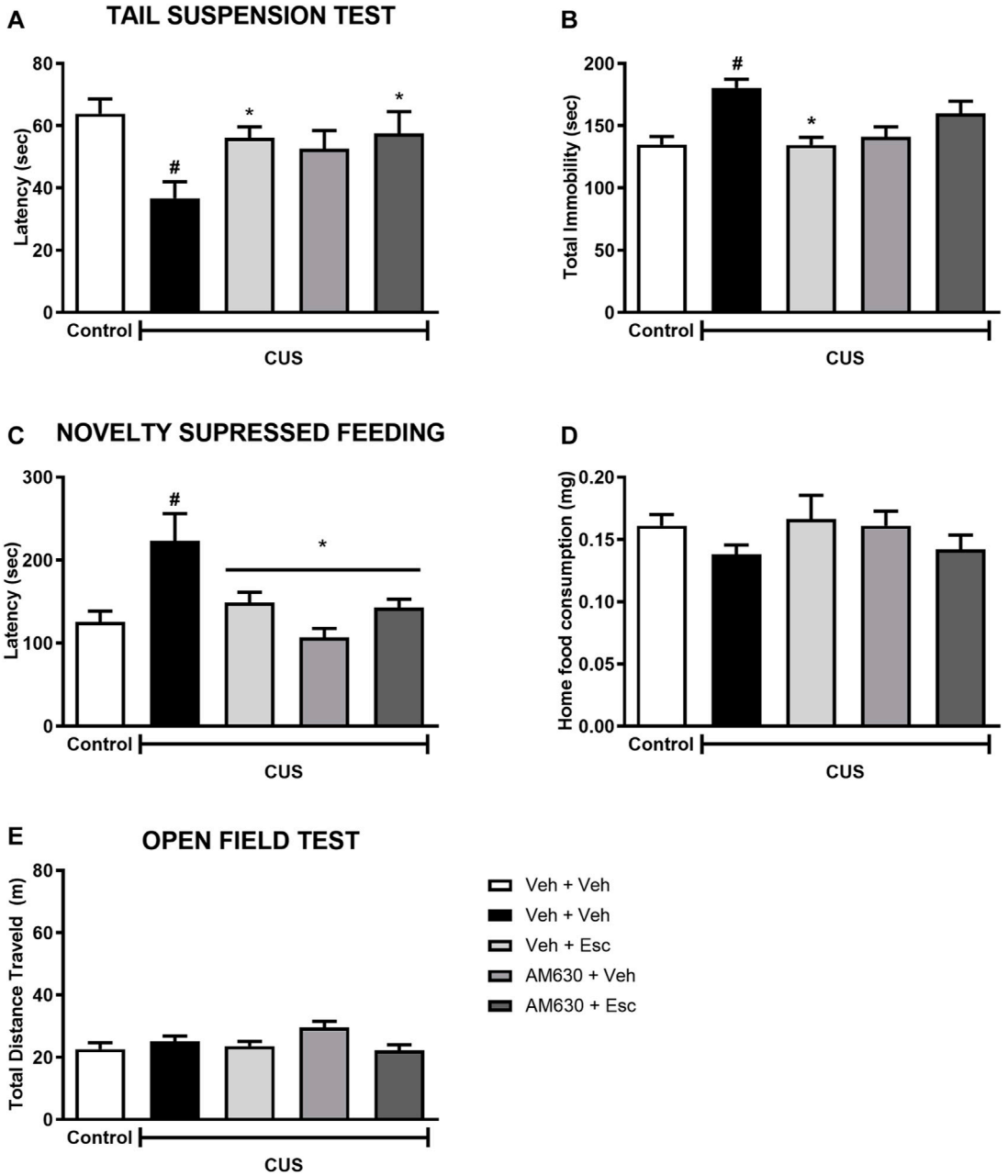


FIGURE 3 | AM630 interferes with the behavioral effects of escitalopram. Figure shows the first immobility episode time in the TST in seconds **(A)**, the total time of immobility in the TST in seconds **(B)**, the latency for first episode of food ingestion in NSF in seconds **(C)**, the total food ingestion in the home cage **(D)**, and the total distance traveled, in meters, by animals submitted to CUS protocol **(E)**. Groups were: non-stressed/saline/saline (n = 10); CUS/Veh + Veh (n = 10); CUS/AM630 + Veh (n = 10); CUS/Veh + Esc (n = 9); and CUS/AM630/Esc (n = 10). ANOVA-TWO WAY was employed and differences were considered statistically significant when $p < 0.05$ (# relative to NS/Veh + Veh and * relative to CUS/Veh + Veh).

(experiment I) or Student's t-Test (Stress effects: Veh control vs. Vehicle stressed group) and Two-way ANOVA (to address the effects of the factors Treatment 1 (Veh or AM630) or Treatment 2 (Veh or Esc) within the stressed group) (experiment II). Differences between groups were considered statistically significant at values of $p < 0.05$. All data are expressed as mean ± SEM.

RESULTS

Chronic Treatment With a CB_2 Antagonist/ Inverse Agonist Induces an Anxiolytic-like Effect in Stressed Mice

Mice exposed to CUS for 21 days and treated with vehicle were more hyponeophagic in the NSF test in comparison to the non-stressed control group (*t*-Student test, $t_{13} = 3.351$, $p = 0.0050$, thus revealing an anxiogenic-like effect of stress exposure. Chronic treatment with AM630 in the doses of 0.03 mg/kg and 0.3 mg/kg significantly decreased the latency for mice to feed in the novel environment (One-way ANOVA followed by Duncan; $F_{2,26} = 3.437$, $p = 0.031$), indicating an anxiolytic-like response induced by the CB_2 receptor inverse agonism. No differences were observed concerning the food consumption at their home-cage (*t*-Student, $t_{13} = 1.407$, $p = 0.183$; One-way ANOVA, $F_{3,26} = 0.528$, $p = 0.674$) (**Figures 2A,B**).

In the TST, on the other hand, there was no difference between the stressed group treated with vehicle and the non-stressed control group concerning the latency for the first immobility episode (*t*-Student, $t_{12} = 0.439$, $p = 0.669$) nor considering the total immobility time (*t*-Student; $t = 1.373$, $p = 0.94$). Chronic treatment with AM630 did not significantly alter any of the behavioral outcomes in the TST compared to the stressed mice treated with vehicle (One-way ANOVA followed by Duncan; Latency for the first immobility episode: $F_{3,27} = 2.532$, $p = 0.078$; Total immobility: $F_{3,27} = 4.502$, $p = 0.011$) (**Figures 2C,D**). Additionally, neither stress (t-Student, $t_{14} = 0.305$, $p = 0.765$) nor AM630 treatment (One-way ANOVA; $F_{3,28} = 0.421$, $p = 0.739$) significantly influenced the locomotor activity of mice in the OF (**Figure 2E**).

Since AM630 induced an anxiolytic-like effect in stressed mice, we next sought to investigate whether chronic inverse agonism of CB_2 receptors could modulate the behavioral effects of the antidepressant escitalopram in stressed mice.

AM630 Prior to Esc Abolishes the Antidepressant Behavior but Does Not Interfere With the Anxiolytic-like Effect Promoted by the Antidepressant and Promotes Complex Modulation of Proteins Related to Neuroplasticity

In the TST, stress significantly decreased the latency for the first immobility episode (**Figure 3A**) whilst also increasing total immobility (**Figure 3B**) (t-Student test, $t_{18} = 3.827$, $p < 0.001$ and $t_{18} = 4.843$, $p < 0.001$, respectively). In stressed mice, the factor treatment 1 (Veh or AM630) did not affect the behavior concerning the latency or the total immobility time (Two-way ANOVA; $F_{1,35} = 2.366$, $p = 0.133$ and $F_{1,35} = 0.766$, $p = 0.387$, respectively). Post-hoc analysis revealed that repeated administration of Esc prevented the effects of CUS in both parameters, as observed in the comparison between CUS-Veh + Veh and CUS-Veh + Esc groups (One-way ANOVA followed by Duncan; Total Latency: $F_{3,35} = 2.933$, $p = 0.047$; Total immobility: $F_{3,35} = 6{,}711$, $p = 0.001$). The pretreatment with AM630 did not affect the Esc antidepressant-like effect in the latency task (Two-way ANOVA, interaction $F_{1,35} = 1.625$, $p = 0.211$). However, in the total immobility episode, the antidepressant-like effect of Esc was attenuated by pre-administration of AM630 since no significant differences was observed between CUS-Veh + Veh and CUS-AM630 + Esc (One-way ANOVA followed by Duncan; $p > 0.05$), indicating that the activation of CB_2 receptors is important for the ability of Esc in decreasing passive coping strategies in the TST.

Concerning the effect of stress in the NSF, it was observed a statistically significant difference between NS-Veh + Veh and CUS-Veh + Veh on the latency to feed in the new environment (**Figure 3C**) (*t*-Student test, $t_{18} = 2.785$, $p = 0.01$). Regarding the treatment effect within the CUS groups, there was a significant effect of treatment 1 and a significant interaction between treatments (Two-way ANOVA; Treatment 1: $F_{1,35} = 10.015$ $p = 0.003$; Treatment 2: $F_{1,35} = 1.015$, $p = 0.321$; Interaction: $F_{1,35} = 8.214$, $p = 0.007$), After post-hoc analysis, in both groups CUS-Veh + Esc and CUS-AM630 + Veh we observed an anxiolytic-like effect, as they showed a decrease in the time to feed in the new environment compared to the CUS-Veh + Veh group (One-way ANOVA followed by Duncan; $F_{3,35} = 6.589$, $p = 0.001$). The anxiolytic-like effect of Esc was not affected by the pre-administration of AM630. As expected, no differences were found in the home cage consumption (**Figure 3D**) in relation to the stress effect ($t_{18} = 1.956$, $p = 0.06$) or the treatments (Two-way ANOVA; Treatment 1: $F_{1,35} = 0{,}004$ $p = 0.95$; Treatment 2: $F_{1,35} = 0{,}14$ $p = 0.71$; Interaction: $F_{1,35} = 3{,}52$ $p = 0.07$).

In the OF test (**Figure 3E**), stress didn't affect the locomotor activity of the animals (t-Student test, $t_{18} = 0.953$, $p = 0.352$). Regarding to the treatments in the CUS animals, the factor Treatment 1 was statistically significant (Two-way ANOVA $F_{1,45} = 6.802$ $p = 0.01$), but in the post-hoc analysis it was not observed any differences in the total distance traveled by the groups, suggesting no hypo/hyperlocomotion induced by any treatment.

We performed analysis aiming to uncover the possible molecular pathways altered by the stress and by the chronic treatment with the antidepressant. A statistically significant a reduction in the protein levels of pro-BDNF (**Figure 4A**) and phospho-mTOR (**Figure 4C**) was found in the hippocampus of stressed mice when compared to NS-Veh + Veh (t-student test, $t_7 = 4.882$, $p = 0.002$; $t_7 = 2.889$, $p = 0.023$ respectively). Hippocampal protein expression of DAGL (**Figure 4D**) ($t_7 = 3.357$, $p = 0.012$) was found to be increased in the CUS-Veh + Veh compared to NS-Veh + Veh. No effects of stress exposure were found in the hippocampal expression of mature BDNF (**Figure 4B**) (students *t*-test, $t_7 = 1.069$, $p = 0.320$). Among the stressed groups, there was a significant effect of treatment 1(AM630) in the protein expression of pro-BDNF (**Figure 4A**) (Two-way ANOVA; $F_{1,13} = 18.314$, $p = 0.001$), but there was no effect of treatment 2 (ESC) ($F_{1,13} = 3.169$, $p = 0.098$), indicating that, while the antidepressant treatment is not able to reverse the stress effects upon hippocampal pro-BDNF expression, chronic blockade of CB_2 receptors *per se* causes a further reduction in pro-BNDF expression. There was also a

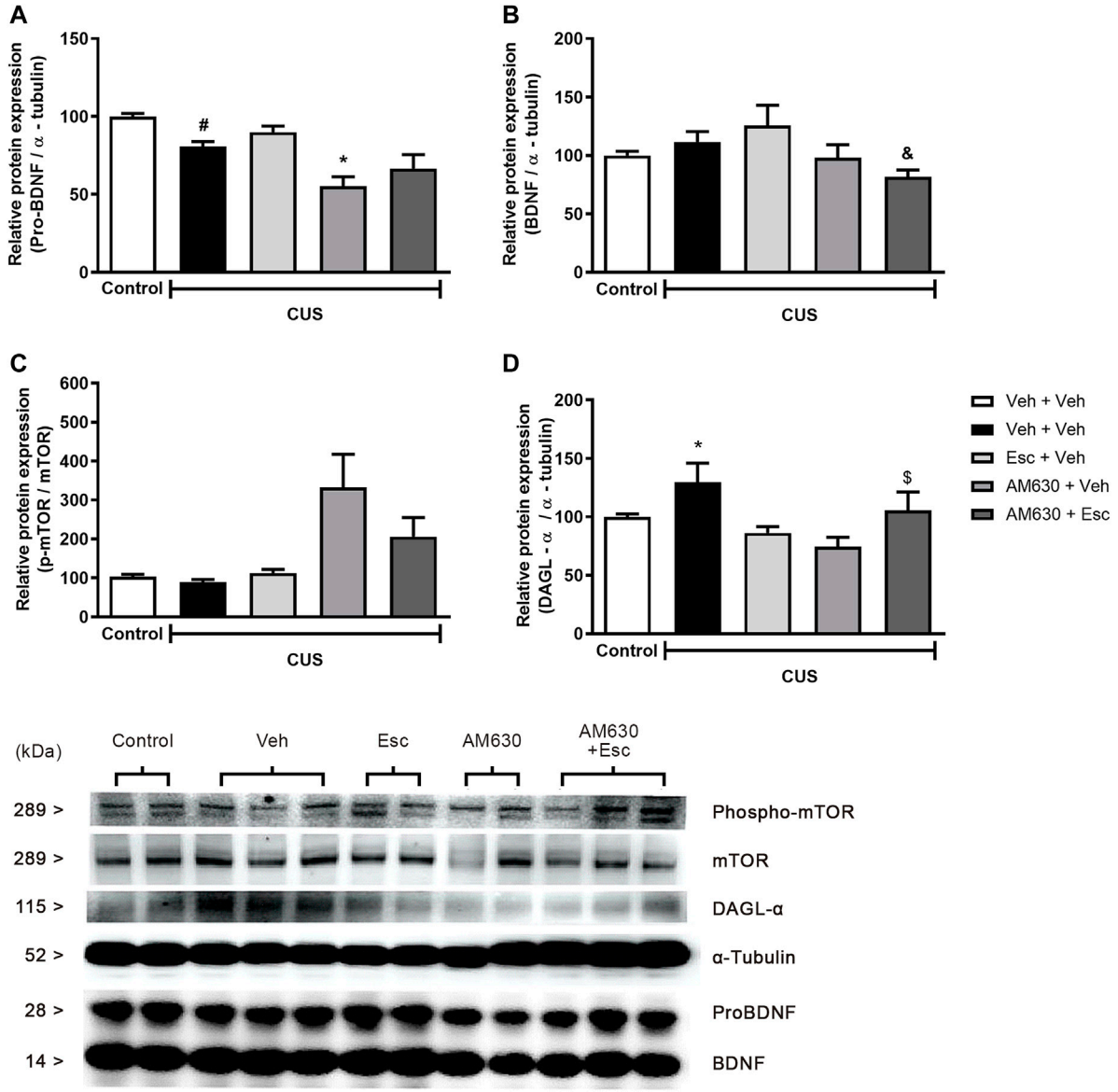


FIGURE 4 | Relative hippocampal protein expression and corticosterone levels of stressed mice treated with ESC in combination or not with AM630. Figure shows the expression of pro-BDNF **(A)**. mature BDNF **(B)**. phospho-mTOR **(C)** and DAGL **(D)**. Representatives of western blot membranes are detailed in the image. Groups were: non-stressed/saline/saline (n = 5); CUS/saline/saline (n = 5); CUS/AM630/saline (n = 4); CUS/saline/ESC (n = 4); and CUS/AM630/ESC (n = 5). ANOVA-two was employed and differences were considered statistically significant when $p < 0,05$. N = 4-5/group. (# relative to NS/Veh + Veh; * relative to CUS/Veh + Veh and & relative to the CUS-AM630 compared to CUS-AM630 + Esc).

significant effect of Treatment 1 on the levels of mature BDNF (**Figure 4B**) (Two-way ANOVA, $F_{1,13} = 6.405$, $p = 0.025$). There was also a significant difference between the CUS-Veh + Esc and the CUS-AM630 + Esc groups (One-way ANOVA followed by Duncan; $F_{3,13} = 2.646$, $p = 0.093$), suggesting that CB_2 chronic blockade interfere in the antidepressant actions. Concerning the levels of phospho-mTOR in the hippocampus of stressed mice, there was a significant effect of Treatment 1 (Two-way ANOVA, $F_{1,13} = 11.827$, $p = 0.004$). One-way ANOVA followed by Duncan indicated that the phospho-mTOR expression was significantly higher in the hippocampus of CUS-AM630 + Veh mice compared to the CUS-Veh + Veh group ($F_{3,13} = 4.823$, $p = 0.018$). DAGL protein expression was not affected by any individual treatment (Two-way ANOVA, Treatment 1: $F_{1,13} = 3.759$, $p = 0.075$; Treatment 2: $F_{1,13} = 1.011$, $p = 0.333$), but there was a significant interaction between treatments ($F_{1,13} = 12.166$, $p = 0.004$). Both CUS-Veh + Esc and CUS-AM630 + Veh groups showed a diminishment in the DAGL levels in the hippocampus (One-way ANOVA followed by Duncan; $F_{3,13} = 5.368$, $p = 0.013$), but this was not observed in the CUS-AM630 + Esc group, showing CB_2 receptor participates in the modulation of endocannabinoid pathways promoted by the anti-stress effects of the antidepressant.

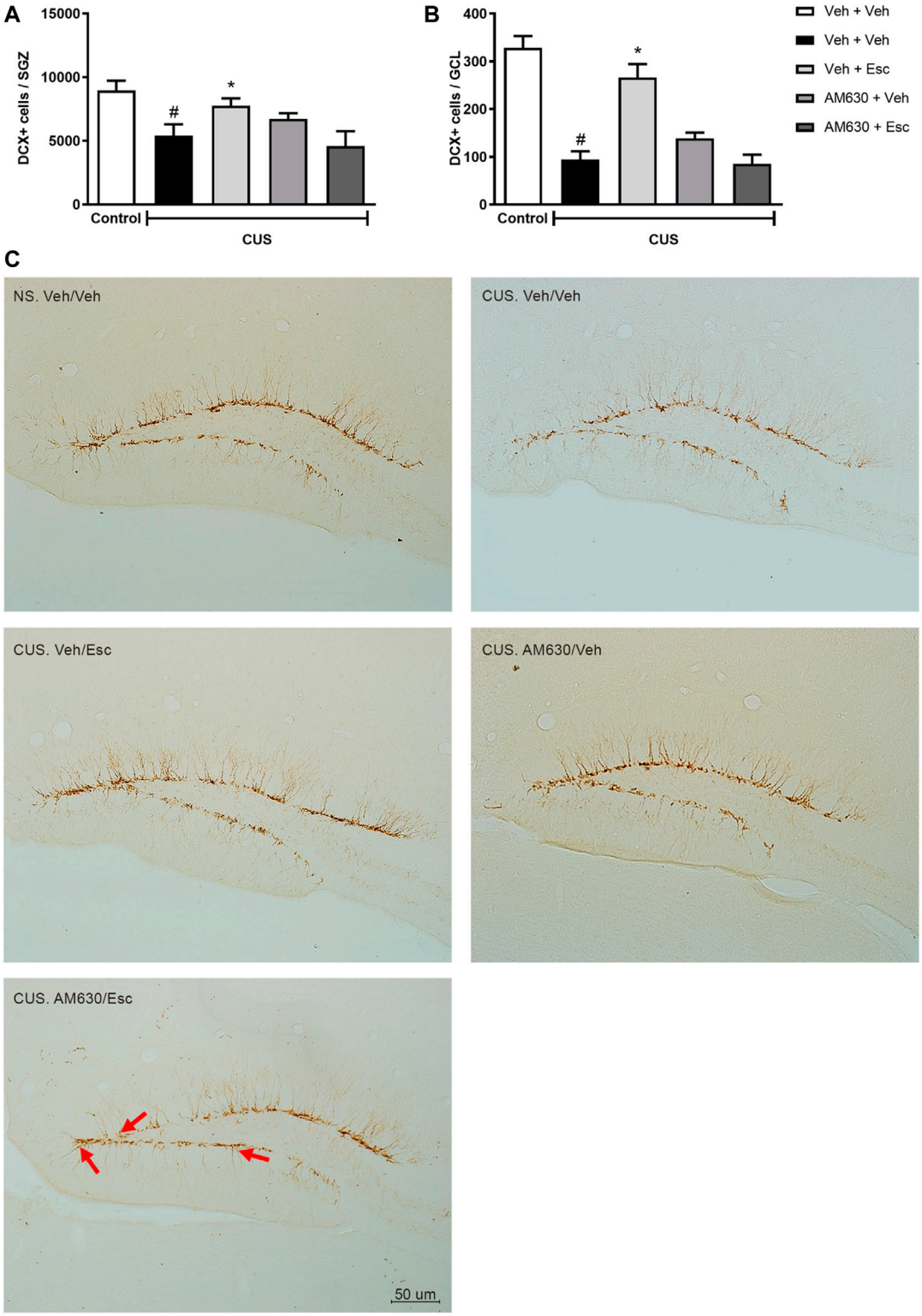


FIGURE 5 | DCX immunostaining in Dentate Gyrus of hippocampus. doublecortin positive cells (DCX+) expression in Subgranullar Zone of Dentate Gyrus (SZG) **(A)**; Granular Cell Layer (CGL) **(B)** and representative photomicrograph of doublecortin positive cells (DCX+) located in the dentate gyrus of the hippocampus **(C)**. Red arrows indicate DCX + cells that have migrated from SGZ to the granular layer of the dentate gyrus of mice. Light Microscope (Olympus B202) at ×20 magnification. Groups were: NS/Veh + Veh ($n = 4$); CUS/ Veh + Veh ($n = 5$); CUS/AM630/Veh ($n = 5$); CUS/Veh + Esc ($n = 5$); and CUS/AM630/Esc ($n = 5$). ANOVA-TWO WAY was employed and differences were considered statistically significant when $p < 0.05$ (# relative to NS/Veh + Veh and * relative to CUS/Veh + Veh).

Pro-Neurogenic Effect of Esc in Stressed Mice is Affected by AM630 Pre-Treatment

CUS exposure induced a decrease in the number of DCX-positive cells in both the SGZ (**Figure 5A**) and the GCL (**Figure 5B**) (*t*-Student test, $t_7 = 2.930$, $p = 0.02$ and $t_7 = 7.924$, $p < 0.001$, respectively), indicating a general reduction of DCX-positive neuroblasts and a decrease in their migration to the GCL. In the CUS groups, there was a significant interaction between treatments regarding the number of DCX-positive cells in the SGZ (Two-way ANOVA; $F_{1,16} = 7.472$, $p = 0.015$). In the GCL, there was a significant effect of treatment 1 (Two-way ANOVA; $F_{1,16} = 11.637$, $p = 0.004$), of treatment 2 ($F_{1,16} = 8.815$, $p = 0.09$), as well as a significant interaction between treatments ($F_{1,16} = 31.945$, $p < 0.001$). Esc treatment in the CUS group significantly attenuated the decrease in the number of DCX positive cells in both SGZ and GCL, as compared to CUS-Veh + Veh (One-way ANOVA followed by Duncan; SGZ: $F_{3,16} = 2.920$, $p = 0.066$; GCL: $F_{3,16} = 17.466$, $p < 0.001$). The pro-neurogenic effect of Esc in the number of DCX-positive cells in the SGZ and GCL was attenuated by pre-administration of AM630, since no significant differences were observed between CUS-Veh + Veh and CUS-AM630 + Esc (One-way ANOVA followed by Duncan). Photomicrography of doublecortin positive cells (DCX+) analyzed in the dentate gyrus of the hippocampus are shown in the **Figure 5C**.

DISCUSSION

In the present study, we suggest that the antidepressant-like behavior and the pro-neurogenic effect promoted by Esc in stressed mice are, at least in part, dependent of CB2 receptors. The pharmacological reduction of CB_2 receptors activity have attenuated the behavioral deficits induced in chronically stressed mice, since the lower doses of AM630 (**Figure 3**) were able to promote anxiolytic-like and antidepressant-like effects, suggesting the CB2 spontaneous activity as an important regulator of behaviors. Interestingly, several lines of evidence demonstrate controversial effects of inverse agonists/antagonists of CB2. The overexpression of CB2 in mice increases resistance to anxiogenic-like stimuli in the hippocampus and amygdala (García-Gutiérrez and Manzanares, 2011). On the other hand, acute administration of CB2 antagonist/inverse agonist was shown to induce anxiogenic-like behavioral, whereas chronic pharmacological blockade of this receptor produced anxiolytic-like effects in parallel with increased expression of the CB2 in the amygdala and prefrontal cortex (García-Gutiérrez et al., 2012). In a recent study, the acute administration of the association of CB2 inverse agonist/antagonist, AM630, and atypical antidepressants (agomelatine and tianeptine) in ineffective doses, promoted antidepressant-like effects in the forced swimming test (Poleszak et al., 2020).

In our model, the pharmacological modulation of the activity of CB_2 receptors with AM630 prior to ESC was not able to prevent the stress-induced depressive-like behavior but promoted anxiolytic-like effect, suggesting the anxiolytic-like effect seems to be dependent of the spontaneous activity of CB2 receptors but not the antidepressant-like behavior. Our findings concerning the behavioral effects of CB_2 modulation are consistent with the molecular results induced by chronic AM630 treatment (**Figure 3**). The mTOR signaling have been shown to be an integrative protein hub that couples environmental cues, such as stress, to the activation of intracellular pathways to assemble and optimize the inflammatory responses (Laplante and Sabatini 2012). mTOR reconfigures the cellular metabolism and regulates translation, cytokine release, macrophage and mitochondrial polarization and cell migration (Laplante and Sabatini 2012). In the CNS, mTORC1 is considered an important inductor of neurogenesis in neurogenic niches and *in vitro* models (Palazuelos et al., 2012). Accordingly, chronic AM630 treatment enhanced the expression of phospho-mTOR in stressed mice, suggesting the recruitment of pro-neuroplastic input aiming to counteract the stress effects.

Recent efforts shed light into the contribution of CB_2 receptor activation during the stress-induced neuroendocrine adaptations (García-Gutiérrez et al., 2010; Zoppi et al., 2014). The increase of the full agonist of CB_2 ligand, 2-AG, is reported as a classical chronic stress-related response in several brain regions: amygdala (Patel et al., 2005a; Hill et al., 2010), pre-frontal cortex (Dubreucq et al., 2012; Patel et al., 2005b), hypothalamus (Dubreucq et al., 2012; Patel et al., 2004), and hippocampus (Dubreucq et al., 2012). Enhanced HPA-axis activation appears to be the primary mechanism by which stress increases 2-AG levels (Morena et al., 2016). In stressed mice, we observed increased hippocampal DAGL protein expression, the main enzyme responsible for the synthesis of 2-AG, which exerts pro-inflammatory actions. This stress-induced effect was prevented by chronic CB_2 blockade, suggesting that coping stress effects might include reduction of 2-AG synthesis and signaling by regulation of DAGL expression via buffering HPA-axis activation. HPA-axis disruption promoted by stress is a key factor related to mood disorders that include depletion of monoamines and growth factors, neuroinflammation and alteration in adult hippocampal neurogenesis (Fujioka 2010; Kohl 2011; Busse 2015; Bai 2019). Hence, the classical cannabinoid role in regulation of anti-inflammatory responses in the CNS is especially important since neuroimmunomodulatory processes have been proposed to underlie the pathophysiology of a variety of stress-related neuropsychiatric disorders (Madrigal et al., 2006; Wager-Smith and Markou, 2011). In this sense, several studies have reported that mice lacking the CB_2 receptor have an exacerbated pro-inflammatory phenotype (Turcote 2016).

Regarding neuroplastic effects of Esc, our results showed increased DCX + cells in the hippocampus of mice chronically treated with the SSRI (**Figure 5**). Preclinical models of chronically stressed and treated with Fluoxetine (FLX), a classical SSRI, have shown a more complex dendritic arborization of DCX +, indicating that the amount of DCX + cells is not necessarily related to antidepressant chronic effects, whereas the microenvironment modulation promoted by this cell subtype might be more relevant to the antidepressant effects than its absolute number (Wang et al., 2008). Additionally, a refined work of Hill and colleagues (2015) with inducible transgenic mice in which the pro-apoptotic gene Bax was deleted from NSC´s,

therefore enhancing adult neurogenesis through decreasing in progenitor cell death, has shown reduced anxiety- and depression-like behaviors in stressed mice (Hill et al., 2015). These data indicates that the increasing in adult neurogenesis is sufficient to promote stress resilience. Moreover, both cannabinoid receptors modulate adult neurogenesis by acting at distinct neurogenic phases (Palazuelos et al., 2006; Palazuelos et al., 2012; Prenderville et al., 2015). CB_2 is expressed on NSCs *in vitro* and *in vivo* models and it plays a role in the regulation of cell proliferation, neuronal differentiation and maturation (Palazuelos et al., 2006; Palazuelos et al., 2012). In our work, we found DCX + cells number to be increased in mice treated with ESC parallel to the antidepressant-like behavior, an effect dampened by the pre-treatment with AM630, suggesting a complex neuroplastic modulation of chronic antidepressant and CB_2 receptor activity during stress. The CB_2 role in neurogenesis was assessed by Mensching and colleagues by using an CB_2-KO mouse model. They reported that CB2-KO did not present alterations in SGZ proliferation nor DCX + cells compared to WT (Mensching et al., 2019). On the other hand, previous studies have shown reduced basal levels of cell proliferation in the SGZ of CB2-KO mice at 2 months of age after exposure to neurotoxic drug Kainic acid, a potent agonist of glutamate receptors (Palazuellos et al., 2012). These results indicate that CB_2 might not regulate basal levels of adult hippocampal neurogenesis, but rather this refined modulation appears to be more significant in the modulation of neurogenesis during dynamically regulated states, such as in neuroinflammation, drug treatment or even stress. Taking this into account, our data indicate that Esc may be up-regulating some important steps of neurogenesis in a CB2-dependent fashion which is reflected by the altered profile of DCX+ in stressed and treated mice.

We found CB2 chronic blockade to dampen BDNF signaling in the hippocampus of stressed mice. The interactions between BDNF signaling and cannabinoid receptors have been shown to independently modulate neurogenesis (Aguado et al., 2005, 2007; Aso 2008), but how they may interact remains poorly understood. *In vitro* approaches performed by Ferreira and colleagues have interestingly demonstrated an interplay between BDNF and cannabinoid receptors, especially regarding the CB_2 receptor as a pivotal modulator of BNDF expression and effects in the DG (Ferreira et al., 2018). Thus, our results may reflect direct AM630 actions on the reduction of BDNF signaling which is required to the neurogenic effects of antidepressants, thereafter, leading to the neurogenic disturbance observed in the group whose received chronic Esc.

Esc is classified as an SSRI because of its mechanism of action, but the monoaminergic actions are not enough to completely explain its behavioral and neuroplastic effects (Delgado, 2000; Dale et al., 2015). There are few evidences investigating the possible supplementary Esc mechanisms of action, but comparisons with other SSRIs are conceivable. For instance, FLX has pro-neurogenic effects assigned to the expression of 5HT1A in mature granule cells of the adult mice DG (Samuels et al., 2015). FLX has been shown to accelerate the maturation of young neurons by promoting a faster transition between the DCX + stage to the type 3 cells (NeuN + stage), possibly because of the 5-HT1A expression in these specific cells (Malberg & Duman, 2003; Wang et al., 2008). Furthermore, the FLX proneurogenic effect was shown to be dependent of mTORC1 signaling (Liu et al., 2015). In our model, phosphorylated mTOR have increased in mice exposed to the AM630 chronic treatment, therefore, it is possible that the sharply increase in the mTORC1 signaling promoted by AM630 might be compensated by the drug combination, resulting in the observed neurogenic imbalance. In this sense, further investigations are need to evince whether Esc and FLX share one or more mechanisms.

Alternatively, the impaired neuroplastic effect of ESC in mice prior treated with AM630 might be attributed to the anti-neurogenic effect of pro-inflammatory state induced by lacking CB_2 signaling. Previous studies reported two specific conditions where DCX expression is regulated non linearly compared to levels of adult hipocampal neurogenesis: in chronic stress model where DCX is upregulated and in inflammation models where DCX is down regulated. Considering these data, in our model, it is possible that the reduction in DCX + cells was promoted by a pro-inflammatory state evoked by the pharmacological blockade of CB_2 receptors. Other approaches such as the determination of the inflammatory profile in the DG will allow to indicate whether the effects of CB_2 receptor manipulations in the behavior and in the number of DCX cells are due to inflammation-related actions on the neurogenic niche or to a direct effect of neuronal CB_2 receptors in the Esc response.

Noteworthy, not only neurons exert important functions, but also glial cells exert pivotal roles in the CNS, as it has been highlighted by several studies in the past few years (Jäkel & Dimou, 2017). CB_2 receptor are expressed mainly in microglia, but also in astrocytes and oligodendrocytes (Scheller & Kirchhoff, 2016; Ilyasov et al., 2018). Therefore, the role of glial cells in the ESC antidepressants and neuroplastic effects should be considered in future neuropharmacological studies.

Some methodological differences in relation to other published studies should be considered, since previous investigations of targeting CB2 to promote behavioral modulations were assessed in non-stressed rodents and the neurobiological basis of the stress are determinant to the responses obtained in psychopharmacology studies with cannabinoids (to detailed information, see Morena et al., 2016).

Despite the relevance of our results, we recognize some limitations of our study. Our control versus CUS-groups that received vehicle as treatments presented different stress-induced changes in behavioral despair in the TST (**Figures 2** vs **Figure 3**). However, it is relevant to mention that there are differences between protocols performed to the dose-response curve experiments and the experiments with the antidepressant/antagonism assay which could change the stress levels of control mice: In the first protocol (dose-response curve of AM630) mice received a single injection per day whereas in the second protocol (escitalopram) mice receive two separate injections daily (1st AM630 or vehicle; 2nd ESC or vehicle). This

difference in handling and number of injections can interfere in the response observed, since it constitutes one more 'layer' of stress to the animals and induces more anxious-like behaviors, specially to groups of control animals (Lapin, 1995; Clarkson et al., 2018). This apparent discrepancy was observed in a recent study published by our group (Fernandes et al., 2021). In addition, chronic unpredictable stress can be used to determine sub-populations of mice that respond different to stress (resilient versus susceptible) and specific molecular markers that could be used as future tools to understand how stress influence behaviors and, translationally, psychiatric disorders (Torrisi et al., 2021; Dziedzicka-Wasylewska et al., 2021). The participation of CB2 receptors in resilience events remain to be elucidated.

Another important limitation of our results relies on the single measure of DCX as a marker of immature neurons survival without the analysis of other phases of the process of adult hippocampal neurogenesis during stress responses possibly under the influence of CB2 receptor (the initial proliferative phase and the expression of survival and mature cells. The migratory ability of DCX + cells is well established in the literature and this feature may be responsible for the complex modulation of the microenvironment during neurogenic events, mediating the connectivity profile of cells in different regions into the DG (Kempermann et al., 2015). However, the precise role of DCX + cells in the neurogenic processes remains under evaluation, which is the reason we choose to investigate this specific cell population in the context of antidepressant chronic treatment. Although the existence of other pharmacological approaches to antagonize more specifically the CB2 receptors in the periphery, such as the SR 144528 (Rinaldi-Carmona et al., 1998), some data has shown pharmacological activity (Rhee and Kim, 2002) and behavioral effects (Hassanzadeh et al., 2016) to be similar to the AM630, highlighting the need of development of new pharmacological compounds to manipulate and study the CB2 receptor role in the SNC.

In summary, our data reveals the relevance of CB_2 receptor activation on the Esc neuroplastic effects and antidepressant-like, but not anxiolytic-like. effects Our results bring new pieces of evidence for an important role of the CB_2 receptor in the mechanism of action of SSRI, supporting the hypothesis that SSRI drugs display CB_2 receptor-dependent neuroplastic effects and behavioral adaptations to promote stress coping. We fully endorse the need of further investigation of parallel mechanisms of action of antidepressants.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethical Committee of Animal Experimentation of the Ribeirão Preto Medical School (FMRP)- USP approved the experimental protocols according to the Brazilian laws and the ARRIVE guide (CEUA/FMRP 032/2015-1, 01/2019).

AUTHOR CONTRIBUTIONS

MR: Conceptualization, Methodology, Formal Analysis, Writing, Review and Editing, Visualization. RA: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Writing, Review and Editing, Visualization. EF: Methodology, Investigation. FS: Methodology, Formal Analysis, Writing, Review and Editing, RO: Conceptualization, Methodology, Investigation. FG: Conceptualization, Resources, Writing Review and editing, Funding acquisition. AC: Conceptualization, Methodology, Formal Analysis, Resources, Writing Original draft, Writing Review and editing, Visualization, Supervision, Project administration, Funding acquisition.

FUNDING

This study was supported by the FAPESP Young research grant (2015/05551-0), the thematic grant (2017/24304-0), the L'Oreal-Unesco-Brazilian Academy of Science for Women in Science Fellowship, and CNPq Universal Grant line A (400033/2016-0).

ACKNOWLEDGMENTS

We would like to thank Eleni Tamburus, America Scopinho and Tadeu Franco Vieira (*in memorian*) for their excellent technical support. We want to express our gratitude to our lab colleagues for keeping an amazing, collaborative and healthy environment.

REFERENCES

Aguado, T., Romero, E., Monory, K., Palazuelos, J., Sendtner, M., and Marsicano, G. (2007). The CB1 Cannabinoid Receptor Mediates Excitotoxicity-Induced Neural Progenitor Proliferation and Neurogenesis. *The Journal of Biological Chemistry* 282 (33), 23892–23898. doi:10.1074/jbc.M700678200

Ashton, J. C., Friberg, D., Darlington, C. L., and Smith, P. F. (2006). Expression of the Cannabinoid CB2 Receptor in the Rat Cerebellum: an Immunohistochemical Study. *Neurosci. Lett.* 396 (2), 113–116. doi:10.1016/j.neulet.2005.11.038

Ashton, J. C., and Glass, M. (2007). The Cannabinoid CB2 Receptor as a Target for Inflammation-dependent Neurodegeneration. *Curr. Neuropharmacol* 5 (2), 73–80. doi:10.2174/157015907780866884

Bai, C. (2019). Retirar por gentileza, nao achei no meu endnoteweb

Benito, C., Núñez, E., Tolón, R. M., Carrier, E. J., Rábano, A., Hillard, C. J., et al. (2003). Cannabinoid CB2 Receptors and Fatty Acid Amide Hydrolase Are Selectively Overexpressed in Neuritic Plaque-Associated Glia in Alzheimer's Disease Brains. *J. Neurosci.* 23 (35), 11136–11141. doi:10.1523/jneurosci.23-35-11136.2003

Benito, C., Tolón, R. M., Pazos, M. R., Núñez, E., Castillo, A. I., and Romero, J. (2008). Cannabinoid CB2 Receptors in Human Brain Inflammation. *Br. J. Pharmacol.* 153 (2), 277–285. doi:10.1038/sj.bjp.0707505

Busse, M., Busse, S., Myint, A. M., Gos, T., Dobrowolny, H., and Müller, U. J. (2015). Decreased quinolinic acid in the hippocampus of depressive patients: evidence for local anti-inflammatory and neuroprotective responses?. *European archives of psychiatry and clinical neuroscience* 265 (4), 321–329. doi:10.1007/s00406-014-0562-0

Campos, A. C., Ortega, Z., Palazuelos, J., Fogaça, M. V., Aguiar, D. C., Díaz-Alonso, J., et al. (2013). The Anxiolytic Effect of Cannabidiol on Chronically Stressed Mice Depends on Hippocampal Neurogenesis: Involvement of the Endocannabinoid System. *Int. J. Neuropsychopharmacol.* 16 (6), 1407–1419. doi:10.1017/S1461145712001502

Campos, A. C., Vaz, G. N., Saito, V. M., and Teixeira, A. L. (2014). Further Evidence for the Role of Interferon-Gamma on Anxiety- and Depressive-like Behaviors: Involvement of Hippocampal Neurogenesis and NGF Production. *Neurosci. Lett.* 578, 100–105. doi:10.1016/j.neulet.2014.06.039

Carlisle, S. J., Marciano-Cabral, F., Staab, A., Ludwick, C., and Cabral, G. A. (2002). Differential Expression of the CB2 Cannabinoid Receptor by Rodent Macrophages and Macrophage-like Cells in Relation to Cell Activation. *Int. Immunopharmacol* 2 (1), 69–82. doi:10.1016/s1567-5769(01)00147-3

Dale, E., Bang-Andersen, B., and Sánchez, C. (2015). Emerging Mechanisms and Treatments for Depression beyond SSRIs and SNRIs. *Biochem. Pharmacol.* 95 (2), 81–97. doi:10.1016/j.bcp.2015.03.011

Delgado, P. L. (2000). Depression: the Case for a Monoamine Deficiency. *J. Clin. Psychiatry* 61 (Suppl. 6), 7–11.

Dubreucq, S., Matias, I., Cardinal, P., Häring, M., Lutz, B., and Marsicano, G. (2012). Genetic dissection of the role of cannabinoid type-1 receptors in the emotional consequences of repeated social stress in mice. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* 37 (8), 1885–1900. doi:10.1038/npp.2012.36

Dziedzicka-Wasylewska, M., Solich, J., Korlatowicz, A., and Faron-Górecka, A. (2021). What Do the Animal Studies of Stress Resilience Teach Us? *Cells* 10 (7). doi:10.3390/cells10071630

Fernandes, G. G., Costa, K. C. M., Scomparin, D. S., Freire, J. B., Guimarães, F. S., and Campos, A. C. (2021). Genetic Ablation of the Inducible Form of Nitric Oxide in Male Mice Disrupts Immature Neuron Survival in the Adult Dentate Gyrus. *Front. Immunol.* 12, 782831. doi:10.3389/fimmu.2021.782831

Franklin, K., and Paxinos, G. (2008). *The Mouse Brain in Stereotaxic Coordinates*. 3rd Editio. USA: Academic Press.

Fujioka, H., and Akema, T. (2010). Lipopolysaccharide acutely inhibits proliferation of neural precursor cells in the dentate gyrus in adult rats. *Brain research* 1352, 35–42. doi:10.1016/j.brainres.2010.07.032

García-Gutiérrez, M. S., García-Bueno, B., Zoppi, S., Leza, J. C., and Manzanares, J. (2012). Chronic Blockade of Cannabinoid CB2 Receptors Induces Anxiolytic-like Actions Associated with Alterations in GABA(A) Receptors. *Br. J. Pharmacol.* 165 (4), 951–964. doi:10.1111/j.1476-5381.2011.01625.x

García-Gutiérrez, M. S., and Manzanares, J. (2011). Overexpression of CB2 Cannabinoid Receptors Decreased Vulnerability to Anxiety and Impaired Anxiolytic Action of Alprazolam in Mice. *J. Psychopharmacol.* 25 (1), 111–120. doi:10.1177/0269881110379507

García-Gutiérrez, M. S., Pérez-Ortiz, J. M., Gutiérrez-Adán, A., and Manzanares, J. (2010). Depression-resistant Endophenotype in Mice Overexpressing Cannabinoid CB(2) Receptors. *Br. J. Pharmacol.* 160 (7), 1773–1784. doi:10.1111/j.1476-5381.2010.00819.x

Gong, J. P., Onaivi, E. S., Ishiguro, H., Liu, Q. R., Tagliaferro, P. A., Brusco, A., et al. (2006). Cannabinoid CB2 Receptors: Immunohistochemical Localization in Rat Brain. *Brain Res.* 1071 (1), 10–23. doi:10.1016/j.brainres.2005.11.035

Gould, T. D., Dao, D. T., and Kovacsics, C. E. (2009). "The Open Field Test," in *Mood anxiety Relat. phenotypes mice*. Editors T. Gould, 42 (Totowa, NJ: Humana Press). doi:10.1007/978-1-60761-303-9_1

Hájos, N., Ledent, C., and Freund, T. F. (2001). Novel Cannabinoid-Sensitive Receptor Mediates Inhibition of Glutamatergic Synaptic Transmission in the hippocampus. *Neuroscience* 106 (1), 1–4. doi:10.1016/s0306-4522(01)00287-1

Hassanzadeh, P., Arbabi, E., Atyabi, F., and Dinarvand, R. (2016). The Endocannabinoid System and NGF Are Involved in the Mechanism of Action of Resveratrol: a Multi-Target Nutraceutical with Therapeutic Potential in Neuropsychiatric Disorders. *Psychopharmacology (Berl)* 233 (6), 1087–1096. doi:10.1007/s00213-015-4188-3

Hill, M. N., McLaughlin, R. J., Bingham, B., Shrestha, L., Lee, T. T., and Gray, J. M. (2015). Endogenous cannabinoid signaling is essential for stress adaptation. Proceedings of the National Academy of Sciences of the United States of America 107 (20), 9406–9411. doi:10.1073/pnas.0914661107

Hill, A. S., Sahay, A., and Hen, R. (2015). Increasing Adult Hippocampal Neurogenesis Is Sufficient to Reduce Anxiety and Depression-like Behaviors. *Neuropsychopharmacology* 40 (10), 2368–2378. doi:10.1038/npp.2015.85

Hill, M. N., Ho, W. S., Sinopoli, K. J., Viau, V., Hillard, C. J., and Gorzalka, B. B. (2006). Involvement of the Endocannabinoid System in the Ability of Long-Term Tricyclic Antidepressant Treatment to Suppress Stress-Induced Activation of the Hypothalamic-Pituitary-Adrenal axis. *Neuropsychopharmacology* 31 (12), 2591–2599. doi:10.1038/sj.npp.1301092

Ilyasov, A. A., Milligan, C. E., Pharr, E. P., and Howlett, A. C. (2018). The Endocannabinoid System and Oligodendrocytes in Health and Disease. *Front. Neurosci.* 12, 733. doi:10.3389/fnins.2018.00733

Jäkel, S., and Dimou, L. (2017). Glial Cells and Their Function in the Adult Brain: A Journey through the History of Their Ablation. *Front Cel Neurosci* 11, 24. doi:10.3389/fncel.2017.00024

Klegeris, A., Bissonnette, C. J., and McGeer, P. L. (2003). Reduction of Human Monocytic Cell Neurotoxicity and Cytokine Secretion by Ligands of the Cannabinoid-type CB2 Receptor. *Br. J. Pharmacol.* 139 (4), 775–786. doi:10.1038/sj.bjp.0705304

Kohl, Z., Winner, B., Ubhi, K., Rockenstein, E., Mante, RM., Münch, M., et al. (2012). Fluoxetine rescues impaired hippocampal neurogenesis in a transgenic A53T synuclein mouse model. *The European journal of neuroscience,* 35 (1), 10–19. doi:10.1111/j.1460-9568.2011.07933.x

Kopschina Feltes, P., Doorduin, J., Klein, H. C., Juárez-Orozco, L. E., Dierckx, R. A., Moriguchi-Jeckel, C. M., et al. (2017). Anti-inflammatory Treatment for Major Depressive Disorder: Implications for Patients with an Elevated Immune Profile and Non-responders to Standard Antidepressant Therapy. *J. Psychopharmacol.* 31 (9), 1149–1165. doi:10.1177/0269881117711708

Kruk-Slomka, M., Michalak, A., and Biala, G. (2015). Antidepressant-like Effects of the Cannabinoid Receptor Ligands in the Forced Swimming Test in Mice: Mechanism of Action and Possible Interactions with Cholinergic System. *Behav. Brain Res.* 284, 24–36. doi:10.1016/j.bbr.2015.01.051

Laplante, M., and Sabatini, D. M. (2012). mTOR signaling in growth control and disease. *Cell* 149 (2), 39. doi:10.1016/j.cell.2012.03.017

Lisboa, S. F., Gomes, F. V., Guimaraes, F. S., and Campos, A. C. (2016). Microglial Cells as a Link between Cannabinoids and the Immune Hypothesis of Psychiatric Disorders. *Frontiers in neurology* 7, 5. doi:10.3389/fneur.2016.00005

Liu, X. L., Luo, L., Mu, R. H., Liu, B. B., Geng, D., Liu, Q., et al. (2015). Fluoxetine Regulates mTOR Signalling in a Region-dependent Manner in Depression-like Mice. *Sci. Rep.* 5, 16024. doi:10.1038/srep16024

Madrigal, J. L., García-Bueno, B., Caso, J. R., Pérez-Nievas, B. G., and Leza, J. C. (2006). Stress-induced oxidative changes in brain. *CNS and neurological disorders drug targets* 5 (5), 561–568. doi:10.2174/187152706778559327

Malberg, J. E., and Duman, R. S. (2003). Cell Proliferation in Adult hippocampus Is Decreased by Inescapable Stress: Reversal by Fluoxetine Treatment. *Neuropsychopharmacology* 28 (9), 1562–1571. doi:10.1038/sj.npp.1300234

Maresz, K., Carrier, E. J., Ponomarev, E. D., Hillard, C. J., and Dittel, B. N. (2005). Modulation of the Cannabinoid CB2 Receptor in Microglial Cells in Response to Inflammatory Stimuli. *J. Neurochem.* 95 (2), 437–445. doi:10.1111/j.1471-4159.2005.03380.x

Mechoulam, R., and Parker, L. A. (2013). The Endocannabinoid System and the Brain. *Annu. Rev. Psychol.* 64, 21–47. doi:10.1146/annurev-psych-113011-143739

Morena, M., Patel, S., Bains, J. S., and Hill, M. N. (2016). Neurobiological Interactions Between Stress and the Endocannabinoid System. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* 41 (1), 80–102. doi:10.1038/npp.2015.166

Onaivi, E. S., Ishiguro, H., Gong, J. P., Patel, S., Meozzi, P. A., Myers, L., et al. (2008). Functional Expression of Brain Neuronal CB2 Cannabinoid Receptors Are Involved in the Effects of Drugs of Abuse and in Depression. *Ann. N. Y Acad. Sci.* 1139, 434–449. doi:10.1196/annals.1432.036

Onaivi, E. S. (2006). Neuropsychobiological Evidence for the Functional Presence and Expression of Cannabinoid CB2 Receptors in the Brain. *Neuropsychobiology* 54 (4), 231–246. doi:10.1159/000100778

Ortega-Alvaro, A., Aracil-Fernández, A., García-Gutiérrez, M. S., Navarrete, F., and Manzanares, J. (2011). Deletion of CB2 Cannabinoid Receptor Induces Schizophrenia-Related Behaviors in Mice. *Neuropsychopharmacology* 36 (7), 1489–1504. doi:10.1038/npp.2011.34

Palazuelos, J., Aguado, T., Egia, A., Mechoulam, R., Guzmán, M., and Galve-Roperh, I. (2006). Non-psychoactive CB2 Cannabinoid Agonists Stimulate Neural Progenitor Proliferation. *FASEB J.* 20 (13), 2405–2407. doi:10.1096/fj.06-6164fje

Palazuelos, J., Ortega, Z., Díaz-Alonso, J., Guzmán, M., and Galve-Roperh, I. (2012). CB2 Cannabinoid Receptors Promote Neural Progenitor Cell Proliferation via mTORC1 Signaling. *J. Biol. Chem.* 287 (2), 1198–1209. doi:10.1074/jbc.M111.291294

Patel, S., Cravatt, B. F., and Hillard, C. J. (2005a). Synergistic interactions between cannabinoids and environmental stress in the activation of the central amygdala. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* 30 (3), 497–507. doi:10.1038/sj.npp.1300535

Patel, S., Roelke, C. T., Rademacher, D. J., and Hillard, C. J. (2005b). Inhibition of restraint stress-induced neural and behavioural activation by endogenous cannabinoid signalling. *The European journal of neuroscience* 21 (4), 1057–1069. doi:10.1111/j.1460-9568.2005.03916.x

Patel, S., Roelke, C. T., Rademacher, D. J., Cullinan, W. E., and Hillard, C. J. (2004). Endocannabinoid signaling negatively modulates stress-induced activation of the hypothalamic-pituitary-adrenal axis. *Endocrinology* 145 (12), 5431–5438. doi:10.1210/en.2004-0638

Poleszak, E., Wośko, S., Sławińska, K., Wyska, E., Szopa, A., Świąder, K., et al. (2020). Influence of the Endocannabinoid System on the Antidepressant Activity of Bupropion and Moclobemide in the Behavioural Tests in Mice. *Pharmacol. Rep.* 72, 1562–1572. doi:10.1007/s43440-020-00088-0

Prenderville, J. A., Kelly, Á. M., and Downer, E. J. (2015). The Role of Cannabinoids in Adult Neurogenesis. *Br. J. Pharmacol.* 172 (16), 3950–3963. doi:10.1111/bph.13186

Rhee, M. H., and Kim, S. K. (2002). SR144528 as Inverse Agonist of CB2 Cannabinoid Receptor. *J. Vet. Sci.* 3 (3), 179–184. doi:10.4142/jvs.2002.3.3.179

Rinaldi-Carmona, M., Barth, F., Millan, J., Derocq, J. M., Casellas, P., Congy, C., et al. (1998). SR 144528, the First Potent and Selective Antagonist of the CB2 Cannabinoid Receptor. *J. Pharmacol. Exp. Ther.* 284 (2), 644–650.

Santarelli, L., Saxe, M., Gross, C., Surget, A., Battaglia, F., Dulawa, S., et al. (2003). Requirement of Hippocampal Neurogenesis for the Behavioral Effects of Antidepressants. *Science* 301 (5634), 805–809. doi:10.1126/science.1083328

Samuels, B. A., Anacker, C., Hu, A., Levinstein, M. R., Pickenhagen, A., Tsetsenis, T., et al. (2015). 5-HT1A Receptors on Mature Dentate Gyrus Granule Cells Are Critical for the Antidepressant Response. *Nat. Neurosci.* 18 (11), 1606–1616. doi:10.1038/nn.4116

Sánchez, C., de Ceballos, M. L., Gomez del Pulgar, T., Rueda, D., Corbacho, C., Velasco, G., et al. (2001). Inhibition of Glioma Growth *In Vivo* by Selective Activation of the CB(2) Cannabinoid Receptor. *Cancer Res.* 61 (15), 5784–5789.

Scheller, A., and Kirchhoff, F. (2016). Endocannabinoids and Heterogeneity of Glial Cells in Brain Function. *Front. Integr. Neurosci.* 10, 24. doi:10.3389/fnint.2016.00024

Seo, M. K., Choi, C. M., McIntyre, R. S., Cho, H. Y., Lee,, C. H., and Mansur, R. B. (2017). Effects of escitalopram and paroxetine on mTORC1 signaling in the rat hippocampus under chronic restraint stress. *BMC neuroscience* 18 (1), 39. doi:10.1186/s12868-017-0357-0

Steru, L., Chermat, R., Thierry, B., and Simon, P. (1985). The Tail Suspension Test: a New Method for Screening Antidepressants in Mice. *Psychopharmacology (Berl)* 85 (3), 367–370. doi:10.1007/BF00428203

Torrisi, S. A., Lavanco, G., Maurel, O. M., Gulisano, W., Laudani, S., Geraci, F., et al. (2021). A Novel Arousal-Based Individual Screening Reveals Susceptibility and Resilience to PTSD-like Phenotypes in Mice. *Neurobiol. Stress* 14, 100286. doi:10.1016/j.ynstr.2020.100286

Turcotte, C., Blanchet, M. R., Laviolette, M., and Flamand, N. (2016). The CB_2 receptor and its role as a regulator of inflammation. *Cellular and molecular life sciences : CMLS* 73 (23), 4449–447.

Tynan, R. J., Weidenhofer, J., Hinwood, M., Cairns, M. J., Day, T. A., and Walker, F. R. (2012). A Comparative Examination of the Anti-inflammatory Effects of SSRI and SNRI Antidepressants on LPS Stimulated Microglia. *Brain Behav. Immun.* 26 (3), 469–479. doi:10.1016/j.bbi.2011.12.011

Wager-Smith, K., and Markou, A. (2011). Depression: a repair response to stress-induced neuronal microdamage that can grade into a chronic neuroinflammatory condition?. *Neuroscience and biobehavioral reviews* 35 (3), 742–764. doi:10.1016/j.neubiorev.2010.09.010

Wang, J. W., David, D. J., Monckton, J. E., Battaglia, F., and Hen, R. (2008). Chronic Fluoxetine Stimulates Maturation and Synaptic Plasticity of Adult-Born Hippocampal Granule Cells. *J. Neurosci.* 28 (6), 1374–1384. doi:10.1523/JNEUROSCI.3632-07.2008

Willner, P., and Mitchell, P. J. (2002). The Validity of Animal Models of Predisposition to Depression. *Behav. Pharmacol.* 13 (3), 169–188. doi:10.1097/00008877-200205000-00001

Wotjak, C. T. (2005). Role of Endogenous Cannabinoids in Cognition and Emotionality. *Mini Rev. Med. Chem.* 5 (7), 659–670. doi:10.2174/1389557054368763

Yiangou, Y., Facer, P., Durrenberger, P., Chessell, I. P., Naylor, A., Bountra, C., et al. (2006). COX-2, CB2 and P2X7-Immunoreactivities Are Increased in Activated Microglial Cells/macrophages of Multiple Sclerosis and Amyotrophic Lateral Sclerosis Spinal Cord. *BMC Neurol.* 6, 12. doi:10.1186/1471-2377-6-12

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 03 February 2022
doi: 10.3389/fphar.2021.804950

A Cannabinoid 2-Selective Agonist Inhibits Allogeneic Skin Graft Rejection *In Vivo*

Senthil Jayarajan, Joseph J. Meissler, Martin W. Adler and Toby K. Eisenstein*

Center for Substance Abuse Research, Lewis Katz School of Medicine at Temple University, Philadelphia, PA, United States

Previous work from our laboratory showed that a CB2 selective agonist, O-1966, blocked the proliferative response of C57BL/6 mouse spleen cells exposed to spleen cells of C3HeB/FeJ mice *in vitro* in the mixed lymphocyte reaction (MLR). The MLR is widely accepted as an *in vitro* correlate of *in vivo* grant rejection. Mechanisms of the immunosuppression induced by the cannabinoid were explored, and it was shown that O-1966 in this *in vitro* assay induced $CD25^{+}Foxp3^{+}$ Treg cells and IL-10, as well as down-regulated mRNA for CD40 and the nuclear form of the transcription factors NF-κB and NFAT in T-cells. The current studies tested the efficacy of O-1966 in prolonging skin grafts *in vivo*. Full thickness flank skin patches (1-cm^2) from C3HeB/FeJ mice were grafted by suturing onto the back of C57BL/6 mice. O-1966 or vehicle was injected intraperitoneally into treated or control groups of animals beginning 1 h pre-op, and then every other day until 14 days post-op. Graft survival was scored based on necrosis and rejection. Treatment with 5 mg/kg of O-1966 prolonged mean graft survival time from 9 to 11 days. Spleens harvested from O-1966 treated mice were significantly smaller than those of vehicle control animals based on weight. Flow cytometry analysis of $CD4^{+}$ spleen cells showed that O-1966 treated animals had almost a 3-fold increase in $CD25^{+}Foxp3^{+}$ Treg cells compared to controls. When dissociated spleen cells were placed in culture *ex vivo* and stimulated with C3HeB/FeJ cells in an MLR, the cells from the O-1966 treated mice were significantly suppressed in their proliferative response to the allogeneic cells. These results support CB2 selective agonists as a new class of compounds to prolong graft survival in transplant patients.

Keywords: graft rejection, CB2 agonist, T reg cells, mixed lymphocyte reaction (MLR), immunosuppresion

OPEN ACCESS

Edited by:
Meliha Karsak,
University Medical Center Hamburg-Eppendorf, Germany

Reviewed by:
Seyfullah Arslan,
Ankara Yildirim Beyazit University, Turkey
María Gómez-Cañas,
Complutense University of Madrid, Spain

***Correspondence:**
Toby K. Eisenstein
tke@temple.edu

Specialty section:
This article was submitted to Experimental Pharmacology and Drug Discovery, a section of the journal Frontiers in Pharmacology

Received: *29 October 2021*
Accepted: *21 December 2021*
Published: *03 February 2022*

Citation:
Jayarajan S, Meissler JJ, Adler MW and Eisenstein TK (2022) A Cannabinoid 2-Selective Agonist Inhibits Allogeneic Skin Graft Rejection In Vivo. Front. Pharmacol. 12:804950. doi: 10.3389/fphar.2021.804950

1 INTRODUCTION

The discovery of the CB2 receptor and its abundance and fairly selective expression on cells of the immune system (Munro et al., 1993; Galiegue et al., 1995) has posed the question of its function on immune responses. Many studies investigating beneficial or detrimental effects of cannabinoids on immune responses and resistance to infection have focused on Δ^9-THC or on the endogenous cannabinoids, 2-arachidonoylglycerol (2-AG) and anandamide. All three of these ligands bind to both CB1 and CB2. To prove that an effect of these agonists occurs via the CB2 receptor, investigators have used selective CB1 and CB2 antagonists, or used CB1 or CB2 receptor knock-out mice. Another approach to probing the role of CB2 receptors in immune responses is to use synthetic, CB2 selective agonists (Huffman et al., 1996; Hanuš et al., 1999; Huffman et al., 1999; Pacher and

Mechoulam, 2011). With these approaches, the majority of studies on cannabinoids have shown them to be anti-inflammatory, immunosuppressive (Croxford and Yamamura, 2005; Klein, 2005; Eisenstein and Meissler, 2015) and to polarize immune responses towards a Th2 phenotype (Newton et al., 1994; Klein et al., 2000; Yuan et al., 2002; Yang et al., 2014). Immunosuppression by Δ^9-THC has been associated with increases in the immunosuppressive and anti-inflammatory cytokines, TGF-β and IL-10 (Zhu et al., 2000), and it was shown that TGF-β induction occurs via the CB2 receptor (Gardner et al., 2002). Δ^9-THC has also been shown to inhibit macrophage presentation of antigen to T cells, which occurred through effects at the CB2 receptor, as macrophages taken from CB2 k/o mice were not suppressed (Buckley et al., 2000). Δ^9-THC and anandamide suppressed *in vitro* antibody formation by mouse splenocytes in a CB2 dependent manner as determined using cannabinoid receptor selective inhibitors (Eisenstein et al., 2007). CB2 selective agonists have been shown to be broadly anti-inflammatory, inhibiting paw edema in a rat carrageenan model, which correlated with reduced neutrophil infiltration and decreased production of reactive oxygen intermediates (Parlar et al., 2018). CB2 agonist have also been reported to inhibit chemotaxis of primary human blood T cells and the human Jurkat cell line to the chemokine CXCL12 (Ghosh et al., 2006; Coopman et al., 2007). Antigen-specific and non-antigen specific T cell proliferation was inhibited by CB2 agonists (Maresz et al., 2007), and by anandamide, acting through the CB2 receptor (Cencioni et al., 2010). Further, CB2 selective agonists have been shown to ameliorate autoimmune reactions in a variety of mouse models that include experimental autoimmune encephalitis (EAE) (a model for multiple sclerosis) (Ni et al., 2004; Maresz et al., 2007), systemic sclerosis (Akhmetshina et al., 2009; Servettaz et al., 2010), autoimmune uveoretinitis (Xu et al., 2007), murine colitis and inflammatory bowel disease (Storr et al., 2009; Singh et al., 2012; Fichna et al., 2014; Leinwand et al., 2017).

A model explored by our laboratory has been to test the effects of CB2 selective agonists on the mixed lymphocyte reaction (MLR). The MLR is accepted as an *in vitro* correlate of *in vivo* graft rejection. Briefly, spleen cells from two histoincompatible mouse strains are placed in culture together. Cells from the stimulator strain are inhibited from dividing by treatment with mitomycin C. After 48 h of incubation the responder strain cells will proliferate, which can be quantitated using tritiated thymidine. Our results have shown that CB2 selective agonists strongly inhibit the MLR. In the *in vitro* cultures IL-2 is inhibited, IL-10 is augmented, and Treg cells are induced (Robinson et al., 2013; Robinson et al., 2015). The present studies examined the effect of injecting a CB2 selective agonist on *in vivo* immune responses, including skin graft rejection in mice, and cytokine and Treg levels in treated, as compared to, animals receiving vehicle. A CB2 agonist was shown to prolong skin graft rejection time and to induce IL-10 and Tregs in the mouse spleen.

2 METHODS

2.1 Cannabinoid

O-1966, a CB2-selective agonist, was a generous gift from Anu Mahadevan (Organix, Woburn, MA). The affinity of O-1966 for CB1 and CB2 cannabinoid receptors was reported previously to be 5,055 ± 984 and 23 ± 2.1 nmol/L, respectively (Wiley et al., 2002). It was shown to stimulate ^{35}S-GTPγS binding with an EC50 of 70 ± 14 nmol/L and an Emax of 74 ± 5 (percent of maximal stimulation produced by the full agonist CP 55,940) (Zhang et al., 2007).

2.2 Mice

Six week-old, specific pathogen-free C3HeB/FeJ and C57BL/6J female mice were purchased from Jackson Laboratories (Bar Harbor, Maine). Animals were housed in the central animal facility of Lewis Katz School of Medicine at Temple University which is AAALAC certified. Treatments were carried out following procedures approved by the University IACUC Committee.

2.3 Experimental Design

2.3.1 Skin Graft Procedure

All surgeries were done under aseptic conditions. Animals were anesthetized with 1–2% isofluorane delivered via nose cone. To test the capacity of O-1966 treatment to inhibit rejection of a skin graft *in vivo*, 1 cm^2 pieces of flank skin were harvested from donor C3HeB/FeJ mice. The flank of recipient C57BL/6J mice was prepared to receive the graft by removing the skin and superficial tissue to create a graft bed slightly larger than the piece to be transferred. The graft was transferred into the bed and sutured in place. The graft was bandaged for 7 days. This procedure followed a standard protocol for carrying out such grafts (Lagodzinski et al., 1990). Animals did not receive post-operative analgesic as per permission from the IACUC, because of concerns that the analgesic might affect immune status which was being monitored as a function of cannabinoid treatment. Doses of O-1966 or vehicle (0.03% ethanol and 0.03% cremophor in saline) were administered by intraperitoneal injection (i.p.) every other day from 1 h before transplantation to post-operative day 14. Three different doses of O-1966 were used in three different cohorts of mice: 1, 5, and 10 μg/kg. Bandages were removed on day 7 and the grafts were monitored daily for rejection. An allograft was considered fully rejected when it was >90% necrotic. In the initial experiments, the three different dosage groups had 8 animals each. The experiment was repeated using just the 5 mg/kg dose, with 9 mice in the cannabinoid group and 9 in the vehicle group, yielding a total of 17 animals in each group when the two experiments at 5 mg/kg were combined. All animals were sacrificed at 14 days post-surgery in order to harvest splenic tissue to assess immune status as described below in **Sections 2.3.2** and **2.3.3**.

2.3.2 One-Way Mixed Lymphocyte Reaction

C57BL/6J mice that had received skin grafts from C3HeB/FeJ mice, with or without treatment with O1966, were sacrificed 14 days after grafting surgery. Their spleens were aseptically removed, and single cell suspensions were obtained by passing spleens through nylon mesh bags (Sefar Inc., Depew, NY) in RPMI-1640 with 5% fetal bovine serum (FBS) containing 50 μM 2-mercaptoethanol (2-Me), and 100 U/ml penicillin

and streptomycin sulfate. All reagents were purchased from Gibco Life Technologies (Carlsbad, CA), with the exception of FBS, which was purchased from HyClone Laboratories (Logan, UT). Red blood cells were lysed by hypotonic shock for 10 s with sterile water. Responder spleen cells from C57BL/6 mice were resuspended in RPMI with 10% FBS, 50 μM 2-Me, and 100 U/ml penicillin and streptomycin sulfate. Splenocytes from C3HeB/FeJ were similarly prepared to serve as the *in vitro* stimulator cells, but they were inactivated by treatment with 50 μg/ml of mitomycin C for 20 min at 37°C. The cells were washed three times to remove mitomycin C from the medium and resuspended to the desired concentration using a Beckman Coulter Z1 Dual Cell and Particle Counter (Beckman Coulter Inc., Indianapolis, IN). Responder cells (8×10^5) and stimulator cells (8×10^5) were co-cultured in 200 μl in 96 well plates for 48 h at 37°C in 5% CO_2. After a 48 h incubation period, half of the cultures were tested to see if they responded to the stimulator cells by cell division in the MLR. The other half of the cells were analyzed by flow cytometry to determine the percentage of $CD25^+Foxp3^+$ Treg cells. To assay the MLR, cells were pulsed with 1 μCi/well [^{3}H]-thymidine and harvested 18 h later onto glass fiber filters (Packard, Downers Grove, IL) using a Packard multichannel harvester, and placed in vials in liquid scintillation solution (Cytoscint, MP-Biomedical, Irvine, CA). [^{3}H]-thymidine incorporation on the filters was measured using a Packard 1900 TR liquid scintillation counter. Data were corrected for background by subtraction of [^{3}H]-thymidine incorporation in the absence of stimulator cells. Results are expressed as a suppression index (SI), where untreated spleen cells are given a value of 1.00 (100%), and responses of cultures receiving treatment with cannabinoids are calculated as:

$$\text{SI} = \frac{\text{Mean counts per minute of cannabinoid treated cultures}}{\text{Mean counts per minute of untreated cultures}}$$

The method for assaying for Treg cells is described below under the section on flow cytometry.

2.3.3 Flow Cytometry

The MLR cultures were harvested at various time points and washed with staining buffer (PBS containing 1% BSA, Sigma, St. Louis, MO). 1×10^6 cells in 1 ml of PBS were added to Falcon™ polystyrene round-bottom tubes (BD Biosciences) and stained with 1 μl of LIVE/DEAD® Dead Cell Stain (Molecular Probes, Inc.) for 30 min on ice. The cells were washed twice with staining buffer and resuspended in 50 μl of staining buffer. To prevent nonspecific binding, the cells were incubated with 1 μg of 2.4G2 antibody specific for Fcγ III/II receptor (BioLegend, San Diego, CA) at 4°C for 5 min. Cells were then incubated with 0.5 μg of fluorophore conjugated rat anti-mouse CD3ε (BioLegend), rat anti-mouse CD4 (BioLegend), or isotype control for 30 min on ice, washed twice with staining buffer and resuspended in PBS with 2% (w/v) paraformaldehyde (Sigma) on ice for 15 min. To assess the percent of Treg cells, the cells were washed three times with PBS and resuspended in 1 ml PBS with 0.5% (v/v) Tween 20 (Sigma), washed three times with staining buffer and resuspended in 100 μl staining buffer containing 0.5 μg rat anti-mouse Foxp3 or isotype control (BioLegend) at room temperature for 30 min. The cells were washed three times with staining buffer, resuspended in 400 μl staining buffer, and analyzed immediately on the LSRII (BD Biosciences, San Jose, CA) and analyzed using FACSDiva software (BD Biosciences) and post-analyzed with FlowJo (Tree Star, Inc., Ashland, OR).

2.4 Statistics

Data were analyzed using GraphPad InStat® (GraphPad Software, Inc., La Jolla, CA). Skin graft rejection data were analyzed using the Log-rank (Mantel-Cox) test. Data for spleen weights and for flow cytometry results were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance was defined as $p < 0.05$.

3 RESULTS

3.1 O-1966 Retards Skin Graft Rejection *In Vivo*

As shown in **Figure 1**, an inverse U-shaped dose response was observed for efficacy of O-1966 in retarding graft rejection. Mice that received either the 1 mg/kg or the 10 mg/kg doses showed no benefit from the CB2 agonist in graft prolongation (**Figures 1A,C**). In contrast, treatment with 5 mg/kg of O-1966 increased the median survival time of the grafts to 11 days compared to a median survival time of 9 days for vehicle-treated mice ($p = 0.0004$) (**Figure 1B**). The final rejection time for all grafts was extended from 11 days in controls to 14 days in the cannabinoid-treated animals.

3.2 O-1966 Treatment Decreases Splenic Weight in Skin Graft Recipients

Mice that are mounting a significant allograft rejection response will show increased splenic weight due to the proliferation of responding T cells. Splenic weight was therefore determined in mice that had received skin grafts, with or without treatment with O-1966. On day 14 post-surgery, the spleens of graft recipient mice which had received the 5 mg/kg dose of O-1966 were removed, weighed, and normalized to their body weight to yield a splenic index. **Figure 2** shows that the splenic indices of mice treated with O-1966 were significantly decreased compared to those of vehicle-treated mice. The splenic index of O-1966-treated mice was not different from that of control mice that did not receive skin grafts. Thus, the cannabinoid prevented the splenomegaly that characterizes animals undergoing skin graft rejection.

3.3 O-1966 Treatment Increases Treg Cells in Skin Graft Recipients

The spleens of the animals that were sacrificed 14 days after surgery and weighed, were further processed to determine the percentage of Treg cells and the levels of CD4 expression on

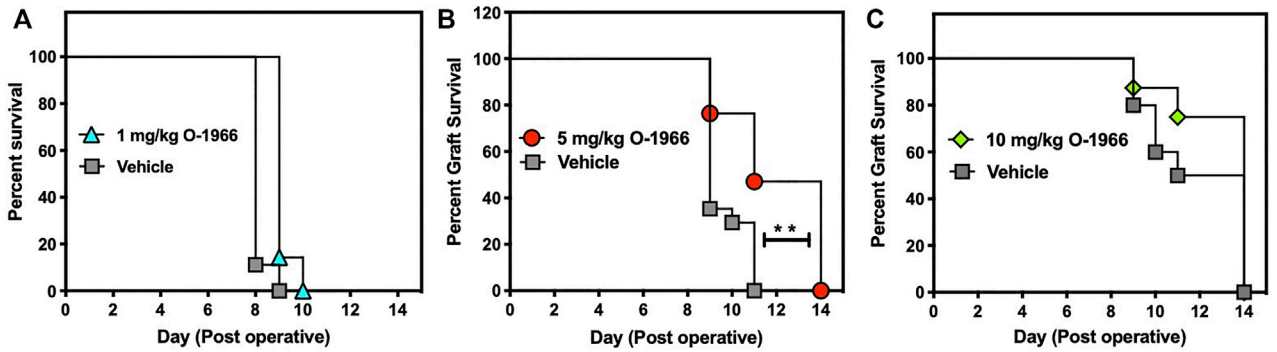


FIGURE 1 | O-1966 treatment prolongs skin graft viability. Donor C3HeB/FeJ flank skin was transplanted to the back of a recipient C57BL/6J mice, sutured, and bandaged. Doses of O-1966 or vehicle (0.03% ethanol and 0.03% cremophor in saline) were injected i.p. every other day, from 1 h pre-op to 14 days post-op. On day 7 bandages were removed and grafts were monitored for rejection. Percent graft survival of mice treated with **(A)** 1 mg/kg O-1966 (△), or vehicle (□), **(B)** 5 mg/kg O-1966 (●) or vehicle (□), or **(C)** 10 mg/kg O-1966 (◇) or vehicle (□). Panels **(A,C)** are results of a single experiment (n = 8 per group), and data in Panel **(B)** are the mean of two experiments (n = 17 per group). Median survival time of vehicle vs. O-1966 treatment at 5 mg/kg, $^{**}p < 0.001$ by the Log rank [Mantel-Cox] test.

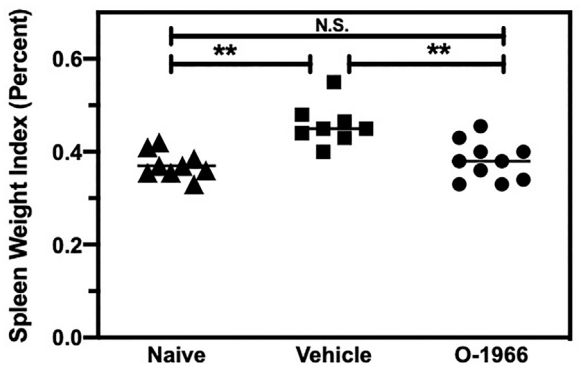


FIGURE 2 | O-1966 decreases spleen weights in skin graft recipient mice. Donor C3HeB/FeJ flank skin was transplanted to the back of a recipient C57BL/6J mice, sutured, and bandaged. Doses of O-1966 (5 mg/kg) or vehicle (0.03% ethanol and 0.03% cremophor in saline) were injected i.p. every other day from 1 h pre-op to 14 days post-op. On post-op day 14, animals were sacrificed and spleens were removed. The spleen weight to body weight ratio was calculated and is expressed as a percentage, of control mice (▲) (n = 9) and grafted mice treated with 5 mg/kg O-1966 (■) (n = 8) or vehicle (●) (n = 10). $^{**}p < 0.001$ for O-1966 vs. vehicle as determined by one-way ANOVA followed by Tukey's multiple comparison test.

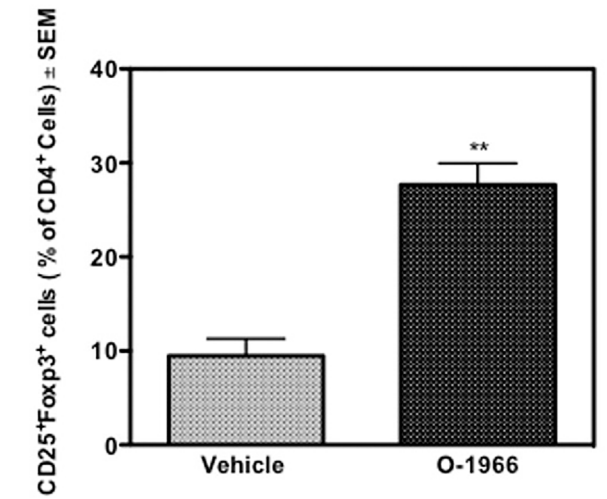


FIGURE 3 | O-1966 treatment increases percentage of splenic Tregs in skin graft recipient mice. Donor C3HeB/FeJ flank skin was transplanted to the back of recipient C57BL/6J mice, sutured, and bandaged. Doses of O-1966 (5 mg/kg) or vehicle (0.03% ethanol and 0.03% cremophor in saline) were injected i.p. every other day from 1 h pre-op to 14 days post-op. Splenocytes were harvested from grafted mice treated with O-1966 (▬) or vehicle (▭) on day 14 and were analyzed by flow cytometry for $CD4^+CD25^+FoxP3^+$ Tregs (n = 17 for both groups). Data show number of Tregs as a percentage of total live $CD4^+$ cells (LIVE/DEAD® dead cell stain negative). Data are mean of two separate experiments. $^{**}p < 0.01$ for O-1966 vs. vehicle as determined by one-way ANOVA followed by Tukey's multiple comparison test.

T cells. **Figure 3** shows that mice treated with O-1966 had 27.7% $CD25^+Foxp3^+$ Tregs in the live $CD4^+$ population, while mice treated with vehicle had only 9.5% $CD25^+Foxp3^+$ Tregs. This result leads to the conclusion that the CB2 selective agonist, O-1966, had the effect of increasing splenic Treg cells in mice that had received skin grafts. It was also found that mice treated with O-1966 had reduced levels of CD4 on the cell surface of $CD3^+$ cells. O-1966 treatment caused a negative shift of fluorescence intensity of these cells. **Figures 4A,B** present the mean fluorescence intensity of CD4 from a representative animal and from all recipient mice (n = 17 for each treatment group), respectively, sand show that the average intensity of CD4 expression on the cell surface is decreased by O-1966 treatment.

3.4 *In Vivo* O-1966 Treatment Suppresses Splenocyte Proliferation *Ex Vivo* in the Mixed Lymphocyte Reaction

The responsiveness of splenocytes from C57BL/6J mice that had received an allograft 14 days prior, and were treated over the 14-day period with either O-1966 or vehicle, were harvested and placed in culture. These C57BL/6J cells were then restimulated *ex*

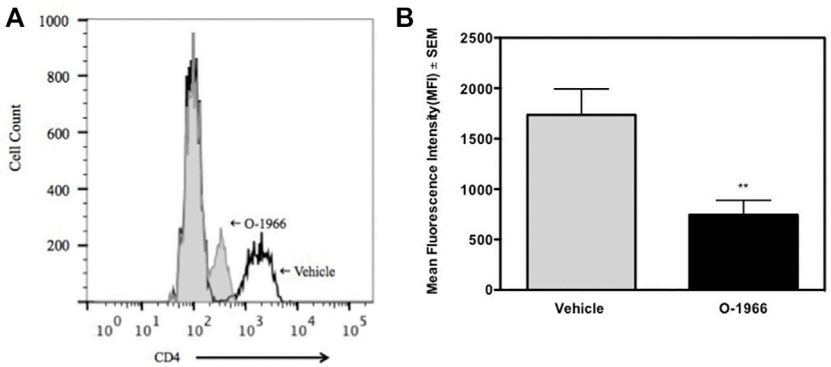


FIGURE 4 | O-1966 treatment decreases CD4 expression in skin graft recipient mice. Donor C3HeB/FeJ flank skin was transplanted to the back of recipient C57BL/6J mice, sutured, and bandaged. Doses of O-1966 (5 mg/kg) or vehicle (0.03% ethanol and 0.03% cremophor in saline) were injected i.p. every other day from 1 h pre-op to 14 days post-op. Splenocytes were harvested on day 14, stained for CD4, and analyzed by flow cytometry. **(A)** Representative histograms of CD4 expression on $CD3^+$ cells from mice treated with O-1966 (gray filled) or vehicle (white filled). **(B)** Mean fluorescence intensity (MFI) of CD4 in $CD3^+CD4^+$ populations from mice treated with O-1966 (■) or vehicle (▭). Data are mean of two experiments (n = 17 for both groups). $^{**}p < 0.01$ for O-1966 vs. vehicle by one-way ANOVA followed by Tukey's multiple comparison test).

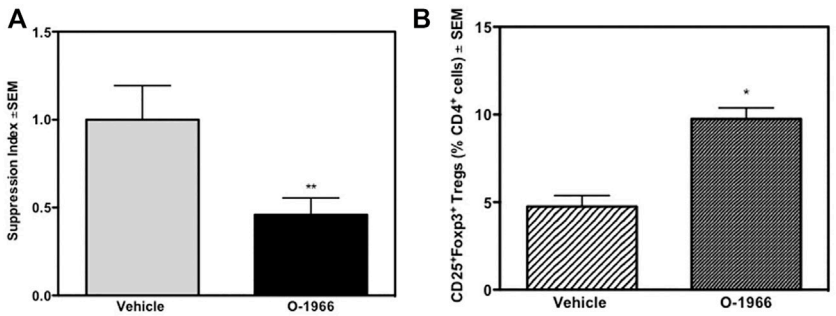


FIGURE 5 | *In vivo* O-1966 treatment decreases proliferation and increases the percentage of Tregs following *ex vivo* stimulation. Donor C3HeB/FeJ flank skin was transplanted to the back of recipient C57BL/6J mice, sutured, and bandaged. Doses of O-1966 (5 mg/kg) or vehicle (0.03% ethanol and 0.03% cremophor in saline) were injected i.p. every other day from 1 h pre-op to 14 days post-op. On post-op day 14, animals were sacrificed and spleens were aseptically removed, restimulated with C3HeB/FeJ splenocytes and put into culture for MLR **(A)** or harvested at 48 h and analyzed by flow cytometry **(B)**. **(A)** Proliferation of cultures with splenocytes from O-1966 treated mice (■) or vehicle treated mice (▭). **(B)** Cultures harvested at 48 h from mice treated with O-1966 (▨) or vehicle (▨) and analyzed by flow cytometry for $CD25^+Foxp3^+$ Tregs (n = 17 for both groups). Data show number of Tregs as a percentage of total live $CD4^+$ cells (LIVE/DEAD® dead cell stain negative). Data are mean of two separate experiments. $^*p < 0.05$, $^{**}p < 0.01$ for O-1966 vs. vehicle by one-way ANOVA followed by Tukey's multiple comparison test).

vivo in an MLR assay with mitomycin-treated C3HeB/FeJ spleen cells, the same haplotype as the tissue that was grafted *in vivo*. As shown in **Figure 5**, splenocytes from mice grafted with C3HeB/FeJ skin and treated with O-1966 *in vivo* had significantly decreased proliferation in response to *ex vivo* stimulation with the C3HeB/FeJ cells.

3.5 *In Vivo* O-1966 Treatment Increases Treg Cells in an *Ex Vivo* Mixed Lymphocyte Reaction

Some of the wells of the *ex vivo* MLR cultures were harvested 48 h after the start of the assay and stained for CD4, CD25 and Foxp3, and analyzed by flow cytometry. **Figure 6A** shows that cultures from mice treated with O-1966 *in vivo*, and restimulated *ex vivo* had double the percentage of $CD25^+Foxp3^+$ Tregs compared to cultures using spleen cells taken from vehicle treated mice, with the percentage increasing from 4.7 to 9.8%. Further, *ex vivo* restimulated cells harvested from cannabinoid-treated mice had reduced levels of CD4 compared to cells from mice treated *in vivo* with vehicle (**Figure 6B**).

4 DISCUSSION

The results of this study extend published *in vitro* studies using the MLR assay (Robinson et al., 2013; Robinson et al., 2015) to show efficacy of a CB2 selective agonist, O-1966, *in vivo*, in retarding rejection of skin grafts in mice and production of an immunosuppressive phenotype in the spleens of grafted animals

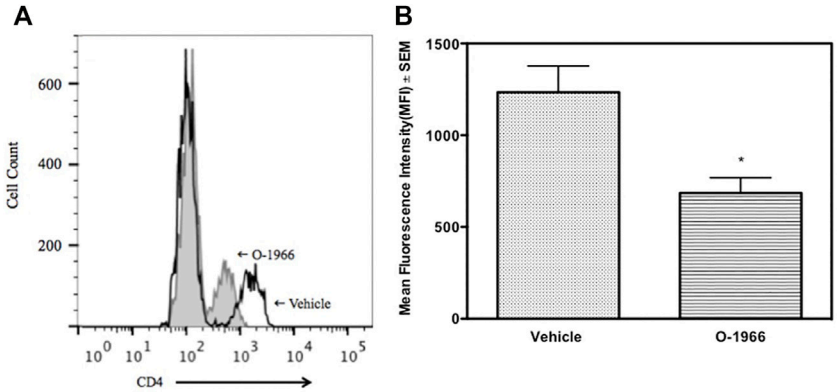


FIGURE 6 | *In vivo* O-1966 treatment decreases CD4 expression following *ex vivo* stimulation. Donor C3HeB/FeJ flank skin was transplanted to the back of recipient C57BL/6J mice, sutured, and bandaged. Doses of O-1966 (5 mg/kg) or vehicle (0.03% ethanol and 0.03% cremophor in saline) were injected i.p. every other day from 1 h pre-op to 14 days post-op. On post-op day 14, animals were sacrificed and spleens were aseptically removed, restimulated with C3HeB/FeJ splenocytes and harvested at 48 h and analyzed by flow cytometry for CD4 expression. **(A)** Representative histogram of CD4 expression on $CD3^+$ cells in cultures of splenocytes from skin graft recipient mice treated with O-1966 (gray filled) or vehicle (white filled). **(B)** Mean fluorescence intensity (MFI) of CD4 in $CD3^+$ populations in cultures from O-1966 treated mice (▬) or vehicle treated mice (▭). Data are representative of two experiments **(A)** or mean of two separate experiments **(B)**. *$p < 0.05$ for O-1966 vs. vehicle by one-way ANOVA followed by Tukey's multiple comparison test).

injected with the cannabinoid. Spleens of grafted mice treated with O-1966 had significantly increased numbers of immunosuppressive Treg cells, which would be expected to dampen immune responses to the graft. The smaller spleen sizes of the grafted, treated animals, also indicates that CB2 administration led to inhibition of immune cell proliferation to the graft. Other investigators have reported that other CB2 selective agonists can reduce spleen weight (Gu et al., 2017). The current observation that spleen cells harvested from grafted, CB2-treated mice were inhibited in their proliferation when placed *ex vivo* in culture with cells of the mouse strain that supplied the graft, is powerful evidence that CB2 can mediate immunosuppression. A mechanism was identified for the immunosuppression, namely the induction of Treg cells. A CB2 receptor agonist has also been shown to induce Treg cells and IL-10 in a murine model of Crohn's disease (Leinwand et al., 2017). Other possible mechanisms for CB2-mediated immunosuppression are suggested by studies from other laboratories and include blockage of T cell receptor signaling (Börner et al., 2009), and inhibition of maturation of T cells *in vivo* (Ziring et al., 2006). Another relevant paper reported that absence of the CB2 receptor resulted in more severe graft-versus-host reactions in a murine model by increasing CD8 cytotoxic T cells (Yuan et al., 2021).

In addition, it has been reported that JWH133, another CB2 selective agonist, protected against a murine model of ulcerative colitis by inducing T cell apoptosis (Rieder et al., 2010; Singh et al., 2012). In our previous *in vitro* studies using the MLR assay, we tested extensively for apoptosis and did not find it using O-1966 or another CB2 selective agonist, JWH-015 (Robinson et al., 2013). Consonant with our findings, lack of a cytotoxic effect on human, primary T cells by anandamide, an endogenous cannabinoid agonist, has been reported (Cencioni et al., 2010). Anandamide and JWH-015 (a CB2 selective agonist) were found to suppress activated T cells from producing IL-2, TNF-α and IFN-γ via action through the CB2 receptor (Cencioni et al., 2010). We had previously reported that O-1966 blocked IL-2 production in the MLR *in vitro* (Robinson et al., 2013). The experiments in this paper have focused on the effects of a CB2 selective agonist on T cells. However, the immunosuppressive capacity of CB2 agonists *in vivo* may reflect actions on macrophages. Several investigators have shown that CB2 agonists can polarize macrophages from an M1 to an M2 anti-inflammatory phenotype, where they produce increased amounts of IL-10 and arginase, and decreased amounts of pro-inflammatory cytokines and chemokines (Braun et al., 2018; Wu et al., 2020). In a mouse model of multiple sclerosis, treatment with a CB2 agonist markedly reduced microglial activation and reduced myeloid progenitor cell recruitment, possibly through altering the pattern of chemokine expression (Palazuelos et al., 2008). In contrast to these results, it has been reported that Δ^9-THC attenuated skin graft rejection in mice via a CB1 mediated induction of myeloid-derived suppressor cells (Sido et al., 2015). Since Δ^9-THC, like anandamide, binds to both CB1 and CB2 receptors, differentiating the receptor mediating the biological effect requires use of selective antagonists and cannabinoid receptor knock-out mice. The reason for the discrepancies in the literature are not readily apparent. CB2 agonists have also been shown to reduce the infarct size in induced stroke (Zhang et al., 2007) by preventing leukocyte extravasation at the site of the injury (Zhang et al., 2009). The effect has been narrowed to show that a CB2 agonist can inhibit neutrophil recruitment to the brain (Murikinati et al., 2010) and decrease the permeability of blood-brain barrier (Ramirez et al., 2012). Another group has also reported that CB2 receptor knock-out mice have defective neutrophil recruitment (Kapellos et al., 2019). A CB2 agonist has been shown to ameliorate sickness behavior induced by bacterial lipopolysaccharide that is mediated by excess production of pro-inflammatory cytokines (Sahu et al., 2019). There are several reports of CB2 attenuating sepsis in animal

models (He et al., 2019). The CB2 receptor has also been reported to be protective against the inflammatory sequelae of several infections including HIV and SARS-CoV2 (Rizzo et al., 2020; Rastegar et al., 2021) and to protect against liver damage due to Concanavalin A in mice, which is a model for hepatotoxicity induced by Hepatitis B infection (Huang et al., 2019). Thus, CB2 agonists may target many different cells in the immune system to reduce inflammation, innate immunity and adaptive immunity. Although the immunosuppressive effect of O-1996 in this study is attributed to increased numbers of Treg cells, it would be appropriate to study whether this cannabinoid and other CB2 selective agonists engage other mechanisms to suppress the immune response.

In regard to the experimental design, experiments with cannabinoids have frequently been subject to criticism about the doses of drugs needed to induce biological effects. O-1966 has an affinity for the CB2 receptor of 23 nM (Wiley et al., 2002). Yet in the present experiments, an inverted U-shaped dose response curve was observed, with enhanced graft survival observed at the 5 mg/kg dose but not at 1 or 10 mg/kg. These higher doses, which do not seem to correlate with affinity constants, are in the range reported by other investigators who have used cannabinoids with activity at the CB2 receptor (Adhikary et al., 2011; Gu et al., 2017).

The potential use of CB2 selective agonists to retard graft rejection is attractive. The increase in mean graft survival time by 2 days is comparable to that achieved in mice using calcineurin inhibitors (Lagodzinski et al., 1990). Current immunosuppressive therapies (calcineurin inhibitors) used to prevent or block tissue rejection in organ transplantation are associated with significant untoward effects. For example, toxicity with chronic tacrolimus use is associated with post-transplantation diabetes mellitus (PTDM) due to the death of pancreatic islet cells (Taylor et al., 2005), and also with kidney damage. Tacrolimus and rapamycin use may induce hypertension linked to hyperkalemia (Hoorn et al., 2011). Up to 50% of transplant patients have renal dysfunction within 5 years of starting immunosuppressive therapy (Naesens et al., 2009; Hoskova et al., 2017). In addition, rapamycin may cause encephalopathies and other central nervous systems deficits including tremors, headache, convulsions and psychosis (Ho et al., 1996). It may be possible to reduce the doses of these standard therapies used to inhibit rejection by combining them in a reduced dose with a CB2 selective agonist like O-1966.

The synthetic cannabinoid studied in this manuscript. O-1966, is a CB2 selective agonist. As CB2 receptors are only sparsely expressed in the neural system, and their main expression is on cells of the immune system, psychoactive effects are not present. Rather, this class of synthetic cannabinoids, CB2 selective agonists, has the potential to be therapeutic agents for conditions where the immune system is over-active, such as graft rejection and autoimmune diseases. The current experiments add to the literature supporting use of this class of compounds to dampen immune responses.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because the original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author. Requests to access the datasets should be directed to TE, tke@temple.edu.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee, Temple University.

AUTHOR CONTRIBUTIONS

SJ carried out the skin grafts; JM helped with the skin graft assessment, *ex vivo* assays, and manuscript preparation; MA participated in experimental design and data interpretation; TE oversaw experimental design, data collection, data interpretation, and manuscript preparation.

FUNDING

This work was supported by Pennsylvania Department of Health grant 4100054871 and NIDA/NIH grants DA13429, and T32-DA07327.

ACKNOWLEDGMENTS

Rebecca Robinson carried out the *ex vivo* assays of immune status while a graduate student in the laboratory of TE. She contributed to the manuscript with material written in her Ph.D. thesis.

REFERENCES

Adhikary, S., Li, H., Heller, J., Skarica, M., Zhang, M., Ganea, D., et al. (2011). Modulation of Inflammatory Responses by a Cannabinoid-2-Selective Agonist after Spinal Cord Injury. *J. Neurotrauma* 28, 2417–2427. doi:10.1089/neu.2011.1853

Akhmetshina, A., Dees, C., Busch, N., Beer, J., Sarter, K., Zwerina, J., et al. (2009). The Cannabinoid Receptor CB2 Exerts Antifibrotic Effects in Experimental Dermal Fibrosis. *Arthritis Rheum.* 60, 1129–1136. doi:10.1002/art.24395

Börner, C., Smida, M., Höllt, V., Schraven, B., and Kraus, J. (2009). Cannabinoid Receptor Type 1- and 2-mediated Increase in Cyclic AMP Inhibits T Cell Receptor-Triggered Signaling. *J. Biol. Chem.* 284, 35450–35460. doi:10.1074/jbc.M109.006338

Braun, M., Khan, Z. T., Khan, M. B., Kumar, M., Ward, A., Achyut, B. R., et al. (2018). Selective Activation of Cannabinoid Receptor-2 Reduces Neuroinflammation after Traumatic Brain Injury via Alternative Macrophage Polarization. *Brain Behav. Immun.* 68, 224–237. doi:10.1016/j.bbi.2017.10.021

Buckley, N. E., McCoy, K. L., Mezey, E., Bonner, T., Zimmer, A., Felder, C. C., et al. (2000). Immunomodulation by Cannabinoids Is Absent in Mice Deficient for the Cannabinoid CB(2) Receptor. *Eur. J. Pharmacol.* 396, 141–149. doi:10.1016/s0014-2999(00)00211-9

Cencioni, M. T., Chiurchiù, V., Catanzaro, G., Borsellino, G., Bernardi, G., Battistini, L., et al. (2010). Anandamide Suppresses Proliferation and

Cytokine Release from Primary Human T-Lymphocytes Mainly via CB2 Receptors. *PLoS One* 5, e8688. doi:10.1371/journal.pone.0008688

Coopman, K., Smith, L. D., Wright, K. L., and Ward, S. G. (2007). Temporal Variation in CB2R Levels Following T Lymphocyte Activation: Evidence that Cannabinoids Modulate CXCL12-Induced Chemotaxis. *Int. Immunopharmacol.* 7, 360–371. doi:10.1016/j.intimp.2006.11.008

Croxford, J. L., and Yamamura, T. (2005). Cannabinoids and the Immune System: Potential for the Treatment of Inflammatory Diseases. *J. Neuroimmunol.* 166, 3–18. doi:10.1016/j.jneuroim.2005.04.023

Eisenstein, T. K., and Meissler, J. J. (2015). Effects of Cannabinoids on T-Cell Function and Resistance to Infection. *J. Neuroimmune Pharmacol.* 10, 204–216. doi:10.1007/s11481-015-9603-3

Eisenstein, T. K., Meissler, J. J., Wilson, Q., Gaughan, J. P., and Adler, M. W. (2007). Anandamide and Delta9-tetrahydrocannabinol Directly Inhibit Cells of the Immune System via CB2 Receptors. *J. Neuroimmunol.* 189, 17–22. doi:10.1016/j.jneuroim.2007.06.001

Fichna, J., Bawa, M., Thakur, G. A., Tichkule, R., Makriyannis, A., McCafferty, D. M., et al. (2014). Cannabinoids Alleviate Experimentally Induced Intestinal Inflammation by Acting at central and Peripheral Receptors. *PLoS One* 9, e109115. doi:10.1371/journal.pone.0109115

Galiègue, S., Mary, S., Marchand, J., Dussossoy, D., Carrière, D., Carayon, P., et al. (1995). Expression of central and Peripheral Cannabinoid Receptors in Human Immune Tissues and Leukocyte Subpopulations. *Eur. J. Biochem.* 232, 54–61. doi:10.1111/j.1432-1033.1995.tb20780.x

Gardner, B., Zu, L. X., Sharma, S., Liu, Q., Makriyannis, A., Tashkin, D. P., et al. (2002). Autocrine and Paracrine Regulation of Lymphocyte CB2 Receptor Expression by TGF-Beta. *Biochem. Biophys. Res. Commun.* 290, 91–96. doi:10.1006/bbrc.2001.6179

Ghosh, S., Preet, A., Groopman, J. E., and Ganju, R. K. (2006). Cannabinoid Receptor CB2 Modulates the CXCL12/CXCR4-Mediated Chemotaxis of T Lymphocytes. *Mol. Immunol.* 43, 2169–2179. doi:10.1016/j.molimm.2006.01.005

Gu, S. M., Lee, H. J., Lee, T. H., Song, Y. J., Kim, Y. H., Han, K. M., et al. (2017). A Synthetic Cannabinoid JWH-210 Reduces Lymphoid Organ Weights and T-Cell Activator Levels in Mice via CB2 Receptors. *Naunyn Schmiedebergs Arch. Pharmacol.* 390, 1201–1209. doi:10.1007/s00210-017-1418-8

Hanus, L., Breuer, A., Tchilibon, S., Shiloah, S., Goldenberg, D., Horowitz, M., et al. (1999). HU-308: a Specific Agonist for CB(2), a Peripheral Cannabinoid Receptor. *Proc. Natl. Acad. Sci. U S A.* 96, 14228–14233. doi:10.1073/pnas.96.25.14228

He, Q., Xiao, F., Yuan, Q., Zhang, J., Zhan, J., and Zhang, Z. (2019). Cannabinoid Receptor 2: a Potential Novel Therapeutic Target for Sepsis. *Acta Clin. Belg.* 74, 70–74. doi:10.1080/17843286.2018.1461754

Ho, S., Clipstone, N., Timmermann, L., Northrop, J., Graef, I., Fiorentino, D., et al. (1996). The Mechanism of Action of Cyclosporin A and FK506. *Clin. Immunol. Immunopathol.* 80, S40–S45. doi:10.1006/clin.1996.0140

Hoorn, E. J., Walsh, S. B., McCormick, J. A., Fürstenberg, A., Yang, C. L., Roeschel, T., et al. (2011). The Calcineurin Inhibitor Tacrolimus Activates the Renal Sodium Chloride Cotransporter to Cause Hypertension. *Nat. Med.* 17, 1304–1309. doi:10.1038/nm.2497

Hoskova, L., Malek, I., Kopkan, L., and Kautzner, J. (2017). Pathophysiological Mechanisms of Calcineurin Inhibitor-Induced Nephrotoxicity and Arterial Hypertension. *Physiolog. Res.* 66, 167–180. doi:10.3549/physiolres.933332

Huang, Z. B., Zheng, Y. X., Li, N., Cai, S. L., Huang, Y., Wang, J., et al. (2019). Protective Effects of Specific Cannabinoid Receptor 2 Agonist GW405833 on Concanavalin A-Induced Acute Liver Injury in Mice. *Acta Pharmacol. Sin.* 40, 1404–1411. doi:10.1038/s41401-019-0213-0

Huffman, J. W., Liddle, J., Yu, S., Aung, M. M., Abood, M. E., Wiley, J. L., et al. (1999). 3-(1',1'-Dimethylbutyl)-1-deoxy-delta8-THC and Related Compounds: Synthesis of Selective Ligands for the CB2 Receptor. *Bioorg. Med. Chem.* 7, 2905–2914. doi:10.1016/s0968-0896(99)00219-9

Huffman, J. W., Yu, S., Showalter, V., Abood, M. E., Wiley, J. L., Compton, D. R., et al. (1996). Synthesis and Pharmacology of a Very Potent Cannabinoid Lacking a Phenolic Hydroxyl with High Affinity for the CB2 Receptor. *J. Med. Chem.* 39, 3875–3877. doi:10.1021/jm960394y

Kapellos, T. S., Taylor, L., Feuerborn, A., Valaris, S., Hussain, M. T., Rainger, G. E., et al. (2019). Cannabinoid Receptor 2 Deficiency Exacerbates Inflammation and Neutrophil Recruitment. *FASEB J.* 33, 6154–6167. doi:10.1096/fj.201802524R

Klein, T. W. (2005). Cannabinoid-based Drugs as Anti-inflammatory Therapeutics. *Nat. Rev. Immunol.* 5, 400–411. doi:10.1038/nri1602

Klein, T. W., Newton, C. A., Nakachi, N., and Friedman, H. (2000). Δ9-Tetrahydrocannabinol Treatment Suppresses Immunity and Early IFN-γ, IL-12, and IL-12 Receptor β2 Responses toLegionella pneumophilaInfection. *J. Immunol.* 164, 6461–6466. doi:10.4049/jimmunol.164.12.6461

Lagodzinski, Z., Górski, A., and Wasik, M. (1990). Effect of FK506 and Cyclosporine on Primary and Secondary Skin Allograft Survival in Mice. *Immunology* 71, 148–150.

Leinwand, K. L., Jones, A. A., Huang, R. H., Jedlicka, P., Kao, D. J., de Zoeten, E. F., et al. (2017). Cannabinoid Receptor-2 Ameliorates Inflammation in Murine Model of Crohn's Disease. *J. Crohns Colitis* 11, 1369–1380. doi:10.1093/ecco-jcc/jjx096

Maresz, K., Pryce, G., Ponomarev, E. D., Marsicano, G., Croxford, J. L., Shriver, L. P., et al. (2007). Direct Suppression of CNS Autoimmune Inflammation via the Cannabinoid Receptor CB1 on Neurons and CB2 on Autoreactive T Cells. *Nat. Med.* 13, 492–497. doi:10.1038/nm1561

Munro, S., Thomas, K. L., and Abu-Shaar, M. (1993). Molecular Characterization of a Peripheral Receptor for Cannabinoids. *Nature* 365, 61–65. doi:10.1038/365061a0

Murikinati, S., Jüttler, E., Keinert, T., Ridder, D. A., Muhammad, S., Waibler, Z., et al. (2010). Activation of Cannabinoid 2 Receptors Protects against Cerebral Ischemia by Inhibiting Neutrophil Recruitment. *FASEB J.* 24, 788–798. doi:10.1096/fj.09-141275

Naesens, N., Kuypers, D. R., and Sarwal, M. (2009). Calcineurin inhibitor nephrotoxicity. *Clin. J. Amer. Soc. Nephrol.* 4, 481–508. doi:10.2215/CJN.04800908

Newton, C. A., Klein, T. W., and Friedman, H. (1994). Secondary Immunity to Legionella pneumophila and Th1 Activity Are Suppressed by delta-9-tetrahydrocannabinol Injection. *Infect. Immun.* 62, 4015–4020. doi:10.1128/IAI.62.9.4015-4020.1994

Ni, X., Geller, E. B., Eppihimer, M. J., Eisenstein, T. K., Adler, M. W., and Tuma, R. F. (2004). Win 55212-2, a Cannabinoid Receptor Agonist, Attenuates Leukocyte/endothelial Interactions in an Experimental Autoimmune Encephalomyelitis Model. *Mult. Scler.* 10, 158–164. doi:10.1191/1352458504ms1009oa

Pacher, P., and Mechoulam, R. (2011). Is Lipid Signaling through Cannabinoid 2 Receptors Part of a Protective System. *Prog. Lipid Res.* 50, 193–211. doi:10.1016/j.plipres.2011.01.001

Palazuelos, J., Davoust, N., Julien, B., Hatterer, E., Aguado, T., Mechoulam, R., et al. (2008). The CB(2) Cannabinoid Receptor Controls Myeloid Progenitor Trafficking: Involvement in the Pathogenesis of an Animal Model of Multiple Sclerosis. *J. Biol. Chem.* 283, 13320–13329. doi:10.1074/jbc.M707960200

Parlar, A., Arslan, S. O., Doğan, M. F., Çam, S. A., Yalçin, A., Elibol, E., et al. (2018). The Exogenous Administration of CB2 Specific Agonist, GW405833, Inhibits Inflammation by Reducing Cytokine Production and Oxidative Stress. *Exp. Ther. Med.* 16, 4900–4908. doi:10.3892/etm.2018.6753

Ramirez, S. H., Haskó, J., Skuba, A., Fan, S., Dykstra, H., McCormick, R., et al. (2012). Activation of Cannabinoid Receptor 2 Attenuates Leukocyte-Endothelial Cell Interactions and Blood-Brain Barrier Dysfunction under Inflammatory Conditions. *J. Neurosci.* 32, 4004–4016. doi:10.1523/JNEUROSCI.4628-11.2012

Rastegar, M., Samadizadeh, S., Yasaghi, M., Moradi, A., Tabarraei, A., Salimi, V., et al. (2021). Functional Variation (Q63R) in the Cannabinoid CB2 Receptor May Affect the Severity of COVID-19: a Human Study and Molecular Docking. *Arch. Virol.* 166, 3117–3126. doi:10.1007/s00705-021-05223-7

Rieder, S. A., Chauhan, A., Singh, U., Nagarkatti, M., and Nagarkatti, P. (2010). Cannabinoid-induced Apoptosis in Immune Cells as a Pathway to Immunosuppression. *Immunobiology* 215, 598–605. doi:10.1016/j.imbio.2009.04.001

Rizzo, M. D., Henriquez, J. E., Blevins, L. K., Bach, A., Crawford, R. B., and Kaminski, N. E. (2020). Targeting Cannabinoid Receptor 2 on Peripheral Leukocytes to Attenuate Inflammatory Mechanisms Implicated in HIV-Associated Neurocognitive Disorder. *J. Neuroimmune Pharmacol.* 15, 780–793. doi:10.1007/s11481-020-09918-7

Robinson, R. H., Meissler, J. J., Breslow-Deckman, J. M., Gaughan, J., Adler, M. W., and Eisenstein, T. K. (2013). Cannabinoids Inhibit T-Cells via Cannabinoid

Receptor 2 in an *In Vitro* Assay for Graft Rejection, the Mixed Lymphocyte Reaction. *J. Neuroimmune Pharmacol.* 8, 1239–1250. doi:10.1007/s11481-013-9485-1

Robinson, R. H., Meissler, J. J., Fan, X., Yu, D., Adler, M. W., and Eisenstein, T. K. (2015). A CB2-Selective Cannabinoid Suppresses T-Cell Activities and Increases Tregs and IL-10. *J. Neuroimmune Pharmacol.* 10, 318–332. doi:10.1007/s11481-015-9611-3

Sahu, P., Mudgal, J., Arora, D., Kinra, M., Mallik, S. B., Rao, C. M., et al. (2019). Cannabinoid Receptor 2 Activation Mitigates Lipopolysaccharide-Induced Neuroinflammation and Sickness Behavior in Mice. *Psychopharmacology (Berl)* 236, 1829–1838. doi:10.1007/s00213-019-5166-y

Servettaz, A., Kavian, N., Nicco, C., Deveaux, V., Chéreau, C., Wang, A., et al. (2010). Targeting the Cannabinoid Pathway Limits the Development of Fibrosis and Autoimmunity in a Mouse Model of Systemic Sclerosis. *Am. J. Pathol.* 177, 187–196. doi:10.2353/ajpath.2010.090763

Sido, J. M., Nagarkatti, P. S., and Nagarkatti, M. (2015). Δ^9-Tetrahydrocannabinol Attenuates Allogeneic Host-Versus-Graft Response and Delays Skin Graft Rejection through Activation of Cannabinoid Receptor 1 and Induction of Myeloid-Derived Suppressor Cells. *J. Leukoc. Biol.* 98, 435–447. doi:10.1189/jlb.3A0155.030RR10.1189/jlb.3A0115-030RR

Singh, U. P., Singh, N. P., Singh, B., Price, R. L., Nagarkatti, M., and Nagarkatti, P. S. (2012). Cannabinoid Receptor-2 (CB2) Agonist Ameliorates Colitis in IL-10(-/-) Mice by Attenuating the Activation of T Cells and Promoting Their Apoptosis. *Toxicol. Appl. Pharmacol.* 258, 256–267. doi:10.1016/j.taap.2011.11.005

Storr, M. A., Keenan, C. M., Zhang, H., Patel, K. D., Makriyannis, A., and Sharkey, K. A. (2009). Activation of the Cannabinoid 2 Receptor (CB2) Protects against Experimental Colitis. *Inflamm. Bowel Dis.* 15, 1678–1685. doi:10.1002/ibd.20960

Taylor, A. L., Watson, C. J. E., and Bradley, J. A. (2005). Immunosuppressive Agents in Solid Organ Transplantation: Mechanisms of Action and Therapeutic Efficacy. *Crit. Rev. Oncology/Hematology* 56, 23–46. doi:10.1016/j.critrevonc.2005.03.012

Wiley, J. L., Beletskaya, I. D., Ng, E. W., Dai, Z., Crocker, P. J., Mahadevan, A., et al. (2002). Resorcinol Derivatives: a Novel Template for the Development of Cannabinoid CB(1)/CB(2) and CB(2)-selective Agonists. *J. Pharmacol. Exp. Ther.* 301, 679–689. doi:10.1124/jpet.301.2.679

Wu, Q., Ma, Y., Liu, Y., Wang, N., Zhao, X., and Wen, D. (2020). CB2R Agonist JWH-133 Attenuates Chronic Inflammation by Restraining M1 Macrophage Polarization via Nrf2/HO-1 Pathway in Diet-Induced Obese Mice. *Life Sci.* 260, 118424. doi:10.106/j.lfs.2020.11842410.1016/j.lfs.2020.118424

Xu, H., Cheng, C. L., Chen, M., Manivannan, A., Cabay, L., Pertwee, R. G., et al. (2007). Anti-inflammatory Property of the Cannabinoid Receptor-2-Selective Agonist JWH-133 in a Rodent Model of Autoimmune Uveoretinitis. *J. Leukoc. Biol.* 82, 532–541. doi:10.1189/jlb.0307159

Yang, X., Hegde, V. L., Rao, R., Zhang, J., Nagarkatti, P. S., and Nagarkatti, M. (2014). Histone Modifications Are Associated with Δ9-tetrahydrocannabinol-mediated Alterations in Antigen-specific T Cell Responses. *J. Biol. Chem.* 289, 18707–18718. doi:10.1074/jbc.M113.545210

Yuan, C. Y., Zhou, V., Sauber, G., Stollenwerk, T., Komorowski, R., Lopez, A., et al. (2021). Signaling through the type 2 cannabinoid receptor regulates the severity of acute and chronic graft-versus-hose disease. *Blood* 137, 1241–1255. doi:10.1182/blood.2020004871

Yuan, M., Kiertscher, S. M., Cheng, Q., Zoumalan, R., Tashkin, D. P., and Roth, M. D. (2002). Delta 9-Tetrahydrocannabinol Regulates Th1/Th2 Cytokine Balance in Activated Human T Cells. *J. Neuroimmunol.* 133, 124–131. doi:10.1016/s0165-5728(02)00370-3

Zhang, M., Adler, M. W., Abood, M. E., Ganea, D., Jallo, J., and Tuma, R. F. (2009). CB2 Receptor Activation Attenuates Microcirculatory Dysfunction during Cerebral Ischemic/reperfusion Injury. *Microvasc. Res.* 78, 86–94. doi:10.1016/j.mvr.2009.03.005

Zhang, M., Martin, B. R., Adler, M. W., Razdan, R. K., Jallo, J. I., and Tuma, R. F. (2007). Cannabinoid CB(2) Receptor Activation Decreases Cerebral Infarction in a Mouse Focal Ischemia/reperfusion Model. *J. Cereb. Blood Flow Metab.* 27, 1387–1396. doi:10.1038/sj.jcbfm.9600447

Zhu, L. X., Sharma, S., Stolina, M., Gardner, B., Roth, M. D., Tashkin, D. P., et al. (2000). Delta-9-tetrahydrocannabinol Inhibits Antitumor Immunity by a CB2 Receptor-Mediated, Cytokine-dependent Pathway. *J. Immunol.* 165, 373–380. doi:10.4049/jimmunol.165.1.373

Ziring, D., Wei, B., Velazquez, P., Schrage, M., Buckley, N. E., and Braun, J. (2006). Formation of B and T Cell Subsets Require the Cannabinoid Receptor CB2. *Immunogenetics* 58, 714–725. doi:10.1007/s00251-006-0138-x

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

REVIEW
published: 04 February 2022
doi: 10.3389/fphar.2021.806417

The Dynamic Role of Microglia and the Endocannabinoid System in Neuroinflammation

Alexander P. Young and Eileen M. Denovan-Wright**

Department of Pharmacology, Dalhousie University, Halifax, NS, Canada

Microglia, the resident immune cells of the brain, can take on a range of pro- or anti-inflammatory phenotypes to maintain homeostasis. However, the sustained activation of pro-inflammatory microglia can lead to a state of chronic neuroinflammation characterized by high concentrations of neurotoxic soluble factors throughout the brain. In healthy brains, the inflammatory processes cease and microglia transition to an anti-inflammatory phenotype, but failure to halt the pro-inflammatory processes is a characteristic of many neurological disorders. The endocannabinoid system has been identified as a promising therapeutic target for chronic neuroinflammation as there is evidence that synthetic and endogenously produced cannabinoids temper the pro-inflammatory response of microglia and may encourage a switch to an anti-inflammatory phenotype. Activation of cannabinoid type 2 (CB_2) receptors has been proposed as the mechanism of action responsible for these effects. The abundance of components of the endocannabinoid system in microglia also change dynamically in response to several brain pathologies. This can impact the ability of microglia to synthesize and degrade endocannabinoids or react to endogenous and exogenous cannabinoids. Cannabinoid receptors also participate in the formation of receptor heteromers which influences their function specifically in cells that express both receptors, such as microglia. This creates opportunities for drug-drug interactions between CB_2 receptor-targeted therapies and other classes of drugs. In this article, we review the roles of pro- and anti-inflammatory microglia in the development and resolution of neuroinflammation. We also discuss the fluctuations observed in the components of the endocannabinoid in microglia and examine the potential of CB_2 receptors as a therapeutic target in this context.

Keywords: neuroinflammation, endocannabinoid system, CB_2 receptor, microglia, MAPK signaling, heteromer, GPCR (G protein coupled receptor)

OPEN ACCESS

Edited by:
Cecilia Hillard,
Medical College of Wisconsin,
United States

Reviewed by:
Julian Romero,
Universidad Francisco de Vitoria,
Spain
Natasha L. Grimsey,
The University of Auckland,
New Zealand

****Correspondence:***
Alexander P. Young
alex.young@dal.ca
Eileen M. Denovan-Wright
emdenova@dal.ca

Specialty section:
This article was submitted to Experimental Pharmacology and Drug Discovery, a section of the journal Frontiers in Pharmacology

Received: *31 October 2021*
Accepted: *24 December 2021*
Published: *04 February 2022*

Citation:
Young AP and Denovan-Wright EM (2022) The Dynamic Role of Microglia and the Endocannabinoid System in Neuroinflammation. Front. Pharmacol. 12:806417. doi: 10.3389/fphar.2021.806417

INTRODUCTION

Neuroinflammation is characterized by sustained activation of microglia which release toxic cytokines that cause widespread damage to the brain. Microglia are recognized as the resident immune cells of the brain and have been identified as active propagators of neuroinflammation throughout the progression of several neurodegenerative diseases (Perry et al., 2010). At rest, microglia secrete neurotrophins and clear debris to support the maintenance of normal brain function (Cherry et al., 2014a). These unreactive microglia survey their environment using scavenger receptors to sense disruptions to local homeostasis (Nimmerjahn et al., 2005). Microglia detect

soluble factors released by neurons, astrocytes, other microglia, and infiltrating peripheral immune cells and may transition toward either an activated M1 (pro-inflammatory) or M2 (anti-inflammatory) phenotype to maintain homeostasis (Chhor et al., 2013). M1 microglia mediate host defense and are characterized by regulated phagocytic activity and the release of pro-inflammatory cytokines such as interleukin (IL)-1β and tumour necrosis factor alpha (TNFα) (Chhor et al., 2013). If these cytokines reach sufficient concentrations, they will trigger neuronal signaling cascades that cause cell impairment or necrosis (Neumann et al., 2002; Bachiller et al., 2018). In a healthy brain, the inflammatory process will halt before this occurs and M1 microglia will transition toward a more anti-inflammatory M2 phenotype to release anti-inflammatory cytokines, clear debris from dead cells, promote angiogenesis, and deposit extracellular matrix (Varin and Gordon, 2009; Cherry et al., 2014b). However, failure to halt the inflammatory process and engage M2 microglia is a common characteristic of several neurological disorders (Cherry et al., 2014b).

Microglia possess the necessary components required to synthesize, degrade, and respond to extracellular endocannabinoids (Stella, 2009; Stella, 2010). The endocannabinoid system comprises the cannabinoid type 1 (CB_1) and type 2 (CB_2) receptors, the endogenous ligands anandamide (AEA) and 2-arachidonylglycerol (2-AG), as well as the enzymes that regulate their production (Lu and Mackie, 2016). Anandamide was the first identified endocannabinoid which is known to bind CB_1 receptors as well as CB_2 receptors with relatively low affinity (Devane et al., 1992; Felder et al., 1993). AEA is synthesized by the enzyme N-acyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) and degraded by fatty acid amide hydrolase (FAAH). The second identified endocannabinoid was 2-arachidonoylglycerol (2-AG) which also activates both CB_1 and CB_2 receptors (Mechoulam et al., 1995; Sugiura et al., 1995; Stella et al., 1997). In human serum, 2-AG is up to 100-fold more abundant than AEA (Hillard et al., 2012). 2-AG is synthesized by diacylglycerol lipase (DAGL) and degraded primarily by monoacylglycerol lipase (MAGL) as well as alpha/beta-hydrolase domain (ABHD) containing enzymes such as ABHD6 and ABHD12 (Di Marzo et al., 1994; Cravatt et al., 2001). Although both endocannabinoids have effects on analgesia, AEA has greater effects on depression and anxiety whereas 2-AG appears to contribute more to the effects on movement and temperature regulation (Kathuria et al., 2003; Gobbi et al., 2005; Long et al., 2009). When both endocannabinoids are elevated through dual blockade of FAAH and MAGL, the effects mimic that of Δ9-tetrahydrocannabinol (Δ^9-THC) from *Cannabis* (Long et al., 2009; Alger and Kim, 2011).

Cannabinoid receptors are G protein-coupled receptors (GPCRs) that typically couple to $G\alpha_i$ but have been observed to couple to $G\alpha_o$ and $G\alpha_s$ under some circumstances (Glass and Felder, 1997; Howlett et al., 2002; Saroz et al., 2019). CB_1 receptors are abundant in central neurons and inhibit transmitter release upon activation (Howlett et al., 2002). CB_2 receptors display a distinct pharmacological profile and are more abundant in peripheral immune cells as well as in microglia (Galiègue et al., 1995; Howlett et al., 2002; Stella, 2009). CB_1 receptors generally exert the psychoactive effects of Δ^9-THC, whereas CB_2 receptors primarily mediate the immunosuppressive and anti-inflammatory effects of select cannabinoid molecules (Bouaboula et al., 1993; Lynn and Herkenham, 1994; Galiègue et al., 1995; Marsicano and Lutz, 1999). Pro-inflammatory and anti-inflammatory microglial phenotypes exhibit changes in the concentration of endocannabinoids as well as differences in the enzymatic machinery to synthesize and metabolize them (Maresz et al., 2005; Mecha et al., 2015). Furthermore, the quantities of the cannabinoid receptors have been observed to fluctuate widely in response to different pro- and anti-inflammatory stimuli. Current data that describe which components of the endocannabinoid system are upregulated or downregulated in each phenotype is useful to understand that the endocannabinoid system is a moving target in the context of neuroinflammation.

Neuroinflammation is a hallmark of aging as well as neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD) (Guzman-Martinez et al., 2019). Each of these neurodegenerative diseases are characterized by overactivation of microglia and have a neuroinflammatory component which could be a common target for therapeutics. The endocannabinoid system has been identified as a promising source of targets for the treatment of such chronic neuroinflammation (Pacher et al., 2006; Ashton and Glass, 2007; Saito et al., 2012). However, the molecular mechanisms that underlie the success of these treatments have not been clearly defined (Tanaka et al., 2020). Cannabinoids appear to dampen the pro-inflammatory microglial phenotype via multiple signaling pathways to regulate the transition from a resting to an anti-inflammatory microglial phenotype. To add an additional layer of complexity, cannabinoid receptors have recently been found to form oligomeric receptor complexes which respond differently to cannabinoids relative to the individual receptors; this may allow for unanticipated drug-drug interactions among CB_2 receptor agonists and other cannabinoids or other classes of drugs that target microglia. In this review, we discuss the roles of pro- and anti-inflammatory microglia in the development and resolution of neuroinflammation. We also discuss the fluctuations observed in the components of the endocannabinoid in microglia and examine the potential of CB_2 receptors as a therapeutic target in this context.

MICROGLIAL PHENOTYPES AND THE ENDOCANNABINOID SYSTEM

The microglial endocannabinoid system changes substantially among different phenotypes (**Figure 1**). At rest, microglia engage in several tasks including surveillance of the brain parenchyma and the maintenance of synapse function, and the abundance of CB_1 and CB_2 receptors is expected to be relatively low (Nimmerjahn et al., 2005; Stella, 2010). Early reports indicated that CB_1 and CB_2 receptor mRNA was undetectable within

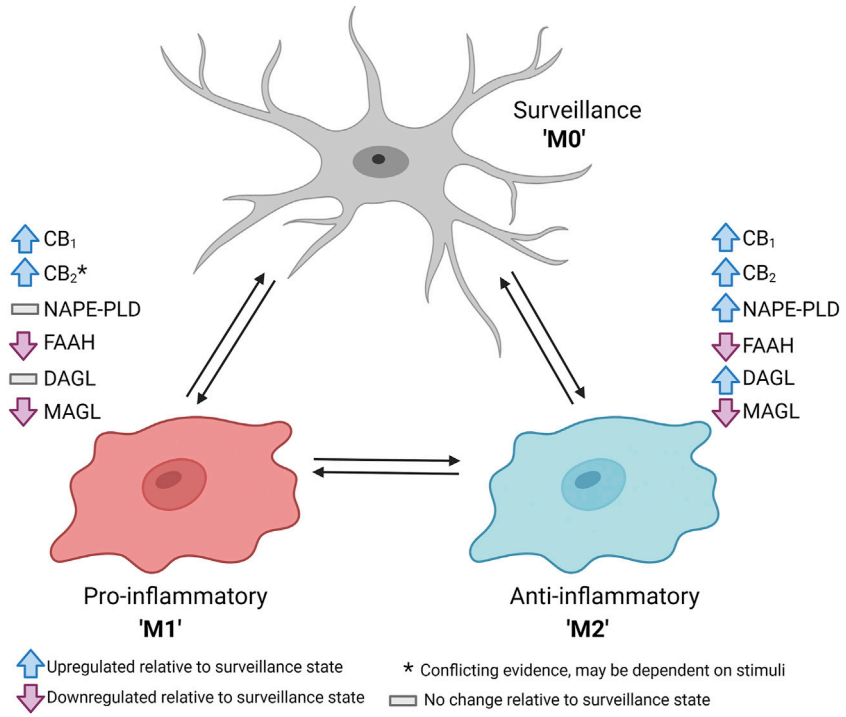


FIGURE 1 | Schematic summary of changes in the components of the endocannabinoid system upon dynamic shift from unreactive or surveillance (M0) phenotype to a pro-inflammatory (M1) or anti-inflammatory (M2) phenotype. Data derived primarily from Maresz et al. (2005), Mecha et al. (2015), and Navarro et al. (2018). Figure created with BioRender.

healthy brain tissue lysate or in isolated resting microglia (Munro et al., 1993; Galiègue et al., 1995; Schatz et al., 1997; Griffin et al., 1999; McCoy et al., 1999; Sugiura et al., 2000; Carlisle et al., 2002). However, other reports have indicated that resting microglia expressed both cannabinoid receptors, although perhaps only in trace amounts (Núñez et al., 2004; Navarro et al., 2018). Unreactive glia have been observed to release both AEA and 2-AG at a ratio of roughly 1:100 (Mecha et al., 2015; Araujo et al., 2019). If endocannabinoids released by resting microglia interacted with local synapses, CB_1 receptors could be activated to inhibit transmitter release from the pre-synaptic neurons *via* modulation of intracellular calcium, cyclic AMP, and inwardly rectifying potassium currents (Howlett et al., 2002). However, it is still uncertain whether endocannabinoids released specifically by microglia directly influence the activity of local synapses.

Under conditions of neuroinflammation, microglia engage in a pro-inflammatory M1 phenotype which includes several changes to their endocannabinoid function. Maresz et al. (2005) initially observed that CB_2 receptor mRNA was upregulated by 100-fold in the central nervous system (CNS) of mice with experimental autoimmune encephalitis. The pattern was consistent in primary mouse microglia treated with interferon-γ (IFNγ). These findings were replicated in immortalized N9 microglia as stimulation with IFNγ and lipopolysaccharide (LPS) caused a 12-fold increase in CB_2 receptor mRNA (Navarro et al., 2018). Conversely, primary rat microglia stimulated with LPS for 6 h exhibited a global downregulation of the components of the endocannabinoid system, including mRNA for CB_1 and CB_2 receptors, NAPE-PLD and FAAH, as well as DAGLα, DAGLβ, and MAGL (Mecha et al., 2015). After 24 h, FAAH and MAGL remained depressed, but the other components returned to baseline which may be indicative of a compensatory mechanism to favour synthesis of endocannabinoids following pro-inflammatory insult. Taken together, it appears that the regulation of CB_2 receptors and other components of the endocannabinoid system in microglia under pro-inflammatory conditions may depend on the type of stimuli or the length of time exposed to such conditions. Interestingly, recent RNA sequencing data revealed that microglia isolated from CB_2 receptor knockout mice failed to transition to an M1 phenotype in response to IFNγ and LPS (Reusch et al., 2021). This is indicative of potential crosstalk between CB_2 receptor-mediated signaling and the effects of toll-like or IFN receptors. Thus, constitutive CB_2 receptor activation may facilitate the initial transition to a pro-inflammatory phenotype.

To assist with the resolution of neuroinflammation, microglia take on an anti-inflammatory M2 phenotype which includes unique changes to microglial endocannabinoid function (Tanaka et al., 2020). Mecha et al. (2015) determined that rat microglia treated with IL-4 and IL-13 for 6 h had an enhanced abundance of mRNA for CB_2 receptors and DAGLα. After 24 h, mRNA for CB_1 receptors and NAPE-PLD became elevated, and

DAGLα had returned to baseline. These cells also exhibited reduced mRNA for FAAH and MAGL in both the 6- and 24-h treatment groups. Furthermore, these M2 microglia also released more AEA and 2-AG (Mecha et al., 2015). This indicates that microglia in an M2 phenotype promote the synthesis of both endocannabinoids and have a lower quantity of degradative enzymes relative to the resting or M1 phenotypes. The same authors also determined that treatment of microglia with endocannabinoids *in vitro* caused an upregulation of both CB_1 and CB_2 receptor mRNA abundance (Mecha et al., 2015). Activation of either CB_1 or CB_2 receptors by endocannabinoids has also been demonstrated to induce a shift toward an M2 phenotype in microglia, with upregulation of mRNA and protein for anti-inflammatory markers such as Arg-1 and SOCS-3 (Correa et al., 2010; Correa et al., 2011; Mecha et al., 2015). Taken together, the current evidence suggests that endocannabinoids promote microglia to shift toward an M2 phenotype which contributes to a feed-forward loop to upregulate cannabinoid receptor expression and release more endocannabinoids. Finally, microglia treated with IL-4 and IL-13 exhibited a substantial increase in Arg-1 mRNA and protein, but the effect was fully blocked by selective antagonists for either CB_1 receptors (AM251) or CB_2 receptors (AM630) (Mecha et al., 2015). Thus, constitutive activity of both CB_1 and CB_2 receptors may be required to enable the transition from an unreactive to an M2 phenotype.

MICROGLIAL PHENOTYPES AND NEUROINFLAMMATION WITH DISEASE AND AGING

Changes in microglial phenotype have been observed in neurodegenerative diseases such as AD, PD, and HD as well as with normal aging. Each of these states also exhibit unique changes to the endocannabinoid system which includes fluctuations in global AEA and 2-AG concentrations as well as changes in CB_1 and CB_2 receptor abundance (**Supplementary Table S1**). Although the components of the endocannabinoid system vary in microglia among pathologies, the cannabinoid receptors have shown promise as therapeutic targets for the treatment of neuroinflammation and neurodegeneration.

Alzheimer's Disease

AD is characterized by the aggregation of amyloid-beta (Aβ) that form extracellular plaques and accumulation of intracellular tau protein that form neurofibrillary tangles (Bloom, 2014). The buildup of cellular plaques and tangles results in the death of affected neurons with a subsequent decline in cognitive function (Selkoe and Hardy, 2016). The cause of AD was initially ascribed to insufficient clearance of Aβ aggregates and hyperphosphorylated tau protein (Murphy and LeVine, 2010). In AD mice, microglia are observed near Aβ plaques at five-fold the normal density, the purpose of which has been proposed to be clearance of Aβ by phagocytosis (Frautschy et al., 1998; D'Andrea et al., 2004). However, current evidence indicates that microglia do not influence the size or number of Aβ plaques in the late stages of AD (Spangenberg et al., 2016). More recent data has demonstrated that ablation of microglia in AD mice using a colony-stimulating factor 1 (CSF1) antagonist prevented the formation of these plaques which indicates that microglia may not be responsible for Aβ clearance but may in fact contribute to the initial deposition of Aβ plaques (Spangenberg et al., 2019). Furthermore, the accumulation of activated non-plaque associated microglia may lead to a sustained localized release of proinflammatory cytokines including IL-1β, IL-6, NO, and TNFα which are also neurotoxic (Benzing et al., 1999; Abbas et al., 2002; Wang et al., 2015). The increased concentration of these cytokines could exacerbate the accumulation of Aβ and produce further damage to the brain (Hickman et al., 2008). The severity of AD dementia has shown to be positively correlated with markers of pro-inflammatory microglial activation (Nordengen et al., 2019). Although originally thought of as a secondary effect of plaque formation, neuroinflammation is now understood to contribute equally to AD progression compared to the canonical protein aggregates (Zhang et al., 2013; Heneka et al., 2015).

Mouse models of AD have consistently demonstrated specific changes in the endocannabinoid system, including upregulation of CB_2 receptors and dysregulation of 2-AG metabolism (Mulder et al., 2011; Cristino et al., 2020). Enhanced CB_2 receptor-like immunoreactivity was found localized within plaque-associated microglia in human AD tissue (Benito et al., 2003). The same pattern of elevated CB_2 receptor-like immunoreactivity in human AD brains was later reported (Halleskog et al., 2011). Western blots from human AD brain lysate later corroborated that CB_2 receptor protein was elevated in the frontal cortex (Solas et al., 2013). Rats that received intracerebral injection of 30 ng of Aβ exhibited 2.7-fold increased CB_2 receptor mRNA abundance (Esposito et al., 2007). Aso et al. (2013) identified that the APP/PS1 mouse model of AD exhibited 1.4-fold increased CB_2 receptor mRNA abundance. Examinations of human AD brains have shown no change in total protein for NAPE-PLD or FAAH (Mulder et al., 2011), although FAAH activity may be selectively upregulated in the plaque-associated glia (Benito et al., 2003). Conversely, substantial changes have been observed in the metabolic enzymes for 2-AG (Mulder et al., 2011). Tissue from human AD brains revealed a positive correlation between disease stage and upregulation of DAGL and MAGL, with no alteration in ABHD6 (Mulder et al., 2011). Isolated membrane and cytosolic fractions from this tissue also exhibited a faster rate of 2-AG degradation compared to control tissue. Concentrations of AEA have been positively correlated with cognitive function in AD patients but negatively correlated with abundance of $Aβ_{42}$ which indicates that AD is also associated with dysregulated AEA production (Jung et al., 2012). Ultimately, brains afflicted with AD exhibit reduced endocannabinoid signaling which is likely caused by enhanced degradation of endocannabinoids without a compensatory increase in the synthetic enzymes.

CB_2 receptor activation has demonstrated potential benefits in several models of AD to dampen neuroinflammation and improve cognition (Martín-Moreno et al., 2012; Cassano et al., 2017; Li et al., 2019). The nonselective cannabinoid agonist, WIN55212-2, dampened the inflammatory response in rats

that received hippocampal injection of Aβ (Fakhfouri et al., 2012). APP/PS1 mice administered a selective CB_2 receptor agonist, JWH-133, exhibited a partial rescue of cognitive deficits as determined by an active avoidance test and a V-maze memory test (Aso et al., 2013). This improvement in cognitive performance was accompanied by a reduction in the pro-inflammatory markers IL-1β, IL-6, and TNFα. However, the drug was only effective when administered at the pre-symptomatic stage. Furthermore, JWH-133 had no effect on the quantity of Aβ in the brain (Aso et al., 2013). Mice that received JWH-133 also exhibited greater numbers of microglia that expressed elevated levels of IL-6 and IL-10 which was indicative of immunoregulatory activity (Aso et al., 2013; Chhor et al., 2013). Activation of microglial CB_2 receptors has also been shown to stimulate phagocytosis of Aβ *in vivo* and *in vitro* (Tolón et al., 2009; Aso et al., 2016). Thus, activation of microglial CB_2 receptors appears to serve a dual purpose to enhance phagocytosis of Aβ plaques and dampen neuroinflammation.

Parkinson's Disease

PD is characterized by motor dysfunction due to damage to dopaminergic neurons of the nigrostriatal pathway. Activated M1 microglia have been determined to be closely associated with neuron damage in human PD brains (McGeer et al., 1988). Imamura et al. (2003) found that the abundance of CD54/$CD11a^+$ microglia was correlated with progressive neurodegeneration in the substantia nigra. Increased proportions of activated microglia were also located in the caudate nucleus, hippocampus, transentorhinal cortex, cingulate cortex, and temporal cortex compared to the healthy control subjects. These activated microglia were also positive for TNFα and IL-6 which was indicative of an M1 phenotype. Subsequent studies have supported the elevated presence of M1 microglia in PD brains (Ouchi et al., 2005; Gerhard et al., 2006). However, quantities of activated microglia were not necessarily correlated with clinical severity (Gerhard et al., 2006). Dopaminergic neurons of the midbrain have been shown to be especially sensitive to the toxic effects of microglial cytokines including TNFα (McGuire et al., 2001). Thus, this population of neurons is highly susceptible to severe damage triggered by neuroinflammation that is characterized by the perpetuating cycle of neuron death with subsequent reactive microglial activation and cytokine release.

There are conflicting reports with respect to fluctuating levels of CB_1 and CB_2 receptors in PD. Elevated CB_1 receptor mRNA has been observed in the caudate putamen but not the substantia nigra of human PD brains (Navarrete et al., 2018). In non-human primates subjected to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicity, CB_1 receptor mRNA was elevated in the globus pallidus and subthalamic nucleus in response to levodopa-induced dyskinesia (Rojo-Bustamante et al., 2018). CB_2 receptor mRNA abundance was also elevated in the substantia nigra but diminished in the caudate putamen in human and mouse tissues (Gómez-Gálvez et al., 2016; Navarrete et al., 2018). Immunofluorescence labeling of human PD brains supported that the elevation in CB_2 receptors was primarily in activated microglia within the substantia nigra pars compacta (Gómez-Gálvez et al., 2016). In the MPTP-induced neurotoxicity model of PD, Price et al. (2009) identified elevated levels of CB_2 receptor protein in the ventral midbrain via western blot and immunofluorescence. This labeling colocalized with CD11b/$CD18^+$ cells which indicated that the CB_2 receptors were indeed expressed in activated microglia. In the reserpine-induced animal model of PD, a substantial increase in 2-AG and AEA was observed in the globus pallidus (Di Marzo et al., 2000). A similar increase in 2-AG was also found in the mouse ventral midbrain using an MPTP-treated mouse model (Mounsey et al., 2015). Elevated AEA was also found in the basal ganglia of rats lesioned with 6-hydroxy dopamine (6-OHDA) (Maccarrone et al., 2003). This was accompanied by reduced FAAH activity in the striatum. Thus, endocannabinoid production appears to be elevated in PD, perhaps as a compensatory mechanism to dampen the associated neuroinflammation.

Activation of microglial CB_2 receptors has been shown to be neuroprotective and improve motor symptoms in several animal models of PD (Price et al., 2009; Chung et al., 2016; Cassano et al., 2017). A naturally occurring CB_2 receptor agonist, β-caryophyllene (BCP), was neuroprotective and dampened the pro-inflammatory response of microglia in rats in a rotenone-induced model of PD (Javed et al., 2016; Ojha et al., 2016). Administration of WIN55,212-2 reduced neuronal death and improved motor symptoms in mice subjected to MPTP-dependent neurotoxicity (Price et al., 2009). Treatment with WIN55,212-2 also reduced the number of M1 microglia in the ventral midbrain. An equal effect was observed within the same study upon administration of a CB_2 receptor-selective agonist, JWH-015. Interestingly, the effects of WIN55,212-2 on microglial activation were completely blocked by the CB_2 receptor-selective inverse agonist, JTE-907. These results were unchanged in CB_1 receptor knockout mice, but the MPTP-dependent neurotoxicity was exacerbated in CB_2 receptor knockout mice. Thus, the effects of WIN55,212-2 were likely mediated solely by CB_2 receptors despite the nonselective nature of the ligand. Taken together, CB_2 receptors, specifically on microglia, may represent a therapeutic target to reduce neuroinflammation and protect neurons through the development of PD.

Huntington's Disease

HD is an inherited disorder that is characterized by the progressive loss of dopaminergic neurons in the indirect pathway of the striatum which causes locomotor and cognitive impairments (Cristino et al., 2020). An increased abundance of activated microglia has been measured in the cortex and striatum in human HD brains compared to aged humans free of neurological disorder (Sapp et al., 2001). There was also a strong positive correlation between disease stage and the accumulation of primed proinflammatory microglia as measured by the abundance of MHC class II antigens (Sapp et al., 2001). Positron emission tomography has been used to measure a marked increase in the binding of radiolabeled PK-11195 in cortical brain regions and in the striatum of patients with HD (Pavese et al., 2006; Yen F. Tai et al., 2007a). As PK-11195 is

known to bind primarily to glial cells in the injured CNS, this would indicate that there was a substantial increase in the abundance of activated microglia in the human HD brain tissue (Cagnin et al., 2002). Pavese et al. (2006) also found that the degree of microglial activation correlated with disease stage which implicated a direct role of microglia in the progression of the disease. This group has used similar methodologies to determine that there was an elevated number of microglia in the striatum and cortex of pre-symptomatic carriers of the mutant *HTT* gene with abnormally expanded CAG repeats (Yen F. Tai et al., 2007b). The elevation in activated microglia was also correlated with decreased binding of ^{11}C-raclopride, indicative of striatal neuron loss. These data indicate that the microglial response and neuronal dysfunction occur in tandem, several years prior to the predicted age of disease onset of HD based on the number of CAG repeats.

HD progression has been characterized by a loss of neuronal CB_1 receptors in several transgenic mouse models as well as in post-mortem human HD brains (Glass et al., 1993; Denovan-Wright and Robertson, 2000; Lastres-Becker et al., 2002; Dowie et al., 2009; Blázquez et al., 2011). Conversely, an upregulation of CB_2 receptors has been observed in the striatum of R6/1 and R6/2 transgenic mice as well as human HD brains (Palazuelos et al., 2009). The immunolabeling revealed colocalization with ionized calcium-binding adapter molecule 1 (Iba1) but not glial fibrillary acidic protein (GFAP) which indicated that the receptors were specifically upregulated in microglia. Male Sprague Dawley rats that received an intrastriatal injection of malonate exhibited a 4-fold increase in CB_2 receptor mRNA within the striatum (Sagredo et al., 2009). Many of the CB_2 receptors were expressed in activated M1 microglia, although astrocytes were also identified as CB_2 receptor positive. However, Dowie et al. (2014) reported that upregulation of CB_2 receptor protein was localized to the vasculature and not microglia or astrocytes in human HD brain tissue. When R6/2 mice were crossed with CB_2 receptor knockout mice, the offspring exhibited aggravated motor symptoms which indicates that constitutive CB_2 receptor activity was beneficial to disease progression in this model (Palazuelos et al., 2009). The striata from these R6/2 mice had higher proportions of M1 microglia with elevated IL-1β, IL-6, TNFα, and iNOS. Given these data, elevated microglial CB_2 receptors may not have been simply induced by the pro-inflammatory state. Thus, CB_2 receptors may regulate microglial activation and play a protective role in the context of HD.

Based on current data, it appears that CB_1 and CB_2 receptors play important roles in HD to control excitotoxicity and neuroinflammation, respectively. Thus, the use of therapeutics to preserve CB_1 receptors and activate CB_2 receptors may be a useful strategy to treat symptoms of HD. One method to preserve neuronal CB_1 receptors appears was through stimulation of the receptors. Laprairie et al. (2013) determined that the selective CB_1 receptor agonist, arachidonyl-2′-chloroethylamide (ACEA), upregulated neuronal expression of CB_1 receptor mRNA and protein in the ST*Hdh*$^{Q7/Q7}$ and ST*Hdh*$^{Q111/Q111}$ cell models of HD. These effects were mediated by NF-κB and Akt downstream of CB_1 receptor activation. Sagredo et al. (2009) found that direct stimulation of CB_1 receptors using ACEA did not improve the survival of striatal projection neurons following an acute neurotoxic malonate lesion in Sprague Dawley rats. There was also no benefit of Δ^9-THC or HU-210 (synthetic nonselective agonist) to preserve CB_1 receptors in the R6/1 mouse model of HD (Dowie et al., 2010). CB_1 receptors are also limited as a therapeutic target due to the psychoactivity associated with global receptor activation (Ashton and Glass, 2007). A method to circumvent these limitations may be to use positive allosteric modulators to enhance CB_1 receptor activation by endogenous cannabinoids. Positive allosteric modulators of CB_1 receptors have shown to improve cell viability in a cell model of HD as well as improve motor coordination and delay symptom onset in R6/2 mice (Laprairie et al., 2019). Furthermore, inhibition of FAAH using URB597 preserved CB_1 receptors in the striatum of R6/1 mice (Dowie et al., 2010). The CB_2 receptor-selective agonist HU-308 has been neuroprotective and reduced the TNFα concentration in rats that received intrastriatal malonate injections (Sagredo et al., 2009). Otherwise, there is limited data to support the use of CB_2 receptor-selective agonists specifically in HD. However, therapeutics that target microglial CB_2 receptors to dampen the neuroinflammatory response have been generally promising for brain diseases with an inflammatory component (Navarro et al., 2016).

Aging

Even in the absence of disease, aging brains exhibit an elevation in the proportions of activated M1 microglia that secrete pro-inflammatory cytokines such as TNFα, IL-1β, and IL-6 in the central nervous system; proportions of M2 microglia are also reduced which leads to a deficiency in anti-inflammatory cytokines such as IL-10 (Ye and Johnson, 1999; Ye and Johnson, 2001; Lukiw, 2004; Streit et al., 2004; Zahn et al., 2007). In brains of aged mice (~20 months old), 25% of microglia have been reported to be MHC class II-positive compared to only 2% of microglia in healthy adult mice (~4 months old) (Henry et al., 2009). Thus, a much larger proportion of microglia in aged mice were primed for pro-inflammatory activity compared to younger mice under otherwise healthy conditions (Norden and Godbout, 2013). A number of studies have demonstrated that aged mice are also more sensitive to inflammatory stimuli compared to adult mice (Wynne et al., 2010; Njie et al., 2012). Sierra et al. (2007) found that aged mice had an enhanced response to LPS injection and had higher expression of TNFα, IL-1β, IL-6, and IL-12 mRNA in microglia compared to adult mice. Henry et al. (2009) also demonstrated that microglia isolated from aged mice exhibited elevated mRNA abundance for proinflammatory cytokine production. There were functional consequences to the elevation of these cytokines as the increased inflammatory properties of aged brains has been associated with psychomotor and cognitive impairment in mice (Weaver et al., 2002; Richwine et al., 2005; Hayashi et al., 2008; Villeda et al., 2011). The exaggerated immune response in elderly populations has also been associated with increased susceptibility to behavioural complications following peripheral immune challenge, including depression and cognitive impairment (Godbout and Johnson, 2006; Godbout and Johnson, 2009; Corona et al., 2012).

The balance of microglial phenotypes has been found to change with age toward an increase in pro-inflammatory phenotypes, with the emergence of some transcriptional phenotypes not observed in younger mice (Hammond et al., 2019). Microglia from aged mice (24-month old) exhibited markers of pro-inflammation, including upregulation of markers of cytokine release and phagocytosis (Raj et al., 2017). Microglia from aged animals also differed substantially from those of young animals in terms of metabolism, potentially due to differences in rapamycin-insensitive companion of mTOR (RICTOR) which was a common upstream regulator of many of the dysregulated metabolic processes (Flowers et al., 2017). Interestingly, cultured BV-2 microglia in the absence of RICTOR exhibited the same phenotype as primary microglia from aged animals. This may indicate that microglial metabolic dysregulation with age can cause multiple phenotypes to converge.

As aged brains exhibit a higher proportion of pro-inflammatory microglia compared to young brains, it would be expected for aged brains to contain elevated levels of CB_2 receptors as well. However, Hodges et al. (2020) reported no statistically significant differences in CB_2 receptor mRNA from the cortex or hypothalamus between young and aged mice. In contrast, Pascual et al. (2014) reported decreased CB_2 receptor abundance in aged rats. Aged rats (24-month old) exhibited a 50% reduction in CB_1 receptor mRNA in the basal ganglia compared to young rats (3-month old) as measured by autoradiography and *in situ* hybridization (Mailleux and Vanderhaeghen, 1992; Romero et al., 1998). However, this early work did not determine whether the loss of CB_1 receptor mRNA occurred only in neurons or in microglia as well.

Stimulation of CB_2 receptors appeared to contribute to the control of neurogenesis in an age-dependent manner (Goncalves et al., 2008). The authors found that a DAGL inhibitor, RHC-80267, inhibited the proliferation of Cor1 neural stem cell line which highlights the importance of 2-AG signaling in the proliferation of cultured neuronal stem cells. Goncalves et al. (2008) also demonstrated that selective antagonists for both CB_1 receptors (AM251) and CB_2 receptors (AM630) inhibited proliferation in the same cell line which suggested that the role of 2-AG in cell proliferation could be mediated by the actions of both CB_1 and CB_2 receptors. These results were consistent when the experiments were repeated in 6-week, 6-month, and 20-month-old female mice. Stimulation of CB_2 receptors *via* JWH-133 also increased the number of neurons in the subventricular zone, and the effects were most pronounced in the aged mice (Goncalves et al., 2008). Thus, the benefits of CB_2 receptor activation could translate to an aging population.

POTENTIAL INFLUENCE OF SEX AND EXERCISE ON ENDOCANNABINOID FUNCTION

There are sex differences in the endocannabinoid system which impacts the responses to cannabinoids. In mice, females have reported higher quantities of both CB_1 and CB_2 receptor mRNA relative to males (Xing et al., 2014). This may partially explain the growing body of evidence that has demonstrated a greater effect of cannabinoids in females for the treatment of pain (Craft et al., 2013; Blanton et al., 2021). Microglia in the spinal cord sensitized male mice to neuropathic and inflammatory pain *via* TLR4, but the effects were not observed in female mice (Sorge et al., 2011). It was later determined that pain hypersensitivity in female mice was mediated by adaptive immune cells and not microglia (Sorge et al., 2015). Female mice also had greater quantities of microglia in the periaqueductal gray region of the brain which is involved in descending pain modulation (Doyle et al., 2017). This difference in number of microglia was proposed to explain the sex differences in the effectiveness of morphine for pain relief. As morphine interacts with microglial TLR4 to initiate a pro-inflammatory response, this may stimulate neuroinflammation which would counteract the analgesic effects (Hutchinson et al., 2010). Therefore, there are apparent inherent sex differences in microglia with respect to pain processing. To our knowledge, the connection between sex differences in cannabinoid receptor quantities and microglial distribution has not been investigated with respect to neuroinflammation. However, this information will be critical to tailor CB_2 receptor-targeted therapies for the treatment of neuroinflammation.

The endocannabinoid system, and especially CB_1 receptors in peripheral tissues, become dysregulated with lifestyle related diseases such as obesity (Matias et al., 2008). High-fat diets associated with obesity lead to higher quantities of circulating endocannabinoids and increased CB_1 receptor activation which drove increased food intake and reduced insulin sensitivity and energy metabolism in skeletal muscle (Pagotto et al., 2006). Selective CB_1 receptor antagonists have been in development as anti-obesity agents, however, these drugs include several negative side effects which has precluded their clinical use (Quarta and Cota, 2020). Interestingly, lifestyle interventions such as consistent exercise appear to counteract the overexpression of CB_1 receptors in peripheral tissues (Heyman et al., 2012). Consistent exercise was also associated with improvements in cognition for AD patients, although a link to endocannabinoid-mediated mechanism has not been established to our knowledge (Meng et al., 2020).

MECHANISMS OF CB_2 RECEPTOR-MEDIATED EFFECTS IN MICROGLIA

Activation of cannabinoid receptors has consistently been observed to dampen the shift of microglia to an M1 phenotype following treatment with a pro-inflammatory stimulus such as LPS or IFNγ by inhibiting the release of soluble factors including NO, TNFα, and IL-6 (Tanaka et al., 2020). Activation of CB_2 receptors also appears to regulate the shift from an unreactive phenotype to an anti-inflammatory M2 phenotype. It is possible that cannabinoids also facilitate a shift from an M1 phenotype directly to an M2 phenotype. Although there is a clear relationship based on the profiles of cytokines released from the microglia, the specific mechanisms and

signaling pathways involved have not been thoroughly examined. Recent evidence has implicated the MAPK pathways as potential targets to explain the relationship between cannabinoid signaling and inflammation.

MAPKs are intracellular signaling proteins that are responsible for many downstream functions and can be subdivided into c-Jun N-terminal Kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 proteins. Each of the MAPK signaling pathways has also been associated with both the pro-inflammatory and anti-inflammatory properties of microglia (Kim et al., 2004; Waetzig et al., 2005; Bachstetter et al., 2011; Li et al., 2019; Chen et al., 2021). As both CB_1 and CB_2 receptors typically couple to $G\alpha_{i/o}$ and $G\beta\gamma$ proteins, activation of these receptors typically initiates phosphorylation of downstream MAPK pathways (Bouaboula et al., 1996; Howlett et al., 2002; Komorowska-Müller and Schmöle, 2020). Correa et al. (2011) found that treatment with AEA dampened the release of pro-inflammatory cytokines IL-12 and IL-23 in a mouse model of multiple sclerosis via the JNK and ERK1/2 pathways, but the effect was only partially mediated by CB_2 receptors. The inhibitory effects of AEA on the release of TNFα, IL-6, and IL-1β were fully blocked by a PKC inhibitor, chelerythrine, which indicated that both CB_1 and CB_2 receptors may have contributed via MAPK signaling (Ma et al., 2015). Recent transcriptomic data using CB_2 receptor knockout microglia demonstrated impaired MAPK signaling which corroborated the involvement of CB_2 receptors in these pathways (Reusch et al., 2021). Activation of CB_2 receptors has also been shown to reduce translocation of NF-κB p65 to the nucleus, perhaps as a downstream consequence of MAPK signaling (Correa et al., 2010; Javed et al., 2016).

Pro-inflammatory stimuli such as LPS and IFNγ have been reported to initiate MAPK signaling in microglia (Frazier et al., 2012; Meng et al., 2014). CB_2 receptor activation diminished the downstream translation of pro-inflammatory cytokine genes in cultured microglia challenged with $A\beta_{1\text{-}42}$ (Ehrhart et al., 2005). Thus, it seems that there is negative cross-talk among CB_2 receptor signaling and LPS- or IFNγ-dependent MAPK signaling. Although ERK phosphorylation was induced independently by LPS and CB_2 receptor activation in cultured BV-2 microglia, co-treatment with LPS and AEA or WIN-55,212,-2 induced a much smaller effect than either stimulus alone (Eljaschewitsch et al., 2006). This appears to be caused by the induction of MAPK phosphatase (MKP)-1/2 which dephosphorylated ERK1/2. As the induction of MKP-1 occurred much faster in the presence of LPS and AEA compared to either compound alone, LPS-mediated ERK phosphorylation was blunted. The effect of AEA was partially blocked by AM251 but fully blocked by AM630 (Eljaschewitsch et al., 2006). This indicated that both CB_1 and CB_2 receptors may have contributed to the inhibition of microglial pro-inflammatory phenotypes. Subsequent work has corroborated that CB_2 receptor activation induced MKP-1 and MKP-3 which inhibited ERK phosphorylation upon LPS stimulation in primary rat microglia (Romero-Sandoval et al., 2009). Thus, it seems likely that CB_2 receptor activation attenuates LPS-induced ERK phosphorylation and downstream transcription of pro-inflammatory genes through the induction of MKP proteins. Further investigation of endocannabinoid-mediated upregulation of MKP proteins may provide important clues into how CB_2 receptor agonism can inhibit the activation of pro-inflammatory microglia.

Acquisition of immunomodulatory M2-like properties in microglia has been observed following CB_2 receptor-dependent MAPK signaling. Cultured BV-2 microglia treated with LPS and IFNγ demonstrated elevated release of the anti-inflammatory cytokine IL-10 release, this was enhanced by co-incubation with AEA in a dose-dependent manner (Correa et al., 2010). A similar effect was observed when the microglia were co-incubated with the CB_2 receptor-selective agonist, JWH-133. These effects were blocked by the CB_2 receptor-selective antagonist, SR144528, but not influenced by the CB_1 receptor-selective antagonist, SR141716A. This indicated that the enhanced effect on IL-10 release was mediated by the activation of CB_2 but not CB_1 receptors. Furthermore, the effects were blocked by the MEK1/2 inhibitor, PD98059, as well as the JNK inhibitor, SP600125. However, the PI3K/Akt inhibitor, Ly294002, had no influence over the effects (Correa et al., 2010). Thus, it appears that CB_2 receptor activation can promote the downstream release of anti-inflammatory factors such as IL-10 via the ERK and JNK MAPK pathways. This could allow microglia in a pro-inflammatory phenotype to also acquire M2-like properties following CB_2 receptor activation.

Non-canonical cAMP-mediated signaling pathways may also contribute to the anti-inflammatory properties of cannabinoids in microglia. CB_2 receptors generally couple to $G\alpha_i$ proteins and do not mediate increased cAMP (Glass and Northup, 1999; Ibsen et al., 2017). However, there is recent evidence to suggest that CB_2 receptors could couple to $G\alpha_s$ proteins in primary human peripheral blood mononuclear cells to elevate cAMP and activate PKA (Saroz et al., 2019). In cultured primary rat microglia treated with thrombin, co-treatment with JWH-133 caused an increase in cAMP accumulation in a dose-dependent manner (Tao et al., 2016). JWH-133 treatment also increased the downstream phosphorylation of PKA as a consequence of elevated cAMP (Tao et al., 2016). This elevation in phosphorylated PKA mediated a reduction in mRNA for pro-inflammatory markers such as CD68, TNFα, IL-1β, and IFNγ. Thrombin generally binds to $G\alpha_i$ protein-coupled receptors such as protease-activated receptor (PAR)-1 and PAR-4 to inhibit cAMP accumulation (Simonds et al., 1989). As both PARs and CB_2 receptors typically inhibit adenylate cyclase, it appears that there is an alteration in the signaling properties when both receptors are co-activated. This observation of cAMP accumulation upon co-treatment with thrombin and JWH-133 could be an early example of $G\alpha_s$ protein-coupled CB_2 receptors in microglia.

POTENTIAL INFLUENCE OF CB_2 RECEPTOR HETEROMERS

Cannabinoid receptors have been found to form oligomeric receptor complexes, known as heteromers, with several other class A GPCRs such as adenosine receptors (Carriba et al., 2007;

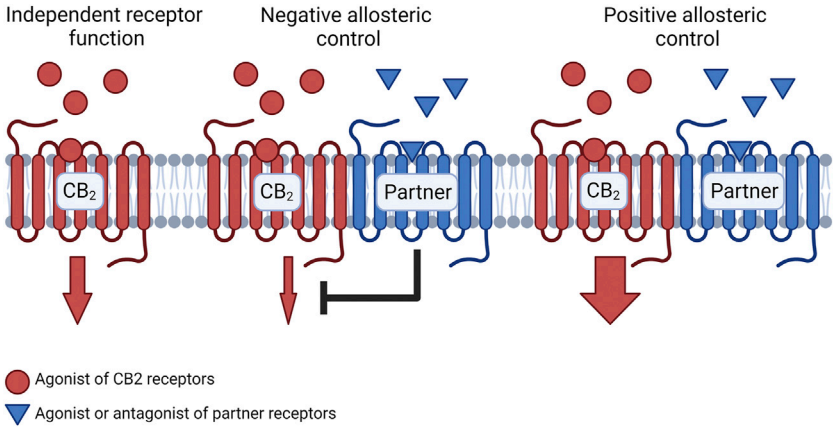


FIGURE 2 | Schematic representation of signaling changes due to CB_2 receptor heteromer formation. When presented with an agonist or antagonist for the partner receptor, the partner receptor may exert negative allosteric control over CB_2 receptors which results in reduced signaling from the CB_2 receptor relative to the CB_2 receptor which does not participate in a heteromeric complex. Conversely, a partner receptor may exert positive allosteric control over the CB_2 receptor which enhances signaling from the CB_2 receptor mediated by the CB_2 receptor agonist. Figure created with BioRender.

Aso et al., 2019; Franco et al., 2019a; Köfalvi et al., 2020) and serotonin receptors (Franco et al., 2019b). Interestingly, cannabinoid receptor heteromers exhibit distinct signaling properties compared to the individual receptors alone (Callén et al., 2012; Balenga et al., 2014; Navarro et al., 2018). CB_2-A_{2A}, CB_2-$5HT_{1A}$, and CB_2-CB_1 heteromers have been observed within microglia and fundamentally alter the microglial response to cannabinoids (Navarro et al., 2018; Franco et al., 2019a; Franco et al., 2019b). Furthermore, these heteromers have been found in different quantities under conditions of neuroinflammation and in response to different cannabinoid treatments (Navarro et al., 2018; Bagher et al., 2020). Currently, there are several established heteromer-dependent mechanisms that can result in either enhanced or diminished CB_2 receptor-mediated signaling (**Figure 2**). These mechanisms will be important to consider through the development of CB_2 receptor-selective molecules for the treatment of neuroinflammation.

Cross-antagonism is a form of allosteric control within CB_2 receptor heteromers that involves diminished signaling from the CB_2 receptors upon antagonism of the partner receptor. This is often a bidirectional phenomenon where an antagonist for the CB_2 receptors would also block activity of the partner receptor. For example, CB_2 receptor-mediated Akt/PKB phosphorylation has been inhibited in the presence of the CB_1 receptor-selective antagonist AM251 in cells that co-expressed both receptors (Callén et al., 2012). Serotonin type 1A ($5HT_{1A}$) receptors exert similar effects as the antagonist WAY-100635 has been observed to diminish CB_2 receptor-mediated ERK phosphorylation when co-administered with the CB_2 receptor agonist PM224 (Franco et al., 2019b). Interestingly, adenosine type 2A (A_{2A}) receptors enhanced CB_2 receptor signaling in microglia where the receptors co-expressed (Franco et al., 2019a). Franco et al. (2019a) determined that treatment of microglia with an A_{2A} receptor antagonist (SCH58621) resulted in an enhanced effect of CB_2 receptor-mediated cAMP inhibition compared to agonism of CB_2 receptors alone. Ultimately, it appears that a blockade of $5HT_{1A}$ receptors diminishes CB_2 receptor-mediated signaling whereas an antagonist for A_{2A} receptors may facilitate an enhanced effect of cannabinoid treatments. Given this contrast, it can be difficult to predict how CB_2 receptor signaling could be affected in the presence of an antagonist for another GPCR that could form heteromers with CB_2 receptors.

When CB_2 receptor heteromers are presented with agonists for both receptors simultaneously, the effects of the ligands may produce different effects compared to either of the agonists alone. Diminished Akt phosphorylation was observed in transfected SH-SY5Y cells upon treatment with a CB_1 receptor agonist (ACEA) and a CB_2 receptor agonist (JWH-133) relative to treatment with either ACEA or JWH-133 alone (Callén et al., 2012). A similarly diminished effect on ERK phosphorylation has been observed upon co-treatment with JWH-133 and an A_{2A} receptor agonist (CGS-21680) (Franco et al., 2019a). In contrast, co-treatment with PM224 and a $5HT_{1A}$ receptor agonist (8-OH-DPAT) produced an enhanced effect on ERK phosphorylation in co-transfected cells compared to either agonist alone (Franco et al., 2019b). Thus, coactivation of CB_2 receptors and A_{2A} receptors may diminish the effects of CB_2 receptor agonists whereas coactivation with $5HT_{1A}$ receptors may lead to enhanced cannabinoid-mediated effects.

RECENT DEVELOPMENTS AND CURRENT CHALLENGES

It has become clear that there is an association between neurodegenerative diseases and the presence of pro-inflammatory microglia which propagate the process of neuroinflammation. However, it has been difficult to

determine whether the microglia are involved in the development of these pathological conditions or simply responding to the damage. It has been proposed that microglia react to neurodegeneration to initiate neuroinflammation which exacerbates the damage, but there is emerging evidence which suggests that aberrant microglial activity could contribute to the development of such diseases. The erasure of microglia from R6/2 HD mice using a CSF1 antagonist promoted the maintenance of cognitive function and striatal neurite density and prevented the onset of some disease symptoms including loss of grip strength and striatal atrophy (Crapser et al., 2020). This would indicate that the microglia within the mouse HD brains induced damage that was ameliorated upon microglial depletion. Microglia expressing mutant huntingtin have been observed to be hyperreactive and released elevated quantities of pro-inflammatory cytokines at baseline (Crotti et al., 2014). This makes it difficult to distinguish between a potential detrimental effect of normal microglia compared to the neuroinflammation initiated by the mutant microglia. Similar benefits have been found upon depletion of microglia in AD mouse models (Spangenberg et al., 2016; Spangenberg et al., 2019). 5xFAD mice that received a CSF1 antagonist for 4 weeks maintained greater neuronal density compared to the vehicle treatment without alterations to Aβ levels (Spangenberg et al., 2016). Similar experiments were performed in younger mice prior to Aβ plaque development. These experiments revealed a lack of Aβ development in animals that received a CSF1 antagonist, however, Aβ plaques began to develop with microglial repopulation upon removal of the drug treatment (Spangenberg et al., 2019). These emerging data demonstrate that diseased microglia likely contribute to the progression of some neurodegenerative diseases, and that early targeting of these cells could be beneficial to prevent these contributions.

Several strategies have been employed to target the endocannabinoid system for the treatment of inflammation and neurodegeneration in humans. These strategies have primarily included combinations of phytocannabinoids, and synthetic CB_2 receptor agonists. The most common method to engage the endocannabinoid system for the treatment of neuroinflammation or neurodegeneration has been with phytocannabinoids, including combinations of Δ^9-THC and cannabidiol (CBD). Sativex™, which combines relatively equal amounts of Δ^9-THC and CBD, has been tested in clinical trials for the treatment of HD (ClinicalTrials.gov identifier: NCT01502046). The results of the pilot cross-over trial indicated that Sativex™ was well tolerated in patients but there was no benefit to the disease progression (López-Sendón Moreno et al., 2016). Although Sativex™ has been approved for the treatment of neuropathic pain in multiple sclerosis, there is no clear clinical evidence that such phytocannabinoid-based drugs are useful specifically for the treatment of neuroinflammation in humans (ClinicalTrials.gov identifier: NCT00391079). Perhaps the most promising preclinical data has emerged from the use of synthetic selective CB_2 receptor agonists to dampen the pro-inflammatory activity of microglia (Tanaka et al., 2020). Anabasum is a novel CB_2 receptor agonist that is currently being trialed as an anti-inflammatory drug for use in cystic fibrosis, systemic sclerosis, dermatomyositis, and systemic lupus erythematosus (Clinicaltrial.gov identifiers: NCT02465450, NCT02465437, NCT02466243). Anabasum has recently demonstrated efficacy against a pro-inflammatory challenge in humans (Motwani et al., 2018). When ultraviolet light-killed *Escherichia coli* were injected intradermally into healthy individuals, anabasum treatment improved clearance of the pro-inflammatory stimulus and inhibited inflammation similar to prednisolone treatment. There was also enhanced biosynthesis of several pro-resolving lipid mediators (Motwani et al., 2018). This indicates that CB_2 receptor agonists have potential to combat peripheral immune challenges in humans. Further work will be required to determine the potential effectiveness to combat inflammation in the brain.

The endocannabinoid system is emerging as a source of many therapeutically relevant targets for the treatment of inflammation and neurodegeneration. To further develop compounds to target the endocannabinoid system for clinical use, there are specific aspects of endocannabinoid function that require further attention. Great focus has been placed on the use of phytocannabinoids which engage an array of targets including CB_1 receptors, $5HT_{1A}$ receptors, and TRPV1 ion channels (Howlett et al., 2002; de Almeida and Devi, 2020). Basic research in the functions of the endocannabinoid system has revealed more specific targets such as CB_2 receptors on microglia for the treatment of neuroinflammation (Ashton and Glass, 2007). There is mounting preclinical evidence for the use of CB_2 receptor agonists to treat chronic and acute inflammation (Komorowska-Müller and Schmöle, 2020). However, there has been very little investigation into the therapeutic window for CB_2 receptor agonists. It is still unclear whether it could be beneficial to pre-treat with CB_2 receptor agonists for any amount of time to delay any potential onset symptoms of neurodegenerative disease. It is also unclear if CB_2 receptor agonists become less effective once a certain degree of neurodegeneration and microglial activation has been reached. Improving our understanding of the temporal therapeutic window of these drugs will be critical to determine their viability in an emergency or clinical setting. We also lack in our basic understanding of the functions of microglial CB_2 receptors as well as how these functions may change throughout the human lifespan or in different microglial populations. For example, in addition to the regulation of immune activity, CB_2 receptors have been implicated in other fundamental functions of microglia such as the regulation of phagocytosis (Ehrhart et al., 2005; Mecha et al., 2015; Guida et al., 2017). Microglia have important roles in pruning of synapses during development and disease (Stevens et al., 2007; Schafer et al., 2012). Thus, activation of CB_2 receptors at specific times could be either beneficial or greatly detrimental to healthy brain development. Ultimately, understanding the function of CB_2 receptors and the endocannabinoid system during these specific timeframes will be critical in the development of effective treatments that regulate microglial activity to dampen inflammation.

AUTHOR CONTRIBUTIONS

The manuscript was conceived and written by AY. The manuscript was critically evaluated and edited by AY and

ED-W. Both authors approved the submission of the manuscript.

FUNDING

This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant (RGPIN-37999-2019) to ED-W and an NSERC CGS-D to AY.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.806417/full#supplementary-material

Supplementary Table S1 | Summary of the key changes that occur in the endocannabinoid system within several *in vivo* models or in human tissue with neurodegenerative diseases. Also noted is the impact of CB_2 receptor activation in the animal model.

REFERENCES

Abbas, N., Bednar, I., Mix, E., Marie, S., Paterson, D., Ljungberg, A., et al. (2002). Up-Regulation of the Inflammatory Cytokines IFN-Gamma and IL-12 and Down-Regulation of IL-4 in Cerebral Cortex Regions of APP(SWE) Transgenic Mice. *J. Neuroimmunol* 126, 50–57. doi:10.1016/s0165-5728(02)00050-4

Alger, B. E., and Kim, J. (2011). Supply and Demand for Endocannabinoids. *Trends Neurosci.* 34, 304–315. doi:10.1016/j.tins.2011.03.003

Araujo, D. J., Tjoa, K., and Saijo, K. (2019). The Endocannabinoid System as a Window into Microglial Biology and its Relationship to Autism. *Front Cel Neurosci* 13, 424. doi:10.3389/fncel.2019.00424

Ashton, J. C., and Glass, M. (2007). The Cannabinoid CB2 Receptor as a Target for Inflammation-Dependent Neurodegeneration. *Curr. Neuropharmacol* 5, 73–80. doi:10.2174/157015907780866884

Aso, E., Juvés, S., Maldonado, R., and Ferrer, I. (2013). CB2 Cannabinoid Receptor Agonist Ameliorates Alzheimer-like Phenotype in AβPP/PS1 Mice. *J. Alzheimers Dis.* 35, 847–858. doi:10.3233/JAD-130137

Aso, E., Andrés-Benito, P., Carmona, M., Maldonado, R., and Ferrer, I. (2016). Cannabinoid Receptor 2 Participates in Amyloid-β Processing in a Mouse Model of Alzheimer's Disease but Plays a Minor Role in the Therapeutic Properties of a Cannabis-Based Medicine. *J. Alzheimers Dis.* 51, 489–500. doi:10.3233/JAD-150913

Aso, E., Fernández-Dueñas, V., López-Cano, M., Taura, J., Watanabe, M., Ferrer, I., et al. (2019). Adenosine A2A-Cannabinoid CB1 Receptor Heteromers in the Hippocampus: Cannabidiol Blunts Δ9-Tetrahydrocannabinol-Induced Cognitive Impairment. *Mol. Neurobiol.* 56, 5382–5391. doi:10.1007/s12035-018-1456-3

Bachiller, S., Jiménez-Ferrer, I., Paulus, A., Yang, Y., Swanberg, M., Deierborg, T., et al. (2018). Microglia in Neurological Diseases: A Road Map to Brain-Disease Dependent-Inflammatory Response. *Front. Cel Neurosci* 12, 488. doi:10.3389/fncel.2018.00488

Bachstetter, A. D., Xing, B., de Almeida, L., Dimayuga, E. R., Watterson, D. M., and Van Eldik, L. J. (2011). Microglial P38α MAPK Is a Key Regulator of Proinflammatory Cytokine Up-Regulation Induced by Toll-Like Receptor (TLR) Ligands or Beta-Amyloid (Aβ). *J. Neuroinflammation* 8, 79. doi:10.1186/1742-2094-8-79

Bagher, A. M., Young, A. P., Laprairie, R. B., Toguri, J. T., Kelly, M. E. M., and Denovan-Wright, E. M. (2020). Heteromer Formation between Cannabinoid Type 1 and Dopamine Type 2 Receptors Is Altered by Combination Cannabinoid and Antipsychotic Treatments. *J. Neurosci. Res.* 98, 2496–2509. doi:10.1002/jnr.24716

Balenga, N. A., Martínez-Pinilla, E., Kargl, J., Schröder, R., Peinhaupt, M., Platzer, W., et al. (2014). Heteromerization of GPR55 and Cannabinoid CB2 Receptors Modulates Signalling. *Br. J. Pharmacol.* 171, 5387–5406. doi:10.1111/bph.12850

Benito, C., Núñez, E., Tolón, R. M., Carrier, E. J., Rábano, A., Hillard, C. J., et al. (2003). Cannabinoid CB2 Receptors and Fatty Acid Amide Hydrolase Are Selectively Overexpressed in Neuritic Plaque-Associated Glia in Alzheimer's Disease Brains. *J. Neurosci.* 23, 11136–11141. doi:10.1523/jneurosci.23-35-11136.2003

Benzing, W. C., Wujek, J. R., Ward, E. K., Shaffer, D., Ashe, K. H., Younkin, S. G., et al. (1999). Evidence for Glial-Mediated Inflammation in Aged APP(SW) Transgenic Mice. *Neurobiol. Aging* 20, 581–589. doi:10.1016/s0197-4580(99)00065-2

Blázquez, C., Chiarlone, A., Sagredo, O., Aguado, T., Pazos, M. R., Resel, E., et al. (2011). Loss of Striatal Type 1 Cannabinoid Receptors Is a Key Pathogenic Factor in Huntington's Disease. *Brain* 134, 119–136. doi:10.1093/brain/awq278

Blanton, H. L., Barnes, R. C., McHann, M. C., Bilbrey, J. A., Wilkerson, J. L., and Guindon, J. (2021). Sex Differences and the Endocannabinoid System in Pain. *Pharmacol. Biochem. Behav.* 202, 173107. doi:10.1016/j.pbb.2021.173107

Bloom, G. S. (2014). Amyloid-β and Tau: The Trigger and Bullet in Alzheimer Disease Pathogenesis. *JAMA Neurol.* 71, 505–508. doi:10.1001/jamaneurol.2013.5847

Bouaboula, M., Rinaldi, M., Carayon, P., Carillon, C., Delpech, B., Shire, D., et al. (1993). Cannabinoid-Receptor Expression in Human Leukocytes. *Eur. J. Biochem.* 214, 173–180. doi:10.1111/j.1432-1033.1993.tb17910.x

Bouaboula, M., Poinot-Chazel, C., Marchand, J., Canat, X., Bourrié, B., Rinaldi-Carmona, M., et al. (1996). Signaling Pathway Associated with Stimulation of CB2 Peripheral Cannabinoid Receptor. Involvement of Both Mitogen-Activated Protein Kinase and Induction of Krox-24 Expression. *Eur. J. Biochem.* 237, 704–711. doi:10.1111/j.1432-1033.1996.0704p.x

Cagnin, A., Gerhard, A., and Banati, R. B. (2002). The Concept of *In Vivo* Imaging of Neuroinflammation with [11C](R)-PK11195 PET. *Ernst Schering Res. Found. Workshop* 12, 179–191. doi:10.1007/978-3-662-05073-6_10

Callén, L., Moreno, E., Barroso-Chinea, P., Moreno-Delgado, D., Cortés, A., Mallol, J., et al. (2012). Cannabinoid Receptors CB1 and CB2 Form Functional Heteromers in Brain. *J. Biol. Chem.* 287, 20851–20865. doi:10.1074/jbc.M111.335273

Carlisle, S. J., Marciano-Cabral, F., Staab, A., Ludwick, C., and Cabral, G. A. (2002). Differential Expression of the CB2 Cannabinoid Receptor by Rodent Macrophages and Macrophage-Like Cells in Relation to Cell Activation. *Int. Immunopharmacol* 2, 69–82. doi:10.1016/S1567-5769(01)00147-3

Carriba, P., Ortiz, O., Patkar, K., Justinova, Z., Stroik, J., Themann, A., et al. (2007). Striatal Adenosine A2A and Cannabinoid CB1 Receptors Form Functional Heteromeric Complexes that Mediate the Motor Effects of Cannabinoids. *Neuropsychopharmacology* 32, 2249–2259. doi:10.1038/sj.npp.1301375

Cassano, T., Calcagnini, S., Pace, L., De Marco, F., Romano, A., and Gaetani, S. (2017). Cannabinoid Receptor 2 Signaling in Neurodegenerative Disorders: From Pathogenesis to a Promising Therapeutic Target. *Front. Neurosci.* 11, 30. doi:10.3389/fnins.2017.00030

Chen, M. J., Ramesha, S., Weinstock, L. D., Gao, T., Ping, L., Xiao, H., et al. (2021). Extracellular Signal-Regulated Kinase Regulates Microglial Immune Responses in Alzheimer's Disease. *J. Neurosci. Res.* 99, 1704–1721. doi:10.1002/jnr.24829

Cherry, J. D., Olschowka, J. A., and O'Banion, M. K. (2014a). Are "resting" microglia more "M2"? *Front. Immunol.* 5, 594. doi:10.3389/fimmu.2014.00594

Cherry, J. D., Olschowka, J. A., and O'Banion, M. K. (2014b). Neuroinflammation and M2 Microglia: The Good, the Bad, and the Inflamed. *J. Neuroinflammation* 11, 98. doi:10.1186/1742-2094-11-98

Chhor, V., Le Charpentier, T., Lebon, S., Oré, M. V., Celador, I. L., Josserand, J., et al. (2013). Characterization of Phenotype Markers and Neuronotoxic Potential of Polarised Primary Microglia *In Vitro*. *Brain Behav. Immun.* 32, 70–85. doi:10.1016/j.bbi.2013.02.005

Chung, Y. C., Shin, W. H., Baek, J. Y., Cho, E. J., Baik, H. H., Kim, S. R., et al. (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. doi:10.1038/emm.2015.100

Corona, A. W., Fenn, A. M., and Godbout, J. P. (2012). Cognitive and Behavioral Consequences of Impaired Immunoregulation in Aging. *J. Neuroimmune Pharmacol.* 7, 7–23. doi:10.1007/s11481-011-9313-4

Correa, F., Hernangómez, M., Mestre, L., Loría, F., Spagnolo, A., Docagne, F., et al. (2010). Anandamide Enhances IL-10 Production in Activated Microglia by

Targeting CB(2) Receptors: Roles of ERK1/2, JNK, and NF-kappaB. *Glia* 58, 135–147. doi:10.1002/glia.20907

Correa, F., Hernangómez-Herrero, M., Mestre, L., Loría, F., Docagne, F., and Guaza, C. (2011). The Endocannabinoid Anandamide Downregulates IL-23 and IL-12 Subunits in a Viral Model of Multiple Sclerosis: Evidence for a Cross-Talk between IL-12p70/IL-23 Axis and IL-10 in Microglial Cells. *Brain Behav. Immun.* 25, 736–749. doi:10.1016/j.bbi.2011.01.020

Craft, R. M., Marusich, J. A., and Wiley, J. L. (2013). Sex Differences in Cannabinoid Pharmacology: A Reflection of Differences in the Endocannabinoid System? *Life Sci.* 92, 476–481. doi:10.1016/j.lfs.2012.06.009

Crapser, J. D., Ochaba, J., Soni, N., Reidling, J. C., Thompson, L. M., and Green, K. N. (2020). Microglial Depletion Prevents Extracellular Matrix Changes and Striatal Volume Reduction in a Model of Huntington's Disease. *Brain* 143, 266–288. doi:10.1093/brain/awz363

Cravatt, B. F., Demarest, K., Patricelli, M. P., Bracey, M. H., Giang, D. K., Martin, B. R., et al. (2001). Supersensitivity to Anandamide and Enhanced Endogenous Cannabinoid Signaling in Mice Lacking Fatty Acid Amide Hydrolase. *Proc. Natl. Acad. Sci. U S A.* 98, 9371–9376. doi:10.1073/pnas.161191698

Cristino, L., Bisogno, T., and Di Marzo, V. (2020). Cannabinoids and the Expanded Endocannabinoid System in Neurological Disorders. *Nat. Rev. Neurol.* 16, 9–29. doi:10.1038/s41582-019-0284-z

Crotti, A., Benner, C., Kerman, B. E., Gosselin, D., Lagier-Tourenne, C., Zuccato, C., et al. (2014). Mutant Huntingtin Promotes Autonomous Microglia Activation via Myeloid Lineage-Determining Factors. *Nat. Neurosci.* 17, 513–521. doi:10.1038/nn.3668

D'Andrea, M. R., Cole, G. M., and Ard, M. D. (2004). The Microglial Phagocytic Role with Specific Plaque Types in the Alzheimer Disease Brain. *Neurobiol. Aging* 25, 675–683. doi:10.1016/j.neurobiolaging.2003.12.026

de Almeida, D. L., and Devi, L. A. (2020). Diversity of Molecular Targets and Signaling Pathways for CBD. *Pharmacol. Res. Perspect.* 8, e00682. doi:10.1002/prp2.682

Denovan-Wright, E. M., and Robertson, H. A. (2000). Cannabinoid Receptor Messenger RNA Levels Decrease in a Subset of Neurons of the Lateral Striatum, Cortex and Hippocampus of Transgenic Huntington's Disease Mice. *Neuroscience* 98, 705–713. doi:10.1016/s0306-4522(00)00157-3

Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., et al. (1992). Isolation and Structure of a Brain Constituent that Binds to the Cannabinoid Receptor. *Science* 258, 1946–1949. doi:10.1126/science.1470919

Di Marzo, V., Fontana, A., Cadas, H., Schinelli, S., Cimino, G., Schwartz, J. C., et al. (1994). Formation and Inactivation of Endogenous Cannabinoid Anandamide in Central Neurons. *Nature* 372, 686–691. doi:10.1038/372686a0

Di Marzo, V., Hill, M. P., Bisogno, T., Crossman, A. R., and Brotchie, J. M. (2000). Enhanced Levels of Endogenous Cannabinoids in the Globus Pallidus Are Associated with a Reduction in Movement in an Animal Model of Parkinson's Disease. *FASEB J.* 14, 1432–1438. doi:10.1096/fj.14.10.1432

Dowie, M. J., Bradshaw, H. B., Howard, M. L., Nicholson, L. F., Faull, R. L., Hannan, A. J., et al. (2009). Altered CB1 Receptor and Endocannabinoid Levels Precede Motor Symptom Onset in a Transgenic Mouse Model of Huntington's Disease. *Neuroscience* 163, 456–465. doi:10.1016/j.neuroscience.2009.06.014

Dowie, M. J., Howard, M. L., Nicholson, L. F., Faull, R. L., Hannan, A. J., and Glass, M. (2010). Behavioural and Molecular Consequences of Chronic Cannabinoid Treatment in Huntington's Disease Transgenic Mice. *Neuroscience* 170, 324–336. doi:10.1016/j.neuroscience.2010.06.056

Dowie, M. J., Grimsey, N. L., Hoffman, T., Faull, R. L., and Glass, M. (2014). Cannabinoid Receptor CB2 Is Expressed on Vascular Cells, but Not Astroglial Cells in the Post-Mortem Human Huntington's Disease Brain. *J. Chem. Neuroanat.* 59-60, 62–71. doi:10.1016/j.jchemneu.2014.06.004

Doyle, H. H., Eidson, L. N., Sinkiewicz, D. M., and Murphy, A. Z. (2017). Sex Differences in Microglia Activity within the Periaqueductal Gray of the Rat: A Potential Mechanism Driving the Dimorphic Effects of Morphine. *J. Neurosci.* 37, 3202–3214. doi:10.1523/JNEUROSCI.2906-16.2017

Ehrhart, J., Obregon, D., Mori, T., Hou, H., Sun, N., Bai, Y., et al. (2005). Stimulation of Cannabinoid Receptor 2 (CB2) Suppresses Microglial Activation. *J. Neuroinflammation* 2, 29. doi:10.1186/1742-2094-2-29

Eljaschewitsch, E., Witting, A., Mawrin, C., Lee, T., Schmidt, P. M., Wolf, S., et al. (2006). The Endocannabinoid Anandamide Protects Neurons during CNS Inflammation by Induction of MKP-1 in Microglial Cells. *Neuron* 49, 67–79. doi:10.1016/j.neuron.2005.11.027

Esposito, G., Iuvone, T., Savani, C., Scuderi, C., De Filippis, D., Papa, M., et al. (2007). Opposing Control of Cannabinoid Receptor Stimulation on Amyloid-Beta-Induced Reactive Gliosis: *In Vitro* and *In Vivo* Evidence. *J. Pharmacol. Exp. Ther.* 322, 1144–1152. doi:10.1124/jpet.107.121566

Fakhfouri, G., Ahmadiani, A., Rahimian, R., Grolla, A. A., Moradi, F., and Haeri, A. (2012). WIN55212-2 Attenuates Amyloid-Beta-Induced Neuroinflammation in Rats through Activation of Cannabinoid Receptors and PPAR-γ Pathway. *Neuropharmacology* 63, 653–666. doi:10.1016/j.neuropharm.2012.05.013

Felder, C. C., Briley, E. M., Axelrod, J., Simpson, J. T., Mackie, K., and Devane, W. A. (1993). Anandamide, an Endogenous Cannabimimetic Eicosanoid, Binds to the Cloned Human Cannabinoid Receptor and Stimulates Receptor-Mediated Signal Transduction. *Proc. Natl. Acad. Sci. U S A.* 90, 7656–7660. doi:10.1073/pnas.90.16.7656

Flowers, A., Bell-Temin, H., Jalloh, A., Stevens, S. M., and Bickford, P. C. (2017). Proteomic Analysis of Aged Microglia: Shifts in Transcription, Bioenergetics, and Nutrient Response. *J. Neuroinflammation.* 14, 96. doi:10.1186/s12974-017-0840-7

Franco, R., Reyes-Resina, I., Aguinaga, D., Lillo, A., Jiménez, J., Raïch, I., et al. (2019a). Potentiation of Cannabinoid Signaling in Microglia by Adenosine A 2A Receptor Antagonists. *Glia* 67, 2410–2423. doi:10.1002/glia.23694

Franco, R., Villa, M., Morales, P., Reyes-Resina, I., Gutiérrez-Rodríguez, A., Jiménez, J., et al. (2019b). Increased Expression of Cannabinoid CB2 and Serotonin 5-HT1A Heteroreceptor Complexes in a Model of Newborn Hypoxic-Ischemic Brain Damage. *Neuropharmacology* 152, 58–66. doi:10.1016/j.neuropharm.2019.02.004

Frautschy, S. A., Yang, F., Irrizarry, M., Hyman, B., Saido, T. C., Hsiao, K., et al. (1998). Microglial Response to Amyloid Plaques in APPsw Transgenic Mice. *Am. J. Pathol.* 152, 307–317.

Frazier, W. J., Xue, J., Luce, W. A., and Liu, Y. (2012). MAPK Signaling Drives Inflammation in LPS-Stimulated Cardiomyocytes: The Route of Crosstalk to G-Protein-Coupled Receptors. *PLOS ONE* 7, e50071. doi:10.1371/journal.pone.0050071

Galiègue, S., Mary, S., Marchand, J., Dussossoy, D., Carrière, D., Carayon, P., et al. (1995). Expression of Central and Peripheral Cannabinoid Receptors in Human Immune Tissues and Leukocyte Subpopulations. *Eur. J. Biochem.* 232, 54–61. doi:10.1111/j.1432-1033.1995.tb20780.x

Gerhard, A., Pavese, N., Hotton, G., Turkheimer, F., Es, M., Hammers, A., et al. (2006). *In Vivo* imaging of Microglial Activation with [11C](R)-Pk11195 PET in Idiopathic Parkinson's Disease. *Neurobiol. Dis.* 21, 404–412. doi:10.1016/j.nbd.2005.08.002

Glass, M., and Felder, C. C. (1997). Concurrent Stimulation of Cannabinoid CB1 and Dopamine D2 Receptors Augments cAMP Accumulation in Striatal Neurons: Evidence for a Gs Linkage to the CB1 Receptor. *J. Neurosci.* 17, 5327–5333. doi:10.1523/jneurosci.17-14-05327.1997

Glass, M., and Northup, J. K. (1999). Agonist Selective Regulation of G Proteins by Cannabinoid CB(1) and CB(2) Receptors. *Mol. Pharmacol.* 56, 1362–1369. doi:10.1124/mol.56.6.1362

Glass, M., Faull, R. L., and Dragunow, M. (1993). Loss of Cannabinoid Receptors in the Substantia Nigra in Huntington's Disease. *Neuroscience* 56, 523–527. doi:10.1016/0306-4522(93)90352-g

Gómez-Gálvez, Y., Palomo-Garo, C., Fernández-Ruiz, J., and García, C. (2016). Potential of the Cannabinoid CB2 Receptor as a Pharmacological Target against Inflammation in Parkinson's Disease. *Prog. Neuro-Psychopharmacology Biol. Psychiatry* 64, 200–208. doi:10.1016/j.pnpbp.2015.03.017

Gobbi, G., Bambico, F. R., Mangieri, R., Bortolato, M., Campolongo, P., Solinas, M., et al. (2005). Antidepressant-Like Activity and Modulation of Brain Monoaminergic Transmission by Blockade of Anandamide Hydrolysis. *Proc. Natl. Acad. Sci. U S A.* 102, 18620–18625. doi:10.1073/pnas.0509591102

Godbout, J. P., and Johnson, R. W. (2006). Age and Neuroinflammation: A Lifetime of Psychoneuroimmune Consequences. *Neurol. Clin.* 24, 521–538. doi:10.1016/j.ncl.2006.03.010

Godbout, J. P., and Johnson, R. W. (2009). Age and Neuroinflammation: A Lifetime of Psychoneuroimmune Consequences. *Immunol. Allergy Clin. North. Am.* 29, 321–337. doi:10.1016/j.iac.2009.02.007

Goncalves, M. B., Suetterlin, P., Yip, P., Molina-Holgado, F., Walker, D. J., Oudin, M. J., et al. (2008). A Diacylglycerol Lipase-CB2 Cannabinoid Pathway Regulates Adult Subventricular Zone Neurogenesis in an Age-Dependent Manner. *Mol. Cel Neurosci* 38, 526–536. doi:10.1016/j.mcn.2008.05.001

Griffin, G., Wray, E. J., Tao, Q., McAllister, S. D., Rorrer, W. K., Aung, M. M., et al. (1999). Evaluation of the Cannabinoid CB2 Receptor-Selective Antagonist, SR144528: Further Evidence for Cannabinoid CB2 Receptor Absence in the Rat Central Nervous System. *Eur. J. Pharmacol.* 377, 117–125. doi:10.1016/S0014-2999(99)00402-1

Guida, F., Luongo, L., Boccella, S., Giordano, M. E., Romano, R., Bellini, G., et al. (2017). Palmitoylethanolamide Induces Microglia Changes Associated with Increased Migration and Phagocytic Activity: Involvement of the CB2 Receptor. *Sci. Rep.* 7, 375. doi:10.1038/s41598-017-00342-1

Guzman-Martinez, L., Maccioni, R. B., Andrade, V., Navarrete, L. P., Pastor, M. G., and Ramos-Escobar, N. (2019). Neuroinflammation as a Common Feature of Neurodegenerative Disorders. *Front. Pharmacol.* 10, 1008. doi:10.3389/fphar.2019.01008

Halleskog, C., Mulder, J., Dahlström, J., Mackie, K., Hortobágyi, T., Tanila, H., et al. (2011). WNT Signaling in Activated Microglia Is Proinflammatory. *Glia* 59, 119–131. doi:10.1002/glia.21081

Hammond, T. R., Dufort, C., Dissing-Olesen, L., Giera, S., Young, A., Wysoker, A., et al. (2019). Single-Cell RNA Sequencing of Microglia throughout the Mouse Lifespan and in the Injured Brain Reveals Complex Cell-State Changes. *Immunity* 50, 253–271. doi:10.1016/j.immuni.2018.11.004

Hayashi, Y., Yoshida, M., Yamato, M., Ide, T., Wu, Z., Ochi-Shindou, M., et al. (2008). Reverse of Age-Dependent Memory Impairment and Mitochondrial DNA Damage in Microglia by an Overexpression of Human Mitochondrial Transcription Factor a in Mice. *J. Neurosci.* 28, 8624–8634. doi:10.1523/JNEUROSCI.1957-08.2008

Heneka, M. T., Carson, M. J., El Khoury, J., Landreth, G. E., Brosseron, F., Feinstein, D. L., et al. (2015). Neuroinflammation in Alzheimer's Disease. *Lancet Neurol.* 14, 388–405. doi:10.1016/S1474-4422(15)70016-5

Henry, C. J., Huang, Y., Wynne, A. M., and Godbout, J. P. (2009). Peripheral Lipopolysaccharide (LPS) challenge Promotes Microglial Hyperactivity in Aged Mice that Is Associated with Exaggerated Induction of Both Pro-Inflammatory IL-1beta and Anti-Inflammatory IL-10 Cytokines. *Brain Behav. Immun.* 23, 309–317. doi:10.1016/j.bbi.2008.09.002

Heyman, E., Gamelin, F. X., Aucouturier, J., and Di Marzo, V. (2012). The Role of the Endocannabinoid System in Skeletal Muscle and Metabolic Adaptations to Exercise: Potential Implications for the Treatment of Obesity. *Obes. Rev.* 13, 1110–1124. doi:10.1111/j.1467-789X.2012.01026.x

Hickman, S. E., Allison, E. K., and El Khoury, J. (2008). Microglial Dysfunction and Defective Beta-Amyloid Clearance Pathways in Aging Alzheimer's Disease Mice. *J. Neurosci.* 28, 8354–8360. doi:10.1523/JNEUROSCI.0616-08.2008

Hillard, C. J., Weinlander, K. M., and Stuhr, K. L. (2012). Contributions of Endocannabinoid Signaling to Psychiatric Disorders in Humans: Genetic and Biochemical Evidence. *Neuroscience* 204, 207–229. doi:10.1016/j.neuroscience.2011.11.020

Hodges, E. L., Marshall, J. P., and Ashpole, N. M. (2020). Age-Dependent Hormesis-Like Effects of the Synthetic Cannabinoid CP55940 in C57BL/6 Mice. *Npj Aging Mech. Dis.* 6, 7. doi:10.1038/s41514-020-0045-7

Howlett, A. C., Barth, F., Bonner, T. I., Cabral, G., Casellas, P., Devane, W. A., et al. (2002). International Union of Pharmacology. XXVII. Classification of Cannabinoid Receptors. *Pharmacol. Rev.* 54, 161–202. doi:10.1124/pr.54.2.161

Hutchinson, M. R., Zhang, Y., Shridhar, M., Evans, J. H., Buchanan, M. M., Zhao, T. X., et al. (2010). Evidence that Opioids May Have Toll-Like Receptor 4 and MD-2 Effects. *Brain Behav. Immun.* 24, 83–95. doi:10.1016/j.bbi.2009.08.004

Ibsen, M. S., Connor, M., and Glass, M. (2017). Cannabinoid CB1 and CB2 Receptor Signaling and Bias. *Cannabis Cannabinoid Res.* 2, 48–60. doi:10.1089/can.2016.0037

Imamura, K., Hishikawa, N., Sawada, M., Nagatsu, T., Yoshida, M., and Hashizume, Y. (2003). Distribution of Major Histocompatibility Complex Class II-Positive Microglia and Cytokine Profile of Parkinson's Disease Brains. *Acta Neuropathol.* 106, 518–526. doi:10.1007/s00401-003-0766-2

Javed, H., Azimullah, S., Haque, M. E., and Ojha, S. K. (2016). Cannabinoid Type 2 (CB2) Receptors Activation Protects against Oxidative Stress and Neuroinflammation Associated Dopaminergic Neurodegeneration in Rotenone Model of Parkinson's Disease. *Front. Neurosci.* 10, 321. doi:10.3389/fnins.2016.00321

Jung, K. M., Astarita, G., Yasar, S., Vasilevko, V., Cribbs, D. H., Head, E., et al. (2012). An Amyloid β42-Dependent Deficit in Anandamide Mobilization Is Associated with Cognitive Dysfunction in Alzheimer's Disease. *Neurobiol. Aging* 33, 1522–1532. doi:10.1016/j.neurobiolaging.2011.03.012

Kathuria, S., Gaetani, S., Fegley, D., Valiño, F., Duranti, A., Tontini, A., et al. (2003). Modulation of Anxiety through Blockade of Anandamide Hydrolysis. *Nat. Med.* 9, 76–81. doi:10.1038/nm803

Kim, S. H., Smith, C. J., and Van Eldik, L. J. (2004). Importance of MAPK Pathways for Microglial Pro-inflammatory Cytokine IL-1 Beta Production. *Neurobiol. Aging* 25, 431–439. doi:10.1016/S0197-4580(03)00126-X

Köfalvi, A., Moreno, E., Cordomí, A., Cai, N.-S., Fernández-Dueñas, V., Ferreira, S. G., et al. (2020). Control of Glutamate Release by Complexes of Adenosine and Cannabinoid Receptors. *BMC Biol.* 18, 9. doi:10.1186/s12915-020-0739-0

Komorowska-Müller, J. A., and Schmöle, A.-C. (2020). CB2 Receptor in Microglia: The Guardian of Self-Control. *Ijms* 22, 19. doi:10.3390/ijms22010019

Laprairie, R. B., Kelly, M. E., and Denovan-Wright, E. M. (2013). Cannabinoids Increase Type 1 Cannabinoid Receptor Expression in a Cell Culture Model of Striatal Neurons: Implications for Huntington's Disease. *Neuropharmacology* 72, 47–57. doi:10.1016/j.neuropharm.2013.04.006

Laprairie, R. B., Bagher, A. M., Rourke, J. L., Zrein, A., Cairns, E. A., Kelly, M. E. M., et al. (2019). Positive Allosteric Modulation of the Type 1 Cannabinoid Receptor Reduces the Signs and Symptoms of Huntington's Disease in the R6/2 Mouse Model. *Neuropharmacology* 151, 1–12. doi:10.1016/j.neuropharm.2019.03.033

Lastres-Becker, I., Berrendero, F., Lucas, J. J., Martín-Aparicio, E., Yamamoto, A., Ramos, J. A., et al. (2002). Loss of mRNA Levels, Binding and Activation of GTP-Binding Proteins for Cannabinoid CB1 Receptors in the Basal Ganglia of a Transgenic Model of Huntington's Disease. *Brain Res.* 929, 236–242. doi:10.1016/s0006-8993(01)03403-5

Li, C., Shi, J., Wang, B., Li, J., and Jia, H. (2019a). CB2 Cannabinoid Receptor Agonist Ameliorates Novel Object Recognition but Not Spatial Memory in Transgenic APP/PS1 Mice. *Neurosci. Lett.* 707, 134286. doi:10.1016/j.neulet.2019.134286

Li, M., Zhang, D., Ge, X., Zhu, X., Zhou, Y., Zhang, Y., et al. (2019b). TRAF6-p38/JNK-ATF2 Axis Promotes Microglial Inflammatory Activation. *Exp. Cel Res* 376, 133–148. doi:10.1016/j.yexcr.2019.02.005

López-Sendón Moreno, J. L., García Caldentey, J., Trigo Cubillo, P., Ruiz Romero, C., García Ribas, G., Alonso Arias, M. A. A., et al. (2016). A Double-Blind, Randomized, Cross-Over, Placebo-Controlled, Pilot Trial with Sativex in Huntington's Disease. *J. Neurol.* 263, 1390–1400. doi:10.1007/s00415-016-8145-9

Long, J. Z., Nomura, D. K., Vann, R. E., Walentiny, D. M., Booker, L., Jin, X., et al. (2009). Dual Blockade of FAAH and MAGL Identifies Behavioral Processes Regulated by Endocannabinoid Crosstalk *In Vivo*. *Proc. Natl. Acad. Sci. U S A.* 106, 20270–20275. doi:10.1073/pnas.0909411106

Lu, H. C., and Mackie, K. (2016). An Introduction to the Endogenous Cannabinoid System. *Biol. Psychiatry* 79, 516–525. doi:10.1016/j.biopsych.2015.07.028

Lukiw, W. J. (2004). Gene Expression Profiling in Fetal, Aged, and Alzheimer hippocampus: A Continuum of Stress-Related Signaling. *Neurochem. Res.* 29, 1287–1297. doi:10.1023/b:nere.0000023615.89699.63

Lynn, A. B., and Herkenham, M. (1994). Localization of Cannabinoid Receptors and Nonsaturable High-Density Cannabinoid Binding Sites in Peripheral Tissues of the Rat: Implications for Receptor-Mediated Immune Modulation by Cannabinoids. *J. Pharmacol. Exp. Ther.* 268, 1612–1623.

Ma, L., Jia, J., Liu, X., Bai, F., Wang, Q., and Xiong, L. (2015). Activation of Murine Microglial N9 Cells Is Attenuated through Cannabinoid Receptor CB2 Signaling. *Biochem. Biophys. Res. Commun.* 458, 92–97. doi:10.1016/j.bbrc.2015.01.073

Maccarrone, M., Gubellini, P., Bari, M., Picconi, B., Battista, N., Centonze, D., et al. (2003). Levodopa Treatment Reverses Endocannabinoid System Abnormalities in Experimental Parkinsonism. *J. Neurochem.* 85, 1018–1025. doi:10.1046/j.1471-4159.2003.01759.x

Mailleux, P., and Vanderhaeghen, J. J. (1992). Age-related Loss of Cannabinoid Receptor Binding Sites and mRNA in the Rat Striatum. *Neurosci. Lett.* 147, 179–181. doi:10.1016/0304-3940(92)90589-y

Maresz, K., Carrier, E. J., Ponomarev, E. D., Hillard, C. J., and Dittel, B. N. (2005). Modulation of the Cannabinoid CB2 Receptor in Microglial Cells in Response to Inflammatory Stimuli. *J. Neurochem.* 95, 437–445. doi:10.1111/j.1471-4159.2005.03380.x

Marsicano, G., and Lutz, B. (1999). Expression of the Cannabinoid Receptor CB1 in Distinct Neuronal Subpopulations in the Adult Mouse Forebrain. *Eur. J. Neurosci.* 11, 4213–4225. doi:10.1046/j.1460-9568.1999.00847.x

Martín-Moreno, A. M., Brera, B., Spuch, C., Carro, E., García-García, L., Delgado, M., et al. (2012). Prolonged Oral Cannabinoid Administration Prevents Neuroinflammation, Lowers β-amyloid Levels and Improves Cognitive Performance in Tg APP 2576 Mice. *J. Neuroinflammation* 9, 8. doi:10.1186/1742-2094-9-8

Matias, I., Petrosino, S., Racioppi, A., Capasso, R., Izzo, A. A., and Di Marzo, V. (2008). Dysregulation of Peripheral Endocannabinoid Levels in Hyperglycemia and Obesity: Effect of High Fat Diets. *Mol. Cel Endocrinol* 286, S66–S78. doi:10.1016/j.mce.2008.01.026

McCoy, K. L., Matveyeva, M., Carlisle, S. J., and Cabral, G. A. (1999). Cannabinoid Inhibition of the Processing of Intact Lysozyme by Macrophages: Evidence for CB2 Receptor Participation. *J. Pharmacol. Exp. Ther.* 289, 1620–1625.

McGeer, P. L., Itagaki, S., Boyes, B. E., and McGeer, E. G. (1988). Reactive Microglia Are Positive for HLA-DR in the Substantia Nigra of Parkinson's and Alzheimer's Disease Brains. *Neurology* 38, 1285–1291. doi:10.1212/wnl.38.8.1285

McGuire, S. O., Ling, Z. D., Lipton, J. W., Sortwell, C. E., Collier, T. J., and Carvey, P. M. (2001). Tumor Necrosis Factor Alpha Is Toxic to Embryonic Mesencephalic Dopamine Neurons. *Exp. Neurol.* 169, 219–230. doi:10.1006/exnr.2001.7688

Mecha, M., Feliú, A., Carrillo-Salinas, F. J., Rueda-Zubiaurre, A., Ortega-Gutiérrez, S., de Sola, R. G., et al. (2015). Endocannabinoids Drive the Acquisition of an Alternative Phenotype in Microglia. *Brain Behav. Immun.* 49, 233–245. doi:10.1016/j.bbi.2015.06.002

Mechoulam, R., Ben-Shabat, S., Hanus, L., Ligumsky, M., Kaminski, N. E., Schatz, A. R., et al. (1995). Identification of an Endogenous 2-monoglyceride, Present in Canine Gut, that Binds to Cannabinoid Receptors. *Biochem. Pharmacol.* 50, 83–90. doi:10.1016/0006-2952(95)00109-d

Meng, A., Zhang, X., and Shi, Y. (2014). Role of P38 MAPK and STAT3 in Lipopolysaccharide-Stimulated Mouse Alveolar Macrophages. *Exp. Ther. Med.* 8, 1772–1776. doi:10.3892/etm.2014.2023

Meng, Q., Lin, M. S., and Tzeng, I. S. (2020). Relationship between Exercise and Alzheimer's Disease: A Narrative Literature Review. *Front. Neurosci.* 14, 131. doi:10.3389/fnins.2020.00131

Motwani, M. P., Bennett, F., Norris, P. C., Maini, A. A., George, M. J., Newson, J., et al. (2018). Potent Anti-Inflammatory and Pro-Resolving Effects of Anabasum in a Human Model of Self-Resolving Acute Inflammation. *Clin. Pharmacol. Ther.* 104, 675–686. doi:10.1002/cpt.980

Mounsey, R. B., Mustafa, S., Robinson, L., Ross, R. A., Riedel, G., Pertwee, R. G., et al. (2015). Increasing Levels of the Endocannabinoid 2-AG Is Neuroprotective in the 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine Mouse Model of Parkinson's Disease. *Exp. Neurol.* 273, 36–44. doi:10.1016/j.expneurol.2015.07.024

Mulder, J., Zilberter, M., Pasquaré, S. J., Alpár, A., Schulte, G., Ferreira, S. G., et al. (2011). Molecular Reorganization of Endocannabinoid Signalling in Alzheimer's Disease. *Brain* 134, 1041–1060. doi:10.1093/brain/awr046

Munro, S., Thomas, K. L., and Abu-Shaar, M. (1993). Molecular Characterization of a Peripheral Receptor for Cannabinoids. *Nature* 365, 61–65. doi:10.1038/365061a0

Murphy, M. P., and LeVine, H. (2010). Alzheimer's Disease and the Amyloid-β Peptide. *J. Alzheimers Dis.* 19, 311–323. doi:10.3233/JAD-2010-1221

Navarrete, F., García-Gutiérrez, M. S., Aracil-Fernández, A., Lanciego, J. L., and Manzanares, J. (2018). Cannabinoid CB1 and CB2 Receptors, and Monoacylglycerol Lipase Gene Expression Alterations in the Basal Ganglia of Patients with Parkinson's Disease. *Neurotherapeutics* 15, 459–469. doi:10.1007/s13311-018-0603-x

Navarro, G., Morales, P., Rodríguez-Cueto, C., Fernández-Ruiz, J., Jagerovic, N., and Franco, R. (2016). Targeting Cannabinoid CB2 Receptors in the Central Nervous System. Medicinal Chemistry Approaches with Focus on Neurodegenerative Disorders. *Front. Neurosci.* 10, 406. doi:10.3389/fnins.2016.00406

Navarro, G., Borroto-Escuela, D., Angelats, E., Etayo, Í., Reyes-Resina, I., Pulido-Salgado, M., et al. (2018). Receptor-Heteromer Mediated Regulation of Endocannabinoid Signaling in Activated Microglia. Role of CB1 and CB2 Receptors and Relevance for Alzheimer's Disease and Levodopa-Induced Dyskinesia. *Brain Behav. Immun.* 67, 139–151. doi:10.1016/j.bbi.2017.08.015

Neumann, H., Schweigreiter, R., Yamashita, T., Rosenkranz, K., Wekerle, H., and Barde, Y. A. (2002). Tumor Necrosis Factor Inhibits Neurite Outgrowth and Branching of Hippocampal Neurons by a Rho-Dependent Mechanism. *J. Neurosci.* 22, 854–862. doi:10.1523/JNEUROSCI.22-03-00854.2002

Nimmerjahn, A., Kirchhoff, F., and Helmchen, F. (2005). Resting Microglial Cells Are Highly Dynamic Surveillants of Brain Parenchyma *In Vivo*. *Science* 308, 1314–1318. doi:10.1126/science.1110647

Njie, E. G., Boelen, E., Stassen, F. R., Steinbusch, H. W., Borchelt, D. R., and Streit, W. J. (2012). *Ex Vivo* cultures of Microglia from Young and Aged Rodent Brain Reveal Age-Related Changes in Microglial Function. *Neurobiol. Aging* 33, 195. doi:10.1016/j.neurobiolaging.2010.05.008

Norden, D. M., and Godbout, J. P. (2013). Review: Microglia of the Aged Brain: Primed to Be Activated and Resistant to Regulation. *Neuropathol. Appl. Neurobiol.* 39, 19–34. doi:10.1111/j.1365-2990.2012.01306.x

Nordengen, K., Kirsebom, B. E., Henjum, K., Selnes, P., Gísladóttir, B., Wettergreen, M., et al. (2019). Glial Activation and Inflammation along the Alzheimer's Disease Continuum. *J. Neuroinflammation* 16, 46–13. doi:10.1186/s12974-019-1399-2

Núñez, E., Benito, C., Pazos, M. R., Barbachano, A., Fajardo, O., González, S., et al. (2004). Cannabinoid CB2 Receptors Are Expressed by Perivascular Microglial Cells in the Human Brain: An Immunohistochemical Study. *Synapse* 53, 208–213. doi:10.1002/syn.20050

Ojha, S., Javed, H., Azimullah, S., and Haque, M. E. (2016). β-Caryophyllene, a Phytocannabinoid Attenuates Oxidative Stress, Neuroinflammation, Glial Activation, and Salvages Dopaminergic Neurons in a Rat Model of Parkinson Disease. *Mol. Cel Biochem* 418, 59–70. doi:10.1007/s11010-016-2733-y

Ouchi, Y., Yoshikawa, E., Sekine, Y., Futatsubashi, M., Kanno, T., Ogusu, T., et al. (2005). Microglial Activation and Dopamine Terminal Loss in Early Parkinson's Disease. *Ann. Neurol.* 57, 168–175. doi:10.1002/ana.20338

Pacher, P., Bátkai, S., and Kunos, G. (2006). The Endocannabinoid System as an Emerging Target of Pharmacotherapy. *Pharmacol. Rev.* 58, 389–462. doi:10.1124/pr.58.3.2

Pagotto, U., Marsicano, G., Cota, D., Lutz, B., and Pasquali, R. (2006). The Emerging Role of the Endocannabinoid System in Endocrine Regulation and Energy Balance. *Endocr. Rev.* 27, 73–100. doi:10.1210/er.2005-0009

Palazuelos, J., Aguado, T., Pazos, M. R., Julien, B., Carrasco, C., Resel, E., et al. (2009). Microglial CB2 Cannabinoid Receptors Are Neuroprotective in Huntington's Disease Excitotoxicity. *Brain* 132, 3152–3164. doi:10.1093/brain/awp239

Pascual, A. C., Gaveglio, V. L., Giusto, N. M., and Pasquaré, S. J. (2014). Cannabinoid Receptor-Dependent Metabolism of 2-Arachidonoylglycerol during Aging. *Exp. Gerontol.* 55, 134–142. doi:10.1016/j.exger.2014.04.008

Pavese, N., Gerhard, A., Tai, Y. F., Ho, A. K., Turkheimer, F., Barker, R. A., et al. (2006). Microglial Activation Correlates with Severity in Huntington Disease: A Clinical and PET Study. *Neurology* 66, 1638–1643. doi:10.1212/01.wnl.0000222734.56412.17

Perry, V. H., Nicoll, J. A., and Holmes, C. (2010). Microglia in Neurodegenerative Disease. *Nat. Rev. Neurol.* 6, 193–201. doi:10.1038/nrneurol.2010.17

Price, D. A., Martinez, A. A., Seillier, A., Koek, W., Acosta, Y., Fernandez, E., et al. (2009). WIN55,212-2, a Cannabinoid Receptor Agonist, Protects against Nigrostriatal Cell Loss in the 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine Mouse Model of Parkinson's Disease. *Eur. J. Neurosci.* 29, 2177–2186. doi:10.1111/j.1460-9568.2009.06764.x

Quarta, C., and Cota, D. (2020). Anti-Obesity Therapy with Peripheral CB1 Blockers: From Promise to Safe(?) Practice. *Int. J. Obes. (Lond)* 44, 2179–2193. doi:10.1038/s41366-020-0577-8

Raj, D., Yin, Z., Breur, M., Doorduin, J., Holtman, I. R., Olah, M., et al. (2017). Increased White Matter Inflammation in Aging– and Alzheimer's Disease Brain. *Front. Mol. Neurosci.* 10, 206. doi:10.3389/fnmol.2017.00206

Reusch, N., Ravichandran, K. A., Olabiyi, B. F., Komorowska-Müller, J. A., Hansen, J. N., Ulas, T., et al. (2021). Cannabinoid Receptor 2 Is Necessary to Induce Toll-Like Receptor-Mediated Microglial Activation. *Glia* 70 (1), 71–88. doi:10.1002/glia.24089

Richwine, A. F., Godbout, J. P., Berg, B. M., Chen, J., Escobar, J., Millard, D. K., et al. (2005). Improved Psychomotor Performance in Aged Mice Fed Diet High in

Antioxidants Is Associated with Reduced *Ex Vivo* Brain Interleukin-6 Production. *Brain Behav. Immun.* 19, 512–520. doi:10.1016/j.bbi.2004.12.005

Rojo-Bustamante, E., Abellanas, M. A., Clavero, P., Thiolat, M. L., Li, Q., Luquin, M. R., et al. (2018). The Expression of Cannabinoid Type 1 Receptor and 2-Arachidonoyl Glycerol Synthesizing/Degrading Enzymes Is Altered in Basal Ganglia during the Active Phase of Levodopa-Induced Dyskinesia. *Neurobiol. Dis.* 118, 64–75. doi:10.1016/j.nbd.2018.06.019

Romero, J., Berrendero, F., Garcia-Gil, L., de la Cruz, P., Ramos, J. A., and Fernández-Ruiz, J. J. (1998). Loss of Cannabinoid Receptor Binding and Messenger RNA Levels and Cannabinoid Agonist-Stimulated [35S]guanylyl-5'O-(thio)-Triphosphate Binding in the Basal Ganglia of Aged Rats. *Neuroscience* 84, 1075–1083. doi:10.1016/S0306-4522(97)00552-6

Romero-Sandoval, E. A., Horvath, R., Landry, R. P., and DeLeo, J. A. (2009). Cannabinoid Receptor Type 2 Activation Induces a Microglial Anti-Inflammatory Phenotype and Reduces Migration via MKP Induction and ERK Dephosphorylation. *Mol. Pain* 5, 25. doi:10.1186/1744-8069-5-25

Sagredo, O., González, S., Aroyo, I., Pazos, M. R., Benito, C., Lastres-Becker, I., et al. (2009). Cannabinoid CB2 Receptor Agonists Protect the Striatum against Malonate Toxicity: Relevance for Huntington's Disease. *Glia* 57, 1154–1167. doi:10.1002/glia.20838

Saito, V. M., Rezende, R. M., and Teixeira, A. L. (2012). Cannabinoid Modulation of Neuroinflammatory Disorders. *Curr. Neuropharmacol* 10, 159–166. doi:10.2174/157015912800604515

Sapp, E., Kegel, K. B., Aronin, N., Hashikawa, T., Uchiyama, Y., Tohyama, K., et al. (2001). Early and Progressive Accumulation of Reactive Microglia in the Huntington Disease Brain. *J. Neuropathol. Exp. Neurol.* 60, 161–172. doi:10.1093/jnen/60.2.161

Saroz, Y., Kho, D. T., Glass, M., Graham, E. S., and Grimsey, N. L. (2019). Cannabinoid Receptor 2 (CB2) Signals via G-Alpha-S and Induces IL-6 and IL-10 Cytokine Secretion in Human Primary Leukocytes. *ACS Pharmacol. Transl Sci.* 2, 414–428. doi:10.1021/acsptsci.9b00049

Schafer, D. P., Lehrman, E. K., Kautzman, A. G., Koyama, R., Mardinly, A. R., Yamasaki, R., et al. (2012). Microglia Sculpt Postnatal Neural Circuits in an Activity and Complement-Dependent Manner. *Neuron* 74, 691–705. doi:10.1016/j.neuron.2012.03.026

Schatz, A. R., Lee, M., Condie, R. B., Pulaski, J. T., and Kaminski, N. E. (1997). Cannabinoid Receptors CB1 and CB2: a Characterization of Expression and Adenylate Cyclase Modulation within the Immune System. *Toxicol. Appl. Pharmacol.* 142, 278–287. doi:10.1006/taap.1996.8034

Selkoe, D. J., and Hardy, J. (2016). The Amyloid Hypothesis of Alzheimer's Disease at 25 years. *EMBO Mol. Med.* 8, 595–608. doi:10.15252/emmm.201606210

Sierra, A., Gottfried-Blackmore, A. C., McEwen, B. S., and Bulloch, K. (2007). Microglia Derived from Aging Mice Exhibit an Altered Inflammatory Profile. *Glia* 55, 412–424. doi:10.1002/glia.20468

Simonds, W. F., Goldsmith, P. K., Codina, J., Unson, C. G., and Spiegel, A. M. (1989). Gi2 Mediates Alpha 2-adrenergic Inhibition of Adenylyl Cyclase in Platelet Membranes: *In Situ* Identification with G Alpha C-Terminal Antibodies. *Proc. Natl. Acad. Sci. U S A.* 86, 7809–7813. doi:10.1073/pnas.86.20.7809

Solas, M., Francis, P. T., Franco, R., and Ramirez, M. J. (2013). CB2 Receptor and Amyloid Pathology in Frontal Cortex of Alzheimer's Disease Patients. *Neurobiol. Aging* 34, 805–808. doi:10.1016/j.neurobiolaging.2012.06.005

Sorge, R. E., LaCroix-Fralish, M. L., Tuttle, A. H., Sotocinal, S. G., Austin, J. S., Ritchie, J., et al. (2011). Spinal Cord Toll-Like Receptor 4 Mediates Inflammatory and Neuropathic Hypersensitivity in Male but Not Female Mice. *J. Neurosci.* 31, 15450–15454. doi:10.1523/JNEUROSCI.3859-11.2011

Sorge, R. E., Mapplebeck, J. C., Rosen, S., Beggs, S., Taves, S., Alexander, J. K., et al. (2015). Different Immune Cells Mediate Mechanical Pain Hypersensitivity in Male and Female Mice. *Nat. Neurosci.* 18, 1081–1083. doi:10.1038/nn.4053

Spangenberg, E. E., Lee, R. J., Najafi, A. R., Rice, R. A., Elmore, M. R., Blurton-Jones, M., et al. (2016). Eliminating Microglia in Alzheimer's Mice Prevents Neuronal Loss without Modulating Amyloid-β Pathology. *Brain* 139, 1265–1281. doi:10.1093/brain/aww016

Spangenberg, E., Severson, P. L., Hohsfield, L. A., Crapser, J., Zhang, J., Burton, E. A., et al. (2019). Sustained Microglial Depletion with CSF1R Inhibitor Impairs Parenchymal Plaque Development in an Alzheimer's Disease Model. *Nat. Commun.* 10, 3758. doi:10.1038/s41467-019-11674-z

Stella, N., Schweitzer, P., and Piomelli, D. (1997). A Second Endogenous Cannabinoid that Modulates Long-Term Potentiation. *Nature* 388, 773–778. doi:10.1038/42015

Stella, N. (2009). Endocannabinoid Signaling in Microglial Cells. *Neuropharmacology* 56, 244–253. doi:10.1016/j.neuropharm.2008.07.037

Stella, N. (2010). Cannabinoid and Cannabinoid-Like Receptors in Microglia, Astrocytes, and Astrocytomas. *Glia* 58, 1017–1030. doi:10.1002/glia.20983

Stevens, B., Allen, N. J., Vazquez, L. E., Howell, G. R., Christopherson, K. S., Nouri, N., et al. (2007). The Classical Complement cascade Mediates CNS Synapse Elimination. *Cell* 131, 1164–1178. doi:10.1016/j.cell.2007.10.036

Streit, W. J., Sammons, N. W., Kuhns, A. J., and Sparks, D. L. (2004). Dystrophic Microglia in the Aging Human Brain. *Glia* 45, 208–212. doi:10.1002/glia.10319

Sugiura, T., Kondo, S., Sukagawa, A., Nakane, S., Shinoda, A., Itoh, K., et al. (1995). 2-Arachidonoylglycerol: A Possible Endogenous Cannabinoid Receptor Ligand in Brain. *Biochem. Biophys. Res. Commun.* 215, 89–97. doi:10.1006/bbrc.1995.2437

Sugiura, T., Kondo, S., Kishimoto, S., Miyashita, T., Nakane, S., Kodaka, T., et al. (2000). Evidence that 2-arachidonoylglycerol but Not N-Palmitoylethanolamine or Anandamide Is the Physiological Ligand for the Cannabinoid CB2 Receptor. Comparison of the Agonistic Activities of Various Cannabinoid Receptor Ligands in HL-60 Cells. *J. Biol. Chem.* 275, 605–612. doi:10.1074/jbc.275.1.605

Tai, Y. F., Pavese, N., Gerhard, A., Tabrizi, S. J., Barker, R. A., Brooks, D. J., et al. (2007a). Imaging Microglial Activation in Huntington's Disease. *Brain Res. Bull.* 72, 148–151. doi:10.1016/j.brainresbull.2006.10.029

Tai, Y. F., Pavese, N., Gerhard, A., Tabrizi, S. J., Barker, R. A., Brooks, D. J., et al. (2007b). Microglial Activation in Presymptomatic Huntington's Disease Gene Carriers. *Brain* 130, 1759–1766. doi:10.1093/brain/awm044

Tanaka, M., Sackett, S., and Zhang, Y. (2020). Endocannabinoid Modulation of Microglial Phenotypes in Neuropathology. *Front. Neurol.* 11, 87. doi:10.3389/fneur.2020.00087

Tao, Y., Li, L., Jiang, B., Feng, Z., Yang, L., Tang, J., et al. (2016). Cannabinoid Receptor-2 Stimulation Suppresses Neuroinflammation by Regulating Microglial M1/M2 Polarization through the cAMP/PKA Pathway in an Experimental GMH Rat Model. *Brain Behav. Immun.* 58, 118–129. doi:10.1016/j.bbi.2016.05.020

Tolón, R. M., Núñez, E., Pazos, M. R., Benito, C., Castillo, A. I., Martínez-Orgado, J. A., et al. (2009). The Activation of Cannabinoid CB2 Receptors Stimulates *In Situ* and *In Vitro* Beta-Amyloid Removal by Human Macrophages. *Brain Res.* 1283, 148–154. doi:10.1016/j.brainres.2009.05.098

Varin, A., and Gordon, S. (2009). Alternative Activation of Macrophages: Immune Function and Cellular Biology. *Immunobiology* 214, 630–641. doi:10.1016/j.imbio.2008.11.009

Villeda, S. A., Luo, J., Mosher, K. I., Zou, B., Britschgi, M., Bieri, G., et al. (2011). The Ageing Systemic Milieu Negatively Regulates Neurogenesis and Cognitive Function. *Nature* 477, 90–94. doi:10.1038/nature10357

Waetzig, V., Czeloth, K., Hidding, U., Mielke, K., Kanzow, M., Brecht, S., et al. (2005). c-Jun N-Terminal Kinases (JNKs) Mediate Pro-Inflammatory Actions of Microglia. *Glia* 50, 235–246. doi:10.1002/glia.20173

Wang, W. Y., Tan, M. S., Yu, J. T., and Tan, L. (2015). Role of Pro-Inflammatory Cytokines Released from Microglia in Alzheimer's Disease. *Ann. Transl Med.* 3, 136. doi:10.3978/j.issn.2305-5839.2015.03.49

Weaver, J. D., Huang, M. H., Albert, M., Harris, T., Rowe, J. W., and Seeman, T. E. (2002). Interleukin-6 and Risk of Cognitive Decline: MacArthur Studies of Successful Aging. *Neurology* 59, 371–378. doi:10.1212/wnl.59.3.371

Wynne, A. M., Henry, C. J., Huang, Y., Cleland, A., and Godbout, J. P. (2010). Protracted Downregulation of CX3CR1 on Microglia of Aged Mice after Lipopolysaccharide Challenge. *Brain Behav. Immun.* 24, 1190–1201. doi:10.1016/j.bbi.2010.05.011

Xing, G., Carlton, J., Jiang, X., Wen, J., Jia, M., and Li, H. (2014). Differential Expression of Brain Cannabinoid Receptors between Repeatedly Stressed Males and Females May Play a Role in Age and Gender-Related Difference in Traumatic Brain Injury: Implications from Animal Studies. *Front. Neurol.* 5, 161. doi:10.3389/fneur.2014.00161

Ye, S. M., and Johnson, R. W. (1999). Increased Interleukin-6 Expression by Microglia from Brain of Aged Mice. *J. Neuroimmunol* 93, 139–148. doi:10.1016/s0165-5728(98)00217-3

Ye, S. M., and Johnson, R. W. (2001). An Age-Related Decline in Interleukin-10 May Contribute to the Increased Expression of Interleukin-6 in Brain of Aged Mice. *Neuroimmunomodulation* 9, 183–192. doi:10.1159/000049025

Zahn, J. M., Poosala, S., Owen, A. B., Ingram, D. K., Lustig, A., Carter, A., et al. (2007). AGEMAP: A Gene Expression Database for Aging in Mice. *Plos Genet.* 3, e201. doi:10.1371/journal.pgen.0030201

Zhang, B., Gaiteri, C., Bodea, L. G., Wang, Z., McElwee, J., Podtelezhnikov, A. A., et al. (2013). Integrated Systems Approach Identifies Genetic Nodes and Networks in Late-Onset Alzheimer's Disease. *Cell* 153, 707–720. doi:10.1016/j.cell.2013.03.030

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

MINI REVIEW
published: 16 February 2022
doi: 10.3389/fphar.2022.852631

The Binding Mode to Orthosteric Sites and/or Exosites Underlies the Therapeutic Potential of Drugs Targeting Cannabinoid CB_2 Receptors

Rafael Franco[1,2,3*†], *Paula Morales*[4†], *Gemma Navarro*[1,5†], *Nadine Jagerovic*[4] *and Irene Reyes-Resina*[1,2]

[1]CiberNed. Network Center for Neurodegenerative Diseases, National Spanish Health Institute Carlos III, Madrid, Spain, [2]Molecular Neurobiology Laboratory, Department of Biochemistry and Molecular Biolomedicine, Universitat de Barcelona, Barcelona, Spain, [3]School of Chemistry, Universitat de Barcelona, Barcelona, Spain, [4]Medicinal Chemistry Institute, Spanish National Research Council, Madrid, Spain, [5]Department of Biochemistry and Physiology, Faculty of Pharmacy and Food Science, Universitat de Barcelona, Barcelona, Spain

The classical terms agonists and antagonists for G protein coupled receptors (GPCRs) have often become misleading. Even the biased agonism concept does not describe all the possibilities already demonstrated for GPCRs. The cannabinoid CB_2 receptor (CB_2R) emerged as a promising target for a variety of diseases. Reasons for such huge potential are centered around the way drugs sit in the orthosteric and/or exosites of the receptor. On the one hand, a given drug in a specific CB_2R conformation leads to a signaling cascade that differs qualitatively and/or quantitatively from that triggered by another drug. On the other hand, a given drug may lead to different signaling outputs in two different tissues (or cell contexts) in which the conformation of the receptor is affected by allosteric effects derived from interactions with other proteins or with membrane lipids. This highlights the pharmacological complexity of this receptor and the need to further unravel the binding mode of CB_2R ligands in order to fine-tune signaling effects and therapeutic propositions.

Keywords: biased agonism, heteromer, health benefits, therapy, functional selectivity, cannabinoid receptor, CB2, allosterism

OPEN ACCESS

Edited by:
Reem Smoum,
Hebrew University of Jerusalem, Israel

Reviewed by:
Carlos Ferrerio-Vera,
Phytoplant Research S.L., Spain

****Correspondence:***
Rafael Franco
rfranco123@gmail.com

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Experimental Pharmacology and Drug Discovery, a section of the journal Frontiers in Pharmacology

Received: *11 January 2022*
Accepted: *20 January 2022*
Published: *16 February 2022*

Citation:
Franco R, Morales P, Navarro G, Jagerovic N and Reyes-Resina I (2022) The Binding Mode to Orthosteric Sites and/or Exosites Underlies the Therapeutic Potential of Drugs Targeting Cannabinoid CB_2 Receptors. Front. Pharmacol. 13:852631. doi: 10.3389/fphar.2022.852631

INTRODUCTION

G protein-coupled receptors (GPCRs) are the target of about 40% of current drugs (Hauser et al., 2017). Although the potential of GPCRs as therapeutic targets is still considered to be high, there have been only a few recent approvals of drugs targeting these receptors. The causes are multifactorial, but perhaps the main one is the increased demands, in terms of efficacy and safety, by regulatory bodies. Functional selectivity is a property of GPCRs that has recently become relevant to overcome the issues related to the lack of success of GPCR-targeted drug candidates (Chang and Bruchas, 2014; Franco et al., 2018). For therapeutic purposes, functional selectivity of a given compound acting on the targeted receptor could achieve the desired effect(s) while minimizing side effects. A simplified version of the full potential of functional selectivity is the concept of biased agonism. Biased agonism is now considered across all platforms developing therapeutic drugs in both industry and academia. A compound selectively modulating a signaling pathway could offer a suitable therapeutic benefit compared to a another agonist that could, in parallel, induce undesired signaling events. The structural features of the cannabinoid receptors

(CBRs) offer more possibilities of biased signaling as the orthosteric site is not open to the extracellular milieu. Here we aim to review the multiple therapeutic possibilities resulting from targeting the cannabinoid receptor type 2 (CB_2R) orthosteric and/or non-orthosteric sites. At present, CB_2R appears as more promising in drug discovery than the cannabinoid receptor type 1 (CB_1R) as some of CB_1R agonists have psychotropic effects and an antagonist approved for human use (for weight control) was withdrawn due to serious side effects (Christensen et al., 2007; Sam et al., 2011). In fact, ligands for CB_2R seem to be generally safe and irrespective of whether they are agonists or antagonists. Safety however will not be considered in the present article.

ORTHOSTERIC AND NON-ORTHOSTERIC SITES IN THE CB_2R

Modes of Ligand Binding to the Orthosteric Site

The canonical Gα protein subunit for CB_1R and CB_2R is Gαi. Therefore, activation of these receptors leads to inactivation of the adenylate cyclase with the subsequent decrease in cAMP and deactivation of protein kinase A-mediated signaling. However, activation of CBRs may also lead to activation of the mitogen-activated protein kinases (MAPK) signaling cascade, regulation of ion channels, and recruitment of ß-arrestins, with subsequent regulation of Tyr kinase activity among others (Alexander et al., 2021).

Binding to GPCRs using radiolabeled compounds leads to detect one or two sites. Two sites reflect two different populations that, in the well-studied adenosine A_1 GPCR, correspond to the receptor uncoupled or coupled to the G protein. Uncoupled receptors display low affinity for agonists whereas G-protein coupled receptors display high affinity. These two affinity sites for the A_1 receptor can only be detected using agonists, i.e. antagonists have similar affinities for G-protein coupled and uncoupled A_1 receptors (see (Casadó et al., 1990) and references therein). To our knowledge radioligand binding to the CB_2R results in the detection of one single population. The two radioligands frequently used for measuring the binding to cannabinoid receptors, [^{3}H]WIN55,212-2 and [^{3}H]CP 55,940, are considered very potent orthosteric agonists of both CBRs, CB_1R and CB_2R. Competition assays using radioligands and non-labeled compounds in heterologous cells expressing CB_2R showed that affinities were consistent, i.e., WIN55,212-2 competed with similar low nanomolar affinity the binding of [^{3}H]WIN55,212-2 and of [^{3}H]CP 55,940. In similar conditions, a naturally occurring cannabinoid, cannabigerol, competed for the binding of [^{3}H]WIN55,212-2 or [^{3}H]CP 55,940 with a K_i in the micromolar range (Navarro et al., 2018b; Navarro et al., 2020b). This result did not fit with the decrease in cytosolic cAMP concentration obtained by nanomolar amounts of the compound. The main difference in the experimental setup was the use of isolated membranes for radioligand binding and of living cells for cAMP level measurements. The availability of novel approaches to obtain reliable receptor binding data in living cells is fortunately increasing, indeed, these methods do not require radiolabeled compounds. On using the SNAP-tag technology in cells expressing the tagged CB_2R and a validated "hot" compound (Martinez-Pinilla et al., 2016), the K_i for cannabigerol competition was 152 nM (Navarro et al., 2018b; Navarro et al., 2020b). These results show that the measured affinity of a given compound depends on the probe used for binding and allows identification of different states of the receptor or different modes to accommodate the ligand within the orthosteric center. In the case of the CB_1R, differences are more extreme as, in radioligand binding assays, natural cannabinoids may compete for the binding of [^{3}H]WIN55,212-2 but not of [^{3}H]CP 55,940. For instance, cannabigerol binding to CB_2R is similar if measured using [^{3}H]WIN55,212-2 or [^{3}H]CP 55,940, whereas there is no significant competition of binding to the CB_1R when [^{3}H]CP 55,940 is used. In summary, cannabigerol binds to a subcompartment of the orthosteric site of the CB_1R, i.e., the orthosteric site of this receptor may be simultaneously occupied by cannabigerol and [^{3}H]CP 55,940. These relatively recent findings add useful information to understand the variety of actions that different cannabinoids exert and also the experimental diversity between laboratories in the values of affinity and potency. This diversity may also underlie the enormous potential of cannabinoid receptors to combat a wide variety of diseases (see (Franco et al., 2020) and references therein).

Identification of Non-Orthosteric Sites

Cannabidiol, one of the main components of *Cannabis Sativa* L. has been instrumental to detect non-orthosteric centers in CBRs. This phytocannabinoid exerts physiological effects via a variety of receptors, located both in the cell surface and inside cells. Apart from interacting with CBRs, it may interact with serotonin and peroxisome proliferator-activated receptors (Banerjee et al., 1975; Russo et al., 2005; O'Sullivan et al., 2009; O'Sullivan and Kendall, 2010; Espejo-Porras et al., 2013; Fernández-Ruiz et al., 2013; De Gregorio et al., 2019; Franco et al., 2019b; Franco et al., 2020; Echeverry et al., 2021). At first cannabidiol was considered an orthosteric ligand able to partially activate cannabinoid receptors although with low potency (McPartland et al., 2007). Recent results in two different laboratories have shown that this compound can interact in an allosteric mode with the two CBRs (Laprairie et al., 2015; Martínez-Pinilla et al., 2017). For both receptors, CB_1R and CB_2R, it acts as a negative allosteric modulator (NAM) when co-administered with an orthosteric ligand. At CB_2R it minimized the effects of JWH133 on the MAP kinase signaling pathway (Martínez-Pinilla et al., 2017). Thus, cannabidiol binds to an allosteric site at nanomolar concentrations while micromolar concentrations are required for significant binding to the orthosteric site. Accordingly, the *in vitro* results depend on the concentration while the *in vivo* actions at moderate doses should be mainly due to its binding to the allosteric site that has been very recently suggested to be close to the receptor entrance (Navarro et al., 2021) (See section: "Structural Insights into CB_2R Binding Modes"). As would be expected from an allosteric mode of action, the binding of the

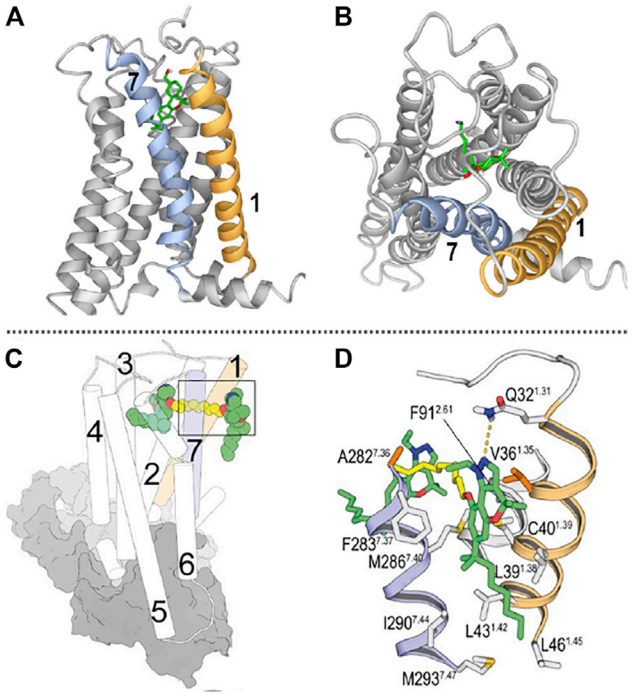


FIGURE 1 | (A) Lateral view of the CB_2R/AM12033 complex from the entrance portal formed by transmembrane helices (TMs) 1 and 7 (shown in orange and blue, respectively). **(B)** View from the outside of the cell of the CB_2R in complex with the agonist AM12033 (PDB-ID 6KPF); ligand access from extracellular is blocked by the N-terminus and the EC loops. **(C)** General view of the binding mode of a CB_2R bitopic ligand [molecule **22** in (Morales et al., 2020)] into the orthosteric site and the vestibule of the CB2R-Gi complex (depicted as cylinders for CB_2R and grey surfaces for Gi). **(D)** Detailed view of the binding mode of ligand **22** into the receptor vestibule obtained during the MD simulations. TMs 1 and 7 are shown in orange and blue, respectively; and the pharmacophore units and spacer of bitopic ligands are shown in green and yellow tubes, respectively. **(C,D)** have been reproduced from our previously reported article (Morales et al., 2020); permitted reproduction under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

compound to the allosteric site causes conformational changes in such a way that biases the effect of orthosteric agonists (Navarro et al., 2018a). A more recent report shows that structural changes in the molecule shifts negative to positive modulation (of the CB_2R) thus confirming its allosteric nature (Navarro et al., 2021).

Novel approaches to achieve signaling diversity and addressing success in drug discovery are attempting the design of bitopic ligands that bind the orthosteric site and an allosteric site (Lane et al., 2013; Mohr et al., 2013; Bradley and Tobin, 2016). By combining experimental and in silico approaches an allosteric site was identified at the entrance of the orthosteric binding site of the ß-adrenergic GPCRs (González et al., 2011). This site has been termed the -extracellular- vestibule (Dror et al., 2011) or entrance (Wang et al., 2013), also metastable (Fronik et al., 2017) or secondary (González et al., 2011) binding site. Exosite is also used to describe such non-orthosteric sites when they are located at the lipidic-receptor interface (Masureel et al., 2018). Bitopic ligands designed according to these findings improve subfamily selectivity (Medina et al., 2014; Masureel et al., 2018); they also offer signaling bias and better off-rates (Valant et al., 2012; Lane et al., 2013). Knowing that unlike GPCRs for polar compounds, CBRs do not have the orthosteric center accessible from the extracellular milieu, we designed bitopic ligands able to enter into the CB_2R orthosteric site but also able to interact with amino acids located at the receptor transmembrane portals (Morales et al., 2020). Signaling assays in the CB_2R wild-type and specific mutants led us to discover the first CB_2R bitopic ligands. These compounds, which consist of two chromenopyrazole moieties linked by methylene spacers of different lengths, can bind to the orthosteric site and to an exosite. Bitopic ligands showed to be CB_2R selective and, as depicted in **Figure 1**, may likely extend from the orthosteric site, the vestibule and an "allosteric exosite" able to accommodate the same moiety that sits in the orthosteric site.

STRUCTURAL INSIGHTS INTO CB_2R BINDING MODES

As previously mentioned, in recent years, the CB_2R has been resolved in its active (Hua et al., 2020; Xing et al., 2020) and inactive (Li et al., 2019) states, enlightening the structural knowledge of crucial domains for G protein activation as well as ligand binding. Not only CB_2R but also CB_1R and other class A lipid GPCRs have structural features that determine the lipophilic nature of their ligands (Hua et al., 2016; Hua et al., 2017; Krishna Kumar et al., 2019; Shao et al., 2019). On the one hand, the extracellular loops and the N-terminus of these receptors are generally structured over the orthosteric binding pocket occluding ligand entrance from the extracellular milieu. Moreover, transmembrane openings have been detected in these receptors acting as portals for lateral access of ligands to the binding crevice. Therefore, hydrophobic ligands such as phytocannabinoids need to diffuse through the lipid membrane to target binding sites. **Figure 1A,B** shows these features in the recently released structure of CB_2R in complex with Gαi.

Class A GPCR allosteric sites are widely distributed in different receptor domains including intracellular, intrahelical or exosites. For instance, the CB_1R has been resolved bound to the NAM ORG27569 and the agonist CP55940 (Shao et al., 2019). This crystal structure revealed the ability of ORG27569 to target an extrahelical exosite within the inner leaflet of the lipid bilayer. Even though few CB_2R allosteric modulators have been reported and none resolved in complex with the receptor, molecular dynamic and mutagenic studies have recently shown the potential allosteric site of CBD in CB_2R (Navarro et al., 2021). This report shows that CBD can bind to an allosteric cavity close to the receptor entrance in a transmembrane portal defined by transmembrane helices 1 and 7. As aforementioned, concomitant binding at orthosteric and allosteric/exosites has been shown at CB_2R with chromenopyrazole bitopic ligands (Morales et al., 2020). Site-directed mutagenesis and molecular dynamic studies

determined key interacting residues at transmembrane helices 1 and 7 which define the entry portal for these ligands (**Figures 1C,D**).

The CB_2R structural understanding gained in the past few years will likely accelerate the rational drug design of CB_2R modulators with optimal activity to address specific physiopathological conditions.

BIDIRECTIONAL INFORMATION EXCHANGE BETWEEN LIGAND AND CB_2RS

On the one hand, functional selectivity can result from different agonists that activate different populations of receptors, but also from agonists that produce different conformational changes in the receptor that allow different qualitative and/or quantitative signaling outputs. On the other hand, a given agonist can give rise to different signaling outputs depending on the conformation of the receptor's orthosteric site, which can vary depending on the cell type and the fate of the cell (Fuxe et al., 1998; Urban et al., 2007; Kenakin and Miller, 2010; Rajagopal et al., 2011; Fuxe et al., 2014; Ladarre et al., 2014; Navarro et al., 2020a; Franco et al., 2021).

By definition, allosterism produces conformational changes that alter the binding of agonists to the orthosteric site and, consequently, also modify (qualitatively or quantitatively) signal transduction. Important to highlight is that allosterism is bidirectional, i.e. an orthosteric compound binding to a receptor leads to conformational changes that likely alter the affinity of the binding of the allosteric compound to the receptor (Christopoulos and Kenakin, 2002; May and Christopoulos, 2003; Smith et al., 2011). In practice this means that if an allosteric compound is suspected on the basis of changes in affinity of radiolabeled compound to the orthosteric site, the orthosteric compound should modify the affinity of the binding of the allosteric compound to the allosteric site. In the field of GPCR, this requirement has made difficult the identification of allosteric compounds, as there are few to none radiolabeled compounds designed to measure binding to allosteric sites. In the case of CB_2R, the discovery of bitopic ligands together with the structure of the receptor leaves no doubt about the possibility of regulating the functionality of the receptor by "touching" allosteric/exosites.

Different Macromolecular Environments of the CB_2R Impact agonist Binding and Effect

Can a given compound be more efficacious at targeting a cell that expresses CB_2R in a particular conformation? and/or can a CB_2R in a particular cell type be more likely to respond to the challenge of a given compound?

The pharmacology of cannabinoid receptors is complex. As discussed above, binding data can depend on the radioligand used as the probe, and the effects of a given compound on a given receptor are not always consistent across laboratories. At present we have enough data to realize that there are many possibilities

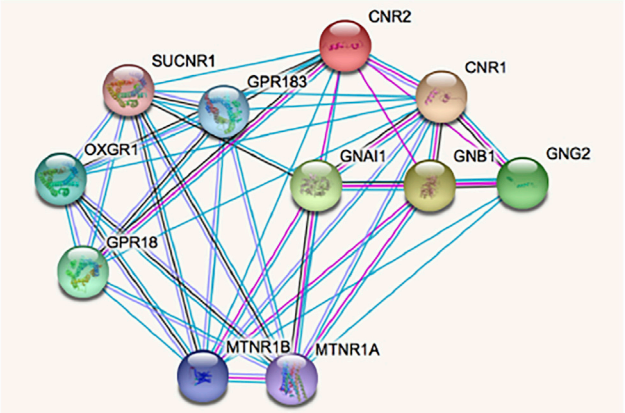


FIGURE 2 | Interactions involving the CB_2R according to STRING database for functional protein association networks. Abbreviations/gene products are: CNR2, CB_2R; CNR1, CB_1R; MTNR1A/1B, Melatonin GPCRs 1A/1B; OXGR1, Alpha-ketoglutarate receptor (a GPCR); SUCNR1, Succinate receptor 1 (a GPCR); GPR18 and GPR183 are orphan GPCRs; GNAI1, Guanine nucleotide-binding protein G(i) subunit alpha-1; GNB1, Guanine nucleotide-binding protein G(i) subunit ß-1; GNG2, Guanine nucleotide-binding protein $G_i/G_s/G_o$ subunit gamma-2.

for CB_2R-mediated responses that may turn into novel and powerful possibilities for drug discovery.

The complex pharmacology of the CB_2R has likely delayed the identification of CB_2R-containing macromolecular complexes, whose occurrence has been demonstrated in natural sources (i.e. not only in heterologous expression systems). Such interactions modify binding and/or function. Current data suggest that the receptor environment modifies the conformation and, accordingly, the binding and effects of orthosteric and non-orthosteric ligands. Interaction of the CB_2R with other GPCRs may be searched in http://www.gpcr-hetnet.com/ (using the gene name: CNR2) (Borroto-Escuela et al., 2014). **Figure 2** shows the STRING analysis of the interactions of the receptor which indicates mandatory interactions with G proteins, and interactions with the CB1R and with other GPCRs. In www.gpcr-hetnet.com and in **Figure 2** interactions of CB_2R with further GPCRs are not yet included (they have not yet been incorporated into the STRING database). Also missing are the recently described interactions of the CB_2R with glutamate N-Methyl-D-Asp (NMDA) ionotropic receptors (Rivas-Santisteban et al., 2021). From a therapeutic perspective, the fact that CB_2R may interact with other receptors that are also targeted by cannabinoids, for instance with GPR18 and GPR55, is of high interest (Balenga et al., 2014; Reyes-Resina et al., 2018; Martínez-Pinilla et al., 2019; Martínez-Pinilla et al., 2020; Rivas-Santisteban et al., 2021).

So far, no major change has been detected concerning the nature of the G protein coupling of CB_2R in a macromolecular environment as it occurs for D_1 and D_2 dopamine receptors. Whereas the D_1 is coupled to Gαs and D_2 to Gαi, the macromolecular complex formed when the two receptors are co-expressed in the same neuron couples to Gαq (Rashid et al.,

2007; Hasbi et al., 2009; George et al., 2014; Perreault et al., 2015). Notwithstanding, conformational changes that affect the binding and signaling outputs produced by a given agonist have been shown in the interactions with the Gαi-coupled CB_1R (Callén et al., 2012; Sierra et al., 2015; Angelats et al., 2018), the Gαs-coupled adenosine A_{2A} receptor (Franco et al., 2019a), and the ionotropic NMDA receptor (Rivas-Santisteban et al., 2021).

In one of the first studies of biased agonism in GPCR heteromers (CB_1R/CB_2R), Navarro and co-workers showed that the allosteric effect of CBD was particularly noteworthy for the endocannabinoid anandamide but also that the effect tested using different agonists was smaller in the heteromer (Navarro et al., 2018a). These results confirmed that CBD acts as an allosteric modulator (for both receptors) also suggesting that the formation of the heteromer leads to conformational changes that make it less sensitive to the action of this phytocannabinoid. There are several examples of conformational changes induced by receptor-receptor interactions, i.e. by heteromer expression (Franco et al., 2007; Ferré et al., 2009; Franco et al., 2016). In the case of the CB_2R, indirect evidence is provided by potentiation of receptor-mediated signaling when forming heteromers with the adenosine A_{2A} receptor (Franco et al., 2019a).

Can Ligands Affect Conformation via Regulation of the CB_2R Context?

The binding of orthosteric and non-orthosteric ligands alters the conformation of the receptor, but can ligands alter the environment? The answer to this question will take time as there is little background on the regulation of, for instance, heteromer formation.

Defining the target in the right context and delineating contextual changes due to ligand-induced regulation of the structure of the CB_2R-contaning macromolecule, may further improve the rational design of therapeutic drugs (orthosteric and non-orthosteric) targeting the CB_2R.

AUTHOR CONTRIBUTIONS

RF, PM, NJ, GN and IR-R have scanned the literature and selected the cited articles. RF, PM and IR-R prepared the first draft. RF, PM, NJ, GN and IR-R agreed on the order and titles of the sections. PM prepared **Figure 1**. IR-R prepared **Figure 2**. All authors contributed to prepare an improved version. All authors edited the manuscript and have read and approved the final version of the review.

FUNDING

This work was partially supported by AARFD-17-503612 grant of the US Alzheimer's Association and by grants SAF 2017-84117-R, RTI 2018-094204-B-I00, RTI 2018-095544-B-I00 and PID 2020-113430RB-I00 funded by Spanish MCIN/AEI/10.13039/501100011033 and, as appropriate, by "ERDF A way of making Europe", by the "European Union" or by the "European Union Next Generation EU/PRTR". PM is a postdoctoral fellow supported by the Juan de la Cierva Programme-MICIU (IJC 2019-042182-I). The research group of the University of Barcelona is considered of excellence (grup consolidat #2017 SGR 1497) by the Regional Catalonian Government, which does not provide any specific funding for reagents or for payment of services or Open Access fees.

REFERENCES

Alexander, S. P., Christopoulos, A., Davenport, A. P., Kelly, E., Mathie, A., Peters, J. A., et al. (2021). The Concise Guide to Pharmacology 2021/22: G Protein-Coupled Receptors. *Br. J. Pharmacol.* 178, S27–S156. doi:10.1111/BPH.15538

Angelats, E., Requesens, M., Aguinaga, D., Kreutz, M. R., Franco, R., and Navarro, G. (2018). Neuronal Calcium and cAMP Cross-Talk Mediated by Cannabinoid CB1 Receptor and EF-Hand Calcium Sensor Interactions. *Front. Cell Dev. Biol.* 6, 67–69. doi:10.3389/fcell.2018.00067

Balenga, N. A., Martínez-Pinilla, E., Kargl, J., Schröder, R., Peinhaupt, M., Platzer, W., et al. (2014). Heteromerization of GPR55 and Cannabinoid CB2 Receptors Modulates Signalling. *Br. J. Pharmacol.* 171, 5387–5406. doi:10.1111/bph.12850

Banerjee, S. P., Snyder, S. H., and Mechoulam, R. (1975). Cannabinoids: Influence on Neurotransmitter Uptake in Rat Brain Synaptosomes. *J. Pharmacol. Exp. Ther.* 194, 74–81. Available at: http://www.ncbi.nlm.nih.gov/pubmed/168349 (Accessed August 15, 2019).

Borroto-Escuela, D. O., Brito, I., Romero-Fernandez, W., Di Palma, M., Oflijan, J., Skieterska, K., et al. (2014). The G Protein-Coupled Receptor Heterodimer Network (GPCR-HetNet) and its Hub Components. *Int. J. Mol. Sci.* 15, 8570–8590. doi:10.3390/ijms15058570

Bradley, S. J., and Tobin, A. B. (2016). Design of Next-Generation G Protein-Coupled Receptor Drugs: Linking Novel Pharmacology and *In Vivo* Animal Models. *Annu. Rev. Pharmacol. Toxicol.* 56, 535–559. doi:10.1146/annurev-pharmtox-011613-140012

Callén, L., Moreno, E., Barroso-Chinea, P., Moreno-Delgado, D., Cortés, A., Mallol, J., et al. (2012). Cannabinoid Receptors CB1 and CB2 Form Functional Heteromers in Brain. *J. Biol. Chem.* 287, 20851–20865. doi:10.1074/jbc.M111.335273

Casadó, V., Cantí, C., Mallol, J., Canela, E. I., Lluis, C., and Franco, R. (1990). Solubilization of A1 Adenosine Receptor from Pig Brain: Characterization and Evidence of the Role of the Cell Membrane on the Coexistence of High- and Low-Affinity States. *J. Neurosci. Res.* 26, 461–473. doi:10.1002/jnr.490260409

Chang, S. D., and Bruchas, M. R. (2014). Functional Selectivity at GPCRs: New Opportunities in Psychiatric Drug Discovery. *Neuropsychopharmacology* 39, 248–249. doi:10.1038/npp.2013.205

Christensen, R., Kristensen, P. K., Bartels, E. M., Bliddal, H., and Astrup, A. (2007). Efficacy and Safety of the Weight-Loss Drug Rimonabant: A Meta-Analysis of Randomised Trials. *Lancet* 370, 1706–1713. doi:10.1016/S0140-6736(07)61721-8

Christopoulos, A., and Kenakin, T. (2002). G Protein-Coupled Receptor Allosterism and Complexing. *Pharmacol. Rev.* 54, 323–374. doi:10.1124/PR.54.2.323

De Gregorio, D., McLaughlin, R. J., Posa, L., Ochoa-Sanchez, R., Enns, J., Lopez-Canul, M., et al. (2019). Cannabidiol Modulates Serotonergic Transmission and Reverses Both Allodynia and Anxiety-Like Behavior in a Model of Neuropathic Pain. *Pain* 160, 136–150. doi:10.1097/j.pain.0000000000001386

Dror, R. O., Pan, A. C., Arlow, D. H., Borhani, D. W., Maragakis, P., Shan, Y., et al. (2011). Pathway and Mechanism of Drug Binding to G-Protein-Coupled Receptors. *Proc. Natl. Acad. Sci. U. S. A.* 108, 13118–13123. doi:10.1073/pnas.1104614108

Echeverry, C., Prunell, G., Narbondo, C., de Medina, V. S., Nadal, X., Reyes-Parada, M., et al. (2021). A Comparative *In Vitro* Study of the Neuroprotective Effect Induced by Cannabidiol, Cannabigerol, and Their Respective Acid Forms:

Relevance of the 5-HT 1A Receptors. *Neurotox. Res.* 39, 335–348. doi:10.1007/S12640-020-00277-Y

Espejo-Porras, F., Fernández-Ruiz, J., Pertwee, R. G., Mechoulam, R., and García, C. (2013). Motor Effects of the Non-Psychotropic Phytocannabinoid Cannabidiol that are Mediated by 5-HT1A Receptors. *Neuropharmacology* 75, 155–163. doi:10.1016/j.neuropharm.2013.07.024

Fernández-Ruiz, J., Sagredo, O., Pazos, M. R., García, C., Pertwee, R., Mechoulam, R., et al. (2013). Cannabidiol for Neurodegenerative Disorders: Important New Clinical Applications for This Phytocannabinoid? *Br. J. Clin. Pharmacol.* 75, 323–333. doi:10.1111/j.1365-2125.2012.04341.x

Ferré, S., Baler, R., Bouvier, M., Caron, M. G., Devi, L. A., Durroux, T., et al. (2009). Building a New Conceptual Framework for Receptor Heteromers. *Nat. Chem. Biol.* 5, 131–134. doi:10.1038/nchembio0309-131

Franco, R., Casadó, V., Cortés, A., Ferrada, C., Mallol, J., Woods, A., et al. (2007). Basic Concepts in G-Protein-Coupled Receptor Homo- and Heterodimerization. *Scientific World J.* 7, 48–57. doi:10.1100/tsw.2007.197

Franco, R., Martínez-Pinilla, E., Lanciego, J. L., and Navarro, G. (2016). Basic Pharmacological and Structural Evidence for Class A G-Protein-Coupled Receptor Heteromerization. *Front. Pharmacol.* 7, 1–10. doi:10.3389/fphar.2016.00076

Franco, R., Aguinaga, D., Jiménez, J., Lillo, J., Martínez-Pinilla, E., and Navarro, G. (2018). Biased Receptor Functionality Versus Biased Agonism in G-Protein-Coupled Receptors. *Biomol. Concepts* 9, 143–154. doi:10.1515/bmc-2018-0013

Franco, R., Reyes-Resina, I., Aguinaga, D., Lillo, A., Jiménez, J., Raïch, I., et al. (2019a). Potentiation of Cannabinoid Signaling in Microglia by Adenosine A2A Receptor Antagonists. *Glia* 67, 2410–2423. doi:10.1002/glia.23694

Franco, R., Villa, M., Morales, P., Reyes-Resina, I., Gutiérrez-Rodríguez, A., Jiménez, J., et al. (2019b). Increased Expression of Cannabinoid CB2 and Serotonin 5-HT1A Heteroreceptor Complexes in a Model of Newborn Hypoxic-Ischemic Brain Damage. *Neuropharmacology* 152, 58–66. doi:10.1016/j.neuropharm.2019.02.004

Franco, R., Rivas-Santisteban, R., Reyes-Resina, I., Casanovas, M., Pérez-Olives, C., Ferreiro-Vera, C., et al. (2020). Pharmacological Potential of Varinic-, Minor-, and Acidic Phytocannabinoids. *Pharmacol. Res.* 158, 104801. doi:10.1016/j.phrs.2020.104801

Franco, R., Rivas-Santisteban, R., Reyes-Resina, I., and Navarro, G. (2021). The Old and New Visions of Biased Agonism Through the Prism of Adenosine Receptor Signaling and Receptor/Receptor and Receptor/Protein Interactions. *Front. Pharmacol.* 11, 628601. doi:10.3389/fphar.2020.628601

Fronik, P., Gaiser, B. I., and Sejer Pedersen, D. (2017). Bitopic Ligands and Metastable Binding Sites: Opportunities for G Protein-Coupled Receptor (GPCR) Medicinal Chemistry. *J. Med. Chem.* 60, 4126–4134. doi:10.1021/acs.jmedchem.6b01601

Fuxe, K., Ferré, S., Zoli, M., and Agnati, L. F. (1998). Integrated Events in central Dopamine Transmission as Analyzed at Multiple Levels. Evidence for Intramembrane Adenosine A2A/Dopamine D2 and Adenosine A1/Dopamine D1 Receptor Interactions in the Basal Ganglia. *Brain Res. Brain Res. Rev.* 26, 258–273. doi:10.1016/S0165-0173(97)00049-0

Fuxe, K., Tarakanov, A., Romero Fernandez, W., Ferraro, L., Tanganelli, S., Filip, M., et al. (2014). Diversity and Bias through Receptor-Receptor Interactions in GPCR Heteroreceptor Complexes. Focus on Examples from Dopamine D2 Receptor Heteromerization. *Front. Endocrinol.* 5, 71–11. doi:10.3389/fendo.2014.00071

George, S. R., Kern, A., Smith, R. G., and Franco, R. (2014). Dopamine Receptor Heteromeric Complexes and Their Emerging Functions. *Prog. Brain Res.* 211, 183–200. doi:10.1016/B978-0-444-63425-2.00008-8

González, A., Perez-Acle, T., Pardo, L., and Deupi, X. (2011). Molecular Basis of Ligand Dissociation in β-Adrenergic Receptors. *PLoS One* 6, e23815. doi:10.1371/journal.pone.0023815

Hasbi, A., Fan, T., Alijaniaram, M., Nguyen, T., Perreault, M. L., O'Dowd, B. F., et al. (2009). Calcium Signaling Cascade Links Dopamine D1-D2 Receptor Heteromer to Striatal BDNF Production and Neuronal Growth. *Proc. Natl. Acad. Sci. U. S. A.* 106, 21377–21382. doi:10.1073/pnas.0903676106

Hauser, A. S., Attwood, M. M., Rask-Andersen, M., Schiöth, H. B., and Gloriam, D. E. (2017). Trends in GPCR Drug Discovery: New Agents, Targets and Indications. *Nat. Rev. Drug Discov.* 16, 829–842. doi:10.1038/nrd.2017.178

Hua, T., Vemuri, K., Pu, M., Qu, L., Han, G. W., Wu, Y., et al. (2016). Crystal Structure of the Human Cannabinoid Receptor CB1. *Cell* 167, 750–762.e14. doi:10.1016/j.cell.2016.10.004

Hua, T., Vemuri, K., Nikas, S. P., Laprairie, R. B., Wu, Y., Qu, L., et al. (2017). Crystal Structures of Agonist-Bound Human Cannabinoid Receptor CB1. *Nature* 547, 468–471. doi:10.1038/nature23272

Hua, T., Li, X., Wu, L., Iliopoulos-Tsoutsouvas, C., Wang, Y., Wu, M., et al. (2020). Activation and Signaling Mechanism Revealed by Cannabinoid Receptor-Gi Complex Structures. *Cell* 180, 655–665. doi:10.1016/j.cell.2020.01.008

Kenakin, T., and Miller, L. J. (2010). Seven Transmembrane Receptors as Shapeshifting Proteins: the Impact of Allosteric Modulation and Functional Selectivity on New Drug Discovery. *Pharmacol. Rev.* 62, 265–304. doi:10.1124/pr.108.000992

Krishna Kumar, K., Shalev-Benami, M., Robertson, M. J., Hu, H., Banister, S. D., Hollingsworth, S. A., et al. (2019). Structure of a Signaling Cannabinoid Receptor 1-G Protein Complex. *Cell* 176, 448–458.e12. doi:10.1016/j.cell.2018.11.040

Ladarre, D., Roland, A. B., Biedzinski, S., Ricobaraza, A., and Lenkei, Z. (2014). Polarized Cellular Patterns of Endocannabinoid Production and Detection Shape Cannabinoid Signaling in Neurons. *Front. Cell. Neurosci.* 8, 426. doi:10.3389/fncel.2014.00426

Lane, J. R., Sexton, P. M., and Christopoulos, A. (2013). Bridging the Gap: Bitopic Ligands of G-Protein-Coupled Receptors. *Trends Pharmacol. Sci.* 34, 59–66. doi:10.1016/j.tips.2012.10.003

Laprairie, R. B., Bagher, A. M., Kelly, M. E., and Denovan-Wright, E. M. (2015). Cannabidiol Is a Negative Allosteric Modulator of the Cannabinoid CB1 Receptor. *Br. J. Pharmacol.* 172, 4790–4805. doi:10.1111/bph.13250

Li, X., Hua, T., Vemuri, K., Ho, J. H., Wu, Y., Wu, L., et al. (2019). Crystal Structure of the Human Cannabinoid Receptor CB2. *Cell* 176, 459–467.e13. doi:10.1016/j.cell.2018.12.011

Martínez-Pinilla, E., Varani, K., Reyes-Resina, I., Angelats, E., Vincenzi, F., Ferreiro-Vera, C., et al. (2017). Binding and Signaling Studies Disclose a Potential Allosteric Site for Cannabidiol in Cannabinoid CB2 Receptors. *Front. Pharmacol.* 8, 744. doi:10.3389/fphar.2017.00744

Martínez-Pinilla, E., Aguinaga, D., Navarro, G., Rico, A. J., Oyarzábal, J., Sánchez-Arias, J. A., et al. (2019). Targeting CB1 and GPR55 Endocannabinoid Receptors as a Potential Neuroprotective Approach for Parkinson's Disease. *Mol. Neurobiol.* 56, 5900–5910. doi:10.1007/s12035-019-1495-4

Martínez-Pinilla, E., Rico, A. J., Rivas-Santisteban, R., Lillo, J., Roda, E., Navarro, G., et al. (2020). Expression of GPR55 and Either Cannabinoid CB1 or CB2 Heteroreceptor Complexes in the Caudate, Putamen, and Accumbens Nuclei of Control, Parkinsonian, and Dyskinetic Non-human Primates. *Brain Struct. Funct.* 225, 2153–2164. doi:10.1007/s00429-020-02116-4

Martínez-Pinilla, E., Rabal, O., Reyes-Resina, I., Zamarbide, M., Navarro, G., Sánchez-Arias, J. A., et al. (2016). Two Affinity Sites of the Cannabinoid Subtype 2 Receptor Identified by a Novel Homogeneous Binding Assay. *J. Pharmacol. Exp. Ther.* 358, 580–587. doi:10.1124/jpet.116.234948

Masureel, M., Zou, Y., Picard, L. P., van der Westhuizen, E., Mahoney, J. P., Rodrigues, J. P. G. L. M., et al. (2018). Structural Insights into Binding Specificity, Efficacy and Bias of a β2AR Partial Agonist. *Nat. Chem. Biol.* 14, 1059–1066. doi:10.1038/s41589-018-0145-x

May, L. T., and Christopoulos, A. (2003). Allosteric Modulators of G-Protein-Coupled Receptors. *Curr. Opin. Pharmacol.* 3, 551–556. doi:10.1016/S1471-4892(03)00107-3

McPartland, J. M., Glass, M., and Pertwee, R. G. (2007). Meta-Analysis of Cannabinoid Ligand Binding Affinity and Receptor Distribution: Interspecies Differences. *Br. J. Pharmacol.* 152, 583–593. doi:10.1038/sj.bjp.0707399

Medina, R. A., Vázquez-Villa, H., Gómez-Tamayo, J. C., Benhamú, B., Martín-Fontecha, M., de la Fuente, T., et al. (2014). The Extracellular Entrance Provides Selectivity to Serotonin 5-HT7 Receptor Antagonists with Antidepressant-like Behavior *In Vivo*. *J. Med. Chem.* 57, 6879–6884. doi:10.1021/jm500880c

Mohr, K., Schmitz, J., Schrage, R., Tränkle, C., and Holzgrabe, U. (2013). Molecular Alliance-From Orthosteric and Allosteric Ligands to Dualsteric/Bitopic Agonists at G Protein Coupled Receptors. *Angew. Chem. Int. Ed. Engl.* 52, 508–516. doi:10.1002/anie.201205315

Morales, P., Navarro, G., Gómez-Autet, M., Redondo, L., Fernández-Ruiz, J., Pérez-Benito, L., et al. (2020). Discovery of Homobivalent Bitopic Ligands of the Cannabinoid CB2 Receptor*. *Chemistry* 26, 15839–15842. doi:10.1002/chem.202003389

Navarro, G., Reyes-Resina, I., Rivas-Santisteban, R., Sánchez de Medina, V., Morales, P., Casano, S., et al. (2018a). Cannabidiol Skews Biased Agonism at Cannabinoid CB1 and CB2 Receptors with Smaller Effect in CB1-CB2 Heteroreceptor Complexes. *Biochem. Pharmacol.* 157, 148–158. doi:10.1016/j.bcp.2018.08.046

Navarro, G., Varani, K., Reyes-Resina, I., Sánchez de Medina, V., Rivas-Santisteban, R., Sánchez-Carnerero Callado, C., et al. (2018b). Cannabigerol Action at Cannabinoid CB1 and CB2 Receptors and at CB1-CB2 Heteroreceptor Complexes. *Front. Pharmacol.* 9, 632. doi:10.3389/fphar.2018.00632

Navarro, G., Gonzalez, A., Campanacci, S., Rivas-Santisteban, R., Reyes-Resina, I., Casajuana-Martin, N., et al. (2020a). Experimental and Computational Analysis of Biased Agonism on Full-Length and a C-Terminally Truncated Adenosine A2A Receptor. *Comput. Struct. Biotechnol. J.* 18, 2723–2732. doi:10.1016/j.csbj.2020.09.028

Navarro, G., Varani, K., Lillo, A., Vincenzi, F., Rivas-Santisteban, R., Raïch, I., et al. (2020b). Pharmacological Data of Cannabidiol- and Cannabigerol-Type Phytocannabinoids Acting on Cannabinoid CB1, CB2 and CB1/CB2 Heteromer Receptors. *Pharmacol. Res.* 159, 104940. doi:10.1016/j.phrs.2020.104940

Navarro, G., Gonzalez, A., Sánchez-Morales, A., Casajuana-Martin, N., Gómez-Ventura, M., Cordomí, A., et al. (2021). Design of Negative and Positive Allosteric Modulators of the Cannabinoid CB2 Receptor Derived from the Natural Product Cannabidiol. *J. Med. Chem.* 64, 9354–9364. doi:10.1021/ACS.JMEDCHEM.1C00561

O'Sullivan, S. E., and Kendall, D. A. (2010). Cannabinoid Activation of Peroxisome Proliferator-Activated Receptors: Potential for Modulation of Inflammatory Disease. *Immunobiology* 215, 611–616. doi:10.1016/J.IMBIO.2009.09.007

O'Sullivan, S. E., Sun, Y., Bennett, A. J., Randall, M. D., and Kendall, D. A. (2009). Time-Dependent Vascular Actions of Cannabidiol in the Rat Aorta. *Eur. J. Pharmacol.* 612, 61–68. doi:10.1016/J.EJPHAR.2009.03.010

Perreault, M. L., Shen, M. Y., Fan, T., and George, S. R. (2015). Regulation of C-Fos Expression by the Dopamine D1-D2 Receptor Heteromer. *Neuroscience* 285, 194–203. doi:10.1016/j.neuroscience.2014.11.017

Rajagopal, S., Ahn, S., Rominger, D. H., Gowen-MacDonald, W., Lam, C. M., Dewire, S. M., et al. (2011). Quantifying Ligand Bias at Seven-Transmembrane Receptors. *Mol. Pharmacol.* 80, 367–377. doi:10.1124/mol.111.072801

Rashid, A. J., So, C. H., Kong, M. M., Furtak, T., El-Ghundi, M., Cheng, R., et al. (2007). D1-D2 Dopamine Receptor Heterooligomers with Unique Pharmacology Are Coupled to Rapid Activation of Gq/11 in the Striatum. *Proc. Natl. Acad. Sci. U. S. A.* 104, 654–659. doi:10.1073/pnas.0604049104

Reyes-Resina, I., Navarro, G., Aguinaga, D., Canela, E. I., Schoeder, C. T., Załuski, M., et al. (2018). Molecular and Functional Interaction between GPR18 and Cannabinoid CB2 G-Protein-Coupled Receptors. Relevance in Neurodegenerative Diseases. *Biochem. Pharmacol.* 157, 169–179. doi:10.1016/j.bcp.2018.06.001

Rivas-Santisteban, R., Lillo, A., Lillo, J., Rebassa, J. B., Contestí, J. S., Saura, C. A., et al. (2021). N-Methyl-D-aspartate (NMDA) and Cannabinoid CB2 Receptors Form Functional Complexes in Cells of the central Nervous System: Insights into the Therapeutic Potential of Neuronal and Microglial NMDA Receptors. *Alzheimers. Res. Ther.* 13 (1), 184. doi:10.1186/S13195-021-00920-6

Russo, E. B., Burnett, A., Hall, B., and Parker, K. K. (2005). Agonistic Properties of Cannabidiol at 5-HT1a Receptors. *Neurochem. Res.* 30, 1037–1043. doi:10.1007/s11064-005-6978-1

Sam, A. H., Salem, V., and Ghatei, M. A. (2011). Rimonabant: From RIO to Ban. *J. Obes.* 2011, 432607. doi:10.1155/2011/432607

Shao, Z., Yan, W., Chapman, K., Ramesh, K., Ferrell, A. J., Yin, J., et al. (2019). Structure of an Allosteric Modulator Bound to the CB1 Cannabinoid Receptor. *Nat. Chem. Biol.* 15, 1199–1205. doi:10.1038/S41589-019-0387-2

Sierra, S., Luquin, N., Rico, A. J., Gómez-Bautista, V., Roda, E., Dopeso-Reyes, I. G., et al. (2015). Detection of Cannabinoid Receptors CB1 and CB2 within Basal Ganglia Output Neurons in Macaques: Changes Following Experimental Parkinsonism. *Brain Struct. Funct.* 220, 2721–2738. doi:10.1007/s00429-014-0823-8

Smith, N. J., Bennett, K. A., and Milligan, G. (2011). When Simple Agonism is Not Enough: Emerging Modalities of GPCR Ligands. *Mol. Cell. Endocrinol.* 331, 241–247. doi:10.1016/J.MCE.2010.07.009

Urban, J. D., Clarke, W. P., von Zastrow, M., Nichols, D. E., Kobilka, B., Weinstein, H., et al. (2007). Functional Selectivity and Classical Concepts of Quantitative Pharmacology. *J. Pharmacol. Exp. Ther.* 320, 1–13. doi:10.1124/jpet.106.104463

Valant, C., Robert Lane, J., Sexton, P. M., and Christopoulos, A. (2012). The Best of Both Worlds? Bitopic Orthosteric/Allosteric Ligands of G Protein-Coupled Receptors. *Annu. Rev. Pharmacol. Toxicol.* 52, 153–178. doi:10.1146/annurev-pharmtox-010611-134514

Wang, C., Jiang, Y., Ma, J., Wu, H., Wacker, D., Katritch, V., et al. (2013). Structural Basis for Molecular Recognition at Serotonin Receptors. *Science* 340, 610–614. doi:10.1126/science.1232807

Xing, C., Zhuang, Y., Xu, T. H., Feng, Z., Zhou, X. E., Chen, M., et al. (2020). Cryo-EM Structure of the Human Cannabinoid Receptor CB2-Gi Signaling Complex. *Cell* 180, 645–654.e13. doi:10.1016/j.cell.2020.01.007

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

PERSPECTIVE
published: 22 February 2022
doi: 10.3389/fphar.2022.826400

Expression and Functions of the CB_2 Receptor in Human Leukocytes

Mélissa Simard[1,2], *Volatiana Rakotoarivelo*[1,2], *Vincenzo Di Marzo*[1,2,3,4,5] *and Nicolas Flamand*[1,2]*

[1]Centre de Recherche de l'Institut Universitaire de Cardiologie et de Pneumologie de Québec, Département of Médecine, Faculté de Médecine, Université Laval, Québec City, QC, Canada, [2]Canada Excellence Research Chair on the Microbiome-Endocannabinoidome Axis in Metabolic Health (CERC-MEND), Université Laval, Québec City, QC, Canada, [3]Endocannabinoid Research Group, Institute of Biomolecular Chemistry, Consiglio Nazionale Delle Ricerche (CNR), Pozzuoli, Italy, [4]Institut sur la Nutrition et les Aliments Fonctionnels, Centre NUTRISS, École de Nutrition, Faculté des Sciences de L'agriculture et de L'alimentation, Université Laval, Québec City, QC, Canada, [5]Joint International Unit Between the Consiglio Nazionale Delle Ricerche (Italy) and Université Laval (Canada) on Chemical and Biomolecular Research on the Microbiome and Its Impact on Metabolic Health and Nutrition (UMI-MicroMeNu), Naples, Italy

The cannabinoid CB_2 receptor was cloned from the promyeloid cell line HL-60 and is notably expressed in most, if not all leukocyte types. This relatively restricted localization, combined to the absence of psychotropic effects following its activation, make it an attractive drug target for inflammatory and autoimmune diseases. Therefore, there has been an increasing interest in the past decades to identify precisely which immune cells express the CB_2 receptor and what are the consequences of such activation. Herein, we provide new data on the expression of both CB_1 and CB_2 receptors by human blood leukocytes and discuss the impact of CB_2 receptor activation in human leukocytes. While the expression of the CB_2 mRNA can be detected in eosinophils, neutrophils, monocytes, B and T lymphocytes, this receptor is most abundant in human eosinophils and B lymphocytes. We also review the evidence obtained from primary human leukocytes and immortalized cell lines regarding the regulation of their functions by the CB_2 receptor, which underscore the urgent need to deepen our understanding of the CB_2 receptor as an immunoregulator in humans.

Keywords: CB2 receptor, eosinophil, neutrophil, monocyte, lymphocyte, inflammation, asthma, allergy

OPEN ACCESS

Edited by:
Pal Pacher,
National Institute on Alcohol Abuse and Alcoholism (NIAAA), United States

Reviewed by:
Valeria Gasperi,
University of Rome Tor Vergata, Italy

***Correspondence:**
Nicolas Flamand
Nicolas.Flamand@criucpq.ulaval.ca

Specialty section:
This article was submitted to Experimental Pharmacology and Drug Discovery, a section of the journal Frontiers in Pharmacology

Received: *30 November 2021*
Accepted: *14 January 2022*
Published: *22 February 2022*

Citation:
Simard M, Rakotoarivelo V, Di Marzo V and Flamand N (2022) Expression and Functions of the CB_2 Receptor in Human Leukocytes. Front. Pharmacol. 13:826400. doi: 10.3389/fphar.2022.826400

INTRODUCTION

The cannabinoid receptors 1 and 2 (CB_1 and CB_2) are two G protein-coupled receptors that function through binding a vast array of ligands including phytocannabinoids and endocannabinoids (Di Marzo et al., 1998; Turcotte et al., 2015). The CB_1 receptor, highly expressed in the brain, was the first cannabinoid receptor identified through its responsiveness to Δ^9-tetrahydrocannabinol (Δ^9-THC) and cloned (Devane et al., 1988; Matsuda et al., 1990). Its activation induces psychotropic effects and its involvement shown in, among others, motor function, cognition and memory (Howlett and Abood 2017). It is also widely recognized as worsening obesity and related diseases (Di Marzo 2018). The CB_2 receptor was later cloned from HL-60 cells and identified on its 44% aminoacid homology with the CB_1, as well as its similar binding profile to the endocannabinoid *N*-arachidonoyl-ethanolamine (AEA) and Δ^9-THC (Munro et al., 1993). Soon after, Galiègue et al. documented that it was expressed by human leukocytes (Galiegue et al., 1995). This consolidated the concept that the CB_2 is the peripheral cannabinoid receptor and, for many, the inflammatory cannabinoid receptor. In fact, the CB_2 receptor has been found in all leukocyte populations tested so far [see

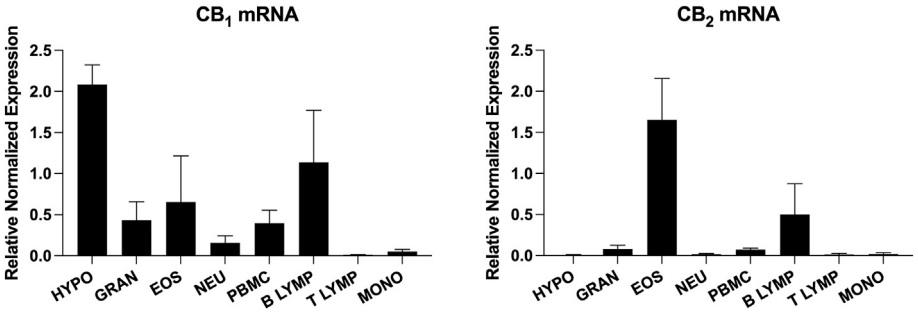


FIGURE 1 | Expression of the CB_1 and CB_2 receptors mRNA in human leukocytes isolated from the blood. Human venous blood was collected from healthy volunteers with the informed consent of all participants in blood collection tubes containing K_3EDTA as anticoagulant. Granulocytes (GRAN), eosinophils (EOS) and neutrophils (NEU) were isolated as in Chouinard et al. (2013). PBMCs were obtained from the PBMC layer and taken as is or otherwise processed for monocyte (MONO), B and T lymphocytes (LYMP) isolation using the EasySep™ monocyte isolation kit, CD19 positive Selection Kit II and CD3 positive selection Kit II respectively, as per the manufacturer's protocol. Purity of the different isolated leukocytes was always >97% with the exception of B Lymphocytes (90%) with MONO being the main contaminant. Hypothalamus (HYPO) samples were obtained from the Douglas-Bell Canada Brain Bank (McGill University, Montréal, Canada). mRNA was next isolated from the different preparations with TRIzol as per the manufacturer's protocol. 500 ng of total RNA was reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) as recommended. qPCR analyses were finally performed on a CFX Connect Real-Time PCR System, using the following primers (forward - reverse): GAPDH (5′-ACATCGCTCAGACACCATG-3′–5′-TGTAGTTGAGGTCAATGAAGGG-3′) 18S (5′-CGCACGGCCGGTACAGTGAA-3′–5′-GGGAGAGGAGCGAGCGACCA-3′) CB_1 (5′-TTCCCTCTTGTGAAGGCACTG-3′–5′-TCTTGACCGTGCTCTTGATGC-3′) and CB_2 (5′-CAAGGCTGTCTTCCTGCTGA-3′–5′-CGGGTGAGCAGAGCTTTGTA-3′). Data represent the mean (±SEM) of 4–6 donors and was obtained using the CFX Maestro Software (Bio-Rad).

(Turcotte et al., 2016) for a review]. However, CB_2 receptor expression is not restricted to leukocytes. It has notably been found in resident immune brain cells (microglia), the kidney, spleen, tonsil, thymus, lung epithelial cells and testes (Sanchez et al., 2001; Brown et al., 2002; Van Sickle et al., 2005; Ellert-Miklaszewska et al., 2007; Zhou et al., 2018; Cakir et al., 2019; Fantauzzi et al., 2020).

EXPRESSION OF THE CB_1 AND CB_2 RECEPTORS BY HUMAN BLOOD LEUKOCYTES

Galiègue et al. paved the way to our understanding of CB_2 expression by human leukocytes by showing its mRNA was expressed in human leukocytes, with the following order of relative abundance: tonsillar B cells > natural killer cells > monocytes ~ granulocytes > T4 lymphocytes > T8 lymphocytes (Galiegue et al., 1995). While very informative and useful, the data from Galiègue et al. did not include eosinophils while including tissue instead of blood B lymphocytes. This was somewhat pointed out in following studies (Turcotte et al., 2016), as it might have led to some inconsistencies. For example, while some documented the expression of the CB_2 receptor in human granulocytes (neutrophils and contaminating eosinophils) (Galiegue et al., 1995; Kurihara et al., 2006), others did not (Oka et al., 2004; Graham et al., 2010). This raised the possibility that contaminating cells might have been responsible for the previously documented CB_2 signal in neutrophils, and possibly other cell types. Noteworthy, it was later reported that eosinophil-depleted neutrophils weakly expressed the CB_2 receptor mRNA, while eosinophils (the main neutrophil suspension contaminant) expressed it at high levels, raising the strong possibility that discrepancies regarding CB_2 expression in neutrophils could be the result of contaminating eosinophils in granulocyte preparations (Chouinard et al., 2013). CB_2 expression was also reported in human eosinophils in other studies (Frei et al., 2016; Larose et al., 2017; Freundt-Revilla et al., 2018; Dothel et al., 2019).

In an attempt to better define CB_2 expression in human blood leukocytes, we revisited its expression by qPCR using mRNA from leukocytes that were isolated from the blood of healthy volunteers. CB_1 receptor expression was assessed in parallel. Hypothalamus samples were utilized as positive controls for the CB_1 receptor. In our hands, all tested leukocytes expressed the CB_1 receptor mRNA although to a lesser extent than hypothalamus samples (**Figure 1A**). In contrast, while we detected the expression of the CB_2 receptor mRNA in all leukocyte and hypothalamus samples, human eosinophils and B lymphocytes displayed the strongest signals (**Figure 1B**). Thus, these cell types are likely the origin of CB_2 expression found in mixed populations such as granulocytes (neutrophils and eosinophils, often abbreviated as PMN) and PBMCs (monocytes, B and T lymphocytes). This underlines the importance of separating granulocytes and PBMCs when studying the CB_2 receptor. The small, but detectable levels of CB_2 receptor mRNA in hypothalamus samples are consistent with other studies reporting its expression in this tissue (Sanchez et al., 2001; Van Sickle et al., 2005; Ellert-Miklaszewska et al., 2007).

FACTORS INFLUENCING CB_2 RECEPTOR EXPRESSION IN HUMAN LEUKOCYTES

Some factors were documented as influencing CB_2 receptor expression in human leukocytes. CB_2 expression can increase

during inflammation as it is the case in eosinophils from symptomatic allergic donors compared to healthy controls (Frei et al., 2016; Larose et al., 2017), in monocytes of patients after ischemic stroke (Greco et al., 2021), in myeloid and plasmacytoid dendritic cells of patients with multiple sclerosis (Chiurchiu et al., 2013; Sanchez Lopez et al., 2015) and in T lymphocytes of Non-Hodgkin's lymphomas (Rayman et al., 2007; Robinson et al., 2013). On the other hand, LPS decreased CB_2 receptor expression in isolated dendritic cells and B lymphocytes (Lee et al., 2001; Do et al., 2004). Finally, the CB_2 receptor was not detected in resting macrophages, was present at high levels in responsive and primed cells and was greatly diminished in fully activated cells (Cabral 2010). The latter observation suggests that the CB_2 receptor might have a time-specific function in macrophages during inflammation.

Numerous CB_2 receptor antibodies have been developed but most (if not all) are failing to provide reliable signals in different applications (immunohistochemistry, cytofluorometry and immunoblot), while not always having been characterized with the appropriate controls (control peptide blockade, CB_2 receptor-devoid cells, cross reactivity). Thus, until a clear consensus is achieved on which antibodies are sufficiently reliable, data on CB_2 protein should be interpreted with caution. With that in mind, the CB_2 receptor protein localization can vary. Indeed, Castaneda et al. reported that the CB_2 receptor protein was found intracellularly in most leukocytes with only B lymphocytes expressing it at the extracellular membrane (Castaneda et al., 2013). CB_2-positive B lymphocytes were mainly located in the mantle of secondary lymphoid follicles, which contain immature B lymphocytes while some positive cells also appeared in the germinal centers of secondary follicles, which contain mature B lymphocytes, suggesting an heterogeneous distribution of the receptor during B lymphocytes maturation stages (Galiegue et al., 1995). Immunohistochemical analysis using an N-terminal specific anti-CB_2 antibody revealed high protein expression in the germinal centers of secondary follicles while a C-terminal specific anti-CB_2 antibody (only recognizing a non-phosphorylated inactive receptor) showed positivity primary follicle, the mantle and marginal zones of the secondary follicles where resting cells reside (Rayman et al., 2004). Therefore, active CB_2 seems mainly present on B lymphocytes in the germinal centers.

IMPACT OF CB_2 RECEPTOR ACTIVATION IN HUMAN LEUKOCYTES

The early studies investigating the roles of the CB_2 receptor, notably those involving *cnr2*-deficient mice, led to the idea that it is mainly anti-inflammatory (Turcotte et al., 2016). However, recent studies are emerging and indicate that the outcome of CB_2 receptor signaling may differ depending on the experimental model/disease. A good example is experimental asthma. Indeed, early work indicated that the CB_2 receptor agonist WIN 55,212-2 inhibited ovalbumin-induced plasma extravasation in guinea pig airways (Fukuda et al., 2010). In contrast, the CB_2 receptor agonist JWH-133 aggravated ovalbumin-induced asthma in mice while having no effect in dinitrofluorobenzene-induced asthma (Bozkurt et al., 2016; Frei et al., 2016). When house dust mites were utilized as allergen, *cnr2*-deficient mice were resistant to allergic responses (Ferrini et al., 2017) while an innate lymphoid cell-2 dependent model involving IL-25, IL-33 and/or *Alternaria alternate* had lower symptoms, decreased eosinophil number, and airway resistance (Hurrell et al., 2021). In humans, CB_2 receptor expression was increased in nasal polyps of aspirin-exacerbated disease patients (Corrado et al., 2018) while being decreased in epithelial cells of asthmatic patients (Fantauzzi et al., 2020).

While we address some leukocytes individually below, the overall impact of CB_2 receptor activation on human leukocytes is summarized in **Table 1**. However, we underscore that the selectivity of the pharmacological tools targeting CB_2 receptors (agonists, antagonists, inverse agonists) has been often questioned, as exemplified by the work of Soethoudt et al. (2017).

Human Eosinophils

Eosinophils participate in innate immunity against parasites and in the development/persistence of diverse inflammatory responses, notably allergies and asthma. Studies involving human eosinophils and CB receptors are scarce. Their treatment with either the endocannabinoid 2-AG and/or CB_2 receptor agonists stimulated their migration or potentiated their migration toward other chemoattractants (Oka et al., 2004; Kishimoto et al., 2006; Larose et al., 2014; Frei et al., 2016). Importantly, these effects were prevented by the CB_2 receptor antagonists AM630 and/or SR144528. Consistent with a CB_2-mediated increased in eosinophil migration, cannabis use has been linked to some cases of acute eosinophilic pneumonia, although no demonstration has proven that this involved the CB_2 receptor (Sauvaget et al., 2010; Liebling and Siu 2013; Natarajan et al., 2013; Ocal et al., 2016; Mull et al., 2020). Interestingly, while JWH-133 led to a moderate chemotactic response in human eosinophils, it had no effect on mouse eosinophils (Frei et al., 2016). Altogether, the current data support that the CB_2 receptor stimulates eosinophil migration. This could eventually lead to increased parasitic defenses but also to a worsening of eosinophils-related inflammatory diseases.

Human B Lymphocytes

B lymphocytes maturation and differentiation are complex processes. Following their activation, naïve cells (spleen marginal zone) proliferate and differentiate into short-lived plasma cells, while cells from the follicles undergo massive proliferation and form germinal centers, where long-lived plasma and memory cells are formed (Basu et al., 2013). Very little is known about the role of the CB_2 receptor in human B lymphocytes but their treatment with CP 55,940 increased their proliferation, a phenomenon blocked by SR144528 (Carayon et al., 1998). In mice, activation of the CB_2 receptor has been associated with B lymphocyte differentiation, migration, proliferation and antibody class switching (Jorda et al., 2002; Tanikawa et al., 2007; Agudelo et al., 2008), suggesting the receptor is part of the B lymphocytes immune programing,

TABLE 1 | CB_2-mediated effects on human leukocytes and related human cell lines.

Leukocytes or cell lines	Agonist		Antagonist or inverse agonist	Effects	Impact on signaling	References
Eosinophils						
Blood	2-AG	1 μM (4 h)	SR144528 (1 μM)	Induce migration in presence of 1 μM NDGA (lipoxygenase inhibitor)		Oka et al. (2004)
		1 μM (1 h)	SR144528 (1 μM)	2-AG-induced migration in presence of 1 μM NDGA is attributed to chemotaxis rather than chemokinesis		Kishimoto et al. (2006)
		3 μM (2 h)	SR144528 (10 μM) AM630 (10 μM)	Induce migration in presence of IL-5	Inhibited by the Lyn inhibitor PP2	Larose et al. (2014)
		250 nM (5 h)	SR144528 (1 μM)	↑ CCL24-induced shape change and migration		Frei et al. (2016)
	CP 55,940	1 μM (2 h)	-	No effect on migration		Larose et al. (2014)
	JWH-133	100–250 nM (5 h)	SR144528 (1 μM)	Induce migration	Migration inhibited by MEK1 inhibitors (U-0126, PD98,059) and the ROCK inhibitor Y-27632	Frei et al. (2016)
				↑ CCL24-induced shape change and migration	Not inhibited by pertussis toxin (PTX; $G\alpha_i$-independant), p38 or PI3K inhibitors	
				↑ CCL24-induced CD11b upregulation	- ↑ Ca^{2+} influx	
				↑ Adhesion to ICAM-1	- Ca^{2+} influx inhibited by the PLC inhibitor U-73122 and the IP3 receptor antagonist 2-APB	
Leukemia EoL-1 cells	2-AG	1 μM (4 h)	SR144528 (1 μM)	Induce migration in presence of 1 μM NDGA	Inhibited by PTX ($G_{i/0}$-dependant)	Oka et al. (2004)
	S-777469	100–500 nM (4 h)	-	↓ 2-AG-induced migration		Haruna et al. (2017)
B lymphocytes						
Blood	CP 55,940	1–100 nM (72 h)	SR144528 (100–300 nM)	↑ Proliferation		Carayon et al. (1998)
Tonsillar	CP 55,940	1–100 nM (72 h)	SR144528 (100–300 nM)	↑ Proliferation of both naïve and germinal centrosome B lymphocytes		Carayon et al. (1998)
	WIN 55,212–2	10 μM (4 h)	SR144528 (10 nM)	No effect		Gustafsson et al. (2006)
Raji cell line	2-AG	300 nM (4 h)	SR144528 (100 nM)	Induce moderate migration		Rayman et al. (2004)
				↑ Migration following stimulation with an anti-sCD40 antibody		
Rec-1 cell line	WIN 55,212–2	10 μM (4 h)	SR144528 (10 nM)	↑ Apoptosis (caspase-3 activity)	- Inhibited by the CB_1 inverse agonist SR141716A and by p38 inhibitors	Gustafsson et al. (2006)
				↑ Ceramide levels (downstream of p38 activation)	- Not inhibited by c-Jun or MEK-1 inhibitors	
SKW 6.4 cell line	-		SR144528 (5–10 μM) AM630 (5 μM)	↓ IL-6 induced secretion of soluble IgM	- Inhibited by the CB_2 agonist HU308	Feng et al. (2014)
				- ↓ IL-6-induced p-STAT3	- Do not degrade IκBα as the NF-κB inhibitor Bay11-7085	
				- ↑ Pax5 (first) and Bcl-6 mRNA levels		
Neutrophils						
Blood	2-AG	1 μM (4 h)	SR144528 (1 μM)	No effect on migration in presence of NDGA		Oka et al. (2004)
		300 nM (20 min)	SR144528 (1 μM)	No motility or morphologic alterations		Kurihara et al. (2006)
	JWH-015	100 nM-10 μM (20 min)	SR144528 (1 μM)	No motility or morphologic alterations		Kurihara et al. (2006)
	JWH-133	1 μM (2 h)	-	No effect on neutrophil function		Zhou et al. (2020)
		100 nM (5 h)	SR144528 (1 μM)	No effect on IL-8-induced migration		Frei et al. (2016)

(Continued on following page)

TABLE 1 | (*Continued*) CB_2-mediated effects on human leukocytes and related human cell lines.

Leukocytes or cell lines	Agonist		Antagonist or inverse agonist	Effects	Impact on signaling	References
		100 nM-1 μM (30 min)	AM630 (500 nM)	↓ LPS-induced VEGF-A ↓ LPS-induced endothelial permeability		Braile et al. (2021)
T lymphocytes						
Blood	AEA	0.5–5 μM (6 h)	SR144528 (1 μM)	↓ Proliferation ↓ IL-2, TNF-α and IFN-γ ↓ IL-17		Cencioni et al. (2010)
	JWH-015	20 μM (1 h)	AM630 (500 nM)	↓ CXCL12-induced chemotaxis		Ghosh et al. (2006)
		250 nM (2 h)	AM630 (500 nM)	↓ Proliferation ↓ IL-2	↓ p-ERK1/2	Borner et al. (2009)
		1 μM (6 h)	SR144528 (1 μM)	↓ Proliferation ↓ IL-2, TNF-α and IFN-γ ↓ IL-17		Cencioni et al. (2010)
		1 μM (1–30 min)	AM630 (1 μM)	↓ HIV-1 infection in primary CD4 T cells		Costantino et al. (2012)
	JWH-133	0.001–10 μM (30 min)	-	↓ CXCL12-induced chemotaxis	↑ p-ERK1/2	Coopman et al. (2007)
		100 nM-1 μM (1–30 min)	AM630 (1 μM)	↓ HIV-1 infection in primary CD4 T cells ↓ Activation of CXCR4 by SDF-1α ↓ Levels of F-actin	↓ p-ERK1/2 and p-Akt	Costantino et al. (2012)
	Δ^9-THC	5 μg/ml (18 h)	SR144528 (1 μM)	↓ Percentage of T lymphocytes expressing IFN-γ ↓ IFN-γ intracellular level detected per cell ↑ IL-4 and IL-5		Yuan et al. (2002)
Jurkat cells	GW 405833	10–40 μM (3–24 h)	AM630 (1 μg/ml)	↓ Cell viability ↑ Cell apoptosis (annexin V)		Huang et al. (2019)
	JWH-015	20 μM (1 h)	AM630 (500 nM)	↓ CXCL12-induced chemotaxis ↓ Transendothelial migration ↓ PMA-induced MMP9	↑ CXCL12-induced p-ERK1/2 Migration not inhibited by the MEK-1 inhibitor PD 98,059	Ghosh et al. (2006)
		250 nM (2 h)	AM630 (500 nM)	↓ anti-CD3/anti-CD28-induced IL-2 production	- ↓ p-ERK1/2 - ↑ p-Lck - ↓ cAMP levels - Increased cAMP levels were inhibited by PTX	Borner et al. (2009)
	LV50	10 μM (4–72 h)	SR144528 (1 μM)	↓ T cell proliferation ↑ Apoptosis		Capozzi et al. (2018)
	Δ^9-THC	1–5 μM (1–2 h)	SR144528 (2 μM)	↓ Cell viability ↑ Apoptosis (Annexin 5) ↑ Ceramide levels Activation of caspase 8 at a post-mitochondrial level		Herrera et al. (2006)
Monocytes						
Blood	2-AG	10 nM–10 μM (4 h)	SR144528 (1 μM)	↑ Migration (chemotaxis toward 2-AG)		Kishimoto et al. (2003)
	(E)-β-caryophyllene	500 nM (18 h)	AM630 (5 μM)	↓ LPS-induced IL-1β and TNFα	↓ LPS-induced p-ERK1/2 and p-JNK1/2	Gertsch et al. (2008)
	JWH-015	5–20 μM (60 min)	SR144528 (1 μM)	↓ CCL2- and CCL3-induced migration ↓ CCR2 and CCR1 mRNA expression ↓ IFNγ-induced ICAM-1 induction	- Inhibited by PI3K and the MEK-1 inhibitors - Not inhibited by the p38 inhibitor SB-203580	Montecucco et al. (2008)
		1–10 μM (20 min)	-	↓ IL-1β		Rizzo et al. (2019)
	JWH-133	1 μM (18 h)	SR144528 (1 μM)	-	↑ p-ERK1/2	Gertsch et al. (2008)
		0.1–10 μM (days 4, 7 and 10)	-			Williams et al. (2014)

(Continued on following page)

TABLE 1 | (*Continued*) CB_2-mediated effects on human leukocytes and related human cell lines.

Leukocytes or cell lines	Agonist		Antagonist or inverse agonist	Effects	Impact on signaling	References
				↓ HIV-1 viral infection during differentiation in monocyte derived macrophages		
U937 cells	2-AG	1 μM (5 min)	SR144528 (3 μM)	↑ Adhesion to fibronectin		Gokoh et al. (2005a)
	CP 55,940	1 nM–1 μM (2 h)	SR144528 (1 μM)	↓ HIV-1 transactivating protein-enhanced adhesion of cells to extracellular matrix protein, such as collagen IV and laminin		Raborn et al. (2014)
	WIN 55,212–2	1–10 μM (2 h)	AM630 (1 μM)	↓ Adhesion to HUVECs		Zhao et al. (2010)
Mast cells						
Endometrial	JWH-015	10^{-8}–10^{-6} M (2 h)	-	↓ Calcium ionophore A23187-induced degranulation		Iuvone et al. (2008)
Macrophages						
Monocyte-derived macrophages (healthy subjects)	JWH-015	50 nM (30 min)	SR144528 (50 nM–0.1 μM)	↓ oxLDL-induced CD36 ↓ oxLDL-induced TNF-α, IL-12 and IL-10		Chiurchiu et al. (2014)
	Lenabasum	0.1–30 μM (Day 0, 3, and 6)	-	No effect		Tarique et al. (2020)
Monocyte-derived macrophages (patients with cystic fibrosis)	Lenabasum	0.1–30 μM (Day 0, 3, and 6)	-	↓ Macrophage polarization into pro-inflammatory M1 phenotype ↓ IL-8 and TNF-α secretion		Tarique et al. (2020)
Lung	JWH-133	1 μM (10 min)	AM630 (0.5 μM)	↓ LPS-induced VEGF-A and VEGF-C ↓ LPS-induced IL-6	↑ p-ERK1/2	Staiano et al. (2016)
HL-60-derived macrophage	2-AG	1 μM (1 min)	SR144528 (1 μM)	Induce morphological changes such as the extension of pseudopods	- Inhibited by PTX ($G_{i/0}$-dependant)	Gokoh et al. (2005b)
				↑ Actin polymerization	- Inhibited by selective chelating agent for intracellular free Ca^{2+} BAPTA-AM - Inhibited by the PI3K inhibitor wortmannin -Not inhibited by the tyrosine kinase inhibitor herbimycin, the MEK-1 inhibitor PD 98,059 or the PKC inhibitor Ro-31–8220	
THP-1-derived macrophage M2	JWH-015	1–5 μM (12 h)	-	↓ Migration of A549 cells	↓ p-ERK1/2 and p-STAT3	Ravi et al. (2016)
Dendritic cells						
Myeloid	AEA	2.5 μM (4 h)	SR144528 (1 μM)	↓ R848-induced TNF-α, IL-12p40, IL-6		Chiurchiu et al. (2013)
	JWH-015	1 μM (4 h)	SR144528 (1 μM)	↓ R848-induced TNF-α, IL-12p40, IL-6		Chiurchiu et al. (2013)
Plasmacytoid (healthy subjects)	AEA	2.5 μM (4 h)	SR144528 (1 μM)	↓ R848-induced TNF-α, IFN-α		Chiurchiu et al. (2013)
	2-AG	10 μM (18 h)	SR144528 (1 μM)	↓ CpGA-induced IFNα ↓ TLR9 activation		Rahaman et al. (2019)
	JWH-015	1 μM (4 h)	SR144528 (1 μM)	↓ R848-induced TNF-α and IFN-α		Chiurchiu et al. (2013)
		0.01–1 μM (5 h)	-	↓ CpG-induced IFNα and TNFα	↓ p-IRF7, p-TBK1, p-NF-κB and p-IKKγ	Henriquez et al. (2019)
	JWH-133	0.001–0.1 μM (5 h)	-	↓ CpG-induced IFNα and TNFα	↓ p-IRF7, p-TBK1, p-NF-κB and p-IKKγ	Henriquez et al. (2019)
Plasmacytoid (patient with multiple sclerosis)	AEA	2.5 μM (4 h)	SR144528 (1 μM)	No effect		Chiurchiu et al. (2013)
	JWH-015	1 μM (4 h)	SR144528 (1 μM)	No effect		Chiurchiu et al. (2013)

playing an important role in B lymphocyte repertoire formation (Pereira et al., 2009).

Human Neutrophils

Neutrophils are first responders of the innate immune system, playing crucial roles in acute inflammatory responses and host defense. They employ several strategies to fight microbes, including the phagocytosis and killing of pathogens with the help of their granule content. Studies showing a CB_2-receptor-mediated effect of human neutrophils were not conclusive and contaminating eosinophils in neutrophil preparations might have caused a red herring situation, eosinophils being responsible for most of the CB_2 receptor signal/effects (**Figure 1** and *Expression of the CB_1 and CB_2 Receptors by Human Blood Leukocytes*). In fact, numerous studies indicated that endocannabinoids as well as selective and non-selective CB_2 receptor agonists do not diminish human neutrophil functions (migration, superoxide generation and degranulation) *via* the CB_2 receptor and when they display an inhibitory effect on their functional responses it is mostly related to a mechanism distinct from the CB_1 and CB_2 receptors (Deusch et al., 2003; Kraft et al., 2004; Oka et al., 2004; McHugh et al., 2008; Chouinard et al., 2011; Montecucco et al., 2012; Zhou et al., 2020), which is consistent with their lack/very low expression of the CB_2 receptor. In contrast, JWH-133 inhibited the release of VEGF-A but not CXCL8 from LPS-stimulated human neutrophils, a phenomenon prevented by the CB_2 receptor antagonist AM630 (Braile et al., 2021).

- *In vivo* studies indicated that mouse neutrophils are more responsive to CB_2 receptor activation than human neutrophils. As such, *Cnr2*$^{-/-}$ mice models reported increased neutrophil numbers at inflammatory sites (Alferink et al., 2016; Kapellos et al., 2017; Kapellos et al., 2019). Accordingly, CB_2 activation by selective agonists suppressed neutrophil recruitment to the inflammation site (Horvath et al., 2012; Andrade-Silva et al., 2016; Wang et al., 2016; Parlar et al., 2018; Kapellos et al., 2019). However, it is not clear whether the reported evidence is a matter of mouse neutrophil responsiveness or of indirect CB_2-dependent effects mediated by other cells (Kraft and Kress 2005). At this point, we cannot exclude that a CB_2-dependent mechanism prevents neutrophil recruitment into by impairing their transmigration into the tissues and by affecting other cells (e.g., endothelial cells) as proposed earlier (Nilsson et al., 2006).

Human T Lymphocytes

Cytotoxic CD8 T lymphocytes are responsible for the elimination of invading/dysfunctional cells while CD4 T lymphocytes produce a myriad of inflammatory mediators and are referred to as helper lymphocytes (Th). Although CB_2 receptor expression was barely detected in circulating T lymphocytes (**Figure 1**), several studies reported that CB_2 receptor expression is increased in activated T lymphocytes and that its activation decreases their proliferation (Borner et al., 2009; Cencioni et al., 2010; Capozzi et al., 2018). This is accompanied with decreased IL-2 production and increased apoptosis (Herrera et al., 2006; Borner et al., 2009; Cencioni et al., 2010; Capozzi et al., 2018; Huang et al., 2019). Interestingly, CB_2 receptor activation seems to exert divergent effects depending on the T lymphocyte subtype with the tendency to decrease human Th1 and Th17 functions, while promoting those of Th2. For instance, Δ^9-THC decreased in a CB_2-dependant manner the percentage of human T lymphocytes expressing IFN-γ, and intracellular levels of IFN-γ per cells (Th1), while increasing levels of IL-4 and IL-5 (Th2) (Yuan et al., 2002). Accordingly, a decrease in IL-17 levels was found in JWH-015-treated T lymphocytes (Cencioni et al., 2010). Finally, the CB_2 agonist Lenabasum reduced TNF-α in both CD8 and CD4 T lymphocytes (Th1). The treatment also decreased IL-17 levels (Th17) as well as Th1 and Th17 respective signature transcription factors T-bet and RORγt (Tiberi et al., 2021).

Human Monocytes

Blood monocytes migrate into tissues where they differentiate into macrophages or convert into non-classical monocytes (Guilliams et al., 2018). 2-AG is a CB_2-dependant human monocyte chemoattractant (Kishimoto et al., 2003) and induces the adhesion of human monocytic U937 cells to fibronectin (Gokoh et al., 2005a). However, JWH-015 decreased the CCL2-and CCL3-induced migration of human monocytes by decreasing their receptors' expression (Montecucco et al., 2008). JWH-015 also reduces human monocyte differentiation and U937 cells adhesion to extracellular matrix proteins, both induced by HIV-1 (Raborn et al., 2014; Williams et al., 2014). Finally, CB_2 receptor engagement in human monocytes was shown to decrease the LPS-induced IL-1β and IL-6 production (Gu et al., 2019; Rizzo et al., 2019).

Human Macrophages

Macrophages are resident cells that are remarkably versatile, exerting important roles in development, homeostasis, tissue repair and immunity. The endocannabinoid 2-AG was found to induce shape changes of HL-60-derived macrophages in a CB_2-dependent manner (Gokoh et al., 2005b). Additionally, CB_2 receptor activation with JWH-015 or JWH-133 decreased the LPS-induced VEGF-A, VEGF-C IL-6 release, as well as the oxLDL-induced release of TNF-α, IL-12 and IL-10 (Chiurchiu et al., 2014; Staiano et al., 2016). In mice, the CB_2 receptor was shown to switch the polarization of M1 macrophage into M2 macrophage (Duerr et al., 2014; Denaes et al., 2016; Du et al., 2018). Such a phenomenon has been partially observed in humans by Tarique et al. who showed that Lenabasum decreased the polarization (M1) of monocyte-derived macrophage obtained from cystic fibrosis patients (Tarique et al., 2020).

Human Mast Cells

Mast cells are strategically located at the interface with the external environment, acting as key initiators of local inflammatory responses (Elieh Ali Komi et al., 2020). The first evidence that they could be regulated by the CB_2 receptor came from the rat basophilic leukemia cell line (RBL-2H3) expressing the CB_2 receptor (Facci et al., 1995). However, while the authors showed that *N*-palmitoyl-ethanolamine (PEA) inhibited serotonin release AEA did not. However, PEA interacts with

PPARα (Lo Verme et al., 2005) and its initial effects are likely linked to PPARα. In humans, the treatment of isolated mast cells with JWH-015 decreased their degranulation *in vitro* (Iuvone et al., 2008).

Human Dendritic Cells

Dendritic cells are sentinels of the immune system bridging the innate and adaptive immunity by ingesting pathogens and transporting antigens to lymphoid tissues. Stimulation of CB_2 receptor with CB_2 receptor agonists reduced their cytokine production. Indeed, AEA and JWH-015 decreased R848-induced levels of TNF-α, IL-12p40 and IL-6 by myeloid dendritic cells while AEA, 2-AG, JWH-015 and JWH-133 decreased levels of R848-and/or CpG-induced IFN-α by plasmacytoid dendritic cells by a mechanisms involving NF-κB and IKKγ signalization (Chiurchiu et al., 2013; Henriquez et al., 2019; Rahaman et al., 2019).

CONCLUSION

It is becoming clear that the CB_2 receptor plays important roles in the regulation of several inflammatory processes. However, while the first studies investigating the role of this receptor in mice led to the concept that its function was mainly anti-inflammatory, new evidence is challenging this concept, notably in allergic diseases, which usually involve cells such as eosinophils and B lymphocytes, whose functional responses to CB_2 receptor activation simulates them, in human-based studies. Moreover, the scarcity of human studies investigating the CB_2 receptor makes our understanding of the latter difficult at this point and underscores the urgency of performing additional work involving human samples/cells to deepen our understanding of CB_2-receptor-driven inflammatory responses and establish to what extent we can translate findings from experimental models to the clinic. It is thus urgent to further characterize the functions of the CB_2 receptor in human leukocytes and inflammatory diseases.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Comité d'éthique de la recherche de l'Institut universitaire de cardiologie et de pneumologie de Québec. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization: MS, VR, VD, and NF; Investigation: MS and VR; Data curation—formal analysis: MS, VR, and NF; Writing—original draft: MS and NF; Writing—review, editing, and revision: MS, VR, VD, and NF.

FUNDING

This work was supported by grants to NF from The Natural Sciences and Engineering Research Council of Canada (RGPIN-2021-03777) and to VD from the Canada Excellence Research Chair on the Microbiome-Endocannabinoidome Axis in Metabolic Health. VR was supported by a post-doctoral award from the Québec Heart and Lung Institute Foundation.

ACKNOWLEDGMENTS

We would like to thank Andréanne Côté and Annie Roy for providing the blood samples.

REFERENCES

Agudelo, M., Newton, C., Widen, R., Sherwood, T., Nong, L., Friedman, H., et al. (2008). Cannabinoid Receptor 2 (CB2) Mediates Immunoglobulin Class Switching from IgM to IgE in Cultures of Murine-Purified B Lymphocytes. *J. Neuroimmune Pharmacol.* 3 (1), 35–42. doi:10.1007/s11481-007-9088-9

Alferink, J., Specht, S., Arends, H., Schumak, B., Schmidt, K., Ruland, C., et al. (2016). Cannabinoid Receptor 2 Modulates Susceptibility to Experimental Cerebral Malaria through a CCL17-dependent Mechanism. *J. Biol. Chem.* 291 (37), 19517–19531. doi:10.1074/jbc.M116.746594

Andrade-Silva, M., Correa, L. B., Candéa, A. L., Cavalher-Machado, S. C., Barbosa, H. S., Rosas, E. C., et al. (2016). The Cannabinoid 2 Receptor Agonist β-caryophyllene Modulates the Inflammatory Reaction Induced by Mycobacterium Bovis BCG by Inhibiting Neutrophil Migration. *Inflamm. Res.* 65 (11), 869–879. doi:10.1007/s00011-016-0969-3

Basu, S., Ray, A., and Dittel, B. N. (2013). Cannabinoid Receptor 2 (CB2) Plays a Role in the Generation of Germinal Center and Memory B Cells, but Not in the Production of Antigen-Specific IgG and IgM, in Response to T-dependent Antigens. *PLoS One* 8 (6), e67587. doi:10.1371/journal.pone.0067587

Börner, C., Smida, M., Höllt, V., Schraven, B., and Kraus, J. (2009). Cannabinoid Receptor Type 1- and 2-mediated Increase in Cyclic AMP Inhibits T Cell Receptor-Triggered Signaling. *J. Biol. Chem.* 284 (51), 35450–35460. doi:10.1074/jbc.M109.006338

Bozkurt, T. E., Kaya, Y., Durlu-Kandilci, N. T., Onder, S., and Sahin-Erdemli, I. (2016). The Effect of Cannabinoids on Dinitrofluorobenzene-Induced Experimental Asthma in Mice. *Respir. Physiol. Neurobiol.* 231, 7–13. doi:10.1016/j.resp.2016.05.012

Braile, M., Cristinziano, L., Marcella, S., Varricchi, G., Marone, G., Modestino, L., et al. (2021). LPS-mediated Neutrophil VEGF-A Release Is Modulated by Cannabinoid Receptor Activation. *J. Leukoc. Biol.* 109 (3), 621–631. doi:10.1002/JLB.3A0520-187R

Brown, S. M., Wager-Miller, J., and Mackie, K. (2002). Cloning and Molecular Characterization of the Rat CB2 Cannabinoid Receptor. *Biochim. Biophys. Acta* 1576 (3), 255–264. doi:10.1016/s0167-4781(02)00341-x

Cakir, M., Tekin, S., Doganyigit, Z., Cakan, P., and Kaymak, E. (2019). The Protective Effect of Cannabinoid Type 2 Receptor Activation on Renal Ischemia-Reperfusion Injury. *Mol. Cel Biochem* 462 (1-2), 123–132.

Capozzi, A., Mattei, V., Martellucci, S., Manganelli, V., Saccomanni, G., Garofalo, T., et al. (2018). Anti-proliferative Properties and Proapoptotic Function of

New CB2 Selective Cannabinoid Receptor Agonist in Jurkat Leukemia Cells. *Int. J. Mol. Sci.* 19 (7), 1958. doi:10.3390/ijms19071958

Carayon, P., Marchand, J., Dussossoy, D., Derocq, J. M., Jbilo, O., Bord, A., et al. (1998). Modulation and Functional Involvement of CB2 Peripheral Cannabinoid Receptors during B-Cell Differentiation. *Blood* 92 (10), 3605–3615. doi:10.1182/blood.v92.10.3605.422k05_3605_3615

Castaneda, J. T., Harui, A., Kiertscher, S. M., Roth, J. D., and Roth, M. D. (2013). Differential Expression of Intracellular and Extracellular CB(2) Cannabinoid Receptor Protein by Human Peripheral Blood Leukocytes. *J. Neuroimmune Pharmacol.* 8 (1), 323–332. doi:10.1007/s11481-012-9430-8

Cencioni, M. T., Chiurchiù, V., Catanzaro, G., Borsellino, G., Bernardi, G., Battistini, L., et al. (2010). Anandamide Suppresses Proliferation and Cytokine Release from Primary Human T-Lymphocytes Mainly via CB2 Receptors. *PLoS One* 5 (1), e8688. doi:10.1371/journal.pone.0008688

Chiurchiù, V., Cencioni, M. T., Bisicchia, E., De Bardi, M., Gasperini, C., Borsellino, G., et al. (2013). Distinct Modulation of Human Myeloid and Plasmacytoid Dendritic Cells by Anandamide in Multiple Sclerosis. *Ann. Neurol.* 73 (5), 626–636. doi:10.1002/ana.23875

Chiurchiù, V., Lanuti, M., Catanzaro, G., Fezza, F., Rapino, C., and Maccarrone, M. (2014). Detailed Characterization of the Endocannabinoid System in Human Macrophages and Foam Cells, and Anti-inflammatory Role of Type-2 Cannabinoid Receptor. *Atherosclerosis* 233 (1), 55–63. doi:10.1016/j.atherosclerosis.2013.12.042

Chouinard, F., Lefebvre, J. S., Navarro, P., Bouchard, L., Ferland, C., Lalancette-Hébert, M., et al. (2011). The Endocannabinoid 2-Arachidonoyl-Glycerol Activates Human Neutrophils: Critical Role of its Hydrolysis and de novo Leukotriene B4 Biosynthesis. *J. Immunol.* 186 (5), 3188–3196. doi:10.4049/jimmunol.1002853

Chouinard, F., Turcotte, C., Guan, X., Larose, M. C., Poirier, S., Bouchard, L., et al. (2013). 2-Arachidonoyl-glycerol- and Arachidonic Acid-Stimulated Neutrophils Release Antimicrobial Effectors against *E. coli*, *S. aureus*, HSV-1, and RSV. *J. Leukoc. Biol.* 93 (2), 267–276. doi:10.1189/jlb.0412200

Coopman, K., Smith, L. D., Wright, K. L., and Ward, S. G. (2007). Temporal Variation in CB2R Levels Following T Lymphocyte Activation: Evidence that Cannabinoids Modulate CXCL12-Induced Chemotaxis. *Int. Immunopharmacol* 7 (3), 360–371. doi:10.1016/j.intimp.2006.11.008

Corrado, A., Battle, M., Wise, S. K., Lee, F. E., Guidot, D. M., DelGaudio, J. M., et al. (2018). Endocannabinoid Receptor CB2R Is Significantly Expressed in Aspirin-Exacerbated Respiratory Disease: a Pilot Study. *Int. Forum Allergy Rhinol* 8 (10), 1184–1189. doi:10.1002/alr.22163

Costantino, C. M., Gupta, A., Yewdall, A. W., Dale, B. M., Devi, L. A., and Chen, B. K. (2012). Cannabinoid Receptor 2-mediated Attenuation of CXCR4-Tropic HIV Infection in Primary CD4+ T Cells. *PLoS One* 7 (3), e33961. doi:10.1371/journal.pone.0033961

Denaës, T., Lodder, J., Chobert, M. N., Ruiz, I., Pawlotsky, J. M., Lotersztajn, S., et al. (2016). The Cannabinoid Receptor 2 Protects against Alcoholic Liver Disease via a Macrophage Autophagy-dependent Pathway. *Sci. Rep.* 6, 28806. doi:10.1038/srep28806

Deusch, E., Kraft, B., Nahlik, G., Weigl, L., Hohenegger, M., and Kress, H. G. (2003). No Evidence for Direct Modulatory Effects of delta 9-tetrahydrocannabinol on Human Polymorphonuclear Leukocytes. *J. Neuroimmunol* 141 (1-2), 99–103. doi:10.1016/s0165-5728(03)00259-5

Devane, W. A., Dysarz, F. A., 3rd, Johnson, M. R., Melvin, L. S., and Howlett, A. C. (1988). Determination and Characterization of a Cannabinoid Receptor in Rat Brain. *Mol. Pharmacol.* 34 (5), 605–613.

Di Marzo, V., Melck, D., Bisogno, T., and De Petrocellis, L. (1998). Endocannabinoids: Endogenous Cannabinoid Receptor Ligands with Neuromodulatory Action. *Trends Neurosci.* 21 (12), 521–528. doi:10.1016/s0166-2236(98)01283-1

Di Marzo, V. (2018). New Approaches and Challenges to Targeting the Endocannabinoid System. *Nat. Rev. Drug Discov.* 17 (9), 623–639. doi:10.1038/nrd.2018.115

Do, Y., McKallip, R. J., Nagarkatti, M., and Nagarkatti, P. S. (2004). Activation through Cannabinoid Receptors 1 and 2 on Dendritic Cells Triggers NF-kappaB-dependent Apoptosis: Novel Role for Endogenous and Exogenous Cannabinoids in Immunoregulation. *J. Immunol.* 173 (4), 2373–2382. doi:10.4049/jimmunol.173.4.2373

Dothel, G., Chang, L., Shih, W., Barbaro, M. R., Cremon, C., Stanghellini, V., et al. (2019). Micro-Opioid Receptor, Beta-Endorphin, and Cannabinoid Receptor-2 Are Increased in the Colonic Mucosa of Irritable Bowel Syndrome Patients. *Neurogastroenterol Motil.* 31 (11), e13688. doi:10.1111/nmo.13688

Du, Y., Ren, P., Wang, Q., Jiang, S. K., Zhang, M., Li, J. Y., et al. (2018). Cannabinoid 2 Receptor Attenuates Inflammation during Skin Wound Healing by Inhibiting M1 Macrophages rather Than Activating M2 Macrophages. *J. Inflamm. (Lond)* 15, 25. doi:10.1186/s12950-018-0201-z

Duerr, G. D., Heinemann, J. C., Suchan, G., Kolobara, E., Wenzel, D., Geisen, C., et al. (2014). The Endocannabinoid-CB2 Receptor axis Protects the Ischemic Heart at the Early Stage of Cardiomyopathy. *Basic Res. Cardiol.* 109 (4), 425. doi:10.1007/s00395-014-0425-x

Elieh Ali Komi, D., Wöhrl, S. L., and Bielory, L. (2020). Mast Cell Biology at Molecular Level: a Comprehensive Review. *Clin. Rev. Allergy Immunol.* 58 (3), 342–365. doi:10.1007/s12016-019-08769-2

Ellert-Miklaszewska, A., Grajkowska, W., Gabrusiewicz, K., Kaminska, B., and Konarska, L. (2007). Distinctive Pattern of Cannabinoid Receptor Type II (CB2) Expression in Adult and Pediatric Brain Tumors. *Brain Res.* 1137 (1), 161–169. doi:10.1016/j.brainres.2006.12.060

Facci, L., Dal Toso, R., Romanello, S., Buriani, A., Skaper, S. D., and Leon, A. (1995). Mast Cells Express a Peripheral Cannabinoid Receptor with Differential Sensitivity to Anandamide and Palmitoylethanolamide. *Proc. Natl. Acad. Sci. U S A.* 92 (8), 3376–3380. doi:10.1073/pnas.92.8.3376

Fantauzzi, M. F., Aguiar, J. A., Tremblay, B. J., Mansfield, M. J., Yanagihara, T., Chandiramohan, A., et al. (2020). Expression of Endocannabinoid System Components in Human Airway Epithelial Cells: Impact of Sex and Chronic Respiratory Disease Status. *ERJ Open Res.* 6 (4). doi:10.1183/23120541.00128-2020

Feng, R., Milcarek, C. A., and Xie, X. Q. (2014). Antagonism of Cannabinoid Receptor 2 Pathway Suppresses IL-6-induced Immunoglobulin IgM Secretion. *BMC Pharmacol. Toxicol.* 15, 30. doi:10.1186/2050-6511-15-30

Ferrini, M. E., Hong, S., Stierle, A., Stierle, D., Stella, N., Roberts, K., et al. (2017). CB2 Receptors Regulate Natural Killer Cells that Limit Allergic Airway Inflammation in a Murine Model of Asthma. *Allergy* 72 (6), 937–947. doi:10.1111/all.13107

Frei, R. B., Luschnig, P., Parzmair, G. P., Peinhaupt, M., Schranz, S., Fauland, A., et al. (2016). Cannabinoid Receptor 2 Augments Eosinophil Responsiveness and Aggravates Allergen-Induced Pulmonary Inflammation in Mice. *Allergy* 71 (7), 944–956. doi:10.1111/all.12858

Freundt-Revilla, J., Heinrich, F., Zoerner, A., Gesell, F., Beyerbach, M., Shamir, M., et al. (2018). The Endocannabinoid System in Canine Steroid-Responsive Meningitis-Arteritis and Intraspinal Spirocercosis. *PLoS One* 13 (2), e0187197. doi:10.1371/journal.pone.0187197

Fukuda, H., Abe, T., and Yoshihara, S. (2010). The Cannabinoid Receptor Agonist WIN 55,212-2 Inhibits Antigen-Induced Plasma Extravasation in guinea Pig Airways. *Int. Arch. Allergy Immunol.* 152 (3), 295–300. doi:10.1159/000283042

Galiègue, S., Mary, S., Marchand, J., Dussossoy, D., Carrière, D., Carayon, P., et al. (1995). Expression of central and Peripheral Cannabinoid Receptors in Human Immune Tissues and Leukocyte Subpopulations. *Eur. J. Biochem.* 232 (1), 54–61. doi:10.1111/j.1432-1033.1995.tb20780.x

Gertsch, J., Leonti, M., Raduner, S., Racz, I., Chen, J. Z., Xie, X. Q., et al. (2008). Beta-caryophyllene Is a Dietary Cannabinoid. *Proc. Natl. Acad. Sci. U S A.* 105 (26), 9099–9104. doi:10.1073/pnas.0803601105

Ghosh, S., Preet, A., Groopman, J. E., and Ganju, R. K. (2006). Cannabinoid Receptor CB2 Modulates the CXCL12/CXCR4-Mediated Chemotaxis of T Lymphocytes. *Mol. Immunol.* 43 (14), 2169–2179. doi:10.1016/j.molimm.2006.01.005

Gokoh, M., Kishimoto, S., Oka, S., Metani, Y., and Sugiura, T. (2005a). 2-Arachidonoylglycerol, an Endogenous Cannabinoid Receptor Ligand, Enhances the Adhesion of HL-60 Cells Differentiated into Macrophage-like Cells and Human Peripheral Blood Monocytes. *FEBS Lett.* 579 (28), 6473–6478. doi:10.1016/j.febslet.2005.10.030

Gokoh, M., Kishimoto, S., Oka, S., Mori, M., Waku, K., Ishima, Y., et al. (2005b). 2-arachidonoylglycerol, an Endogenous Cannabinoid Receptor Ligand, Induces Rapid Actin Polymerization in HL-60 Cells Differentiated into Macrophage-like Cells. *Biochem. J.* 386 (Pt 3), 583–589. doi:10.1042/BJ20041163

Graham, E. S., Angel, C. E., Schwarcz, L. E., Dunbar, P. R., and Glass, M. (2010). Detailed Characterisation of CB2 Receptor Protein Expression in Peripheral Blood Immune Cells from Healthy Human Volunteers Using Flow Cytometry. *Int. J. Immunopathol Pharmacol.* 23 (1), 25–34. doi:10.1177/039463201002300103

Greco, R., Demartini, C., Zanaboni, A., Tumelero, E., Elisa, C., Persico, A., et al. (2021). Characterization of CB2 Receptor Expression in Peripheral Blood Monocytes of Acute Ischemic Stroke Patients. *Transl Stroke Res.* 12 (4), 550–558. doi:10.1007/s12975-020-00851-8

Gu, Z., Singh, S., Niyogi, R. G., Lamont, G. J., Wang, H., Lamont, R. J., et al. (2019). Marijuana-Derived Cannabinoids Trigger a CB2/PI3K Axis of Suppression of the Innate Response to Oral Pathogens. *Front. Immunol.* 10, 2288. doi:10.3389/fimmu.2019.02288

Guilliams, M., Mildner, A., and Yona, S. (2018). Developmental and Functional Heterogeneity of Monocytes. *Immunity* 49 (4), 595–613. doi:10.1016/j.immuni.2018.10.005

Gustafsson, K., Christensson, B., Sander, B., and Flygare, J. (2006). Cannabinoid Receptor-Mediated Apoptosis Induced by R(+)-methanandamide and Win55,212-2 Is Associated with Ceramide Accumulation and P38 Activation in Mantle Cell Lymphoma. *Mol. Pharmacol.* 70 (5), 1612–1620. doi:10.1124/mol.106.025981

Haruna, T., Soga, M., Morioka, Y., Imura, K., Furue, Y., Yamamoto, M., et al. (2017). The Inhibitory Effect of S-777469, a Cannabinoid Type 2 Receptor Agonist, on Skin Inflammation in Mice. *Pharmacology* 99 (5-6), 259–267. doi:10.1159/000455916

Henriquez, J. E., Crawford, R. B., and Kaminski, N. E. (2019). Suppression of CpG-ODN-Mediated IFNα and TNFα Response in Human Plasmacytoid Dendritic Cells (pDC) by Cannabinoid Receptor 2 (CB2)-specific Agonists. *Toxicol. Appl. Pharmacol.* 369, 82–89. doi:10.1016/j.taap.2019.02.013

Herrera, B., Carracedo, A., Diez-Zaera, M., Gómez del Pulgar, T., Guzmán, M. G., and Velasco, G. (2006). The CB2 Cannabinoid Receptor Signals Apoptosis via Ceramide-dependent Activation of the Mitochondrial Intrinsic Pathway. *Exp. Cel Res* 312 (11), 2121–2131. doi:10.1016/j.yexcr.2006.03.009

Horváth, B., Magid, L., Mukhopadhyay, P., Bátkai, S., Rajesh, M., Park, O., et al. (2012). A New Cannabinoid CB2 Receptor Agonist HU-910 Attenuates Oxidative Stress, Inflammation and Cell Death Associated with Hepatic Ischaemia/reperfusion Injury. *Br. J. Pharmacol.* 165 (8), 2462–2478. doi:10.1111/j.1476-5381.2011.01381.x

Howlett, A. C., and Abood, M. E. (2017). CB1 and CB2 Receptor Pharmacology. *Adv. Pharmacol.* 80, 169–206. doi:10.1016/bs.apha.2017.03.007

Huang, Z. B., Zheng, Y. X., Li, N., Cai, S. L., Huang, Y., Wang, J., et al. (2019). Protective Effects of Specific Cannabinoid Receptor 2 Agonist GW405833 on Concanavalin A-Induced Acute Liver Injury in Mice. *Acta Pharmacol. Sin* 40 (11), 1404–1411. doi:10.1038/s41401-019-0213-0

Hurrell, B. P., Helou, D. G., Shafiei-Jahani, P., Howard, E., Painter, J. D., Quach, C., et al. (2021). Cannabinoid Receptor II Engagement Promotes ILC2 Expansion and Enhances ILC2-dependent Airway Hyperreactivity. *J. Allergy Clin. Immunol.*.

Iuvone, T., De Filippis, D., Di Spiezio Sardo, A., D'Amico, A., Simonetti, S., Sparice, S., et al. (2008). Selective CB2 Up-Regulation in Women Affected by Endometrial Inflammation. *J. Cel Mol Med* 12 (2), 661–670. doi:10.1111/j.1582-4934.2007.00085.x

Jordà, M. A., Verbakel, S. E., Valk, P. J., Vankan-Berkhoudt, Y. V., Maccarrone, M., Finazzi-Agrò, A., et al. (2002). Hematopoietic Cells Expressing the Peripheral Cannabinoid Receptor Migrate in Response to the Endocannabinoid 2-arachidonoylglycerol. *Blood* 99 (8), 2786–2793. doi:10.1182/blood.v99.8.2786

Kapellos, T. S., Recio, C., Greaves, D. R., and Iqbal, A. J. (20172017). Cannabinoid Receptor 2 Modulates Neutrophil Recruitment in a Murine Model of Endotoxemia. *Mediators Inflamm.* 2017, 4315412. doi:10.1155/2017/4315412

Kapellos, T. S., Taylor, L., Feuerborn, A., Valaris, S., Hussain, M. T., Rainger, G. E., et al. (2019). Cannabinoid Receptor 2 Deficiency Exacerbates Inflammation and Neutrophil Recruitment. *FASEB J.* 33 (5), 6154–6167. doi:10.1096/fj.201802524R

Kishimoto, S., Gokoh, M., Oka, S., Muramatsu, M., Kajiwara, T., Waku, K., et al. (2003). 2-arachidonoylglycerol Induces the Migration of HL-60 Cells Differentiated into Macrophage-like Cells and Human Peripheral Blood Monocytes through the Cannabinoid CB2 Receptor-dependent Mechanism. *J. Biol. Chem.* 278 (27), 24469–24475. doi:10.1074/jbc.M301359200

Kishimoto, S., Oka, S., Gokoh, M., and Sugiura, T. (2006). Chemotaxis of Human Peripheral Blood Eosinophils to 2-arachidonoylglycerol: Comparison with Other Eosinophil Chemoattractants. *Int. Arch. Allergy Immunol.* 140 Suppl 1 (Suppl. 1), 3–7. doi:10.1159/000092704

Kraft, B., and Kress, H. G. (2005). Indirect CB2 Receptor and Mediator-dependent Stimulation of Human Whole-Blood Neutrophils by Exogenous and Endogenous Cannabinoids. *J. Pharmacol. Exp. Ther.* 315 (2), 641–647. doi:10.1124/jpet.105.084269

Kraft, B., Wintersberger, W., and Kress, H. G. (2004). Cannabinoid Receptor-independent Suppression of the Superoxide Generation of Human Neutrophils (PMN) by CP55 940, but Not by Anandamide. *Life Sci.* 75 (8), 969–977. doi:10.1016/j.lfs.2004.02.007

Kurihara, R., Tohyama, Y., Matsusaka, S., Naruse, H., Kinoshita, E., Tsujioka, T., et al. (2006). Effects of Peripheral Cannabinoid Receptor Ligands on Motility and Polarization in Neutrophil-like HL60 Cells and Human Neutrophils. *J. Biol. Chem.* 281 (18), 12908–12918. doi:10.1074/jbc.M510871200

Larose, M. C., Archambault, A. S., Provost, V., Laviolette, M., and Flamand, N. (2017). Regulation of Eosinophil and Group 2 Innate Lymphoid Cell Trafficking in Asthma. *Front. Med. (Lausanne)* 4, 136. doi:10.3389/fmed.2017.00136

Larose, M. C., Turcotte, C., Chouinard, F., Ferland, C., Martin, C., Provost, V., et al. (2014). Mechanisms of Human Eosinophil Migration Induced by the Combination of IL-5 and the Endocannabinoid 2-Arachidonoyl-Glycerol. *J. Allergy Clin. Immunol.* 133 (5), 1480–1483. doi:10.1016/j.jaci.2013.12.1081

Lee, S. F., Newton, C., Widen, R., Friedman, H., and Klein, T. W. (2001). Downregulation of Cannabinoid Receptor 2 (CB2) Messenger RNA Expression during *In Vitro* Stimulation of Murine Splenocytes with Lipopolysaccharide. *Adv. Exp. Med. Biol.* 493, 223–228. doi:10.1007/0-306-47611-8_26

Liebling, P. D., and Siu, S. (2013). A Novel Cause of Eosinophilic Pneumonia: Recreational Marijuana Exposure. *J. Bronchology Interv. Pulmonol* 20 (2), 183–185. doi:10.1097/LBR.0b013e31828caa0d

Lo Verme, J., Fu, J., Astarita, G., La Rana, G., Russo, R., Calignano, A., et al. (2005). The Nuclear Receptor Peroxisome Proliferator-Activated Receptor-Alpha Mediates the Anti-inflammatory Actions of Palmitoylethanolamide. *Mol. Pharmacol.* 67 (1), 15–19. doi:10.1124/mol.104.006353

Matsuda, L. A., Lolait, S. J., Brownstein, M. J., Young, A. C., and Bonner, T. I. (1990). Structure of a Cannabinoid Receptor and Functional Expression of the Cloned cDNA. *Nature* 346 (6284), 561–564. doi:10.1038/346561a0

McHugh, D., Tanner, C., Mechoulam, R., Pertwee, R. G., and Ross, R. A. (2008). Inhibition of Human Neutrophil Chemotaxis by Endogenous Cannabinoids and Phytocannabinoids: Evidence for a Site Distinct from CB1 and CB2. *Mol. Pharmacol.* 73 (2), 441–450. doi:10.1124/mol.107.041863

Montecucco, F., Burger, F., Mach, F., and Steffens, S. (2008). CB2 Cannabinoid Receptor Agonist JWH-015 Modulates Human Monocyte Migration through Defined Intracellular Signaling Pathways. *Am. J. Physiol. Heart Circ. Physiol.* 294 (3), H1145–H1155. doi:10.1152/ajpheart.01328.2007

Montecucco, F., Di Marzo, V., da Silva, R. F., Vuilleumier, N., Capettini, L., Lenglet, S., et al. (2012). The Activation of the Cannabinoid Receptor Type 2 Reduces Neutrophilic Protease-Mediated Vulnerability in Atherosclerotic Plaques. *Eur. Heart J.* 33 (7), 846–856. doi:10.1093/eurheartj/ehr449

Mull, E. S., Erdem, G., Nicol, K., Adler, B., and Shell, R. (2020). Eosinophilic Pneumonia and Lymphadenopathy Associated with Vaping and Tetrahydrocannabinol Use. *Pediatrics* 145 (4). doi:10.1542/peds.2019-3007

Munro, S., Thomas, K. L., and Abu-Shaar, M. (1993). Molecular Characterization of a Peripheral Receptor for Cannabinoids. *Nature* 365 (6441), 61–65. doi:10.1038/365061a0

Natarajan, A., Shah, P., Mirrakhimov, A. E., and Hussain, N. (20132013). Eosinophilic Pneumonia Associated with Concomitant Cigarette and Marijuana Smoking. *BMJ Case Rep.*. doi:10.1136/bcr-2013-009001

Nilsson, O., Fowler, C. J., and Jacobsson, S. O. (2006). The Cannabinoid Agonist WIN 55,212-2 Inhibits TNF-Alpha-Induced Neutrophil Transmigration across ECV304 Cells. *Eur. J. Pharmacol.* 547 (1-3), 165–173. doi:10.1016/j.ejphar.2006.07.016

Öcal, N., Doğan, D., Çiçek, A. F., Yücel, O. E., and Tozkoparan, E. (2016). Acute Eosinophilic Pneumonia with Respiratory Failure Induced by Synthetic Cannabinoid Inhalation. *Balkan Med. J.* 33 (6), 688–690. doi:10.5152/balkanmedj.2016.151145

Oka, S., Ikeda, S., Kishimoto, S., Gokoh, M., Yanagimoto, S., Waku, K., et al. (2004). 2-arachidonoylglycerol, an Endogenous Cannabinoid Receptor Ligand, Induces the Migration of EoL-1 Human Eosinophilic Leukemia Cells and Human

Peripheral Blood Eosinophils. *J. Leukoc. Biol.* 76 (5), 1002–1009. doi:10.1189/jlb.0404252

Parlar, A., Arslan, S. O., Doğan, M. F., Çam, S. A., Yalçin, A., Elibol, E., et al. (2018). The Exogenous Administration of CB2 Specific Agonist, GW405833, Inhibits Inflammation by Reducing Cytokine Production and Oxidative Stress. *Exp. Ther. Med.* 16 (6), 4900–4908. doi:10.3892/etm.2018.6753

Pereira, J. P., An, J., Xu, Y., Huang, Y., and Cyster, J. G. (2009). Cannabinoid Receptor 2 Mediates the Retention of Immature B Cells in Bone Marrow Sinusoids. *Nat. Immunol.* 10 (4), 403–411. doi:10.1038/ni.1710

Raborn, E. S., Jamerson, M., Marciano-Cabral, F., and Cabral, G. A. (2014). Cannabinoid Inhibits HIV-1 Tat-Stimulated Adhesion of Human Monocyte-like Cells to Extracellular Matrix Proteins. *Life Sci.* 104 (1-2), 15–23. doi:10.1016/j.lfs.2014.04.008

Rahaman, O., Bhattacharya, R., Liu, C. S. C., Raychaudhuri, D., Ghosh, A. R., Bandopadhyay, P., et al. (2019). Cutting Edge: Dysregulated Endocannabinoid-Rheostat for Plasmacytoid Dendritic Cell Activation in a Systemic Lupus Endophenotype. *J. Immunol.* 202 (6), 1674–1679. doi:10.4049/jimmunol.1801521

Ravi, J., Elbaz, M., Wani, N. A., Nasser, M. W., and Ganju, R. K. (2016). Cannabinoid Receptor-2 Agonist Inhibits Macrophage Induced EMT in Non-small Cell Lung Cancer by Downregulation of EGFR Pathway. *Mol. Carcinog* 55 (12), 2063–2076. doi:10.1002/mc.22451

Rayman, N., Lam, K. H., Laman, J. D., Simons, P. J., Löwenberg, B., Sonneveld, P., et al. (2004). Distinct Expression Profiles of the Peripheral Cannabinoid Receptor in Lymphoid Tissues Depending on Receptor Activation Status. *J. Immunol.* 172 (4), 2111–2117. doi:10.4049/jimmunol.172.4.2111

Rayman, N., Lam, K. H., Van Leeuwen, J., Mulder, A. H., Budel, L. M., Löwenberg, B., et al. (2007). The Expression of the Peripheral Cannabinoid Receptor on Cells of the Immune System and Non-Hodgkin's Lymphomas. *Leuk. Lymphoma* 48 (7), 1389–1399. doi:10.1080/10428190701377030

Rizzo, M. D., Crawford, R. B., Bach, A., Sermet, S., Amalfitano, A., and Kaminski, N. E. (2019). Δ9-Tetrahydrocannabinol Suppresses Monocyte-Mediated Astrocyte Production of Monocyte Chemoattractant Protein 1 and Interleukin-6 in a Toll-Like Receptor 7-Stimulated Human Coculture. *J. Pharmacol. Exp. Ther.* 371 (1), 191–201. doi:10.1124/jpet.119.260661

Robinson, R. H., Meissler, J. J., Breslow-Deckman, J. M., Gaughan, J., Adler, M. W., and Eisenstein, T. K. (2013). Cannabinoids Inhibit T-Cells via Cannabinoid Receptor 2 in an *In Vitro* Assay for Graft Rejection, the Mixed Lymphocyte Reaction. *J. Neuroimmune Pharmacol.* 8 (5), 1239–1250. doi:10.1007/s11481-013-9485-1

Sánchez, C., de Ceballos, M. L., Gomez del Pulgar, T., Rueda, D., Corbacho, C., Velasco, G., et al. (2001). Inhibition of Glioma Growth *In Vivo* by Selective Activation of the CB(2) Cannabinoid Receptor. *Cancer Res.* 61 (15), 5784–5789.

Sánchez López, A. J., Román-Vega, L., Ramil Tojeiro, E., Giuffrida, A., and García-Merino, A. (2015). Regulation of Cannabinoid Receptor Gene Expression and Endocannabinoid Levels in Lymphocyte Subsets by Interferon-β: a Longitudinal Study in Multiple Sclerosis Patients. *Clin. Exp. Immunol.* 179 (1), 119–127. doi:10.1111/cei.12443

Sauvaget, E., Dellamonica, J., Arlaud, K., Sanfiorenzo, C., Bernardin, G., Padovani, B., et al. (2010). Idiopathic Acute Eosinophilic Pneumonia Requiring ECMO in a Teenager Smoking Tobacco and Cannabis. *Pediatr. Pulmonol* 45 (12), 1246–1249. doi:10.1002/ppul.21314

Soethoudt, M., Grether, U., Fingerle, J., Grim, T. W., Fezza, F., de Petrocellis, L., et al. (2017). Cannabinoid CB2 Receptor Ligand Profiling Reveals Biased Signalling and Off-Target Activity. *Nat. Commun.* 8, 13958. doi:10.1038/ncomms13958

Staiano, R. I., Loffredo, S., Borriello, F., Iannotti, F. A., Piscitelli, F., Orlando, P., et al. (2016). Human Lung-Resident Macrophages Express CB1 and CB2 Receptors Whose Activation Inhibits the Release of Angiogenic and Lymphangiogenic Factors. *J. Leukoc. Biol.* 99 (4), 531–540. doi:10.1189/jlb.3HI1214-584R

Tanikawa, T., Kurohane, K., and Imai, Y. (2007). Induction of Preferential Chemotaxis of Unstimulated B-Lymphocytes by 2-arachidonoylglycerol in Immunized Mice. *Microbiol. Immunol.* 51 (10), 1013–1019. doi:10.1111/j.1348-0421.2007.tb03985.x

Tarique, A. A., Evron, T., Zhang, G., Tepper, M. A., Morshed, M. M., Andersen, I. S. G., et al. (2020). Anti-inflammatory Effects of Lenabasum, a Cannabinoid Receptor Type 2 Agonist, on Macrophages from Cystic Fibrosis. *J. Cyst Fibros* 19 (5), 823–829. doi:10.1016/j.jcf.2020.03.015

Tiberi, M., Evron, T., Saracini, S., Boffa, L., Mercuri, N. B., Chintalacharuvu, S. R., et al. (2021). Potent T Cell-Mediated Anti-inflammatory Role of the Selective CB2 Agonist Lenabasum in Multiple Sclerosis. *Neuropathol. Appl. Neurobiol.*.

Turcotte, C., Blanchet, M. R., Laviolette, M., and Flamand, N. (2016). The CB2 Receptor and its Role as a Regulator of Inflammation. *Cell Mol Life Sci* 73 (23), 4449–4470. doi:10.1007/s00018-016-2300-4

Turcotte, C., Chouinard, F., Lefebvre, J. S., and Flamand, N. (2015). Regulation of Inflammation by Cannabinoids, the Endocannabinoids 2-Arachidonoyl-Glycerol and Arachidonoyl-Ethanolamide, and Their Metabolites. *J. Leukoc. Biol.* 97 (6), 1049–1070. doi:10.1189/jlb.3RU0115-021R

Van Sickle, M. D., Duncan, M., Kingsley, P. J., Mouihate, A., Urbani, P., Mackie, K., et al. (2005). Identification and Functional Characterization of Brainstem Cannabinoid CB2 Receptors. *Science* 310 (5746), 329–332. doi:10.1126/science.1115740

Wang, L. L., Zhao, R., Li, J. Y., Li, S. S., Liu, M., Wang, M., et al. (2016). Pharmacological Activation of Cannabinoid 2 Receptor Attenuates Inflammation, Fibrogenesis, and Promotes Re-epithelialization during Skin Wound Healing. *Eur. J. Pharmacol.* 786, 128–136. doi:10.1016/j.ejphar.2016.06.006

Williams, J. C., Appelberg, S., Goldberger, B. A., Klein, T. W., Sleasman, J. W., and Goodenow, M. M. (2014). Δ(9)-Tetrahydrocannabinol Treatment during Human Monocyte Differentiation Reduces Macrophage Susceptibility to HIV-1 Infection. *J. Neuroimmune Pharmacol.* 9 (3), 369–379. doi:10.1007/s11481-014-9527-3

Yuan, M., Kiertscher, S. M., Cheng, Q., Zoumalan, R., Tashkin, D. P., and Roth, M. D. (2002). Delta 9-Tetrahydrocannabinol Regulates Th1/Th2 Cytokine Balance in Activated Human T Cells. *J. Neuroimmunol* 133 (1-2), 124–131. doi:10.1016/s0165-5728(02)00370-3

Zhao, Y., Yuan, Z., Liu, Y., Xue, J., Tian, Y., Liu, W., et al. (2010). Activation of Cannabinoid CB2 Receptor Ameliorates Atherosclerosis Associated with Suppression of Adhesion Molecules. *J. Cardiovasc. Pharmacol.* 55 (3), 292–298. doi:10.1097/FJC.0b013e3181d2644d

Zhou, L., Zhou, S., Yang, P., Tian, Y., Feng, Z., Xie, X. Q., et al. (2018). Targeted Inhibition of the Type 2 Cannabinoid Receptor Is a Novel Approach to Reduce Renal Fibrosis. *Kidney Int.* 94 (4), 756–772. doi:10.1016/j.kint.2018.05.023

Zhou, X., Yang, L., Fan, X., Zhao, X., Chang, N., Yang, L., et al. (2020). Neutrophil Chemotaxis and NETosis in Murine Chronic Liver Injury via Cannabinoid Receptor 1/Gαi/o/ROS/P38 MAPK Signaling Pathway. *Cells* 9 (2). doi:10.3390/cells9020373

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 28 February 2022
doi: 10.3389/fphar.2022.812745

Candidate Therapeutics by Screening for Multitargeting Ligands: Combining the CB2 Receptor With CB1, PPARγ and 5-HT4 Receptors

*Shayma El-Atawneh and Amiram Goldblum**

Molecular Modelling and Drug Design Lab, Institute for Drug Research and Fraunhofer Project Center for Drug Discovery and Delivery, Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem, Israel

OPEN ACCESS

Edited by:
Frank Park, University of Tennessee Health Science Center (UTHSC), United States

Reviewed by:
Nadine Jagerovic, Spanish National Research Council (CSIC), Spain
Kirk Hevener, University of Tennessee Health Science Center (UTHSC), United States

***Correspondence:**
Amiram Goldblum
amiramg@ekmd.huji.ac.il

Specialty section:
This article was submitted to Experimental Pharmacology and Drug Discovery, a section of the journal Frontiers in Pharmacology

Received: *10 November 2021*
Accepted: *13 January 2022*
Published: *28 February 2022*

Citation:
El-Atawneh S and Goldblum A (2022) Candidate Therapeutics by Screening for Multitargeting Ligands: Combining the CB2 Receptor With CB1, PPARγ and 5-HT4 Receptors. Front. Pharmacol. 13:812745. doi: 10.3389/fphar.2022.812745

In recent years, the cannabinoid type 2 receptor (CB2R) has become a major target for treating many disease conditions. The old therapeutic paradigm of "one disease-one target-one drug" is being transformed to "complex disease-many targets-one drug." Multitargeting, therefore, attracts much attention as a promising approach. We thus focus on designing single multitargeting agents (MTAs), which have many advantages over combined therapies. Using our ligand-based approach, the "Iterative Stochastic Elimination" (ISE) algorithm, we produce activity models of agonists and antagonists for desired therapeutic targets and anti-targets. These models are used for sequential virtual screening and scoring large libraries of molecules in order to pick top-scored candidates for testing *in vitro* and *in vivo*. In this study, we built activity models for CB2R and other targets for combinations that could be used for several indications. Those additional targets are the cannabinoid 1 receptor (CB1R), peroxisome proliferator-activated receptor gamma (PPARγ), and 5-Hydroxytryptamine receptor 4 (5-HT4R). All these models have high statistical parameters and are reliable. Many more CB2R/CBIR agonists were found than combined CB2R agonists with CB1R antagonist activity (by 200 fold). CB2R agonism combined with PPARγ or 5-HT4R agonist activity may be used for treating Inflammatory Bowel Disease (IBD). Combining CB2R agonism with 5-HT4R generates more candidates (14,008) than combining CB2R agonism with agonists for the nuclear receptor PPARγ (374 candidates) from an initial set of ~2.1 million molecules. Improved enrichment of true vs. false positives may be achieved by requiring a better ISE score cutoff or by performing docking. Those candidates can be purchased and tested experimentally to validate their activity. Further, we performed docking to CB2R structures and found lower statistical performance of the docking ("structure-based") compared to ISE modeling ("ligand-based"). Therefore, ISE modeling may be a better starting point for molecular discovery than docking.

Keywords: cannabinoid receptors 2 (CB2R), multitargeting, ISE, virtual screening, inflammation, neuroprotective, IBD—inflammatory bowel diseases

1 INTRODUCTION

The cannabinoid receptors (CBRs) consist of cannabinoid receptors 1 (CB1R) and 2 (CB2R), which are members of the lipid class A G protein-coupled receptors (GPCRs) family. The CBRs participate in many physiological processes, including mood regulation, cognitive function, neuroprotection, nociception, cell growth and proliferation, appetite, and lipid metabolism (Stasiulewicz et al., 2020). Both are expressed in the central nervous system (CNS) and in peripheral tissues. CB2Rs have lower expression levels than CB1Rs in the CNS and are primarily expressed in immune cells (Wu, 2019). Their different expression regions in the brain suggest a neuroprotective role of CB2R, avoiding CB1R mediated side-effects (Deng et al., 2015). Moreover, CB2R expression can be upregulated in the brain under some pathological conditions (e.g., addiction, inflammation, anxiety), suggesting CB2R involvement in various psychiatric and neurological disorders (Wu, 2019).

In the brain, CB2R is proposed as a potential target for attenuating inflammation associated with neurodegenerative diseases (Alzheimer's disease (AD), Parkinson's disease (PD), and others) (Cassano et al., 2017; Bie et al., 2018; Kelly et al., 2020; Mecha et al., 2020). Several selective CB2R agonists exhibited analgesic activity in preclinical models of acute inflammatory, chronic, and neuropathic pain (Murineddu et al., 2013; Soliman et al., 2021). Its role is also investigated in mental disorders like schizophrenia, depression, anxiety, and addictions (García-Gutiérrez et al., 2010; García-Gutiérrez and Manzanares, 2010; Ortega-Alvaro et al., 2011; ZX et al., 2011; Jordan and Xi, 2019; ME et al., 2019). Other potential therapeutic areas of CB2Rs were explored: anti-cancer (Guzmán, 2003; Fernández-Ruiz et al., 2007), epilepsy (Ji et al., 2021), osteoporosis (Idris et al., 2005; Rossi et al., 2011), atopic dermatitis (Maekawa et al., 2006), (NCT00697710), ischemia/reperfusion injury (Bátkai et al., 2007; Rajesh et al., 2007), atherosclerosis (Mach et al., 2008), gastrointestinal inflammation (Wright et al., 2008) and disorders of reproduction (Maccarrone, 2008).

In the past 2 decades, treating multifactorial illnesses, i.e., infections, cancer, and CNS disorders, shifted towards multitargeting (Csermely et al., 2005; Hopkins et al., 2006; Boran and Iyengar, 2010; L.; Bolognesi, 2013; Bolognesi and Cavalli, 2016; Zhou et al., 2019). Simultaneous modulation of multiple targets may have better efficacy and safety profile than single targeted drugs, and the number of multitargeting new molecular entities is increasing over the years (Ramsay et al., 2018). The design of multitargeting agents (MTAs) assigns desired therapeutic targets and avoids targets associated with side effects ("anti targets"). In principle, MTA can be a single compound or a combination of compounds, each directed to a different target ("cocktails" or as a co-formulated drug-device), and both are used in the clinic. Despite the highly significant therapeutic relevance of combinatorial therapy (Conway and Cohen, 2010; Morphy, 2010; Wright, 2010; Modi et al., 2011; Lu et al., 2012), single MTA has substantial advantages over combination therapy: 1) more predictable pharmacokinetic profile 2) avoiding drug-drug interactions 3) easier dose regimen and higher compliance 4) enabling to overcome mutations in relevant diseases such as cancer, viral and bacterial ailments 5) simultaneous presence of the molecule in tissues where it is expected to affect and 6) an easier regulatory process (Hopkins, 2008; Anighoro et al., 2014).

Targets from different protein superfamilies may challenge the design of such MTAs, lacking shared/similar ligands or common structural motifs, which are sometimes the cause of side-effects (Morphy et al., 2004). Therefore such different targets may be of more interest. Nevertheless, single MTAs have been discovered (Ryckmans et al., 2002; Natesan Murugesan et al., 2004; Omar et al., 2018).

The broad involvement of CB2R in various disorders makes it a valuable target for multitargeting therapies while combining its modulation with affecting other relevant proteins in each disease. Several studies proposed its combination with other targets such as acetylcholinesterase (AChE) and butyrylcholinesterase for AD (Gonzalez-Naranjo et al., 2013; Dolles et al., 2016, 2018; González-Naranjo et al., 2019). Suggestions were also raised to find dual CB2R/histone deacetylases and CB2R/σ receptor compounds for treating cancer and neurodegenerative diseases (Mangiatordi et al., 2020), and to develop multitargeting analgesics (Maione et al., 2013). Here we shall focus on several possibilities of multitargeting CB2R with other targets.

1.1 Combined Effects of CB2 and CB1 Receptors

The CBRs play a critical role in several human physiological and pathological conditions. However, the CNS side effects of CB1R ligands may limit the therapeutic use of such agents if they cross the Blood-Brain Barrier (BBB). That is the case of the CB1R inverse agonists Rimonabant and Taranabant (Moreira and Crippa, 2009; Martín-García et al., 2010). To overcome the central effects, peripheral CB1R antagonists were developed (Chorvat, 2013; El-Atawneh et al., 2019; Quarta and Cota, 2020). Another option is to develop pure antagonists (An et al., 2020; Stasiulewicz et al., 2020). Agonists of the CBRs may be used to treat anxiety (Stasiulewicz et al., 2020) or as analgesics, anti-inflammatory, neuroprotective and anti-emetic compounds (An et al., 2020). Peripheral CB1R antagonists combined with CB2R agonists may be used for treating liver diseases (Mallat et al., 2011) and diabetic complications (Gruden et al., 2016). This dual activity may be useful in treating obesity, abolishing diabetes-induced albuminuria, inflammation, tubular injury, and renal fibrosis (Barutta et al., 2017). Combining CB1R antagonism with CB2R agonism in the brain is shown to have a synergistic effect on reward processing (Gobira et al., 2019). Another option is to design selective CB2R agonists to benefit from their nociception and neuroinflammation role without psychoactive effects (Hollinshead et al., 2013; Verty et al., 2015; Poleszak et al., 2020). CB2R selective agonists are investigated to treat pain, inflammation, arthritis, addictions, cancer besides their neuroprotective role (An et al., 2020).

1.2 Combined Effects at CB2R, PPARγ, and 5-HT4R

CB2R could be targeted with other receptors to attenuate inflammation for several autoimmune and inflammatory conditions. The peroxisome proliferator-activated receptor

(PPAR)-γ is a nuclear receptor that plays a crucial role in regulating lipid metabolism and glucose homeostasis. It associated with metabolic disorders, such as atherosclerosis, obesity, metabolic syndrome, dyslipidemias, type 2 diabetes, and cancer (Decara et al., 2020). PPARγ agonists have been shown to prevent inflammation, dermal fibrosis, and lipoatrophy in preclinical models of systemic sclerosis (SSc) (Wei et al., 2010). SSc is an orphan autoimmune multi-organic disease that affects the connective tissue. Dual CB2/PPARγ agonists such as VCE-004.8 and JBT-101 (Ajulemic acid, Lenabasum) have alleviated skin fibrosis and inflammation in SSc models (Rio et al., 2018; García-Martín et al., 2019). JBT-101 is in clinical trials for SSc (NCT03398837), dermatomyositis (NCT03813160), and cystic fibrosis (NCT02465450). Additionally, PPARγ agonists can suppress the pro-inflammatory cytokines associated with chronic diseases such as Inflammatory Bowel Disease (IBD).

IBD, including ulcerative colitis (UC) and Crohn's disease (CD), has been considered one of the most prevalent GI diseases with accelerating incidence in newly industrialized countries. Yet it lacks effective drug targets and medications (Seyedian et al., 2019). As a lifelong disease, therapy aims to induce remission in the short term and maintain remission in the long term. New drugs have diverse mechanisms of action, targeting mainly the inflammation pathways. The current anti-inflammatory small molecules used to treat IBD are associated with several side effects (5-amino salicylate and its prodrugs such as Olsalazine and Balsalazide), with more severe toxicity (Azathioprine, Mercaptopurine, Methotrexate) or with known long term negative impacts of steroid hormones (glucocorticoids). Biological drugs are expensive, require more intensive medical attention in a clinic or at home (self-injections), and, in the case of TNFalpha antibodies, elicit resistance by immune system response (Torres et al., 2020). Although the mechanism by which PPARγ acts on the pathogenesis of IBD has not been clarified (Decara et al., 2020), natural and chemical PPARγ ligands have ameliorated the fibrotic process in preliminary clinical trials and experimental models of intestinal fibrosis (Vetuschi et al., 2018). Moreover, many studies showed the anti-inflammatory role of PPARγ activation in intestinal tissues in UC and CD (Decara et al., 2020).

Recent investigations suggest that serotonin (5-HT) can influence the development and severity of inflammation within the gut, particularly in the setting of IBD. 5-HT influences every major function inherent to the gut, including motility, secretion, blood flow, and sensation (Coates et al., 2017). Alterations in its receptor activity in disease conditions may result in many problematic symptoms, including abdominal pain, diarrhea, or constipation (Coates et al., 2017). The 5-HT4 receptor (5-HT4R) mediates enteric neuron survival and neurogenesis of adult mice (Liu et al., 2009). It promotes the reconstruction of an enteric neural circuit leading to the recovery of the defecation reflex in the distal gut (Matsuyoshi et al., 2010). 5-HT4R activation maintains motility in healthy colons of mice and guinea pigs and reduces inflammation in colons of mice with colitis (Spohn et al., 2016). PPARγ and 5-HT4R agonists may be combined with CB2R as a potential therapy for IBD (Turcotte et al., 2016). A peripheral CB2R agonist (Olorinab) reached phase II trials for abdominal pain in CD (NCT03155945) and irritable bowel syndrome (NCT04043455).

1.3 Multitargeting in Silico

Computational methods allow us to examine options for designing or discovering multitargeting candidates in a reliable, fast, and low-cost manner (Sliwoski et al., 2014; Zhang et al., 2017). Screening candidates for binding against several targets to find single MTA differs from designing compounds based on conjugated pharmacophores by merging/fusing/linking molecules (Morphy and Rankovic, 2005; Zhou et al., 2019), which could take longer to synthesize and might increase the molecular weight and affect the drug-likeness properties.

Our research combines ligand and structure-based methods. Our algorithm for solving complex combinatorial problems, the 'Iterative stochastic elimination algorithm' (ISE) (Stern and Goldblum, 2014; El-Atawneh and Goldblum, 2017), has been applied in recent years to molecular discovery (Zatsepin et al., 2016; Da'adoosh et al., 2019; El-Atawneh et al., 2019), including one example of multitargeting modeling: modeling the properties of molecules that may be remotely loaded to nanoliposomes and the properties that enable them to be stable inside the nanoliposomes, in a biological fluid (Cern et al., 2017). Molecules that had high scores in both loading and stability models were chosen. For any discovery of MTAs, virtual screening (VS) by separate ligand-based models is performed in sequential order.

After finding top candidate ligands, it is helpful to examine the structural aspects, since our classifications are based on physicochemical properties and not on structural elements. Molecules with similar properties might have different structures and sizes. Thus, we dock the top candidates to the target protein if such a structure has been reported. Structures of CB2R were deposited recently in the Protein Data Bank (PDB), one with a bound antagonist (PDB code 5ZTY) (Li et al., 2019) and the other with an agonist (PDB code 6KPC) (Hua et al., 2020), which makes structure-based design feasible (Tuccinardi et al., 2006; Cichero et al., 2011). CB2R shares 44% sequence identity and 68% similarity with CB1R in the transmembrane regions (Munro et al., 1993). The antagonist-binding pockets in both receptors are quite distinct, while the agonist-binding pockets in CB1R and CB2R, including side-chain rotamers, of the key residues involved in ligands interactions are almost identical (Li et al., 2019; Hua et al., 2020), which might be the source of cross-reactivity between their ligands and difficulty in attaining selectivity. There are also CB1R and PPARγ structures, with agonists and antagonists in both. Yet, there is no published atomic-level structure of 5-HT4R, but ligand-based modeling for 5-HT4R with ISE is possible due to its many known ligands.

2 METHODS

2.1 Data Sets

2.1.1 Learning\Training Sets

Compounds with reported activity, agonists (EC_{50} values) and antagonists (k_i or IC_{50} values) at the different receptors were taken from the ChEMBL database (http://www.ebi.ac.uk/ChEMBLdb/) (Bento et al., 2014). Duplicates were removed

based on their simplified molecular input line entry specification (SMILES notation). Molecules with undefined potency values, error comments, and a confidence score below seven (reported at ChEMBL) were excluded, as well as molecules that are active above 100 μM. The active molecules were diluted with random molecules assumed to be inactive ("decoys") with a ratio of 1:100 (active: inactive) (Tropsha, 2010). Randoms were picked from the ZINC database (Sterling and Irwin, 2015), based on the "applicability domain" (APD) of the actives (Netzeva et al., 2005). The application of APD for picking randoms imposes to discover differences between active and inactive molecules with some basic similarities, thus making the task of classification more difficult. We apply APD by selecting random molecules for which the values of molecular weight (MW), calculated lipophilic character (clogP), hydrogen bond acceptors (HBA), and hydrogen bond donors (HBD) are within the average ± two standard deviations for these variables of the active molecules.

2.1.2 Screening Set

The Enamine HTS Collection (Enamine HTS Collection 2021), consisting of 2,159,632 compounds was used for VS in both ligand and structure-based methods.

2.2 Datasets Preparation

All molecules were prepared by the "Molecular Database Wash" (v. 2011.10) (Molecular Operating Environment, 2021). This includes hydrogen adjustment, removing minor components, determining the protonation state, enumeration of ionization states, and tautomer forms. Mutagenic and reactive molecules (based on calculated descriptors by MOE) were removed from the learning sets.

2.3 Descriptors Calculation

The standard descriptors we calculated for building the models are the 2-dimensional (2D) descriptors by QuaSAR- MOE (v.2011.10) with 186 descriptors. The complete descriptors list is given at (http://www.cadaster.eu/sites/cadaster.eu/files/challenge/descr.htm). Descriptors with low variance (Smialowski et al., 2010), or highly correlated descriptors (Pearson correlation coefficient > 0.9), were excluded, using the Knime platform (v. 4.0.1) (Berthold et al., 2008) to exclude out of two highly correlated descriptors the one which has greater similarity to other descriptors. We have also tested the performance of 3D descriptors for CB2R (see results and discussion).

2.4 Activity Models Constructed by the Iterative Stochastic Elimination Algorithm

Our generic ISE algorithm has been applied to many problems related to drug discovery and has been presented in reviews, with details of the mathematical and statistical criteria to distinguish between two activities based on physicochemical properties (descriptors) of known active vs. inactive compounds (Stern and Goldblum, 2014; El-Atawneh and Goldblum, 2017). For each model, five cross-validations were performed (James et al., 2013), with 4 out of the five-folds producing the model, and the fifth fold was used as a test set. We include some of the main details of model construction and screening in Supplementary Data **section 1.1**.

2.5 Tanimoto Fingerprint Similarity

The "Atom-pair" fingerprints for the active molecules were generated using RDKit toolkit (RDKit, 2018) (in Knime platform v. 4.0.1) (Berthold et al., 2008). The "Tanimoto similarity coefficient" (Tc) for the fingerprints is based on the CDK toolkit.

2.6 Docking

The two structures of CB2R were downloaded from the PDB (5ZTY (Li et al., 2019) and 6KPC (Hua et al., 2020)), and prepared by the "Protein Preparation Wizard" (Schrödinger Suit 2019-3) (Madhavi Sastry et al., 2013). For 5ZTY, we allowed C-OH rotations of SER90, THR114, TYR190; for 6KPC, we allowed such rotations of TYR25, SER90, THR114, TYR190, and SER285 for the grid construction. Alanine (ALA) scan was performed to assign the critical residues in the binding site of the two structures for 23 residues detected by PDBsum (Laskowski, 2009). The screened molecules were prepared using "LigPrep" (Schrödinger Release, 2018), with default settings, except the chirality option that was set to "Generate all combinations" for the Enamine database (5,024,833 entries were generated). Molecular docking was performed with Glide HTVS and SP (Richard A. Friesner et al., 2006).

In the docking analysis, we examined the geometric character of binding by requiring the docked molecules to be in contact with residues that were found to be "hot spots" by performing a virtual ALA scan.

3 RESULTS

3.1 Ligand-Based Approach

3.1.1 Iterative Stochastic Elimination Algorithm Activity Models

We constructed several models for each target based on the relevant molecular activity reported by ChEMBL. There are molecules reported as partial agonists and inverse agonists for the CB2R (access date: January/2016), and those were excluded from the present study. Some models were constructed with a subset of highly active molecules (i.e., activity values less than 5 nM or 10 nM) from the larger set of reported activities. We choose the best-performing model based on Matthews Correlation Coefficient (MCC, **Supplementary Data S1.1**) (Matthews, 1975), Area under the ROC curve (AUC), and the Enrichment Factor (EF, **Supplementary Data S1.1**) (**Table 1**). Only ten molecules were reported with IC_{50} activity for 5-HT4R (access date: December/2017), so we used the reported K_i values for constructing the antagonist models (reported for 227 molecules). For PPARγ (access date: February/2018) and 5-HT4R agonist models, we built only one model based on the available data. The PPARγ antagonist models (access date: October/2021) have similar performance, and we chose the K_i model because it has a better EF value. All models have good

TABLE 1 | Models of agonists and antagonists for the four receptors[a].

	Model	# Actives	# Randoms	Top MCC	Mean MCC[c]	AUC	EF[d]	# Filters
CB2R agonists	Model 1 (Actives < 100 μM)	1254	100000	0.61	0.57	0.87	11 (38)	3911
	Model 2 (Actives < 5 nM)[b]	275	30000	0.73	0.70	0.90	17 (54)	2933
CB2R antagonists	Model 1 (IC_{50} values, Actives < 100 μM)	689	70000	0.64	0.57	0.85	18 (71)	1738
	Model 2 (IC_{50} values, Actives < 50 nM)	198	22000	0.73	0.69	0.91	8 (34)	3832
	Model 3 (K_i values, Actives < 100 μM)[b]	2437	200000	0.67	0.63	0.92	17 (56)	2747
CB1R agonists	Model 1 (Actives < 100 μM)	513	53000	0.66	0.62	0.89	11 (23)	3273
	Model 2 (Actives < 100 nM)	183	25000	0.8	0.77	0.90	11 (26)	2951
	Model 3 (Actives < 50 nM)[b]	127	13000	0.83	0.79	0.92	12 (27)	2509
CB1R antagonists	Model 1 (Actives < 100 μM)	973	93000	0.7	0.65	0.9	14 (33)	2231
	Model 2 (IC_{50} values, Actives < 10 nM)[b]	296	33000	0.78	0.75	0.92	25 (50)	1399
	Model 3 (K_i values, Actives < 10 nM)	332	35000	0.75	0.7	0.91	20 (65)	1960
PPARγ agonists	Model 1 (Actives < 10 nM)[b]	243	50000	0.91	0.89	0.96	62 (130)	3299
PPARγ antagonists	Model 1 (IC_{50} values, Actives < 10 nM)	194	20000	0.91	0.86	0.98	37 (74)	2677
	Model 2 (K_i values, Actives < 100 nM)[b]	168	17000	0.93	0.91	0.96	71 (98)	682
5-HT4R agonists	Model 1 (Actives < 100 μM)[b]	155	35000	0.94	0.92	0.98	37 (94)	3122
5-HT4R antagonists	Model 1 (K_i values, Actives < 100 μM)	227	50000	0.85	0.81	0.96	20 (61)	1035
	Model 2 (K_i values, Actives < 50 nM)[b]	148	35000	0.94	0.92	0.98	29 (52)	1475

[a]*For each model, we present the number of active and random molecules used to generate the model, the top and average MCC of the filters, the AUC and EF values of the test set. Besides the number of the total filters generated by each model.*
[b]*The chosen models for VS.*
[c]*Mean MCC of the top 1000 filters.*
[d]*EF values above index cutoff = 0.7 are given in parenthesis.*
 # = number.

mean MCC values > 0.65, AUC > 0.9, and EF values vary from 12 to 71 with a positive (> 0.0) index cutoff. The learning sets' similarity is low for all chosen models (average Tc ≤ 0.5, **Supplementary Table S1**).

All constructed Models are presented in **Table 1**. The models used for screening are marked. Models constructed on the basis of active molecules with highest affinity (Nanmolar range) have better statistical parameters than those constructed on the basis of 100 μM activities, and were thus used for screening. That is the case of CB2R/CB1R/PPARγ agonists and antagonists, and 5HT4R antagonists. Only a single model of actives with lesser activity, of 5HT4R agonists, was used for screening. However the number of molecules with lesser affinity among the 155 used for modeling is small: only 5 molecules have EC_{50} values between 1 and 100 μM. Also, the 5HT4R model for agonists is the one with best statistical parameters compared to all other GPCR models for actives up to 100 μM.

3.1.1.1 Performance of 3D Descriptors

Taking the learning set of the chosen 2D-based CB2R agonist model (Model 2- with 275 active molecules < 5 nM diluted with 30,000 randoms), we built 3D and the 2D/3D combined descriptors' based models. The ISE agonist model based on 2D descriptors performed better than the 3D, and the 2D/3D combined descriptors by MCC, AUC, and EF (**Supplementary Table S2**). The 3D model has a lower mean MCC (0.5) and AUC (0.85) than the combined 2D/3D model.

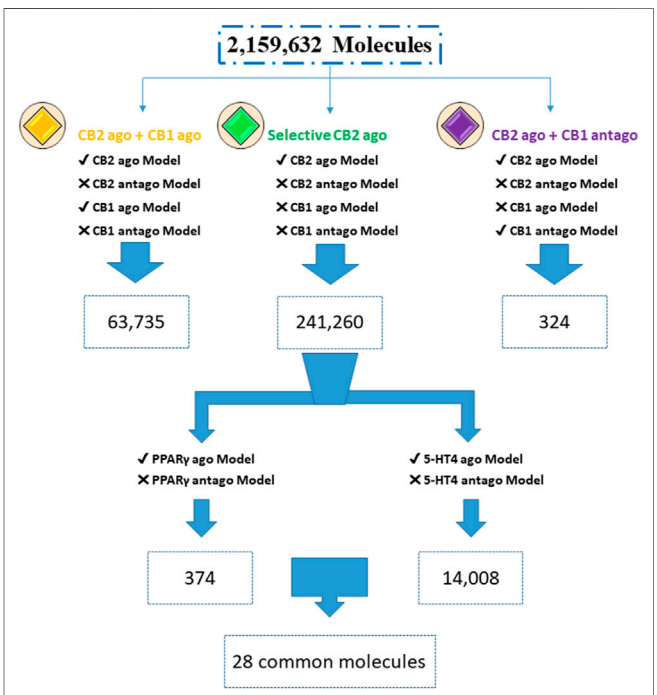


FIGURE 1 | Screening for multitargeted candidates. Enamine database (2,159,632 compounds) was screened through agonist (ago) and antagonist (antago) ISE models. Numbers are of molecules with a positive index for models with a "✓" symbol, while failing to pass the models is marked by "X" (due to a negative index).

3.2 Multitargeting Candidates

To find multitargeting candidates for the different indications, we performed hierarchical VS. First, focusing on the CBRs, we screened the Enamine database (DB) through the different CBR activity models, considering desired activity, i.e., of CB2R agonists, and the unwanted activity as anti targets. Molecules with a positive index pass the model, and those with a negative score

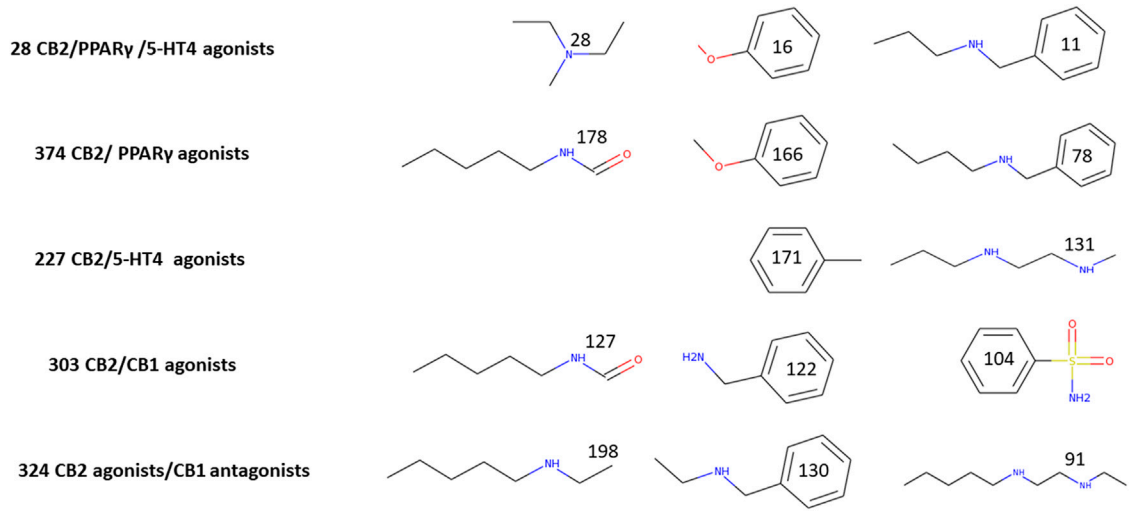


FIGURE 2 | Major common multitargeted substructures. The numbers on each substructure indicate the number of molecules that include it. We chose 303 top candidates (with index score >0.7) for assigning substructures to CB2R/CB1R agonists and 227 top candidates (Index > 0.7) for the substructures of CB2/5-HT4 agonists. Other substructures were assigned for sets with an index > 0.0.

are considered to fail. We found 241,260 CB2 selective agonists (about 11% of the dataset); those molecules passed the CB2R agonist model and did not pass the CB2R antagonist model. They also did not pass the CB1R agonist and antagonist models. Adding the CB1R agonists or antagonists to CB2 agonists, we found many less candidates (63,735 and 324, respectively), as shown in **Figure 1**. Raising the index cutoff above 0.0 reduces these numbers.

Looking for additional activities of the selective CB2R agonists, we screened those 241,260 candidates through the PPARγ and 5-HT4R agonist models (**Figure 1**). To avoid anti-targets we screened the same set by the antagonist models of PPARγ and 5-HT4R. This yielded 374 CB2R and PPARγ agonists, and 14,008 candidates for CB2R and 5-HT4R agonism with no antagonism at any of the three receptors. We found 28 candidate agonists for simultaneously hitting all the three targets of CB2R, PPARγ, and 5-HT4R. All the mentioned hit sets are internally diverse, as well as being diverse (by Tanimoto criteria) towards the actives used for model construction: comparisons yield a low average Tanimoto coefficient of Tc ≤ 0.4 (**Supplementary Table S3**).

3.2.1 Common Substructures for the Multitargeting Hits

Common substructures could be used to explain why molecules are candidates for binding and activating different receptors. We examined that possibility for each multitargeting set. To perform that task, we used Canvas (v. 4.2.012, Schrödinger Suit 2019-4) to find the maximum common substructure. In **Figure 2**, we display the major common substructures for five different groups: agonists of all three receptors, CB2R/PPARγ, CB2R/5-HT4R as well as CB2R/CB1R agonists and CB2Ragonists/CB1R antagonists. A larger scope of common substructures is presented in **Supplementary Figure S1**.

Figure 2 presents major substructure elements of top multitargeted screened molecules. It is easy to detect some of the fragments which appear in more than 20% of each multitargeted group: tertiary and secondary amines, benzylamine, anisol, alkyl chains with amines or amide, and benzenesulfonamide. It is noteworthy that all the 28 CB2R/PPARγ/5-HT4R multitargeted candidates have a tertiary amine moiety, which is not abundant in either CB2R/PPARγ or CB2R/5-HT4R. Two fragments of CB2R/PPARγ—anisol and N-butylbenzylamine contribute to the triple multitargeting, while the only fragment of the CB2R/5-HT4R in the triple target is a phenyl ring. All three structures common to CB2R agonists/CB1R antagonists are secondary amines. Only a single secondary amine is among the main fragments of CB2R/CB1R agonists, and the two others are an aromatic sulfonamide and an amide of N-pentylamine.

3.3 Structure-Based Confirmation of CB2R Ligands

The structures of CB2R (6KPC (Hua et al., 2020) with an agonist and 5ZTY (Li et al., 2019) with an antagonist) have similar binding pockets and binding residues (Li et al., 2019; Hua et al., 2020) (**Supplementary Table S4**). Similarity is also observed between the CB2R and CB1R binding pockets (Li et al., 2019). This creates an obstacle to distinguishing between agonist and antagonist activity for the CB2R if we consider docking alone. We examined the binding residues in both structures by applying a virtual ALA scan (Schrödinger Suit 2019-3) (Madhavi Sastry et al., 2013) for 23 residues in the binding site (**Supplementary Table S4**). AM12033 (6KPC-CB2R agonist) has 19 interactions, mainly with hydrophobic and aromatic residues and 3 H-bonds, with LEU 182 and SER285.

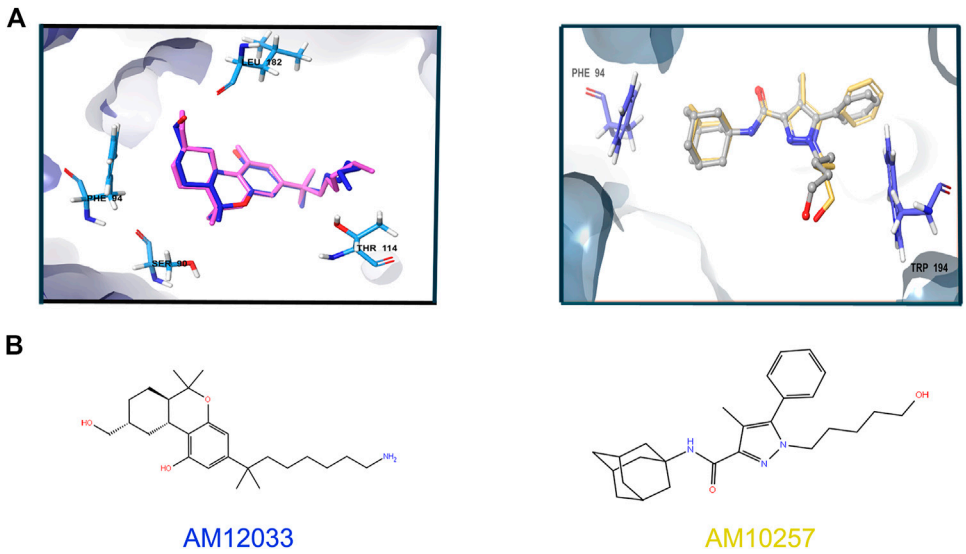


FIGURE 3 | Superimposition of the redocked ligands at 6KPC and 5ZTY. **(A)** Left: relevant residues at 6KPC are shown in azure sticks (SER90, PHE94, LEU182, THR114 and LEU182). The redocked agonist (AM12033, docking score = −12.2 kcal/mol)—blue aligned with the original ligand (pink), with RMSD = 0.94. Right: relevant residues (PHE94 and TRP194) at 5ZTY are shown in blue sticks. The redocked antagonist (AM10257, docking score = −10.8 kcal/mol)—yellow aligned with the original ligand (gray), with RMSD = 1.5. **(B)** 2D representation of the agonist and antagonist ligands.

AM10257 (5ZTY- CB2R antagonist) has 16 interactions with no H-bonds (as shown in PDBsum (Laskowski, 2009)).

The calculated stability for the 23 residues (by virtual ALA scan) does not differ dramatically between 6KPC and 5ZTY. The considered contacts in the 6KPC agonist structure in order to suggest more successful docked ligands are: hydrogen bonding with LEU182 and SER285, and Van der Waals (VDW) interactions with the following: TYR25, PHE87, PHE91, PHE94, ILE110, PHE183, TYR190, LEU191, TRP194, LEU262, MET265, PHE281.

3.3.1 Docking Validation

To choose one out of the two structures for detecting agonists and/or antagonists of CB2R, we constructed similar grids for the docking region in both structures, 6KPC and 5ZTY. We then redocked the ligands in both structures and performed cross-docking between the two. For 6KPC, the agonist, AM12033, got a better docking score (−12.2 kcal/mol) than the antagonist AM10257 (−8.7 kcal/mol). However, in 5ZTY, both agonist and antagonist got similar docking scores (−9.8 and −10.8 kcal/mol, respectively). The redocked positions of the agonist and antagonist are shown in **Figure 3**.

To further examine the binding of ligands to both structures, we docked overall 23 known ligands of CB2R and of CB1R with different selectivities (**Supplementary Table S5**) (An et al., 2020). Docking scores are not correlated with experimental K_i values (An et al., 2020) in **Supplementary Table S5**. Detailed interactions with binding site residues for the 19 ligands that passed docking to the 6KPC structure are listed in **Supplementary Table S6**. None of the interactions can be related to a specific activity. This is also seen in **Supplementary Figure S2**, where the best-docked ligand of each activity type is compared to the 6KPC ligand (AM12033). Finally, we screened the learning set of the CB2R agonist modeling (275 active molecules and 30,000 randoms), resulting in a very low AUC for docking to both 6KPC and 5ZTY: 0.45 and 0.44, respectively. The ISE model, however, got an AUC of 0.9. Due to the success in redocking an agonist, and the need for discovering agonists, we continued all docking experiments with 6KPC.

3.4 Virtual Screening: Ligand-Based vs. Structure-Based Methods

We compared ligand (ISE) and structure-based (docking) methods by performing VS of the Enamine DB (2,159,632 compounds) for CB2R. ISE screening is extremely fast compared to docking (**Figure 4**). A positive index in screening by the CB2R agonist model was assigned to 241,260 molecules. We pick molecules with higher indexes and better EF values to improve the quality of our candidates, thus resulting in fewer molecules. For example, with a high index cutoff ≥ 0.7, 41,102 molecules pass, and the EF equals 54. That EF is only 17 at a lower index cutoff >0.0 (for 241,260 molecules). Docking was applied to the ISE candidates with a positive index: SP docking to the 6KPC structure found 238,718 molecules with docking scores of 6.6 to −12.8 kcal/mol. Filtration was based on docking scores ≤ −9 kcal/mol and hydrogen bonds with LEU182 and SER285, to a final set of 131 candidates.

Docking to CB2R was performed in two stages with the same 6KPC structure. First, HTVS docking was executed for the whole Enamine DB. The docked poses have a docking score range from 10.4 to −12.5 kcal/mol. Molecules with docking scores of less than −9 kcal/mol were further docked by the SP protocol (130,358 molecules). Most of these molecules (130,080) passed SP with a 5.7 to −12.9 kcal/mol docking score. By picking those with a score better than −9 kcal/mol and hydrogen bonds with LEU182 and SER285, only 73 molecules remain. Ten out of the 73 docking hits

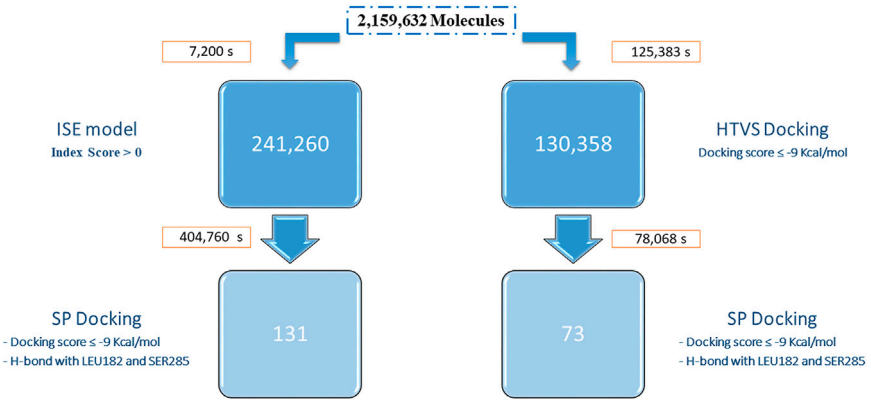


FIGURE 4 | Workflows of VS of the Enamine database by ISE **(left)** and by docking **(right)**. The screening times (in seconds) and the number of candidates are indicated for each step. The SP docking for the ISE hits was performed for 857,546 entries (generated by ligprep from the 241,260 candidates). The docking protocol (HTVS, on the right) was performed for 5,026,503 entries (generated by ligprep). Only 130,358 molecules passed the score filtration, and those continued to SP docking.

have positive ISE index scores. Only nine molecules are shared between the two SP screenings. Both sets are diverse from the known active CB2R agonists, and from each other (average Tc ~0.3).

4 DISCUSSION

The CBRs exert many physiological functions and are thus considered valuable therapeutic targets. CB2R, in particular, gains more attention due to its protective actions, involved in many pathological conditions such as cancer, CNS disorders, and a variety of disorders in the cardiovascular, gastrointestinal, and reproductive systems (Pacher and Mechoulam, 2011), while being devoid of psychoactive effects associated with the CB1R central activation. Finding single multitargeting agents (Morphy et al., 2004; Morphy and Rankovic, 2005; Zhang et al., 2017) for CB2R combined with other targets such as CB1R, PPARγ, and the 5-HT4R is not a trivial endeavor but one worth pursuing. Searching by virtual screening may suggest candidates in a shorter time than by *in vitro* screening and allows to test vast numbers of compounds. Our approach is to begin by constructing models for the binding or function of molecules at specific targets based on previously published results ("ligand-based" modeling). Our main tool for modeling is our ISE algorithm. The number of molecules for each model should not be less than a few dozens. Multitargeting requires to construct models for each of the relevant targets and anti-targets. If these models are of good quality, they may be used for VS, scoring, and sorting millions of molecules in a short time.

Here we present activity models built by the ISE algorithm for agonists and antagonists at each target. All models are statistically valid and should be useful (**Table 1**). The algorithm generates filters based on the ranges of physicochemical properties (computed) of known active molecules and randoms. Those filters are used for scoring by VS. It is noteworthy that the PPARγ and 5-HT4R models perform better than the models of CBRs. Their active sets are more similar (by Tc) than those of the CBRs, as shown in **Supplementary Table S1**. With an average Tc~0.5, these sets of agonists may still be considered to be diverse. For VS, we use filters with top MCC values up to 20% below the maximal value or just the best 1,000 filters.

Choosing between 2D and 3D descriptors depends on the problem we want to solve. Even though 3D descriptors are more representative, they don't yield better results, as have been studied in a large number and diverse range of applications over the past decades (Ekins et al., 2007). Some studies have shown that combining 2D and 3D molecular descriptors may improve models' performance (Yera et al., 2011; Kombo et al., 2013). But for the CB2R agonist model, both the 3D-based and combined 2D/3D models have lower performance than the 2D-based model as shown in **section 3.1.1.1**.

Screening through ISE models was performed to find MTAs for several target combinations which reflect different indications (**Figure 1**). First, we screened through CBR models, which are involved in many pathological disorders. CB2R selective agonists have neuroprotective and anti-inflammatory effects (An et al., 2020). It is possible to reduce the number of molecules by increasing the cutoff index above 0.0. The higher that index, there will be less molecules to test further—but the enrichment factor, with more "true positives" will be greater. By performing SP docking of 241,260 molecules, subsequent to ISE modeling, we got 131 candidates (**Figure 4**). We got more candidates when combining CB2R agonists with CB1R agonist activity (63,735) rather than with CB1R antagonist activity 324) (**Figure 1**). That may be due to the high degree of structural similarity in the orthosteric binding pockets between agonist-bound CB2R and CB1R structures (Shahbazi et al., 2020).

Combining CB2R ligands that are active at CB1R might elicit central side effects associated with the CB1R. Therefore, it is important to limit CB1R activity to the periphery and avoid central activities, either agonistic or antagonistic. By applying criteria for peripheral action of CB1R ligands, it is possible to combine with CB2R ligands, particularly the combination of CB2R agonists/CB1R antagonists. Those candidates may be

tested for multiple metabolic disorders, such as obesity and renal fibrosis (Barutta et al., 2017).

4.1 Some Implications of Ligand-Based Multitargeting

Multitargeting by ISE could be based on molecules with known activities on two or more targets. One publication mentions the construction of such a database, but it is not accessible (Chen et al., 2017). It is highly unlikely that enough molecules will be found to enable ISE modeling. Therefore, in the main spirit of ISE, each "variable" (in that case, a target, with many ligands as its "values") requires separate model construction. Screening and scoring through any single model reduce the molecular library size by 10-fold or more. In HTS, it is common to discover 1 out of 1,000 molecules tested for activity. However, that is a real activity *in vitro*, while we only suggest candidates for *in vitro* testing, which may include false positives. Therefore their numbers are much larger.

As we add more targets and anti-targets, the number of candidates decreases: we found, among our ~2.1 million screened molecules, only 374 candidates for combined (simultaneous) CB2R and PPARγ agonism, which may be tested for SSc (Wei et al., 2010), dermatomyositis, cystic fibrosis, and IBD (Decara et al., 2020). Adding 5-HT4R agonists reduces that number to 28, while CB2R and 5-HT4R agonists that could be valuable for IBD have 14,008 candidates. The much larger number of shared molecules that could hit CB2R and 5-HT4R (compared to sharing between CB2R and PPARγ) reflects the fact that both are aminergic GPCRs of the A family with 27% sequence similarity, as calculated by blastp (McGinnis and Madden, 2004), and may have a greater chance for ligand cross-reactivity (Yang et al., 2021). PPARγ belongs to a different family of cytoplasmic nuclear receptors. Moreover, only 60 molecules are shared between PPARγ and 5-HT4R agonists (without screening through CB2R models).

Screening by ISE models has already succeeded in achieving "scaffold hopping" (Zatsepin et al., 2016; Da'adoosh et al., 2019; El-Atawneh et al., 2019) due to the use of physicochemical properties rather than of structures. Even in those cases of greater similarity among the actives (agonists of PPARγ (0.52) and of 5-HT4R (0.5), **Supplementary Table S1**), the top screened candidates are varied among themselves, i.e., Tc = 0.4 for the 28 multitargeted agonists of CB2R/PPARγ/5-HT4R. That is also the case of screened molecules vs. actives in the learning sets (all results in **Supplementary Table S3**).

The main substructure elements presented in **Figure 2** may help to understand how it is possible that a single molecule binds to different binding sites: the amine moieties—frequently two amines in a molecule—are singly charged, and the first protonation reduces the pKa of the other amine. Amine protonation prevails in four out of the five multitargeted sets, except for CB2R/CB1R agonists in which a negative charge on the oxygen of the amides may have a leading role. It is also clear from the difference between the coupling of CB2R agonists with either CB1R agonists or antagonists, that it is possible to separate between these multitarget pairs. It would still be impossible to suggest a synthesis of multitargeted compounds based on these major fragments, but it is easy to pick molecules that contain these fragments for each multitargeted alternative by requiring to include these substructures with their statistical weight as in **Figure 2** or even better, as in **Supplementary Figure S1**. None of these moieties resemble the structures of known cannabinoid ligands (classical, non-classical, amino-alkylindoles, and those with the eicosanoid group).

4.2 The Impact of Structure-Based Modeling

Structures of CB2R have been recently deposited in the PDB (Li et al., 2019; Hua et al., 2020) and enable to perform structure-based studies—docking, pharmacophore, and molecular dynamics. The similarity between CB2R agonist/antagonist complexes and CB1R and CB2R structures make it challenging to design ligands with high selectivity (Hua et al., 2020). Docking is considered a time-consuming approach, as shown in **Figure 4**. Screening by docking has been shown to be much less reliable statistically than our ligand-based approach for CB2R agonism. Our ISE models screen molecules based on their properties and not on structural elements. That may result in top screened molecules having similar properties but different sizes and volumes, which may or may not be accommodated by the targets. Some of these molecules might not fit into binding sites and will be rejected. The results of our CB2R modeling confirm our preferable sequence of actions: ligand-based modeling should be followed by structure-based testing, which is better than structure-based docking alone.

Virtual ALA scan was used in this and other of our studies for picking "hot spots"—the main residues that contribute to the binding of smaller or larger ligands (i.e., including protein-protein interactions). Those "hot spots" determine the region of the grids for screening by docking and provide the initial geometric criteria that are applied prior to considering the docking scores. In ALA scan, we replace a larger side chain (of 18 amino acids, except for GLY and ALA) with a shorter one. We do not however apply any minimization or dynamics to that change, which positions a methyl group in the Cβ position, with tetrahedral angles vis-à-vis Cα, in place of a longer side chain, leaving some "void". No other side chain position is modified around the virtually mutated one. This protocol is due to our wish to discover molecules that replace an existing ligand/protein with an exact similar conformation of side chains in the protein target, as in the PDB, in order to promote competition. That is clearly not the case with genetically mutated ALA scan. In that *in vitro* experiment, other side chains could change their conformations in the vicinity and more remote from the ALA mutated position. *In vitro* ALA scan may even change conformations of the main protein chain. Therefore, it is rewarding if mutagenesis studies support some of our results such as for PHE87, PHE91, PHE94, HIS95 (Li et al., 2019), and TRP194 (Zhang et al., 2011). TYR190 mutation to Ile resulted in a loss of ligand recognition and function (McAllister et al., 2002).

This is a theoretical study, which includes statistics (AUC, EF) that clarify what are the chances for discovering multitargeted actives. Naturally, the next step is to pick top candidates from each set for biochemical experiments. Our multitargeting results also suggest which multitargeting sets have a greater chance to be experimentally confirmed. Previously, we published our theoretical predictions and experimental validations of the binding of 8 molecules

out of 15 predicted candidates (picked by ISE modeling from a library of 1.8 million) (El-Atawneh et al., 2019). Finally, only *in vitro* testing of candidates predicted by each method *in silico* will confirm or refute the VS results conducted by ISE and docking approaches.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

SE-A performed the research and wrote the first draft of this article. AG revised the article and developed the ISE algorithm.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.812745/full#supplementary-material

REFERENCES

An, D., Peigneur, S., Hendrickx, L. A., and Tytgat, J. (2020). Targeting Cannabinoid Receptors: Current Status and Prospects of Natural Products. *Int. J. Mol. Sci.* 21 (21), 5064. doi:10.3390/IJMS21145064

Anighoro, A., Bajorath, J., and Rastelli, G. (2014). Polypharmacology: Challenges and Opportunities in Drug Discovery. *J. Med. Chem.* 57, 7874–7887. doi:10.1021/jm5006463

Barutta, F., Grimaldi, S., Gambino, R., Vemuri, K., Makriyannis, A., Annaratone, L., et al. (2017). Dual Therapy Targeting the Endocannabinoid System Prevents Experimental Diabetic Nephropathy. *Nephrol. Dial. Transpl.* 32, 1655–1665. doi:10.1093/ndt/gfx010

Bátkai, S., Osei-Hyiaman, D., Pan, H., El-Assal, O., Rajesh, M., Mukhopadhyay, P., et al. (2007). Cannabinoid-2 Receptor Mediates protection against Hepatic Ischemia/reperfusion Injury. *FASEB J.* 21, 1788–1800. doi:10.1096/fj.06-7451com

Bento, A. P., Gaulton, A., Hersey, A., Bellis, L. J., Chambers, J., Davies, M., et al. (2014). The ChEMBL Bioactivity Database: an Update. *Nucleic Acids Res.* 42, D1083–D1090. doi:10.1093/nar/gkt1031

Berthold, M. R., Cebron, N., Dill, F., Gabriel, T. R., Kötter, T., Meinl, T., et al. (2008). *KNIME: The Konstanz Information Miner*. Berlin, Heidelberg: Springer, 319–326. doi:10.1007/978-3-540-78246-9_38

Bie, B., Wu, J., Foss, J. F., and Naguib, M. (2018). An Overview of the Cannabinoid Type 2 Receptor System and its Therapeutic Potential. *Curr. Opin. Anaesthesiol.* 31, 407–414. doi:10.1097/ACO.0000000000000616

Bolognesi, M. L., and Cavalli, A. (2016). Multitarget Drug Discovery and Polypharmacology. *ChemMedChem* 11, 1190–1192. doi:10.1002/CMDC.201600161

Bolognesi, M. L. (2013). Polypharmacology in a Single Drug: Multitarget Drugs. *Curr. Med. Chem.* 20, 1639–1645. doi:10.2174/0929867311320130004

Boran, A. D., and Iyengar, R. (2010). Systems Approaches to Polypharmacology and Drug Discovery. *Curr. Opin. Drug Discov. Devel.* 13, 297–309.

Cassano, T., Calcagnini, S., Pace, L., De Marco, F., Romano, A., and Gaetani, S. (2017). Cannabinoid Receptor 2 Signaling in Neurodegenerative Disorders: From Pathogenesis to a Promising Therapeutic Target. *Front. Neurosci.* 11, 30. doi:10.3389/FNINS.2017.00030

Cern, A., Marcus, D., Tropsha, A., Barenholz, Y., and Goldblum, A. (2017). New Drug Candidates for Liposomal Delivery Identified by Computer Modeling of Liposomes' Remote Loading and Leakage. *J. Control Release* 252, 18–27. doi:10.1016/j.jconrel.2017.02.015

Chen, C., Wu, M., Cen, S., Wu, J., and Zhou, J. (2017). MTLD, a Database of Multiple Target Ligands, the Updated Version. *Molecules* 22. doi:10.3390/molecules22091375

Chorvat, R. J. (2013). Peripherally Restricted CB1 Receptor Blockers. *Bioorg. Med. Chem. Lett.* 23, 4751–4760. doi:10.1016/j.bmcl.2013.06.066

Cichero, E., Ligresti, A., Allarà, M., Di Marzo, V., Lazzati, Z., D'Ursi, P., et al. (2011). Homology Modeling in Tandem with 3D-QSAR Analyses: A Computational Approach to Depict the Agonist Binding Site of the Human CB2 Receptor. *Eur. J. Med. Chem.* 46, 4489–4505. doi:10.1016/J.EJMECH.2011.07.023

Coates, M. D., Tekin, I., Vrana, K. E., and Mawe, G. M. (2017). Review Article: the many Potential Roles of Intestinal Serotonin (5-hydroxytryptamine, 5-HT) Signalling in Inflammatory Bowel Disease. *Aliment. Pharmacol. Ther.* 46, 569–580. doi:10.1111/APT.14226

Conway, D., and Cohen, J. A. (2010). Combination Therapy in Multiple Sclerosis. *Lancet Neurol.* 9, 299–308. doi:10.1016/S1474-4422(10)70007-7

Csermely, P., Agoston, V., Pongor, S., Ágoston, V., and Pongor, S. (2005). The Efficiency of Multi-Target Drugs: the Network Approach Might Help Drug Design. *Trends Pharmacol. Sci.* 26, 178–182. doi:10.1016/j.tips.2005.02.007

Da'adoosh, B., Marcus, D., Rayan, A., King, F., Che, J., and Goldblum, A. (2019). Discovering Highly Selective and Diverse PPAR-delta Agonists by Ligand Based Machine Learning and Structural Modeling. *Sci. Rep.* 9, 1106. doi:10.1038/s41598-019-38508-8

Decara, J., Rivera, P., López-Gambero, A. J., Serrano, A., Pavón, F. J., Baixeras, E., et al. (2020). Peroxisome Proliferator-Activated Receptors: Experimental Targeting for the Treatment of Inflammatory Bowel Diseases. *Front. Pharmacol.* 11, 730. doi:10.3389/FPHAR.2020.00730

Del Rio, C., Cantarero, I., Palomares, B., Gómez-Cañas, M., Fernández-Ruiz, J., Pavicic, C., et al. (2018). VCE-004.3, a Cannabidiol Aminoquinone Derivative, Prevents Bleomycin-Induced Skin Fibrosis and Inflammation through PPARγ- and CB2 Receptor-dependent Pathways. *Br. J. Pharmacol.* 175, 3813–3831. doi:10.1111/BPH.14450

Deng, L., Guindon, J., Cornett, B. L., Makriyannis, A., Mackie, K., and Hohmann, A. G. (2015). Chronic Cannabinoid Receptor 2 Activation Reverses Paclitaxel Neuropathy without Tolerance or Cannabinoid Receptor 1-dependent Withdrawal. *Biol. Psychiatry* 77, 475–487. doi:10.1016/J.BIOPSYCH.2014.04.009

Dolles, D., Hoffmann, M., Gunesch, S., Marinelli, O., Möller, J., Santoni, G., et al. (2018). Structure-Activity Relationships and Computational Investigations into the Development of Potent and Balanced Dual-Acting Butyrylcholinesterase Inhibitors and Human Cannabinoid Receptor 2 Ligands with Pro-cognitive *In Vivo* Profiles. *J. Med. Chem.* 61, 1646–1663. doi:10.1021/ACS.JMEDCHEM.7B01760

Dolles, D., Nimczick, M., Scheiner, M., Ramler, J., Stadtmüller, P., Sawatzky, E., et al. (2016). Aminobenzimidazoles and Structural Isomers as Templates for Dual-Acting Butyrylcholinesterase Inhibitors and hCB2 R Ligands to Combat Neurodegenerative Disorders. *ChemMedChem* 11, 1270–1283. doi:10.1002/CMDC.201500418

Ekins, S., Mestres, J., and Testa, B. (2007). *In Silico* pharmacology for Drug Discovery: Methods for Virtual Ligand Screening and Profiling. *Br. J. Pharmacol.* 152, 9–20. doi:10.1038/sj.bjp.0707305

El-Atawneh, S., Hirsch, S., Hadar, R., Tam, J., and Goldblum, A. (2019). Prediction and Experimental Confirmation of Novel Peripheral Cannabinoid-1 Receptor Antagonists. *J. Chem. Inf. Model.* 59, 3996–4006. doi:10.1021/acs.jcim.9b00577

El-Atawneh, S., and Goldblum, A. (2017). Iterative Stochastic Elimination for Discovering Hits and Leads. *Chim. Oggi-chemistry TODAY* 35, 41–46.

Enamine HTS Collection (2021). HTS Collection. Available at: https://enamine.net/ (Accessed December 15, 2021).

Fernández-Ruiz, J., Romero, J., Velasco, G., Tolón, R. M., Ramos, J. A., and Guzmán, M. (2007). Cannabinoid CB2 Receptor: a New Target for Controlling Neural Cell Survival? *Trends Pharmacol. Sci.* 28, 39–45. doi:10.1016/J.TIPS.2006.11.001

Friesner, R. A., Murphy, R. B., Repasky, M. P., Frye, L. L., Greenwood, J. R., Halgren, T. A., et al. (2006). Extra Precision glide: Docking and Scoring

Incorporating a Model of Hydrophobic Enclosure for Protein-Ligand Complexes. *J. Med. Chem.* 49 (21), 6177–6196. doi:10.1021/JM051256O

García-Gutiérrez, M., Pérez-Ortiz, J., Gutiérrez-Adán, A., and Manzanares, J. (2010). Depression-resistant Endophenotype in Mice Overexpressing Cannabinoid CB2 Receptors. *Br. J. Pharmacol.* 160, 1773–1784. doi:10.1111/J.1476-5381.2010.00819.X

García-Gutiérrez, M. S., and Manzanares, J. (2010). Overexpression of CB2 Cannabinoid Receptors Decreased Vulnerability to Anxiety and Impaired Anxiolytic Action of Alprazolam in Mice. *J. Psychopharmacol.* 25, 111–120. doi:10.1177/0269881110379507

García-Martín, A., Garrido-Rodríguez, M., Navarrete, C., Caprioglio, D., Palomares, B., DeMesa, J., et al. (2019). Cannabinoid Derivatives Acting as Dual PPARγ/CB2 Agonists as Therapeutic Agents for Systemic Sclerosis. *Biochem. Pharmacol.* 163, 321–334. doi:10.1016/J.BCP.2019.02.029

Gobira, P. H., Oliveira, A. C., Gomes, J. S., da Silveira, V. T., Asth, L., Bastos, J. R., et al. (2019). Opposing Roles of CB1 and CB2 Cannabinoid Receptors in the Stimulant and Rewarding Effects of Cocaine. *Br. J. Pharmacol.* 176, 1541–1551. doi:10.1111/BPH.14473

González-Naranjo, P., Campillo, N. E., Páez, J. A., and Paez, A. (2013). Multitarget Cannabinoids as Novel Strategy for Alzheimer Disease. *Curr. Alzheimer Res.* 10, 229–239. doi:10.2174/1567205011310030002

González-Naranjo, P., Pérez-Macias, N., Pérez, C., Roca, C., Vaca, G., Girón, R., et al. (2019). Indazolylketones as New Multitarget Cannabinoid Drugs. *Eur. J. Med. Chem.* 166, 90–107. doi:10.1016/J.EJMECH.2019.01.030

Gruden, G., Barutta, F., Kunos, G., and Pacher, P. (2016). Role of the Endocannabinoid System in Diabetes and Diabetic Complications. *Br. J. Pharmacol.* 173, 1116–1127. doi:10.1111/BPH.13226

Guzmán, M. (2003). Cannabinoids: Potential Anticancer Agents. *Nat. Rev. Cancer* 3, 745–755. doi:10.1038/nrc1188

Hollinshead, S. P., Tidwell, M. W., Palmer, J., Guidetti, R., Sanderson, A., Johnson, M. P., et al. (2013). Selective Cannabinoid Receptor Type 2 (CB2) Agonists: Optimization of a Series of Purines Leading to the Identification of a Clinical Candidate for the Treatment of Osteoarthritic Pain. *J. Med. Chem.* 56, 5722–5733. doi:10.1021/JM400305D

Hopkins, A. L., Mason, J. S., and Overington, J. P. (2006). Can We Rationally Design Promiscuous Drugs? *Curr. Opin. Struct. Biol.* 16, 127–136. doi:10.1016/j.sbi.2006.01.013

Hopkins, A. L. (2008). Network Pharmacology: the Next Paradigm in Drug Discovery. *Nat. Chem. Biol.* 4, 682–690. doi:10.1038/nchembio.118

Hua, T., Li, X., Wu, L., Iliopoulos-Tsoutsouvas, C., Wang, Y., Wu, M., et al. (2020). Activation and Signaling Mechanism Revealed by Cannabinoid Receptor-Gi Complex Structures. *Cell* 180, 655–e18. e18. doi:10.1016/J.CELL.2020.01.008

Idris, A. I., van 't Hof, R. J., Greig, I. R., Ridge, S. A., Baker, D., Ross, R. A., et al. (2005). Regulation of Bone Mass, Bone Loss and Osteoclast Activity by Cannabinoid Receptors. *Nat. Med.* 11, 774–779. doi:10.1038/nm1255

James, G., Witten, D., Hastie, T., and Tibshirani, R. (2013). *An Introduction to Statistical Learning with Applications in R*. Springer, 1–14. doi:10.1007/978-1-4614-7138-7_1

Ji, X., Zeng, Y., and Wu, J. (2021). The CB2 Receptor as a Novel Therapeutic Target for Epilepsy Treatment. *Int. J. Mol. Sci.* 22, 8961. doi:10.3390/IJMS22168961

Jordan, C. J., and Xi, Z. X. (2019). Progress in Brain Cannabinoid CB2 Receptor Research: From Genes to Behavior. *Neurosci. Biobehav. Rev.* 98, 208–220. doi:10.1016/J.NEUBIOREV.2018.12.026

Kelly, R., Joers, V., Tansey, M. G., McKernan, D. P., and Dowd, E. (2020). Microglial Phenotypes and Their Relationship to the Cannabinoid System: Therapeutic Implications for Parkinson's Disease. *Molecules* 25, 453. doi:10.3390/MOLECULES25030453

Kombo, D. C., Tallapragada, K., Jain, R., Chewning, J., Mazurov, A. A., Speake, J. D., et al. (2013). 3D Molecular Descriptors Important for Clinical success. *J. Chem. Inf. Model.* 53, 327–342. doi:10.1021/CI300445E/SUPPL_FILE/CI300445E_SI_005.PDF

Laskowski, R. A. (2009). PDBsum New Things. *Nucleic Acids Res.* 37, D355–D359. doi:10.1093/nar/gkn860

Li, X., Hua, T., Vemuri, K., Ho, J. H., Wu, Y., Wu, L., et al. (2019). Crystal Structure of the Human Cannabinoid Receptor CB2. *Cell* 176, 459–e13. e13. doi:10.1016/j.cell.2018.12.011

Liu, M. T., Kuan, Y. H., Wang, J., Hen, R., and Gershon, M. D. (2009). 5-HT4 Receptor-Mediated Neuroprotection and Neurogenesis in the Enteric Nervous System of Adult Mice. *J. Neurosci.* 29, 9683–9699. doi:10.1523/JNEUROSCI.1145-09.2009

Lu, X., Xiao, L., Wang, L., and Ruden, D. M. (2012). Hsp90 Inhibitors and Drug Resistance in Cancer: the Potential Benefits of Combination Therapies of Hsp90 Inhibitors and Other Anti-cancer Drugs. *Biochem. Pharmacol.* 83, 995–1004. doi:10.1016/j.bcp.2011.11.011

Maccarrone, M. (2008). CB2 Receptors in Reproduction. *Br. J. Pharmacol.* 153, 189–198. doi:10.1038/sj.bjp.0707444

Mach, F., Montecucco, F., and Steffens, S. (2008). Cannabinoid Receptors in Acute and Chronic Complications of Atherosclerosis. *Br. J. Pharmacol.* 153, 290–298. doi:10.1038/sj.bjp.0707517

Maekawa, T., Nojima, H., Kuraishi, Y., and Aisaka, K. (2006). The Cannabinoid CB2 Receptor Inverse Agonist JTE-907 Suppresses Spontaneous Itch-Associated Responses of NC Mice, a Model of Atopic Dermatitis. *Eur. J. Pharmacol.* 542, 179–183. doi:10.1016/J.EJPHAR.2006.05.040

Maione, S., Costa, B., and Di Marzo, V. (2013). Endocannabinoids: A Unique Opportunity to Develop Multitarget Analgesics. *Pain* 154, S87–S93. doi:10.1016/J.PAIN.2013.03.023

Mallat, A., Teixeira-Clerc, F., Deveaux, V., Manin, S., and Lotersztajn, S. (2011). The Endocannabinoid System as a Key Mediator during Liver Diseases: New Insights and Therapeutic Openings. *Br. J. Pharmacol.* 163, 1432–1440. doi:10.1111/J.1476-5381.2011.01397.X

Mangiatordi, G. F., Intranuovo, F., Delre, P., Abatematteo, F. S., Abate, C., Niso, M., et al. (2020). Cannabinoid Receptor Subtype 2 (CB2R) in a Multitarget Approach: Perspective of an Innovative Strategy in Cancer and Neurodegeneration. *J. Med. Chem.* 63, 14448–14469. doi:10.1021/ACS.JMEDCHEM.0C01357

Martín-García, E., Burokas, A., Martín, M., Berrendero, F., Rubí, B., Kiesselbach, C., et al. (2010). Central and Peripheral Consequences of the Chronic Blockade of CB1cannabinoid Receptor with Rimonabant or Taranabant. *J. Neurochem.* 112, 1338–13351. doi:10.1111/J.1471-4159.2009.06549.X

Matsuyoshi, H., Kuniyasu, H., Okumura, M., Misawa, H., Katsui, R., Zhang, G. X., et al. (2010). A 5-Ht(4)-Receptor Activation-Induced Neural Plasticity Enhances *In Vivo* Reconstructs of Enteric Nerve Circuit Insult. *Neurogastroenterol. Motil.* 22, 806–e226. doi:10.1111/J.1365-2982.2010.01474.X

Matthews, B. W. (1975). Comparison of the Predicted and Observed Secondary Structure of T4 Phage Lysozyme. *Biochim. Biophys. Acta* 405, 442–451. doi:10.1016/0005-2795(75)90109-9

McAllister, S. D., Tao, Q., Barnett-Norris, J., Buehner, K., Hurst, D. P., Guarnieri, F., et al. (2002). A Critical Role for a Tyrosine Residue in the Cannabinoid Receptors for Ligand Recognition. *Biochem. Pharmacol.* 63, 2121–2136. doi:10.1016/S0006-2952(02)01031-6

McGinnis, S., and Madden, T. L. (2004). BLAST: at the Core of a Powerful and Diverse Set of Sequence Analysis Tools. *Nucleic Acids Res.* 32, W20–W25. doi:10.1093/NAR/GKH435

Mecha, M., Carrillo-Salinas, F. J., Feliú, A., Mestre, L., and Guaza, C. (2020). Perspectives on Cannabis-Based Therapy of Multiple Sclerosis: A Mini-Review. *Front. Cell. Neurosci.* 14, 34. doi:10.3389/FNCEL.2020.00034

Modi, S., Stopeck, A., Linden, H., Solit, D., Chandarlapaty, S., Rosen, N., et al. (2011). HSP90 Inhibition Is Effective in Breast Cancer: a Phase II Trial of Tanespimycin (17-AAG) Plus Trastuzumab in Patients with HER2-Positive Metastatic Breast Cancer Progressing on Trastuzumab. *Clin. Cancer Res.* 17, 5132–5139. doi:10.1158/1078-0432.CCR-11-0072

Molecular Operating Environment (MOE) (2021). *Molecular Operating Environment*. Montreal, QC, Canada: Chemical Computing Group Inc.

Moreira, F. A., and Crippa, J. A. (2009). The Psychiatric Side-Effects of Rimonabant. *Braz. J. Psychiatry* 31, 145–153. doi:10.1590/S1516-44462009000200012

Morphy, R., Kay, C., and Rankovic, Z. (2004). From Magic Bullets to Designed Multiple Ligands. *Drug Discov. Today* 9, 641–651. doi:10.1016/S1359-6446(04)03163-0

Morphy, R., and Rankovic, Z. (2005). Designed Multiple Ligands. An Emerging Drug Discovery Paradigm. *J. Med. Chem.* 48, 6523–6543. doi:10.1021/jm058225d

Morphy, R. (2010). Selectively Nonselective Kinase Inhibition: Striking the Right Balance. *J. Med. Chem.* 53, 1413–1437. doi:10.1021/jm901132v

Munro, S., Thomas, K. L., and Abu-Shaar, M. (1993). Molecular Characterization of a Peripheral Receptor for Cannabinoids. *Nature* 365, 61–65. doi:10.1038/365061a0

Murineddu, G., Deligia, F., Dore, A., Pinna, G., Asproni, B., and Pinna, G. A. (2013). Different Classes of CB2 Ligands Potentially Useful in the Treatment of Pain. *Recent Pat. CNS Drug Discov.* 8, 42–69. doi:10.2174/1574889811207990016

Murugesan, N., Gu, Z., Fadnis, L., Tellew, J. E., Baska, R. A., Yang, Y., et al. (2004). Dual Angiotensin II and Endothelin A Receptor Antagonists: Synthesis of 2'-substituted N-3-Isoxazolyl Biphenylsulfonamides with Improved Potency and Pharmacokinetics. *J. Med. Chem.* 48, 171–179. doi:10.1021/JM049548X

Netzeva, T. I., Worth, A., Aldenberg, T., Benigni, R., Cronin, M. T., Gramatica, P., et al. (2005). Current Status of Methods for Defining the Applicability Domain of (Quantitative) Structure-Activity Relationships. The Report and Recommendations of ECVAM Workshop 52. *Altern. Lab. Anim.* 33, 155–173. doi:10.1177/026119290503300209

Omar, Y. M., Abdu-Allah, H. H. M., and Abdel-Moty, S. G. (2018). Synthesis, Biological Evaluation and Docking Study of 1,3,4-Thiadiazole-Thiazolidinone Hybrids as Anti-inflammatory Agents with Dual Inhibition of COX-2 and 15-LOX. *Bioorg. Chem.* 80, 461–471. doi:10.1016/J.BIOORG.2018.06.036

Ortega-Alvaro, A., Aracil-Fernández, A., García-Gutiérrez, M. S., Navarrete, F., and Manzanares, J. (2011). Deletion of CB2 Cannabinoid Receptor Induces Schizophrenia-Related Behaviors in Mice. *Neuropsychopharmacology* 36, 1489–1504. doi:10.1038/npp.2011.34

Pacher, P., and Mechoulam, R. (2011). Is Lipid Signaling through Cannabinoid 2 Receptors Part of a Protective System? *Prog. Lipid Res.* 50, 193–211. doi:10.1016/J.PLIPRES.2011.01.001

Poleszak, E., Wośko, S., Sławińska, K., Wyska, E., Szopa, A., Sobczyński, J., et al. (2020). Ligands of the CB2 Cannabinoid Receptors Augment Activity of the Conventional Antidepressant Drugs in the Behavioural Tests in Mice. *Behav. Brain Res.* 378, 112297. doi:10.1016/J.BBR.2019.112297

Quarta, C., and Cota, D. (2020). Anti-obesity Therapy with Peripheral CB1 Blockers: from Promise to Safe(?) Practice. *Int. J. Obes. (Lond)* 44, 2179–2193. doi:10.1038/s41366-020-0577-8

Rajesh, M., Pan, H., Mukhopadhyay, P., Bátkai, S., Osei-Hyiaman, D., Haskó, G., et al. (2007). Cannabinoid-2 Receptor Agonist HU-308 Protects against Hepatic Ischemia/reperfusion Injury by Attenuating Oxidative Stress, Inflammatory Response, and Apoptosis. *J. Leukoc. Biol.* 82, 1382–1389. doi:10.1189/jlb.0307180

Ramsay, R. R., Popovic-Nikolic, M. R., Nikolic, K., Uliassi, E., and Bolognesi, M. L. (2018). A Perspective on Multi-Target Drug Discovery and Design for Complex Diseases. *Clin. Transl. Med.* 7, 3. doi:10.1186/s40169-017-0181-2

RDKit (2018). Open-Source Cheminformatics Software. Available at: http://www.rdkit.org/ (Accessed March 27, 2018).

Rossi, F., Bellini, G., Luongo, L., Torella, M., Mancusi, S., De Petrocellis, L., et al. (2011). The Endovanilloid/endocannabinoid System: A New Potential Target for Osteoporosis Therapy. *Bone* 48, 997–1007. doi:10.1016/J.BONE.2011.01.001

Ryckmans, T., Balançon, L., Berton, O., Genicot, C., Lamberty, Y., Lallemand, B., et al. (2002). First Dual NK(1) Antagonists-Serotonin Reuptake Inhibitors: Synthesis and SAR of a New Class of Potential Antidepressants. *Bioorg. Med. Chem. Lett.* 12, 261–264. doi:10.1016/S0960-894X(01)00727-2

Sastry, G. M., Adzhigirey, M., Day, T., Annabhimoju, R., and Sherman, W. (2013). Protein and Ligand Preparation: Parameters, Protocols, and Influence on Virtual Screening Enrichments. *J. Comput. Aided. Mol. Des.* 27, 221–234. doi:10.1007/s10822-013-9644-8

Schrödinger Release (2018). *Schrödinger Release 2018-2*. New York, NY: LigPrep, Schrödinger, LLCLigPrep.

Seyedian, S. S., Nokhostin, F., and Malamir, M. D. (2019). A Review of the Diagnosis, Prevention, and Treatment Methods of Inflammatory Bowel Disease. *J. Med. Life* 12, 113–122. doi:10.25122/JML-2018-0075

Shahbazi, F., Grandi, V., Banerjee, A., and Trant, J. F. (2020). Cannabinoids and Cannabinoid Receptors: The Story So Far. *iScience* 23, 101301. doi:10.1016/J.ISCI.2020.101301

Sliwoski, G., Kothiwale, S., Meiler, J., and Lowe, E. W. (2014). Computational Methods in Drug Discovery. *Pharmacol. Rev.* 66, 334–395. doi:10.1124/pr.112.007336

Sloan, M. E., Grant, C. W., Gowin, J. L., Ramchandani, V. A., and Le Foll, B. (2019). Endocannabinoid Signaling in Psychiatric Disorders: a Review of Positron Emission Tomography Studies. *Acta Pharmacol. Sin.* 40, 342–350. doi:10.1038/S41401-018-0081-Z

Smialowski, P., Frishman, D., and Kramer, S. (2010). Pitfalls of Supervised Feature Selection. *Bioinformatics* 26, 440–443. doi:10.1093/bioinformatics/btp621

Soliman, N., Haroutounian, S., Hohmann, A. G., Krane, E., Liao, J., Macleod, M., 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. doi:10.1097/J.PAIN.0000000000002269

Spohn, S. N., Bianco, F., Scott, R. B., Keenan, C. M., Linton, A. A., O'Neill, C. H., et al. (2016). Protective Actions of Epithelial 5-Hydroxytryptamine 4 Receptors in Normal and Inflamed Colon. *Gastroenterology* 151, 933–e3. e3. doi:10.1053/J.GASTRO.2016.07.032

Stasiulewicz, A., Znajdek, K., Grudzień, M., Pawiński, T., and Sulkowska, A. J. I. (2020). A Guide to Targeting the Endocannabinoid System in Drug Design. *Int. J. Mol. Sci.* 21, 1. doi:10.3390/IJMS21082778

Sterling, T., and Irwin, J. J. (2015). ZINC 15--Ligand Discovery for Everyone. *J. Chem. Inf. Model.* 55, 2324–2337. doi:10.1021/acs.jcim.5b00559

Stern, N., and Goldblum, A. (2014). Iterative Stochastic Elimination for Solving Complex Combinatorial Problems in Drug Discovery. *Isr. J. Chem.* 54, 1338–1357. doi:10.1002/ijch.201400072

Torres, J., Bonovas, S., Doherty, G., Kucharzik, T., Gisbert, J. P., Raine, T., et al. (2020). ECCO Guidelines on Therapeutics in Crohn's Disease: Medical Treatment. *J. Crohns Colitis* 14, 4–22. doi:10.1093/ecco-jcc/jjz180

Tropsha, A. (2010). Best Practices for QSAR Model Development, Validation, and Exploitation. *Mol. Inform.* 29, 476–488. doi:10.1002/minf.201000061

Tuccinardi, T., Ferrarini, P. L., Manera, C., Ortore, G., Saccomanni, G., and Martinelli, A. (2006). Cannabinoid CB2/CB1 Selectivity. Receptor Modeling and Automated Docking Analysis. *J. Med. Chem.* 49, 984–994. doi:10.1021/JM050875U

Turcotte, C., Blanchet, M. R., Laviolette, M., and Flamand, N. (2016). The CB2 Receptor and its Role as a Regulator of Inflammation. *Cell. Mol. Life Sci.* 73, 4449–4470. doi:10.1007/s00018-016-2300-4

Verty, A. N., Stefanidis, A., McAinch, A. J., Hryciw, D. H., and Oldfield, B. (2015). Anti-Obesity Effect of the CB2 Receptor Agonist JWH-015 in Diet-Induced Obese Mice. *PLoS One* 10, e0140592. doi:10.1371/JOURNAL.PONE.0140592

Vetuschi, A., Pompili, S., Gaudio, E., Latella, G., and Sferra, R. (2018). PPAR-γ with its Anti-inflammatory and Anti-fibrotic Action Could Be an Effective Therapeutic Target in IBD. *Eur. Rev. Med. Pharmacol. Sci.* 22, 8839–8848. doi:10.26355/EURREV_201812_16652

Wei, J., Bhattacharyya, S., and Varga, J. (2010). Peroxisome Proliferator-Activated Receptor γ: Innate protection from Excessive Fibrogenesis and Potential Therapeutic Target in Systemic Sclerosis. *Curr. Opin. Rheumatol.* 22, 671–676. doi:10.1097/BOR.0B013E32833DE1A7

Wright, J. J. (2010). Combination Therapy of Bortezomib with Novel Targeted Agents: an Emerging Treatment Strategy. *Clin. Cancer Res.* 16, 4094–4104. doi:10.1158/1078-0432.CCR-09-2882

Wright, K. L., Duncan, M., and Sharkey, K. A. (2008). Cannabinoid CB2 Receptors in the Gastrointestinal Tract: a Regulatory System in States of Inflammation. *Br. J. Pharmacol.* 153, 263–270. doi:10.1038/sj.bjp.0707486

Wu, J. (2019). Cannabis, Cannabinoid Receptors, and Endocannabinoid System: Yesterday, Today, and Tomorrow. *Acta Pharmacol. Sin.* 40, 297–299. doi:10.1038/s41401-019-0210-3

Xi, Z.-X., Peng, X.-Q., Song, R., Zhang, H.-Y., Liu, Q.-R., Yang, H.-J., et al. (2011). Brain Cannabinoid CB2 Receptors Modulate Cocaine's Actions in Mice. *Nat. Neurosci.* 14, 1160–1166. doi:10.1038/NN.2874

Yang, D., Zhou, Q., Labroska, V., Qin, S., Darbalaei, S., Wu, Y., et al. (2021). G Protein-Coupled Receptors: Structure- and Function-Based Drug Discovery. *Sig Transduct Target. Ther.* 6, 1–27. doi:10.1038/s41392-020-00435-w

Yera, E. R., Cleves, A. E., and Jain, A. N. (2011). Chemical Structural Novelty: On-Targets and Off-Targets. *J. Med. Chem.* 54, 6771–6785. doi:10.1021/jm200666a

Zatsepin, M., Mattes, A., Rupp, S., Finkelmeier, D., Basu, A., Burger-Kentischer, A., et al. (2016). Computational Discovery and Experimental Confirmation of TLR9 Receptor Antagonist Leads. *J. Chem. Inf. Model.* 56, 1835–1846. doi:10.1021/acs.jcim.6b00070

Zhang, W., Pei, J., and Lai, L. (2017). Computational Multitarget Drug Design. *J. Chem. Inf. Model.* 57, 403–412. doi:10.1021/acs.jcim.6b00491

Zhang, Y., Xie, Z., Wang, L., Schreiter, B., Lazo, J. S., Gertsch, J., et al. (2011). Mutagenesis and Computer Modeling Studies of a GPCR Conserved Residue W5.43(194) in Ligand Recognition and Signal Transduction for CB2 Receptor. *Int. Immunopharmacol.* 11, 1303–1310. doi:10.1016/J.INTIMP.2011.04.013

Zhou, J., Jiang, X., He, S., Jiang, H., Feng, F., Liu, W., et al. (2019). Rational Design of Multitarget-Directed Ligands: Strategies and Emerging Paradigms. *J. Med. Chem.* 62, 8881–8914. doi:10.1021/ACS.JMEDCHEM.9B00017

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 22 March 2022
doi: 10.3389/fphar.2022.858215

Impact of the Endocannabinoid System on Bone Formation and Remodeling in p62 KO Mice

Christina Keller[1], *Timur Alexander Yorgan*[2], *Sebastian Rading*[1], *Thorsten Schinke*[2] *and Meliha Karsak*[1]*

[1]Neuronal and Cellular Signal Transduction, Center for Molecular Neurobiology Hamburg (ZMNH), University Medical Center Hamburg-Eppendorf, Hamburg, Germany, [2]Department of Osteology and Biomechanics, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

Several studies have shown that the G-protein coupled cannabinoid receptor CB2 and its interaction partner p62 are molecularly involved in bone remodeling processes. Pharmacological activation of the CB2 receptor enhanced bone volume in postmenopausal osteoporosis and arthritis models in rodents, whereas knockout or mutation of the p62 protein in aged mice led to Paget's disease of bone-like conditions. Studies of pharmacological CB2 agonist effects on bone metabolism in p62 KO mice have not been performed to date. Here, we assessed the effect of the CB2-specific agonist JWH133 after a short-term (5 days in 3-month-old mice) or long-term (4 weeks in 6-month-old mice) treatment on structural, dynamic, and cellular bone morphometry obtained by µCT of the femur and histomorphometry of the vertebral bodies in p62 KO mice and their WT littermates *in vivo*. A genotype-independent stimulatory effect of CB2 on bone formation, trabecular number, and trabecular thickness after short-term treatment and on tissue mineral density after long-term treatment was detected, indicating a weak osteoanabolic function of this CB2 agonist. Moreover, after short-term systemic CB2 receptor activation, we found significant differences at the cellular level in the number of osteoblasts and osteoclasts only in p62 KO mice, together with a weak increase in trabecular number and a decrease in trabecular separation. Long-term treatment showed an opposite JWH133 effect on osteoclasts in WT versus p62 KO animals and decreased cortical thickness only in treated p62 KO mice. Our results provide new insights into CB2 receptor signaling *in vivo* and suggest that CB2 agonist activity may be regulated by the presence of its macromolecular binding partner p62.

Keywords: cannabinoid receptor, p62 (sequestosome 1(SQSTM1), JWH133 (PubChem CID: 6918505), bone, osteoclast (OC), osteoblast (OB), CB2, Paget's disease of bone (PDB)

OPEN ACCESS

Edited by:
Manuela Marcoli,
University of Genoa, Italy

Reviewed by:
Elena Tsourdi,
Technical University Dresden, Germany
Yankel Gabet,
Tel Aviv University, Israel

***Correspondence:**
Meliha Karsak
mkarsak@uke.de

Specialty section:
This article was submitted to Experimental Pharmacology and Drug Discovery, a section of the journal Frontiers in Pharmacology

Received: *19 January 2022*
Accepted: *28 February 2022*
Published: *22 March 2022*

Citation:
Keller C, Yorgan TA, Rading S, Schinke T and Karsak M (2022) Impact of the Endocannabinoid System on Bone Formation and Remodeling in p62 KO Mice. Front. Pharmacol. 13:858215. doi: 10.3389/fphar.2022.858215

INTRODUCTION

Bone is a highly dynamic organ that responds to mechanical stress and is constantly remodeled (Ozcivici et al., 2010). Imbalanced activity of osteoblasts (bone-forming cells), osteoclasts (bone-resorbing cells), and osteocytes can cause a variety of skeletal disorders. Diseases of the skeletal system have a high prevalence and great impact on the healthcare system (Gennari et al., 2019).

Understanding the mode of action of bone cells and the effects of regulatory molecules and signaling pathways that control these cell types is of particular importance for future treatments.

Osteoblasts, osteoclasts, and osteocytes express the G-protein coupled cannabinoid receptors, CB1 and CB2, with a higher predominance of CB2 receptors (Idris et al., 2005; Ofek et al., 2006). The endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are produced locally and are degraded by specific enzymes in bone cells (Pertwee, 2015). However, the role of the endocannabinoid system (ECS) in bone remodeling, bone homeostasis, and bone diseases is not fully understood. Mice deficient in CB2 developed an accelerated bone loss with age (Ofek et al., 2006; Sophocleous et al., 2011; Sophocleous et al., 2014a; Sophocleous et al., 2014b). CB2 KO mice on the C57BL/6 background showed decreased trabecular bone volume at the femur and tibia as early as 8 weeks of age (females) (Ofek et al., 2006). This phenotype became even more pronounced in 1-year-old females and males with an osteoporosis-like phenotype with decreased osteoclast number and increased mineral apposition and bone formation rate (Ofek et al., 2006). The age-related osteoporosis in association with increased bone turnover was independently confirmed in the C57BL/6 CB2 KO strain (Sophocleous et al., 2017). However, different mouse lines, gender, and age of the mice contributed to some discrepant results (Ofek et al., 2006; Sophocleous et al., 2014a; Sophocleous et al., 2014b). Thus, the genetic background of the mice was found to influence bone parameters together with CB2 deletion. Studies in 3-month-old females have identified a high bone mass in CB2 KO mice on a CD1 background with increased trabecular bone volume and decreased bone formation rate in the tibia and femur compared with wild-type mice (Sophocleous et al., 2014a). The phenotype in 1-year-old female animals showed a greater loss of trabecular bone volume at the tibial metaphysis, which was associated with a decreased bone formation rate. No genotype-dependent difference was observed in the femur in these old animals. Also, young males showed no difference in the trabecular bone phenotype in CB2 KO animals on the CD1 background (Sophocleous et al., 2014a). Detailed studies to identify molecular explanations for differential findings using gene expression arrays revealed specific differences in gene expression that may contribute to the phenotypes of different CB2 KO mouse strains (Sophocleous et al., 2014b).

Pharmacological blockage of CB1 and CB2 protected mice from ovariectomy-induced bone loss (Sophocleous et al., 2022). CB receptor antagonists primarily mediate inhibition of bone resorption rather than activation of bone formation (Idris et al., 2005). In contrast, CB2 activation had also been described to protect female mice from ovariectomy-induced osteoporosis (Ofek et al., 2006; Sophocleous et al., 2011). It needs to be clarified whether the protective effect of CB2 activation on ovariectomy-induced bone loss is mediated by an inhibitory effect on bone resorption (Rea et al., 2013) or by a stimulatory effect on bone formation (Sophocleous et al., 2011). Stimulation of the CB2 receptor by its selective agonist JWH133 decreased the release of RANK-L and consequently the number and differentiation of osteoclasts, leading to increased mineralization of bone marrow cells from healthy human donors (Rossi et al., 2015). In an *in vivo* model of collagen-induced arthritis (CIA) in mice, loss of trabecular bone parameters, including bone volume, was significantly prevented by JWH133 treatment (Zhu et al., 2019), but osteoclast-mediated osteolysis induced by breast cancer cells was enhanced in a corresponding mouse model (Sophocleous et al., 2015).

To better understand the CB2 receptor signaling pathways, we have previously performed a screen for protein–protein interactions using tandem mass spectrometry. Here, we identified p62 (sequestosome 1, SQSTM1) as an interaction partner for the G-protein coupled CB2 receptor (Sharaf et al., 2019). p62 is a signaling scaffold protein and signaling hub with multiprotein domains that mediate its interactions with various binding partners, implicating the protein in numerous signaling pathways that influence processes such as cell differentiation, survival, osteoclastogenesis, inflammation, obesity, and autophagy (Moscat et al., 2007; Sanchez-Martin and Komatsu, 2018). Binding to the CB2 receptor is mediated *via* the ZZ-type zinc finger (ZZ) domain (Sharaf et al., 2019). The ubiquitin-associated (UBA) domain of the p62 protein clusters mutations identified in patients with familial and sporadic Paget's disease of bone (PDB) (Morissette et al., 2006; Falchetti et al., 2009), which is characterized by focal and disorganized increases in bone turnover (Roodman and Windle, 2005) and excessive bone-resorbing activity of abnormal osteoclasts (Chamoux et al., 2009). As an autophagy receptor, p62 binds cargo proteins and sequester them to autophagosomes for lysosomal hydrolysis and for the N-degron pathway through the ZZ-domain (Cha-Molstad et al., 2017).

For several years, significant efforts have been made to study p62 in bone diseases (Komatsu et al., 2012; Rea et al., 2013; Sanchez-Martin and Komatsu, 2018). In particular, genetic studies revealed the p.P392L variant of p62 in age-related PDB, leading to increased osteoclastogenic activity (Hiruma et al., 2008). In knockin mouse models of this variant, no histomorphological differences were observed (Hiruma et al., 2008), but Pagetic-like bone lesions were identified (Kurihara et al., 2011; Daroszewska et al., 2018). In mice with a deletion of p62, several results have been published without presenting a clear and congruent bone phenotype (Duran et al., 2004; Zach et al., 2018; Agas et al., 2020). However, it has been predominantly shown that p62 KO mice have an increase in trabecular bone (Zach et al., 2018; Agas et al., 2020). Zach et al. specifically identified an age-dependent phenotype. While 3- and 6-month-old animals showed no changes, older p62 KO mice developed exaggerated bone turnover (a hallmark of PDB) and increased trabecular number along with increased tartrate-resistant acid phosphate (TRAP) activity of distal femur osteoclasts (Zach et al., 2018). The results of the work of Agas et al. (2020) showed an increase in trabecular number and a decrease in trabecular separation as early as two months of age in p62 KO mice (Agas et al., 2020). However, in both studies, the total bone mass of p62 KO mice was similar compared to WT mice (Zach et al., 2018; Agas et al., 2020). In another work, the p62 KO mice did not show any bone phenotype in

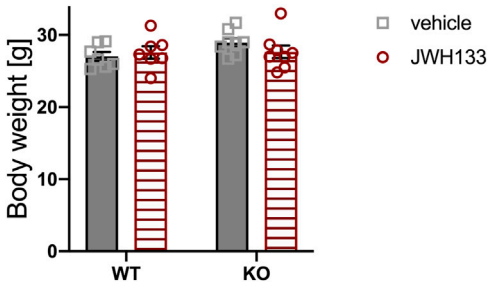


FIGURE 1 | Body weight of p62 KO and WT littermates is not different at the age of 3 months. Male mice were aged 3 months at the beginning of the experiment and were treated for 5 days with vehicle or JWH133. Body weight of mice was similar between all experimental groups. Data were analyzed by using ordinary two-way ANOVA. All error bars show mean ± SEM. Squares and circles represent individual data points. WT vehicle $N = 7$, KO vehicle $N = 8$, WT JWH133 $N = 8$, KO JWH133 $N = 8$.

histomorphometric studies (Duran et al., 2004; Rodriguez et al., 2006). Structural analyses of the long bones of 6- to 8-week-old p62 KO mice revealed normal bone physiology of the tibia and femur. Only after *in vivo* treatment with calciotropic hormone PTHrP, which induces osteoclastogenesis *via* the RANK-L pathway, an increase in osteoclast number was observed in WT but not in p62 KO mice (Duran et al., 2004). Past results argue for the importance of p62 in bone metabolism. Most importantly, p62 is an interaction partner for a number of proteins that play critical roles in bone, such as TRAF 6, RIP1, and aPKC (Sanz et al., 2000; Lamark et al., 2003; Berkamp et al., 2020). It is well known that the binding of p62 to TRAF6 modulates RANK/RANK-L signaling and the NF–κB pathway, thereby regulating osteoclastogenesis (Mcmanus and Roux, 2012) and resulting in increased osteoclastogenesis in p62 KO mice (Duran et al., 2004). Thus, the question arises whether also other interaction proteins act in an altered manner in the absence of p62.

In this work, we explored the possibility that the interaction of CB2 with p62 regulates its function in bone physiology. Therefore, we aimed to investigate whether CB2 activation functions differently in p62 KO mice. We characterized the femur and vertebral bodies of p62 KO and WT mice after short- and long-term *in vivo* activation of CB2 receptors and found slightly different effects of JWH133 on bone homeostasis in p62 KO animals.

RESULTS

Increased Trabecular Number in the Femurs of Young p62 KO Mice

We hypothesized that the interaction of p62 with CB2 is vital for bone cell differentiation and activation and, therefore, may influence bone remodeling. We combined genetic and pharmacological approaches to investigate the role of the interaction between p62 and CB2 on bone cells and bone remodeling under physiological conditions. For this purpose, 12–13-week-old male mice (WT vehicle $N = 7$, WT JWH133 $N = 7$; KO vehicle $N = 8$, KO JWH133 $N = 8$) were subcutaneously injected with either vehicle or the CB2 agonist JWH133 for a short duration of 5 days. The body weight of the mice was monitored to assess health status and to detect possible effects of treatment. The weight of p62 KO mice was comparable with their WT littermates and was not affected by treatment (**Figure 1**).

The distal femoral metaphysis and mid-diaphysis of the mice were measured by μCT to examine bone structure (**Figure 2A**). Analysis revealed similar trabecular bone volume (BV/TV) in p62 KO and WT mice and no effect of CB2 agonist treatment (**Figure 2B**). However, the trabecular number (Tb.N) was significantly increased in vehicle-treated p62 KO mice compared with vehicle-treated WT mice (KO vehicle, $4.14\ mm^{-1} \pm 0.13\ mm^{-1}$, $N = 8$; WT vehicle, $4.74\ mm^{-1} \pm 0.14\ mm^{-1}$, $N = 7$: $p = 0.005$; **Figure 2C**) and accordingly resulted in reduced trabecular separation (Tb.Sp) (KO vehicle, 209.31 μm ± 7.28 μm, $N = 8$; WT vehicle, 240.64 m ± 8.89 μm, $N = 7$: $p = 0.02$) (**Figure 2D**). All other parameters were similar between genotypes and were not affected by treatment with JHW133 (**Figures 2E–I**). To further investigate the trabecular bone volume, the lumbar vertebrae of the mice were analyzed by structural histomorphometry.

JWH133 Increased Trabecular Bone Volume in Lumbar Vertebral Bodies With a Stronger Effect in p62 KO Mice

For histomorphometric evaluation of the bone structure, von Kossa/van Gieson stains of undecalcified spine sections of the same animal groups were examined (vertebral bodies L3 and L4) (**Figure 3A**). The parameter bone volume per tissue volume (BV/TV) was comparable between genotypes (**Figure 3B**). However, JWH133 injections for five consecutive days resulted in an increase in bone volume in both WT and p62 KO mice, representing a significant effect of treatment (**Figure 3B**). This change in total bone mass was caused by an increase in trabecular number and a corresponding decrease in trabecular separation in p62 KO mice (**Figures 3C,D**). In contrast, trabecular thickness (Tb.Th) was comparable between p62 KO and their WT littermates (**Figure 3E**). However, a weak trend of the observed treatment effect led to an increase in trabecular thickness in both genotypes (**Figure 3E**). In contrast to μCT, the histomorphometric analysis includes not only mineralized bone but also osteoid. Osteoid is deposited by osteoblasts and is the portion of bone that is not yet mineralized. Osteoid volume per bone volume (OV/BV) was slightly increased by the treatment in p62 KO mice. However, no significant effect of genotype, treatment, or interaction was detected due to the high variability within the measurement, especially in the WT vehicle group (**Figure 3F**). These results indicate that increased trabecular bone volume of the lumbar vertebrae of p62 KO

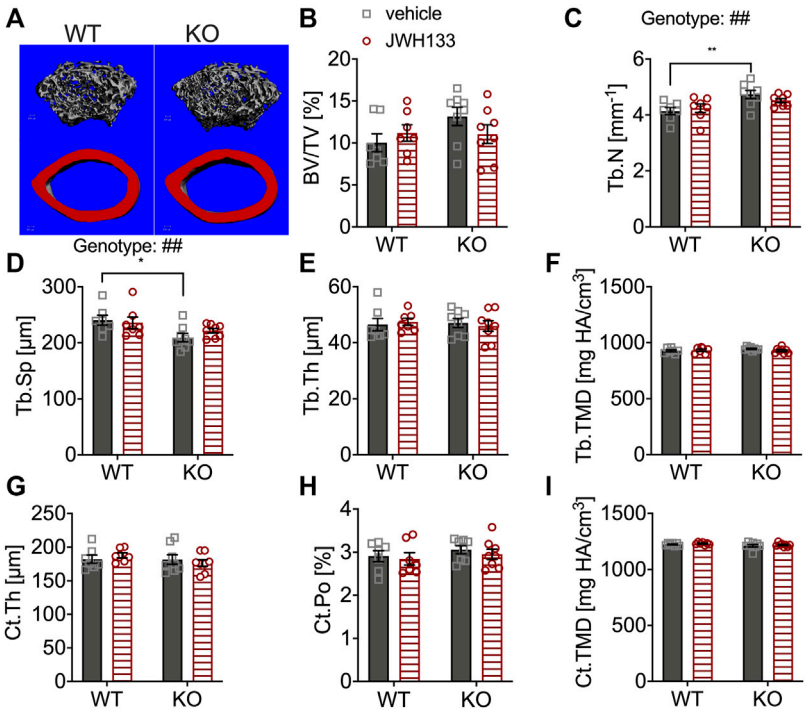


FIGURE 2 | µCT of femurs showed the increased trabecular number and correspondingly reduced spacing in p62 KO mice. **(A)** Trabecular (grey) and cortical (red) bone structure of the femur of WT and p62 KO mice. **(B)** Bone volume per total bone volume (BV/TV) was similar between WT and p62 KO mice and showed no effect of treatment. **(C)** Trabecular number (Tb.N) was significantly increased in vehicle-treated p62 KO mice compared to vehicle-treated WT mice. No effect of genotype was observed after treatment. **(D)** Trabecular spacing (Tb.Sp) was reduced in vehicle-treated p62 KO mice compared to WT mice. **(E)** Trabecular thickness (Tb.Th) showed no difference between p62 KO and WT mice nor an effect of treatment. **(F)** Tissue mineral density (TMD) of trabecular bone was similar between genotypes and showed no effect of treatment. **(G)** Cortical thickness (Ct.Th) of the femur was not influenced by the treatment and showed no difference between genotypes. **(H)** Cortical porosity (Ct. Po) and **(I)** tissue mineral density (TMD) of cortical bone were similar between genotypes and not influenced by the treatment with JWH133. Male mice were aged 3 months by the beginning of the experiment and were treated for 5 days with vehicle or JWH133. Data were analyzed by using ordinary two-way ANOVA and Bonferroni adjusted p values, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. All error bars show mean ± SEM. Squares and circles represent individual data points. WT vehicle $N = 7$, KO vehicle $N = 8$, WT JWH133 $N = 8$, KO JWH133 $N = 8$.

mice is primarily due to increased trabecular number after CB2 agonist treatment, suggesting increased osteoblast activity or decreased resorption by osteoclasts as a result of CB2 agonist treatment. To further investigate this effect, we used dynamic and cellular histomorphometry.

Bone Formation and Mineralization Were Similar Between Genotypes and Not Affected by JWH133

Calcein labels were measured in the lumbar vertebrae for a more detailed analysis of bone formation. Mineralization (mineral apposition rate; MAR) of the lumbar vertebrae was not affected by treatment with JWH133, and no differences in mineralizing surface (MS/BS) or bone formation rate (BFR/BS) were observed between p62 KO and WT mice (**Figures 4A–C**), suggesting normal osteoblast activity. Overall, bone formation and mineralization were not affected by CB2 agonist treatment and were comparable between p62 KO mice and their WT littermates. To further analyze the cause of the increased trabecular bone volume and osteoid deposition after treatment in the mice, cellular histomorphometry was performed.

JWH133 Treatment Increased the Number and Surface of Osteoblasts and Osteoclasts in Young p62 KO Mice

The number of osteoblasts was slightly reduced in vehicle-treated p62 KO mice compared with WT control mice (vehicle), but did not reach significance (WT vehicle, 7.99 mm^{-1} ± 1.2 mm^{-1}, $N = 7$; KO vehicle 3.63 mm^{-1} ± 0.54 mm^{-1}, $N = 8$: $p = 0.10$) (**Figure 5A**). However, treatment with JWH133 significantly increased the number of osteoblasts in p62 KO mice compared to p62 KO control mice (vehicle) (**Figure 5A**: KO vehicle, 3.63 mm^{-1} ± 0.54 mm^{-1}, $N = 8$; KO JWH133, 9.84 mm^{-1} ± 2.1 mm^{-1}, $N = 8$: $p = 0.01$). This increase in osteoblast number was not present in WT mice, resulting in a significant interaction effect (interaction = $F_{(1.26)} = 5.6$, $p = 0.03$) and a weak trend in treatment (treatment = $F_{(1.26)} = 3.3$, $p = 0.08$) (**Figure 5A**). In addition, the percentage

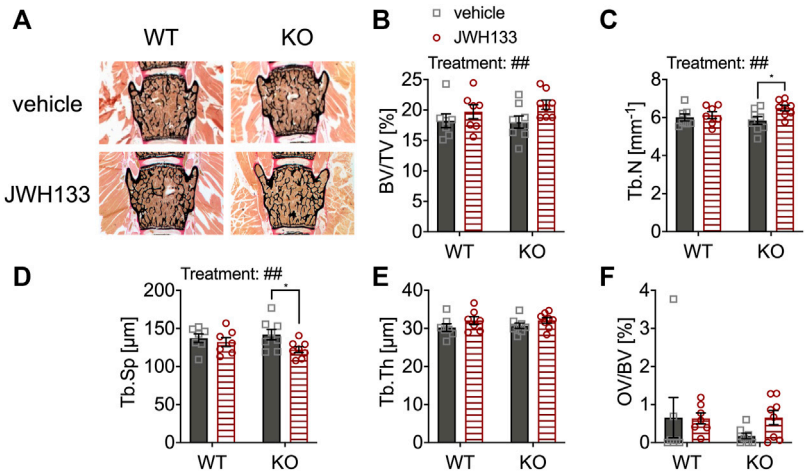


FIGURE 3 | JWH133 increased trabecular bone volume in mice. **(A)** Undecalcified vertebral bodies (L4) stained after von Kossa/van Gieson (black = mineralized bone, red = osteoid) of WT and p62 KO mice that were treated with either vehicle or JWH133. **(B)** Bone volume per tissue volume (BV/TV) was similar between genotypes but was significantly increased by the treatment. **(C)** Trabecular number (Tb.N) was significantly increased by the treatment, and Bonferroni post hoc testing revealed a significant increase in JWH133-treated p62 KO mice compared to vehicle-treated p62 KO mice. **(D)** Trabecular separation (Tb.Sp) was significantly reduced by JWH133, and Bonferroni post hoc testing revealed a significant reduction in JWH133-treated p62 KO mice compared to vehicle-treated p62 KO mice. **(E)** Trabecular thickness (Tb.Th) was similar between genotypes and showed a weak trend of the treatment to increase the thickness. **(F)** Osteoid volume per bone volume (OV/BV) was increased in p62 KO mice after JWH133 treatment but did not reach significance due to high variability. Male mice were aged 3 months by the beginning of the experiment and were treated for 5 days with vehicle or JWH133. Data were analyzed by using ordinary two-way ANOVA and Bonferroni adjusted *p* values, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. All error bars show mean ± SEM. Squares and circles represent individual data points. WT vehicle $N = 7$, KO vehicle $N = 8$, WT JWH133 $N = 8$, KO JWH133 $N = 8$.

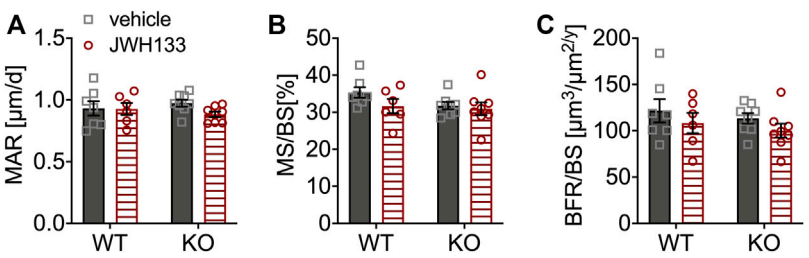


FIGURE 4 | Bone formation and mineralization were similar between genotypes and not affected by JWH133. **(A)** Mineral apposition rate (MAR) of p62 KO and WT mice treated with JWH133 was comparable. **(B)** Mineral surface per bone surface (MS/BS) showed no difference by comparing for genotype and treatment. **(C)** Bone formation rate per bone surface (BRF/BS) showed no effect of treatment and was comparable between genotypes. Male mice were aged 3 months by the beginning of the experiment and were treated for 5 days with vehicle or JWH133. Data were analyzed by using ordinary two-way ANOVA and Bonferroni adjusted *p*values, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. All error bars show mean ± SEM. Squares and circles represent individual data points. WT vehicle $N = 7$, KO vehicle $N = 8$, WT JWH133 $N = 8$, KO JWH133 $N = 8$.

of bone surface occupied by osteoblasts was reduced in vehicle-treated p62 KO mice compared with vehicle-treated WT mice but did not reach significance. Treatment of mice with JWH133 significantly increased the percentage of osteoblasts per bone surface in p62 KO mice (**Figure 5B**: KO vehicle, 4.0 ± 0.6%, $N = 8$; KO JWH133, 10.7 ± 2.4%, $N = 7$: $p = 0.01$), while WT mice were not affected, resulting in a significant effect of interaction (interaction = $F_{(1.26)} = 4.5$, $p = 0.04$) and trend in the effect of treatment (treatment = $F_{(1.26)} = 3.9$, $p = 0.06$) (**Figure 5B**). Next, osteoclasts were analyzed, revealing nearly identical numbers of cells in vehicle-treated p62 KO and WT mice (**Figure 5C**). Treatment with JWH133 significantly increased the number of osteoclasts in p62 KO mice (**Figure 5C**: p62 KO vehicle, 1.78 mm^{-1} ± 0.30 mm^{-1}, $N = 8$; KO JWH133, 3.58 mm^{-1} ± 0.73 mm^{-1}, $N = :$ $p = 0.02$) and very slightly in WT mice, still resulting in a significant effect of treatment (**Figure 5C**). Correspondingly, the percentage of bone surface occupied by osteoclasts was similar in vehicle-treated groups of both genotypes. Again, a statistically significant treatment effect was observed due to an increase in osteoclast surface area in p62 KO mice (p62 KO vehicle, 5.1 ± 1.0%, $N = 8$; KO JWH133, 9.3 ± 1.7%, $N = 8$: $p = 0.04$) and a weak effect was detected in WT mice (**Figure 5D**: treatment $F_{(1,26)} = 6.8$, $p = 0.01$: genotype $F_{(1,26)} = 1.2$, $p = 0.29$: interaction $F_{(1,26)} = 0.55$, $p = 0.46$).

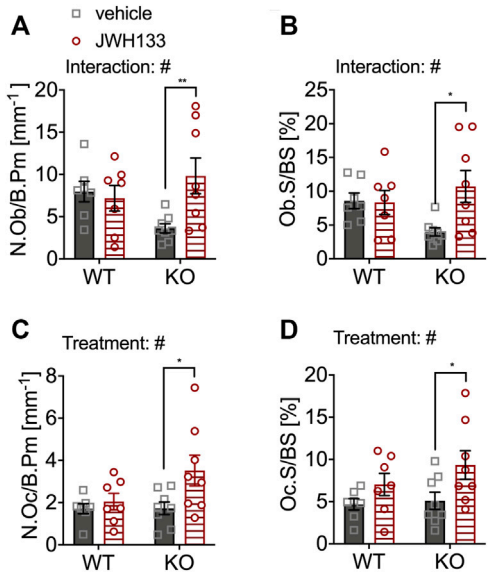


FIGURE 5 | JWH133 treatment increased the number and surface of osteoblasts and osteoclasts in p62 KO mice. **(A)** Number of osteoblasts per bone perimeter (N.Ob/B.Pm) was weakly reduced in p62 KO mice compared to WT mice. The treatment was only effective in p62 KO mice as it increased the number of osteoblasts. **(B)** Osteoblast surface per bone surface (Ob.S/BS) was mildly reduced in p62 KO mice compared to WT mice. The treatment was only effective in p62 KO mice as it increased the surface of osteoblasts. **(C)** Number of osteoclasts per bone perimeter (N.Oc/B.Pm) was similar between genotypes but was significantly increased in p62 KO mice after JWH133 treatment. **(D)** Surface of osteoclasts per bone surface (Oc.S./BS) was similar between genotypes but was significantly increased in p62 KO mice after JWH133 treatment. Male mice were aged 3 months by the beginning of the experiment and were treated for 5 days with vehicle or JWH133. Data were analyzed by using ordinary two-way ANOVA and Bonferroni adjusted *p* values, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. All error bars show mean ± SEM. Squares and circles represent individual data points. WT vehicle *N* = 7, KO vehicle *N* = 8, WT JWH133 *N* = 8, KO JWH133 *N* = 8.

Prolonged JWH133 Treatment in Aged Mice Did Not Lead to Detectable Changes in Trabecular and Cortical Bone of the Femur (µCT)

To test whether prolongation of treatment with the CB2 agonist affects bone mass at a structural level detectable by µCT or structural histomorphometry, a second group of male mice was treated with either vehicle (WT *N* = 8, KO *N* = 9) or JWH133 (WT *N* = 8, KO *N* = 8) for a period of 4 weeks. Body weight of 6-month-old p62 KO mice was significantly increased at the beginning of the experiment compared with their WT littermates (**Figure 6A**). However, treatment with the CB2 agonist did not affect the body weight of p62 KO and WT mice fed with a standard diet. Moreover, the body weight of the mice remained constant throughout the experiment, indicating good health.

The distal femur of the mice was measured by µCT analysis. The trabecular bone of p62 KO mice and their WT littermates was comparable between genotypes and was not affected by the treatment, as all trabecular bone parameters were comparable (**Figures 6B–F**). Next, cortical bone was examined and decreased cortical thickness was observed in p62 KO mice compared with WT mice, indicating an effect of genotype that became significant in JWH133-treated mice (Ct.Th, **Figure 6G**: p62 KO vehicle, 185.0 µm ± 2.0 µm, *N* = 9, WT vehicle, 188.4 µm ± 8.8 µm, *N* = 8: $p = 0.49$; KO JWH133, 179 µm ± 5.1 µm, *N* = 8, WT JWH133, 186.8 µm ± 6.6 µm, *N* = 8: $p = 0.03$). Cortical porosity (Ct.Po, **Figure 6H**) and cortical tissue mineral density (Ct.TMD, **Figure 6I**) were comparable between genotypes and were not affected by the treatment.

Taken together, treatment with JWH133 did not induce structural changes in the femur, at least to an extent that could be detected by µCT analysis. In addition, cortical thickness was lower in p62 KO mice compared with their WT littermates at 6 months of age; this difference was not observed in 3-month-old p62 KO mice.

JWH133 Increased Tissue Mineral Density in the Vertebrae of Aged Mice After a Prolonged Treatment Duration

Long bones (femurs) and irregular bones (vertebral bodies) are distinct and different bone types (Kappen et al., 2007; Berendsen and Olsen, 2015). The biomechanical loading of these bones differs significantly in mice, with vertebral bodies experiencing less force than the femora. This difference in mechanical stimuli may also lead to altered reactions to factors that can directly or indirectly modulate or influence the response to a stimulus. We expected to find structural changes after the 4 weeks treatment with a CB2 agonist, and as vertebral bodies might respond differently to the treatment, an additional µCT-analysis on the spongy bone of the lumbar vertebral body 5 (L5) was performed. All measured trabecular parameters were analyzed, and no differences were observed between genotypes and treatment groups (**Figures 7A–E**). Only for tissue mineral density (TMD), JWH133 resulted in a significant increase in both p62 KO and WT mice (**Figure 7F**: p62 KO vehicle, 809.1 mg HA/cm^3 ± 21.2 mg HA/cm^3, *N* = 9, KO JWH133, 859.0 mg HA/cm^3 ± 28.5 mg HA/cm^3, *N* = 8: $p < 0.001$; WT vehicle, 802.0 mg HA/cm^3 ± 30.7 mg HA/cm^3, *N* = 7, WT JWH133, 847.0 mg HA/cm^3 ± 18.0 mg HA/cm^3, *N* = 7: $p = 0.005$).

Structural Bone Parameters Were Not Affected by Prolonged Treatment With JWH133 in Aged Mice

Since short-term treatment with JWH133 resulted in an increase in osteoid in p62 KO mice, structural histomorphometry was performed on lumbar vertebrae (L1-L4) of the animals after four weeks of treatment with JWH133. Consistent with the µCT data, all structural histomorphometric parameters (**Figures 8A–E**) were unaffected by treatment and showed no influence of genotype.

Prolonged Treatment With JWH133 Showed an Opposing Effect on Osteoclasts of 6-Month-Old WT and p62 KO Mice

For short-term treatment, an increase in bone cells was detected by treatment with the CB2 agonist only in young p62 KO mice. In

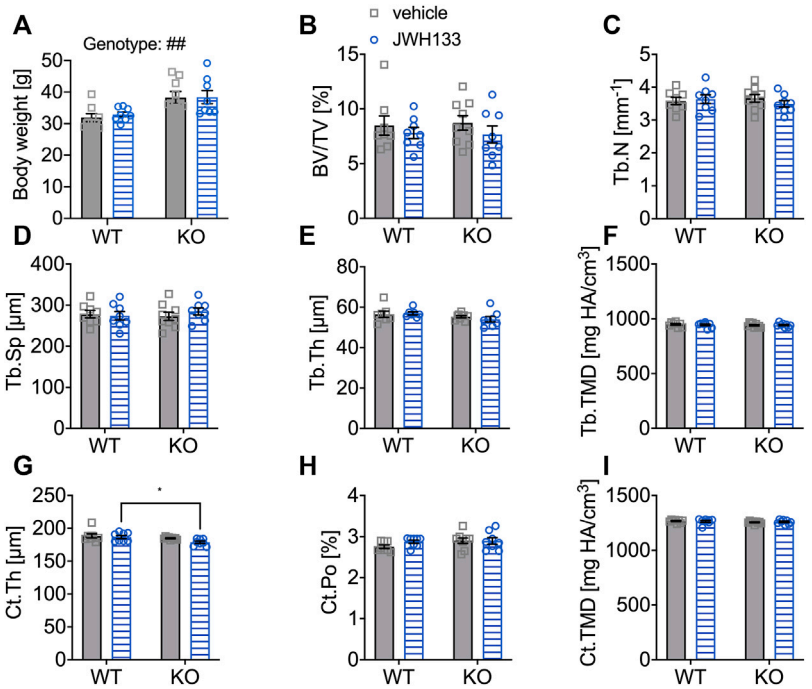


FIGURE 6 | Aged mice did not respond with detectable changes in femoral trabecular and cortical bone (µCT) after a prolonged treatment duration with JWH133. **(A)** Male mice were aged 6 months by the beginning of the experiment. Body weight of p62 KO animals was significantly increased compared to WT mice at the beginning of the experiment. **(B)** Bone volume per tissue volume (BV/TV) was similar between WT and p62 KO mice and showed no effect of treatment. **(C)** Trabecular number (Tb.N) was comparable between genotypes without an effect of treatment. **(D)** Trabecular spacing (Tb.Sp) showed no difference between genotype or treatment. **(E)** Trabecular thickness (Tb.Th) was comparable for genotype and treatment. **(F)** Tissue mineral density (TMD) of the femur was not influenced by the treatment and showed no difference between genotypes. **(G)** Cortical thickness (Ct.Th) was lower in p62 KO mice leading to a significant effect of genotype. **(H)** Cortical porosity (Ct.Po) and **(I)** tissue mineral density (Ct.TMD) of cortical bone were similar between genotypes and not influenced by the treatment with JWH133. Data were analyzed by using ordinary two-way ANOVA and Bonferroni adjusted *p* values, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. All error bars show mean ± SEM. Squares and circles represent individual data points. WT vehicle $N = 8$, KO vehicle $N = 9$, WT JWH133 $N = 8$, KO JWH133 $N = 8$.

the 6-month-old age groups, treated for 4 weeks with the specific CB2 agonist, the number of osteoblasts per bone perimeter (N.Ob/B.Pm) was not affected by treatment and was comparable between genotypes (**Figure 9A**). The number of osteoclasts (N.Oc/B.Pm) was significantly increased in vehicle-treated p62 KO mice compared to WT control mice (p62 KO vehicle, 4.45 mm^{-1} ± 0.25 mm^{-1}, $N = 8$; WT vehicle, 3.48 mm^{-1} ± 0.27 mm^{-1}, $N = 8$: $p = 0.04$). Treatment had an opposite effect on genotypes, as the number of osteoclasts was very slightly increased in WT mice (WT vehicle, 3.48 mm^{-1} ± 0.27 mm^{-1}, $N = 8$; WT JWH133, 4.10 mm^{-1} ± 0.37 mm^{-1}, $N = 8$: $p = 0.27$) and decreased in p62 KO mice (KO vehicle, 4.45 mm^{-1} ± 0.25 mm^{-1}, $N = 8$; KO JWH133, 3.99 mm^{-1} ± 0.20 mm^{-1}, $N = 8$: $p = 0.52$), still resulting in a trend of an interaction, while no effect of genotype or treatment was observed (**Figure 9B**). The correlation between the bone cell surface and cell number provides information about the activity status of the cells. The percentage of osteoblasts per bone surface area (Ob.S/BS) was comparable between genotypes and was not affected by treatment (**Figure 9C**). In osteoclasts, treatment had an opposite effect on genotypes, as the percentage of osteoclasts per bone surface (Oc.S/BS) was weakly increased in WT mice (WT vehicle, 7.56 ± 0.57%, $N = 8$; WT JWH133, 9.41 ± 1.19%, $N = 8$: $p = 0.27$) and slightly decreased in p62 KO mice (KO vehicle, 10.3 ± 0.74%, $N = 8$; KO JWH133, 8.39 ± 0.65%, $N = 8$: $p = 0.52$), resulting in a significant effect of interaction, while no effect of genotype or treatment was observed (**Figure 9D**).

In summary, 6-month-old p62 KO mice showed a reduced cortical thickness of the femur (µCT) and a tendency toward reduced trabecular bone in the lumbar vertebral body L5 compared with their WT littermates. However, the differences were slight and significant only when the vehicle and JWH133 treatment groups were included. No significant structural bone phenotype was observed in 6-month-old p62 KO mice. Long-term treatment showed an enhancing effect of JWH133 on mineral density in the vertebral body independent of genotype. However, CB2 receptor activation led to an opposing effect between p62 KO and WT mice at the cellular level.

DISCUSSION

P62 KO Mice Display a Normal Bone Turnover

General characterization of the bone phenotype of 3-month-old vehicle-treated p62 KO mice revealed an increased trabecular

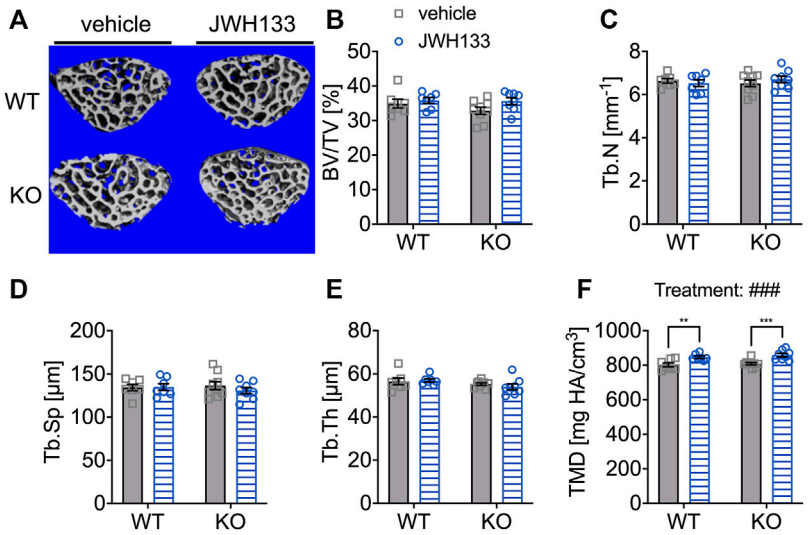


FIGURE 7 | Prolonged treatment with JWH133 increased tissue mineral density in the vertebrae of aged mice. **(A)** Representative image of the vertebral trabecular bone compartment (L5) analyzed by µCT of p62 KO and WT mice treated with vehicle or JWH133. **(B)** Bone volume per tissue volume (BV/TV) was similar between WT and p62 KO mice showed no effect of treatment. **(C)** Trabecular number (Tb.N) was comparable between genotypes showed no effect of treatment. **(D)** Trabecular spacing (Tb.Sp) showed no difference between genotype or treatment. **(E)** Trabecular thickness (Tb.Th) was not different. **(F)** Tissue mineral density (TMD) was significantly influenced by the treatment with JWH133 and affected p62 KO and WT mice. Data were analyzed by using ordinary two-way ANOVA and Bonferroni adjusted *p* values, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. All error bars show mean ± SEM. Squares and circles represent individual data points. WT vehicle $N = 7$, KO vehicle $N = 9$, WT JWH133N = 8, KO JWH133 $N = 8$.

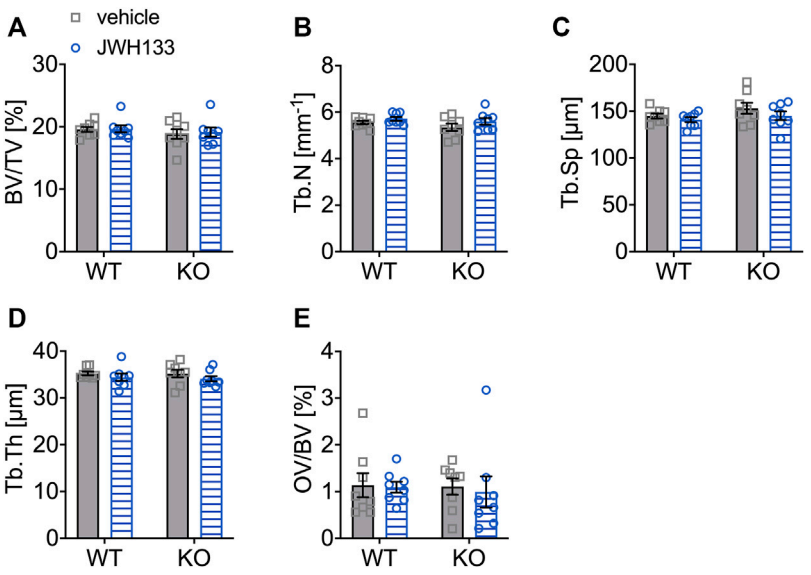


FIGURE 8 | Structural bone parameters were not affected by a 4-week treatment with JWH133 in aged mice. **(A)** Bone volume per tissue volume (BV/TV) was similar between WT and p62 KO mice and showed no effect of treatment. **(B)** Trabecular number (Tb.N) was comparable between genotypes and treatment. **(C)** Trabecular spacing (Tb.Sp) showed no difference between genotype or treatment. **(D)** Trabecular thickness (Tb.Th) was comparable for genotype and treatment. **(E)** Osteoid volume per bone volume (OV/BV) was similar in p62 KO and WT mice and not influenced by the treatment with JWH133. Data were analyzed by using ordinary two-way ANOVA. All error bars show mean ± SEM. Squares and circles represent individual data points. WT vehicle $N = 8$, KO vehicle $N = 8$, WT JWH133 $N = 8$, KO JWH133 $N = 8$.

number (Tb.N) in femoral microstructures visualized by µCT. Despite this finding, the overall bone volume was similar between p62 KO and WT mice, suggesting a weak effect of Tb.N on total bone volume in p62 KO mice. Moreover, static histomorphometry of vertebral bodies revealed similar Tb.N and total bone volume in both genotypes, and dynamic and

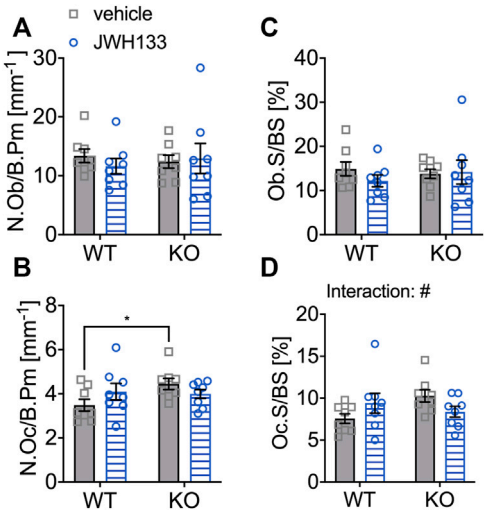


FIGURE 9 | 6-month aged WT and p62 KO mice showed an opposing effect on osteoclasts after prolonged treatment with JWH133. **(A)** Number of osteoblasts per bone perimeter (N.Ob/B.Pm) was comparable in vehicle- and JWH133-treated mice and showed no effect of genotype or treatment. **(B)** Number of osteoclasts per bone perimeter (N.Oc/B.Pm) was significantly increased in p62 KO mice (vehicle) compared to WT mice (vehicle). The treatment affected genotypes in an opposite way. **(C)** Osteoblast surface per bone surface (Ob.S/BS) was not altered between genotypes and was not affected by the treatment. **(D)** Osteoclasts per bone surface (Oc.S/BS) were weakly increased in WT mice and reduced in p62 KO mice by the treatment with JWH133 leading to a significant effect of interaction. Data were analyzed by using ordinary two-way ANOVA and Bonferroni adjusted *p* values, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. All error bars show mean ± SEM. Squares and circles represent individual data points. WT vehicle $N = 8$, KO vehicle $N = 8$, WT JWH133 $N = 8$, KO JWH133 $N = 8$.

cellular histomorphometry of vertebral bodies showed no differences between p62 KO and WT mice at 3 or 6 months of age, indicating normal bone turnover in p62 KO mice.

The observation that 6-month-old vehicle-treated p62 KO mice had significantly increased numbers of osteoclasts compared with vehicle-treated WT mice did not result in changes in the measured static and dynamic parameters. Because cortical thickness and trabecular bone parameters were similar in vehicle-treated p62 KO and WT mice, this suggests that overall resorption activity was not affected by the loss of p62 at this age. We must consider that the vehicle solution injections may have influenced our obtained data on the overall p62 KO phenotype, but this possibility can be at least partially excluded, as the results are largely consistent with previous publications (Duran et al., 2004; Zach et al., 2018). Thus, our results are consistent with the observations of previous histomorphometric measurements on long bones from another p62 KO mouse line, where a weak but statistically non-significant increase in bone volume, trabecular number, and correspondingly decreased trabecular separation was observed in the tibia and femur of 6- to 8-week-old p62 KO mice (Duran et al., 2004). This result was also confirmed in p62 KO mice at 3 and 6 months of age, which had comparable trabecular numbers to WT mice (Zach et al., 2018). In contrast, a recent publication showed significantly increased trabecular number and correspondingly decreased trabecular separation in μCT of the tibia in young p62 KO mice (8–9 weeks), while total bone volume was again unaffected (Agas et al., 2020).

Aging has a pro-osteoclastogenic effect that is exacerbated by the p62 P394L mutation in a mouse model (Daroszewska et al., 2018). Not only in mice carrying the PDB mutation but also in 15-month-old p62 KO mice, an age-dependent (a hallmark of PDB) exaggerated bone turnover was detected in the distal femora, as indicated by an increased trabecular number accompanied by increased TRAP activity of osteoclasts (Zach et al., 2018). Again, in these aged p62 KO mice, the increase in trabecular number had no effect on total bone mass (Zach et al., 2018). A major limitation of this previous work is that the number of mice studied was relatively small, N = 3-4, and thus, the samples analyzed may not be representative. Despite some conflicting results, our observations together with previous publications indicate that the *in vivo* role of p62 in bone physiology is minor and may be age-dependent.

Inconsistencies between studies may be caused by differences between mouse strains and their effects on skeletal microstructure (Papageorgiou et al., 2020). Of note, all studies presented analyzed p62 KO mice on a C57BL/6 background, but used different substrains and different lines (Duran et al., 2004; Zach et al., 2018; Agas et al., 2020). Although genetic differences within the C57BL/6 family are small, there are differences in trabecular indices, bone formation, and bone cell indices (Simon et al., 2013; Sankaran et al., 2017).

CB2 Agonist JWH133 Had Slight Osteoanabolic Effects in p62 KO Mice

Treatment with JWH133 for 5 days in 3-month-old male p62 KO and WT mice showed a slight effect on bone volume (structural histomorphometry of vertebrae). Otherwise, CB2 activation had almost no effect in healthy WT mice but modulated the bone cell differentiation in p62 KO animals. These results might indicate that p62 as a macromolecular effector and interaction partner influences the function of CB2 receptors. To date, the effects of CB2 signaling in bone have been studied in disease models or estrogen deficiency mimicking postmenopausal osteoporosis (Sophocleous et al., 2022). However, these studies provided evidence that CB2 signaling affects both osteoclast formation and osteogenesis in mice, with some conflicting results (Idris et al., 2005; Ofek et al., 2006; Sophocleous et al., 2011).In an ovariectomy-induced model, the CB2 agonist HU-308 had an osteoanabolic effect and attenuated bone loss in C3H mice (Ofek et al., 2006), which was partly confirmed in C57BL/6 mice using a tenfold lower dose of HU-308 (Sophocleous et al., 2011). Furthermore, CB2 activation in rats reduced bone resorption in a breast cancer–induced (Lozano-Ondoua et al., 2013) and in an osteoarthritis model (Mlost et al., 2021). In a rheumatoid arthritis model, JWH133 suppressed osteoclast formation and differentiation *in vitro* and *in vivo* (Zhu et al., 2019). In contrast, in mice, JWH133 stimulated osteoclast formation (Idris et al., 2008) and enhanced breast cancer cell-induced osteoclastogenesis and osteolysis (Sophocleous et al., 2015).

In the present study, we detected a significant increase in the number of trabeculae and a corresponding decrease in trabecular separation after short-term treatment with JWH133 in the spine of young p62 KO mice only. The amount of osteoid was also slightly increased but showed high variation in p62 KO mice after agonist treatment. These results suggest that the CB2 agonist JWH133 significantly affects bone cell differentiation in young p62 KO mice, resulting in high bone turnover associated with increased osteoid volume and trabecular number. Treatment with JWH133 may lead to a shift in the balance between osteoblast and osteoclast activity. Consistent with this, the number and surface area of osteoblasts and osteoclasts were increased after short-term treatment with JWH133 only in p62 KO. Osteoid deposition may have been responsible for the increase in trabecular bone volume in p62 KO mice after treatment with CB2 agonists. The increase in osteoid may also explain why these changes were not detected by μCT, since μCT only measures the mineralized bone. Mineral apposition rate and bone formation rate were not affected by genotype or treatment, which could be due to the short treatment period before sacrifice, which did not allow sufficient time for the bone to mineralize.

The differential effects of JWH133 treatment in young WT and p62 KO animals might be due to variable CB2 protein levels because p62 is involved in protein degradation as a cargo receptor for autophagy (Pankiv et al., 2007). Assuming that CB2-mediated autophagy depends on the presence and activity of p62, CB2 protein levels might be higher in the absence of p62, and accordingly, a stimulatory CB2 response might be enhanced and promote mineralization in p62 KO mice. Interestingly, a remarkably high expression of CB2 was found in osteoclasts from patients with PDB *in vitro* (Paoletta et al., 2021), so it would also be possible that the strong response in the young p62 KO animals is due to increased CB2 receptor levels. A functional link between CB2 receptor and autophagy has been demonstrated in previous studies showing that CB2 agonists have the potential to promote autophagy (Ke et al., 2016; Wu et al., 2018). Moreover, *in vitro* studies showed that osteogenic differentiation induced by CB2 receptor agonists HU-308 and JWH133 was inhibited when autophagy was blocked (Xu et al., 2020). Further possible explanation for the different responses between p62 KO and WT mice could be altered receptor internalization of CB2 receptors.

Thus, it would be possible that in young p62 WT animals, the CB2 receptors were internalized and, hence, not accessible to the ligand. In contrast, CB2 receptors in young p62 KO mice might be more localized at the plasma membrane, allowing short-term stimulation to produce a stronger effect. Internalization of CB2 receptors is dependent on β-arrestin2, and internalized CB2 receptors colocalized with the early endosome and were recycled to the cell surface after agonist removal (Chen et al., 2014). Recycling of internalized CB2 receptors is assumed to be mediated by proteasome degradation (Chen et al., 2014). Because p62 has been shown not only to be involved in protein degradation but also to interact with β-arrestin2 (Woo et al., 2020), it may be possible that CB2 internalization and recycling are impaired under p62 KO conditions, which needs to be investigated in future work. Of note, JWH133 is significantly biased towards G-protein signaling over β-arrestin coupling and cAMP signaling on the mouse CB2 receptor (Soethoudt et al., 2017).

In a second approach, we aimed to prolong treatment with JWH133 to increase the state of high bone turnover in aged p62 KO mice and induce further changes in bone structure detectable by μCT. An effect on osteoclast number and activity was also observed in these 6-month-old animals after 4 weeks of treatment with JWH133. Here, a slight genotype-dependent effect of CB2 agonist treatment was observed. CB2 activation resulted in a slight decrease in osteoclast number in p62 KO mice and a weak increase in WT littermates. However, this opposite effect of JWH133 on osteoclasts in p62 KO mice compared with WT littermates did not result in differences in bone structure visible on μCT of the femur. In addition, all forms of static and dynamic histomorphometry (vertebral bodies) were similar between p62 KO and WT mice. It is possible that the experimental design was a limitation or that compensatory mechanisms were activated during our *in vivo* experiments. During the short-term treatment, mice were administered daily injections for 5 days. During the long-term treatment experiment, the mice received injections 3 times per week for a period of 4 weeks, so the bioavailability of JWH133 may have been too low, or the receptors may have been desensitized and internalized during the long-term treatment (Udoh et al., 2019; Capote et al., 2021; Patel et al., 2021) and not available for the agonist at the membrane at the right time. Another limiting factor may have been the different ages of the mice used in the short-term and long-term treatment experiments (Willinghamm et al., 2010; Daroszewska et al., 2018). In the older animals, our results could be explained by low CB2 receptor expression, as the number of cannabinoid receptors on bone cells might also be altered at older ages, as has been shown for skeletal muscle (Dalle and Koppo, 2021).

Consistent with our findings that p62 balances CB2 signaling, previous publications have shown that CB2 receptor activation affects the RANK-L (receptor activator of NF-κB ligand) pathway, in which p62 plays an important role (Mcmanus and Roux, 2012). In a mouse model of rheumatoid arthritis, JWH133 suppressed RANK-L-induced IKKα/β phosphorylation, resulting in inhibition of NF-kB signaling activation in WT osteoclasts (Zhu et al., 2019). Of note, this signaling pathway is critical for osteoclastogenesis and inhibition of IKK activation and NF-κB nuclear translocation is impaired in p62 KO animals (Duran et al., 2004). The interplay between CB2 receptor activation and p62 protein levels and their effect on osteoclastogenesis could have direct implications for experimental outcomes. We, therefore, speculate that loss or differential p62 levels in the experimental conditions of previous studies may have contributed to the published paradoxical reports of the effects of CB2 activation on osteoclastogenesis (Bab and Zimmer, 2008; Sophocleous et al., 2015; Zhu et al., 2019).

In conclusion, we hypothesize that the signaling and function of the CB2 receptor are modulated by its interaction with the macromolecular-effector protein p62 *via* influencing the protein levels of this GPCR by either internalization or degradation. Our results demonstrate a molecular link of the

endocannabinoid system with p62, as treatment with CB2 receptor agonists resulted in slightly different effects on bone remodeling, bone cell number, and activity in the absence of p62. Although the observed differences are slight, our results suggest an interplay between these two proteins and their signaling complexes. Future studies should investigate whether this molecular link affects bone processes under pathological conditions or at older ages and is thus involved, for example, in disorganized bone turnover or osteoclast activity.

MATERIALS AND METHODS

Drugs

JWH133 (Tocris) was injected s.c. in a concentration of 5 µg/g body weight in a DMSO/Tween 80/NaCl (0.9%) solution (ratio of 1:1:18). Calcein was applied i.p. in a 16 mM NaCl (0.9%)/ NaHCO3 buffered solution.

Mice

Knockout-first p62 mice (C57BL/6N-Sqstm1tm1a (KOMP)Wtsi) were available from the KOMP directory on a C57BL/6N background (ID: 41073) and carried a promoter-driven selection cassette (lacZ and neomycin). In our animal facility, the mice were crossed with C57BL/6J mice (Charles River) for >6 generations to produce fertile offspring that grew normally. Animals were kept in a 12 h:12 h light–dark cycle, with a room temperature of 22°C and 55% humidity and housed with ad libitum access to food and water. All experimental procedures were kept and tested according to the German and European Community laws on the protection of experimental animals and approved by the Behörde für Gesundheit und Verbraucherschutz of the City of Hamburg (project identification code number 139/ 15 and154/16).

Short-Term 5-Day Treatment With CB2 Agonist

Two groups of p62 KO and WT littermate mice were used for this experiment. Starting on day 0, mice were injected with calcein (30 mg/kg, ip) and additionally injected with either vehicle or JWH133 (5 mg/kg, s.c.) during days 1–5. Calcein was injected a second time on day 7. Calcein fluorescently labeled newly mineralized bone and determined bone formation rate. All animals were sacrificed on day 9. During the experiment, mice were monitored daily, and the body weight was determined to monitor the health state.

Long-Term Treatment

Male mice were injected with either JWH133 or vehicle three times per week over a time period of 4 weeks. Vehicle or JWH133 (5 mg/kg) was subcutaneously injected into the 6-month-old animals. Calcein at a concentration of 30 mg/kg was injected intraperitoneally 9 and 2 days prior to the end of the experiment. All animals were sacrificed on day 31 and organs were harvested. Mice were monitored daily, and the body weight was measured before each injection to monitor the health state of mice during the experiment.

Preparation of Mice for µCT and Histomorphometry

Mice were anesthetized by 80%/20% (v/v) CO_2/O_2 inhalation followed by 100% CO_2 to sacrifice the animals. Skin, fat, and organs were removed. The whole mice (muscle and bone) were fixed in 3,5% PFA for at least 24 h and were then transferred to 80% ethanol until used.

Microcomputed Tomography (µCT)

This technique is used to image the three-dimensional structure of the cortical and trabecular bone of small rodents. A custom-made sample holder was used to image 12 femurs at the same time (designed by Dr. Timur Yorgan, Institute of Osteology and Biomechanics at the UKE). The right femur of each mouse was used and scanned with a voxel resolution of 10 µm using µCT 40 desktop cone-beam µCT (Scanco Medical, Bruttisellen, Switzerland). Trabecular bone was analyzed in the distal metaphysis with a volume of 2,500 µm–500 µm proximal to the distal growth plate. Cortical bone was also analyzed in a 1 mm long section of the mid-diaphysis. A threshold value of 300 was used for cortical bone evaluation, and a value of 250 was used for trabecular bone.

Histomorphometry

Static, dynamic, and cellular histomorphometry were already established in the laboratory of Prof. Dr. Michael Amling (UKE, Department of Osteology and Biomechanics). For non-decalcified histology vertebral bodies L1 to L5 were first dehydrated in increasing alcohol concentrations (1–5 h 70% EtOH, two times 1 h 80% EtOH, four times 1 h 96% EtOH, four times 1 h 96% EtOH). Afterward, the samples were incubated for 24 h in an infiltration solution (1,000 ml methyl methacrylate (MMA) destabilized, 3,3g benzoyl peroxide, 100 ml nonylphenol) at 4°C and then transferred to incubation solution II for another 24 h. Next, the samples were embedded in methyl methacrylate and sectioned at 4 µm thickness (for structural and cellular histomorphometry) and 12 µm thickness (dynamic histomorphometry) in the sagittal plane on a Microtec rotation microtome (Techno-Med GmbH, Bielefeld, Germany). 80% isopropyl alcohol and dibutyl ether was applied for stretching. Finally, the slides were dried at 60°C overnight.

Kossa/van Gieson Staining

Kossa/van Gieson staining was used for structural histomorphometry and to stain mineralized bone matrix black and osteoid red. To remove pMMA from the samples, they were incubated three times in 2-methoxyethylacetate. Afterwards, the slides were rehydrated in descending alcohol concentrations (two times 2 min 100% ethanol, 2 min 96% ethanol, 2 min 80% ethanol, 2 min 70% ethanol, and 2 min 50% ethanol) and rinsed with water. The samples were stained subsequently with 3% silver nitrate and rinsed with water. Then, they were stained in soda-formol solution and rinsed with water. Next, they were

stained in 5% sodium thiosulfate and van Gieson solution with interspersed water rinsing steps. The slides were dehydrated in increasing alcohol concentrations and incubated three times in xylene for 5 min. The slides were mounted with DPX mounting solution and covered with a coverslip.

Toluidine Blue Staining

Additional staining with 1% toluidine blue was used for cellular histomorphometry. Depending on the amount of RNA and DNA within the different tissues and cellular compartments, diverse shades of blue were obtained. The plastic was removed by 2-methoxyethylacetate (incubation for three times and 5 min) and rehydration in descending alcohol concentrations (2 times 2 min 100% ethanol, 2 min 96% ethanol, 2 min 80% ethanol, 2 min 70% ethanol, 2 min 50% ethanol). After rinsing in water, the sections were stained in toluidine blue staining solution for 30 min, followed by water and dehydration through ascending alcohol concentrations. After incubation in xylene for 5 min (three times), the slides were mounted with DPX mounting solution and covered with a coverslip.

Histomorphometric Quantification

Structural histomorphometry was performed on van Kossa/van Gieson stained slides of the lumbar vertebral. The parameters bone volume per tissue volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular spacing (Tb.Sp) were analyzed using Bioquant software. For dynamic histomorphometry, calcein bands were analyzed by the OsteoMeasure histomorphometry system (Osteometrics Inc., United States) on non-stained 12 µm thick lumbar vertebral sections. The mineral apposition rate (MAR), mineral surface per bone surface (MS/BS), and bone formation rate per bone surface (BFR/BS) were determined. Cellular histomorphometry was performed using toluidine blue-stained slides of lumbar vertebral sections. Cellular parameters such as osteoblast surface per bone surface (Ob.S/BS), osteoclast surface per bone surface (Oc.S/BS), number of osteoblasts per bone perimeter (N.Ob/B.Pm), and number of osteoclasts per bone perimeter (N.Oc/B.Pm) were examined using the OsteoMeasure histomorphometry system (Osteometrics Inc., United States).

Statistics

The two-tailed unpaired t-test was used to make comparisons between WT with p62 KO mice in one variable ($*p < 0.05$, $**p < 0.01$, and $***p < 0.001$). Two-way repeated measurement ANOVA (two-way ANOVA) was applied to subjects to follow the time course. If the analysis of variance showed a significant effect of genotype, interaction, treatment, or time ($\#p < 0.05$, $\#\#p < 0.01$, and $\#\#\#p < 0.001$), then Bonferroni's multiple comparison post hoc testing was applied ($*p < 0.05$, $**p < 0.01$, and $***p < 0.001$). The statistical analysis and the graphs were made with GraphPad Prism version 7 (GraphPad Software California, United States). Numerical values are presented as mean ± SEM, and n refers to the number of mice used in this experiment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The study involving animals was reviewed and approved by the Behörde für Gesundheit und Verbraucherschutz of the City of Hamburg.

AUTHOR CONTRIBUTIONS

CK, TY, and SR performed the experiments. CK and MK conceptualized and designed the study, conducted data analysis and interpretation, and wrote the manuscript. TY and TS contributed to the scientific discussion and interpretation of the data. All the authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This work was funded by the DFG, Grant Number KA 2306/3-1.

ACKNOWLEDGMENTS

The authors would like to thank the technical assistance of the Institute of Osteology and Biomechanics for their great help in bone characterization, the UC Davis group for providing the KOMP p62 mouse embryos, and all former and current lab members of the AG Karsak.

REFERENCES

Agas, D., Amaroli, A., Lacava, G., Yanagawa, T., and Sabbieti, M. G. (2020). Loss of P62 Impairs Bone Turnover and Inhibits PTH-Induced Osteogenesis. *J. Cel Physiol* 235, 7516–7529. doi:10.1002/jcp.29654

Bab, I., and Zimmer, A. (2008). Cannabinoid Receptors and the Regulation of Bone Mass. *Br. J. Pharmacol.* 153, 182–188. doi:10.1038/sj.bjp.0707593

Berendsen, A. D., and Olsen, B. R. (2015). Bone Development. *Bone* 80, 14–18. doi:10.1016/j.bone.2015.04.035

Berkamp, S., Mostafavi, S., and Sachse, C. (2020). Structure and Function of p62/SQSTM1 in the Emerging Framework of Phase Separation. *FEBS J.* 288 (24), 6927–6941. doi:10.1111/febs.15672

Capote, A. E., Batra, A., Warren, C. M., Chowdhury, S. A. K., Wolska, B. M., Solaro, R. J., et al. (2021). B-arrestin-2 Signaling Is Important to Preserve Cardiac Function during Aging. *Front. Physiol.* 12, 696852. doi:10.3389/fphys.2021.696852

Cha-Molstad, H., Yu, J. E., Feng, Z., Lee, S. H., Kim, J. G., Yang, P., et al. (2017). p62/SQSTM1/Sequestosome-1 Is an N-Recognin of the N-End Rule Pathway Which Modulates Autophagosome Biogenesis. *Nat. Commun.* 8, 102. doi:10.1038/s41467-017-00085-7

Chamoux, E., Couture, J., Bisson, M., Morissette, J., Brown, J. P., and Roux, S. (2009). The P62 P392L Mutation Linked to Paget's Disease Induces Activation of Human Osteoclasts. *Mol. Endocrinol.* 23, 1668–1680. doi:10.1210/me.2009-0066

Chen, X., Zheng, C., Qian, J., Sutton, S. W., Wang, Z., Lv, J., et al. (2014). Involvement of β-arrestin-2 and Clathrin in Agonist-Mediated Internalization of the Human Cannabinoid CB2 Receptor. *Curr. Mol. Pharmacol.* 7, 67–80. doi:10.2174/1874467207666140714115824

Dalle, S., and Koppo, K. (2021). Cannabinoid Receptor 1 Expression Is Higher in Muscle of Old vs. Young Males, and Increases upon Resistance Exercise in Older Adults. *Sci. Rep.* 11, 18349. doi:10.1038/s41598-021-97859-3

Daroszewska, A., Rose, L., Sarsam, N., Charlesworth, G., Prior, A., Rose, K., et al. (2018). Zoledronic Acid Prevents Pagetic-like Lesions and Accelerated Bone Loss in the p62P394L Mouse Model of Paget's Disease. *Dis. Model. Mech.* 11, dmm035576. doi:10.1242/dmm.035576

Durán, A., Serrano, M., Leitges, M., Flores, J. M., Picard, S., Brown, J. P., et al. (2004). The Atypical PKC-Interacting Protein P62 Is an Important Mediator of RANK-Activated Osteoclastogenesis. *Dev. Cel* 6, 303–309. doi:10.1016/s1534-5807(03)00403-9

Falchetti, A., Di Stefano, M., Marini, F., Ortolani, S., Ulivieri, M. F., Bergui, S., et al. (2009). Genetic Epidemiology of Paget's Disease of Bone in italy: Sequestosome1/p62 Gene Mutational Test and Haplotype Analysis at 5q35 in a Large Representative Series of Sporadic and Familial Italian Cases of Paget's Disease of Bone. *Calcif Tissue Int.* 84, 20–37. doi:10.1007/s00223-008-9192-8

Gennari, L., Rendina, D., Falchetti, A., and Merlotti, D. (2019). Paget's Disease of Bone. *Calcif Tissue Int.* 104, 483–500. doi:10.1007/s00223-019-00522-3

Hiruma, Y., Kurihara, N., Subler, M. A., Zhou, H., Boykin, C. S., Zhang, H., et al. (2008). A SQSTM1/p62 Mutation Linked to Paget's Disease Increases the Osteoclastogenic Potential of the Bone Microenvironment. *Hum. Mol. Genet.* 17, 3708–3719. doi:10.1093/hmg/ddn266

Idris, A. I., Sophocleous, A., Landao-Bassonga, E., Van't Hof, R. J., and Ralston, S. H. (2008). Regulation of Bone Mass, Osteoclast Function, and Ovariectomy-Induced Bone Loss by the Type 2 Cannabinoid Receptor. *Endocrinology* 149, 5619–5626. doi:10.1210/en.2008-0150

Idris, A. I., Van 'T Hof, R. J., Greig, I. R., Ridge, S. A., Baker, D., Ross, R. A., et al. (2005). Regulation of Bone Mass, Bone Loss and Osteoclast Activity by Cannabinoid Receptors. *Nat. Med.* 11, 774–779. doi:10.1038/nm1255

Kappen, C., Neubüser, A., Balling, R., and Finnell, R. (2007). Molecular Basis for Skeletal Variation: Insights from Developmental Genetic Studies in Mice. *Birth Defects Res. B Dev. Reprod. Toxicol.* 80, 425–450. doi:10.1002/bdrb.20136

Ke, P., Shao, B. Z., Xu, Z. Q., Wei, W., Han, B. Z., Chen, X. W., et al. (2016). Activation of Cannabinoid Receptor 2 Ameliorates DSS-Induced Colitis through Inhibiting NLRP3 Inflammasome in Macrophages. *PLoS One* 11, e0155076. doi:10.1371/journal.pone.0155076

Komatsu, M., Kageyama, S., and Ichimura, Y. (2012). p62/SQSTM1/A170: Physiology and Pathology. *Pharmacol. Res.* 66, 457–462. doi:10.1016/j.phrs.2012.07.004

Kurihara, N., Hiruma, Y., Yamana, K., Michou, L., Rousseau, C., Morissette, J., et al. (2011). Contributions of the Measles Virus Nucleocapsid Gene and the SQSTM1/p62(P392L) Mutation to Paget's Disease. *Cell Metab* 13, 23–34. doi:10.1016/j.cmet.2010.12.002

Lamark, T., Perander, M., Outzen, H., Kristiansen, K., Øvervatn, A., Michaelsen, E., et al. (2003). Interaction Codes within the Family of Mammalian Phox and Bem1p Domain-Containing Proteins. *J. Biol. Chem.* 278, 34568–34581. doi:10.1074/jbc.M303221200

Lozano-Ondoua, A. N., Hanlon, K. E., Symons-Liguori, A. M., Largent-Milnes, T. M., Havelin, J. J., Ferland, H. L., 3rd, et al. (2013). Disease Modification of Breast Cancer-Induced Bone Remodeling by Cannabinoid 2 Receptor Agonists. *J. Bone Miner. Res.* 28 (1), 92–107. doi:10.1002/jbmr.1732

Mcmanus, S., and Roux, S. (2012). The Adaptor Protein p62/SQSTM1 in Osteoclast Signaling Pathways. *J. Mol. Signal.* 7, 1. doi:10.1186/1750-2187-7-1

Mlost, J., Kostrzewa, M., Borczyk, M., Bryk, M., Chwastek, J., Korostyński, M., et al. (2021). CB2 Agonism Controls Pain and Subchondral Bone Degeneration Induced by Mono-Iodoacetate: Implications GPCR Functional Bias and Tolerance Development. *Biomed. Pharmacother.* 136, 111283. doi:10.1016/j.biopha.2021.111283

Morissette, J., Laurin, N., and Brown, J. P. (2006). Sequestosome 1: Mutation Frequencies, Haplotypes, and Phenotypes in Familial Paget's Disease of Bone. *J. Bone Miner Res.* 21 (Suppl. 2), P38–P44. doi:10.1359/jbmr.06s207

Moscat, J., Diaz-Meco, M. T., and Wooten, M. W. (2007). Signal Integration and Diversification through the P62 Scaffold Protein. *Trends Biochem. Sci.* 32, 95–100. doi:10.1016/j.tibs.2006.12.002

Ofek, O., Karsak, M., Leclerc, N., Fogel, M., Frenkel, B., Wright, K., et al. (2006). Peripheral Cannabinoid Receptor, CB2, Regulates Bone Mass. *Proc. Natl. Acad. Sci. U S A.* 103, 696–701. doi:10.1073/pnas.0504187103

Ozcivici, E., Luu, Y. K., Adler, B., Qin, Y. X., Rubin, J., Judex, S., et al. (2010). Mechanical Signals as Anabolic Agents in Bone. *Nat. Rev. Rheumatol.* 6, 50–59. doi:10.1038/nrrheum.2009.239

Pankiv, S., Clausen, T. H., Lamark, T., Brech, A., Bruun, J. A., Outzen, H., et al. (2007). p62/SQSTM1 Binds Directly to Atg8/LC3 to Facilitate Degradation of Ubiquitinated Protein Aggregates by Autophagy. *J. Biol. Chem.* 282, 24131–24145. doi:10.1074/jbc.M702824200

Paoletta, M., Moretti, A., Liguori, S., Di Paola, A., Tortora, C., Argenziano, M., et al. (2021). Role of the Endocannabinoid/Endovanilloid System in the Modulation of Osteoclast Activity in Paget's Disease of Bone. *Int. J. Mol. Sci.* 22, 10158. doi:10.3390/ijms221810158

Papageorgiou, M., Föger-Samwald, U., Wahl, K., Kerschan-Schindl, K., and Pietschmann, P. (2020). Age- and Strain-Related Differences in Bone Microstructure and Body Composition during Development in Inbred Male Mouse Strains. *Calcif Tissue Int.* 106, 431–443. doi:10.1007/s00223-019-00652-8

Patel, M., Matti, C., Grimsey, N. L., Legler, D. F., Javitch, J. A., Finlay, D. B., et al. (2021). Delineating the Interactions between the Cannabinoid CB2 Receptor and its Regulatory Effectors; Beta-Arrestins and G Protein-Coupled Receptor Kinases. *Br. J. Pharmacol.* 1, 1. doi:10.1111/bph.15748

Pertwee, R. G. (2015). Endocannabinoids and Their Pharmacological Actions. *Handb Exp. Pharmacol.* 231, 1–37. doi:10.1007/978-3-319-20825-1_1

Rea, S. L., Walsh, J. P., Layfield, R., Ratajczak, T., and Xu, J. (2013). New Insights into the Role of Sequestosome 1/p62 Mutant Proteins in the Pathogenesis of Paget's Disease of Bone. *Endocr. Rev.* 34, 501–524. doi:10.1210/er.2012-1034

Rodriguez, A., Durán, A., Selloum, M., Champy, M. F., Diez-Guerra, F. J., Flores, J. M., et al. (2006). Mature-onset Obesity and Insulin Resistance in Mice Deficient in the Signaling Adapter P62. *Cel Metab* 3, 211–222. doi:10.1016/j.cmet.2006.01.011

Roodman, G. D., and Windle, J. J. (2005). Paget Disease of Bone. *J. Clin. Invest.* 115, 200–208. doi:10.1172/JCI24281

Rossi, F., Bellini, G., Tortora, C., Bernardo, M. E., Luongo, L., Conforti, A., et al. (2015). CB(2) and TRPV(1) Receptors Oppositely Modulate *In Vitro* Human Osteoblast Activity. *Pharmacol. Res.* 99, 194–201. doi:10.1016/j.phrs.2015.06.010

Sánchez-Martín, P., and Komatsu, M. (2018). p62/SQSTM1 - Steering the Cell through Health and Disease. *J. Cel Sci* 131, jcs222836. doi:10.1242/jcs.222836

Sankaran, J. S., Varshney, M., and Judex, S. (2017). Differences in Bone Structure and Unloading-Induced Bone Loss between C57BL/6N and C57BL/6J Mice. *Mamm. Genome* 28, 476–486. doi:10.1007/s00335-017-9717-4

Sanz, L., Diaz-Meco, M. T., Nakano, H., and Moscat, J. (2000). The Atypical PKC-Interacting Protein P62 Channels NF-kappaB Activation by the IL-1-TRAF6 Pathway. *EMBO J.* 19, 1576–1586. doi:10.1093/emboj/19.7.1576

Sharaf, A., Mensching, L., Keller, C., Rading, S., Scheffold, M., Palkowitsch, L., et al. (2019). Systematic Affinity Purification Coupled to Mass Spectrometry Identified P62 as Part of the Cannabinoid Receptor CB2 Interactome. *Front. Mol. Neurosci.* 12, 224. doi:10.3389/fnmol.2019.00224

Simon, M. M., Greenaway, S., White, J. K., Fuchs, H., Gailus-Durner, V., Wells, S., et al. (2013). A Comparative Phenotypic and Genomic Analysis of C57BL/6J and C57BL/6N Mouse Strains. *Genome Biol.* 14, R82. doi:10.1186/gb-2013-14-7-r82

Soethoudt, M., Grether, U., Fingerle, J., Grim, T. W., Fezza, F., De Petrocellis, L., et al. (2017). Cannabinoid CB2 Receptor Ligand Profiling Reveals Biased Signalling and Off-Target Activity. *Nat. Commun.* 8, 13958. doi:10.1038/ncomms13958

Sophocleous, A., Idris, A. I., and Ralston, S. H. (2014a). Genetic Background Modifies the Effects of Type 2 Cannabinoid Receptor Deficiency on Bone Mass and Bone Turnover. *Calcif Tissue Int.* 94, 259–268. doi:10.1007/s00223-013-9793-8

Sophocleous, A., Landao-Bassonga, E., Van't Hof, R. J., Idris, A. I., and Ralston, S. H. (2011). The Type 2 Cannabinoid Receptor Regulates Bone Mass and Ovariectomy-Induced Bone Loss by Affecting Osteoblast Differentiation and Bone Formation. *Endocrinology* 152, 2141–2149. doi:10.1210/en.2010-0930

Sophocleous, A., Marino, S., Kabir, D., Ralston, S. H., and Idris, A. I. (2017). Combined Deficiency of the Cnr1 and Cnr2 Receptors Protects against Age-Related Bone Loss by Osteoclast Inhibition. *Aging Cell* 16, 1051–1061. doi:10.1111/acel.12638

Sophocleous, A., Marino, S., Logan, J. G., Mollat, P., Ralston, S. H., and Idris, A. I. (2015). Bone Cell-Autonomous Contribution of Type 2 Cannabinoid Receptor to Breast Cancer-Induced Osteolysis. *J. Biol. Chem.* 290, 22049–22060. doi:10.1074/jbc.M115.649608

Sophocleous, A., Sims, A. H., Idris, A. I., and Ralston, S. H. (2014b). Modulation of Strain-specific Differences in Gene Expression by Cannabinoid Type 2 Receptor Deficiency. *Calcif Tissue Int.* 94, 423–432. doi:10.1007/s00223-013-9823-6

Sophocleous, A., Yiallourides, M., Zeng, F., Pantelas, P., Stylianou, E., Li, B., et al. (2022). Association of Cannabinoid Receptor Modulation with normal and Abnormal Skeletal Remodelling: A Systematic Review and Meta-Analysis of *In Vitro*, *In Vivo* and Human Studies. *Pharmacol. Res.* 175, 105928. doi:10.1016/j.phrs.2021.105928

Udoh, M., Santiago, M., Devenish, S., Mcgregor, I. S., and Connor, M. (2019). Cannabichromene Is a Cannabinoid CB2 Receptor Agonist. *Br. J. Pharmacol.* 176, 4537–4547. doi:10.1111/bph.14815

Willinghamm, M. D., Brodt, M. D., Lee, K. L., Stephens, A. L., Ye, J., and Silva, M. J. (2010). Age-related Changes in Bone Structure and Strength in Female and Male BALB/c Mice. *Calcif Tissue Int.* 86, 470–483. doi:10.1007/s00223-010-9359-y

Woo, J. A., Liu, T., Fang, C. C., Castaño, M. A., Kee, T., Yrigoin, K., et al. (2020). β-Arrestin2 Oligomers Impair the Clearance of Pathological Tau and Increase Tau Aggregates. *Proc. Natl. Acad. Sci. U S A.* 117, 5006–5015. doi:10.1073/pnas.1917194117

Wu, A., Hu, P., Lin, J., Xia, W., and Zhang, R. (2018). Activating Cannabinoid Receptor 2 Protects against Diabetic Cardiomyopathy through Autophagy Induction. *Front. Pharmacol.* 9, 1292. doi:10.3389/fphar.2018.01292

Xu, A., Yang, Y., Shao, Y., Wu, M., and Sun, Y. (2020). Activation of Cannabinoid Receptor Type 2-induced Osteogenic Differentiation Involves Autophagy Induction and P62-Mediated Nrf2 Deactivation. *Cell Commun Signal* 18, 9. doi:10.1186/s12964-020-0512-6

Zach, F., Polzer, F., Mueller, A., and Gessner, A. (2018). p62/sequestosome 1 Deficiency Accelerates Osteoclastogenesis *In Vitro* and Leads to Paget's Disease-like Bone Phenotypes in Mice. *J. Biol. Chem.* 293, 9530–9541. doi:10.1074/jbc.RA118.002449

Zhu, M., Yu, B., Bai, J., Wang, X., Guo, X., Liu, Y., et al. (2019). Cannabinoid Receptor 2 Agonist Prevents Local and Systemic Inflammatory Bone Destruction in Rheumatoid Arthritis. *J. Bone Miner Res.* 34, 739–751. doi:10.1002/jbmr.3637

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Pharmacology

ORIGINAL RESEARCH
published: 21 April 2022
doi: 10.3389/fphar.2022.803331

Modulation of Morphine Analgesia, Antinociceptive Tolerance, and Mu-Opioid Receptor Binding by the Cannabinoid CB2 Receptor Agonist O-1966

*Zachary W. Reichenbach[1,2], Kelly DiMattio[1], Suren Rajakaruna[3], David Ambrose[3], William D. Cornwell[3], Ronald J. Tallarida[1], Thomas Rogers[3], Lee-Yuan Liu-Chen[1], Ronald F. Tuma[1] and Sara Jane Ward[1]**

[1]Center for Substance Abuse Research (CSAR), Department of Neural Sciences, Lewis Katz School of Medicine, Temple University, Philadelphia, PA, United States, [2]Department of Gastroenterology and Hepatology, Temple University Hospital, Philadelphia, PA, United States, [3]Center for Inflammation, Translational, and Clinical Lung Research, Lewis Katz School of Medicine, Temple University, Philadelphia, PA, United States

OPEN ACCESS

Edited by:
Salvatore Salomone,
University of Catania, Italy

Reviewed by:
Erin Bobeck,
Utah State University, United States
Susruta Majumdar,
St. Louis College of Pharmacy,
United States

***Correspondence:**
Sara Jane Ward
sara.ward@temple.edu

Specialty section:
This article was submitted to Experimental Pharmacology and Drug Discovery, a section of the journal Frontiers in Pharmacology

Received: 27 October 2021
Accepted: 31 March 2022
Published: 21 April 2022

Citation:
Reichenbach ZW, DiMattio K, Rajakaruna S, Ambrose D, Cornwell WD, Tallarida RJ, Rogers T, Liu-Chen L-Y Tuma RF and Ward SJ (2022) Modulation of Morphine Analgesia, Antinociceptive Tolerance, and Mu-Opioid Receptor Binding by the Cannabinoid CB2 Receptor Agonist O-1966. Front. Pharmacol. 13:803331. doi: 10.3389/fphar.2022.803331

Acutely, non-selective cannabinoid (CB) agonists have been shown to increase morphine antinociceptive effects, and we and others have also demonstrated that non-selective CB agonists attenuate morphine antinociceptive tolerance. Activation of cannabinoid CB2 receptors reverses allodynia and hyperalgesia in models of chronic pain, and co-administration of morphine with CB2 receptor selective agonists has been shown to be synergistic. CB2 receptor activation has also been shown to reduce morphine-induced hyperalgesia in rodents, an effect attributed to CB2 receptor modulation of inflammation. In the present set of experiments, we tested both the acute and chronic interactions between morphine and the CB2 receptor selective agonist O-1966 treatments on antinociception and antinociceptive tolerance in C57Bl6 mice. Co-administration of morphine and O-1966 was tested under three dosing regimens: simultaneous administration, morphine pre-treated with O-1966, and O-1966 pre-treated with morphine. The effects of O-1966 on mu-opioid receptor binding were determined using [3H]DAMGO and [^{35}S]GTPγS binding assays, and these interactions were further examined by FRET analysis linked to flow cytometry. Results yielded surprising evidence of interactions between the CB2 receptor selective agonist O-1966 and morphine that were dependent upon the order of administration. When O-1966 was administered prior to or simultaneous with morphine, morphine antinociception was attenuated and antinociceptive tolerance was exacerbated. When O-1966 was administered following morphine, morphine antinociception was not affected and antinociceptive tolerance was attenuated. The [^{35}S]GTPγS results suggest that O-1966 interrupts functional activity of morphine at the mu-opioid receptor, leading to decreased potency of morphine to produce acute thermal antinociceptive effects and potentiation of morphine antinociceptive tolerance. However, O-1966 administered after morphine blocked morphine hyperalgesia and led to

an attenuation of morphine tolerance, perhaps due to well-documented anti-inflammatory effects of CB2 receptor agonism.

Keywords: morphine, CB2 receptor agonist, antinociception, antinociceptive tolerance, inflammation

INTRODUCTION

Cannabinoid receptor agonists produce antinociception in a variety of animal models, and the majority of these effects appear to be mediated by CB1 receptors. Interactions between cannabinoid and opioid receptor systems remain an area of intense research, especially in light of the mounting importance of identifying safer and more effective pain therapies that may be able to reduce opioid use and associated harms. Acutely, the non-selective CB agonists Δ9-tetrahydrocannabinol (THC) and CP-55,940 have been shown to increase morphine antinociceptive effects (Smith et al., 1998; Manzanares et al., 1999; Finn et al., 2004; Tham et al., 2005; Vigano et al., 2005; Maguire and France 2018). We and others have also demonstrated that non-selective CB agonists attenuate morphine antinociceptive tolerance (Cichewicz et al., 2001; Cichewicz and Welch 2003; Fischer et al., 2010). The CB1 receptor is abundantly expressed throughout the central nervous system and identified as the cannabinoid receptor responsible for the "psychoactive" effects of non-selective cannabinoid agonists such as THC; therefore, it is presumed that these CB agonist effects on morphine tolerance are associated with their actions on the CB1 receptor. However, this remains to be demonstrated empirically.

Relative to CB1 receptors, detection of CB2 receptors in the CNS of naïve animals remains relatively low to absent, and by and large CB2 receptor activation does not lead to the range of CNS effects associated with CB1 receptor activation, such as euphoria, changes in mood, and alterations in cognition. However, CB2 receptor expression is upregulated within the CNS in animal models of chronic inflammatory or neuropathic pain (Zhang et al., 2003; Wotherspoon et al., 2005; Beltramo et al., 2006), and activation of CB2 receptors reverses allodynia and hyperalgesia in these models (Guindon and Hohmann 2008; Rahn et al., 2011). In addition, co-administration of morphine with CB2 receptor selective agonists synergistically inhibits inflammatory, post-operative and neuropathic pain in rodent models (Grenald et al., 2017; Yuill et al., 2017; Iyer et al., 2020) and reduces morphine-induced thermal hyperalgesia in rats (Tumati et al., 2012). While a preponderance of studies has demonstrated that tolerance is associated with a significant reduction in functional surface μ opioid receptors (Williams et al., 2013). Other studies have suggested that morphine tolerance is due at least in part to direct microglial activation and the release of proinflammatory cytokines (Hutchinson et al., 2007, see Hutchinson et al., 2011 for review). Our laboratory has extensively characterized the protective and anti-inflammatory effects of the CB2 receptor agonist O-1966 in several rodent models of CNS injury (Zhang et al., 2007; Adhikary et al., 2011; Elliott et al., 2011; Amenta et al., 2012; Ramirez et al., 2012; Ronca et al., 2015). As CB2 receptor activation has been shown to significantly modulate inflammatory responses, including inhibition of microglial activation, we hypothesized that CB2 receptor activation may lead to attenuation of morphine antinociceptive tolerance.

In the present set of experiments, we tested both the acute and chronic interactions between morphine and O-1966 treatments alone and in combination on antinociception and antinociceptive tolerance and hyperalgesia in C57Bl6 mice using a standard hot plate assay. Based on previous research, we hypothesized that O-1966 would be devoid of acute antinociceptive effects but would attenuate morphine antinociceptive tolerance. Because our first results from our acute hotplate experiments revealed an unpredicted attenuating effect of O-1966 on acute morphine antinociception, we proceeded in these acute studies as well as the tolerance studies to test administration of morphine and O-1966 under three dosing regimens: concurrent administration, morphine pre-treated with O-1966, and O-1966 pre-treated with morphine. Based on the results of these experiments revealing that the order of drug administration had dramatic effects on how these two drugs affected morphine analgesia and analgesic tolerance, we further tested the hypothesis that select interactive effects between O-1966 and morphine were a result of direct effects of this CB2 receptor agonist on the μ opioid receptor. The effects of O-1966 on mu-opioid receptor binding were determined using [3H]DAMGO and [^{35}S]GTPγS binding assays. Lastly, as our behavioral data revealed that O-1966 could attenuate morphine antinociception but also facilitate morphine tolerance, we tested the hypothesis that O-1966 was interfering with mu-opioid receptor homodimerizationvia FRET analysis linked to flow cytometry.

MATERIALS AND METHODS

Drugs

For *in vivo* experiments, O-1966 (Organix Laboratories, Massachusetts, USA) and SR144528 (RTI) were prepared in ethanol:Cremophor:Saline (1:1:18). Morphine was dissolved in 0.9% saline. All injections were given i. p. in a volume of 10 ml/kg. For *in vitro* experiments, O-1966 and SR144528 were dissolved in DMSO (final concentration 2% in assays) and morphine was dissolved in Milli-Q water. The affinity of O-1966 for CB1 and CB2 cannabinoid receptors was reported previously to be 5055 ± 984 and 23 ± 2.1 nmol/L, respectively (Wiley et al., 2002).

Animals

All experiments were conducted in 7 to 8-week-old male C57BL/6 mice weighing 18–23 g (Taconic Laboratories, New York, USA). Studies were conducted in accordance with the guidelines approved by the Institutional Animal Care and Use Committee at Temple University. Animals were housed under a 12 h light/dark cycle with lights on at 07:00 h and maintained on

a regular chow diet and had access to food and water *ad libitum* throughout the study. All experimental groups were n = 8/ treatment condition.

Measurement of Hot Plate Withdrawal Latency

Nociception was analyzed by means of a hot plate analgesia meter (Columbus Instruments, Columbus, OH). Mice were placed on a hot plate maintained at 54.0 ± 0.5°C. The latency to hind paw lick, hind paw lift, hind paw flutter, mouse shuffle, or mouse jump was measured to the nearest 0.1 s as described in Fischer et al., 2010. A maximal cutoff of 30 s was utilized to prevent injury to the paw tissue. Immediately after the end of the trial, mice were returned to their home cage. The latency to respond at 54°C was measured twice at 2 and 1.5 h prior to the beginning of drug administration, and these data were averaged to yield one baseline value. Following baseline latency measurements, multiple 30 min cycles were run and drugs and drug mixtures were administered cumulatively. During this procedure, cumulative doses of morphine, O-1966, or their combination were administered during the first min of each cycle (i.e., 30-min inter-injection interval), increasing in one-half log unit increments, and antinociceptive measurements were determined during the last minute of each cycle. Latencies obtained following drug administration were reported as Percent Maximal Possible Effect (%MPE). The following formula was utilized to calculate such:

$$\%MPE = \frac{(Experimental\ Latency - Average\ Baseline\ Latency)}{(Maximal\ Cut\ Off\ Time - Average\ Basline\ Latency)} \times 100$$

The antinociceptive effects of 1) morphine alone, 2) O-1966 alone, 3) their simultaneous administration, and 4) their simultaneous administration following CB2 antagonist treatment, were assessed in the same group of mice, with a 1 week washout period separating each drug or drug combination testing. In a separate group of mice, the antinociceptive effects of 1) morphine alone, 2) O-1966 administration followed 15 min later by morphine administration, and 3) morphine administration followed 15 min later by O-1966 administration were assessed with a 1-week washout period separating each drug or drug combination testing.

Induction of Morphine Antinociceptive Tolerance

One day following assessment of hot plate withdrawal latencies and the generation of baseline morphine dose-response curves, separate groups of mice were treated twice daily separated by 10 h for 5 days, as described in Fischer et al., 2010, with two vehicle regimens (saline, cremophor vehicle), two morphine alone dosing regimens (32 mg/kg, 100 mg/kg), and three morphine + O-1966 dosing regimens (simultaneous, O-1966 followed 15 min by morphine, morphine followed 15 min by O-1966). Reassessment of hot plate withdrawal latencies and morphine dose response curves began 14 h after the last tolerance regimen injections.

In Vitro Materials

[Tyrosyl-3, 5-^{3}H(N)]-DAMGO (56 Ci/mmol) and [^{35}S]GTPγS (1,250 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA); sucrose, bovine serum albumin (BSA), phenylmethylsulfonyl fluoride, GDP and GTPγS were purchased from Sigma-Aldrich (St. Louis, MO). DMEM/F12, trypsin and penicillin/streptomycin were purchased from Gibco Life Technologies (Grand Island, NY). The following reagents were purchased from the indicated companies: geneticin (G418), Cellgro Mediatech, Inc. (Herndon, VA); EcoScint scintillation fluid, National Diagnostics (Atlanta, GA); fetal bovine serum (FBS), Atlanta Biologicals (Atlanta, GA). Naloxone and morphine were generously provided by the National Institute on Drug Abuse (Bethesda, MD).

Cell Lines and Membrane Preparation

The following is a modified procedure from Wang et al. (Wang et al., 2005). CHO cells stably transfected with the rat mu-opioid receptor were established previously (Chen et al., 1995). Cells were cultured in 100-mm culture dishes in Dulbecco's modified Eagle's medium/F-12 HAM supplemented with 10% FBS, 0.3 mg/ml geneticin, 100 units/ml penicillin, and 100 g/ml streptomycin in a humidified atmosphere consisting of 5% CO2 and 95% air at 37 °C. Membranes were prepared according to a modified procedure of Zhu et al. (1997). Cells were washed twice and harvested in 1x PBS containing 0.5 mM EDTA and centrifuged at 500 *g* for 3 min. The cell pellet was suspended in lysis buffer (25 mM Tris, pH 7.4, 1 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride), passed through a 26 3/8-gauge needle 10 times and then centrifuged at 46,000 g for 30 min. The pellet was rinsed twice with lysis buffer and resuspended in 50 mM Tris-HCl buffer/0.32 M sucrose (pH 7.4), aliquoted and frozen in dry ice/ethanol, and stored at 80°C. All procedures were performed at 4 °C.

Receptor Binding Assays

The binding affinity of O-1966 to rMOR was determined by competitive inhibition of [^{3}H]DAMGO binding to CHO-rMOR membranes was performed with [^{3}H]DAMGO at a concentration close to its K_d value (2 nM), using six concentrations (0.1 nM–1 μM) of unlabeled O-1966. The reaction was performed in 50 mM Tris-HCl buffer containing 1 nM EGTA and 0.1% (w/v) BSA (pH 7.4) at room temperature for 1 h in duplicate in a volume of 1 ml with 15–25 μg of membrane protein. Naloxone (10 μM) was used to define nonspecific binding. The reaction was terminated by filtration of bound and free [^{3}H]DAMGO with GF/B filters presoaked with 50 mM Tris, pH 7.4, 0.1 mg/ml BSA, and 0.2% polyethyleneimine under reduced pressure. The filter was washed with ice-cold buffer containing 100 mM Tris (pH 7.6) and 0.154 M NaCl and radioactivity in filters were determined by liquid scintillation counting. This binding was repeated three times and data were analyzed and the K_i value of O-1966 was determined with GraphPad Prism Software.

Ligand-Stimulated [35S]GTPγS Binding

To determine the effects of CB2 compounds on G protein activation at the mu-opioid receptor by morphine, we used clonal Chinese hamster ovary cells stably expressing the rat MOR (CHO-rMOR) due to their lack of endogenous cannabinoid receptors [^{35}S]GTPγS binding was performed as previously described following a modified protocol (Zhu et al., 1997). Briefly, membranes (containing 10 μg protein) were incubated with 10 μM GDP and ~0.4 nM [^{35}S]GTPγS in reaction buffer (50 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA) in the following two paradigms in a final volume of 0.5 ml:

Morphine pretreatment

0.5 μM morphine for 10 min at 30°C followed by 1 nM–10 μM CB2 compound (O-1966, SR144528, or O-1966 + SR144528).

CB2 pretreatment

1 nM–10 μM CB2 compound (O-1966, SR144528, or O-1966 + SR144528) for 10 min at 30°C followed by 0.5 μM morphine.

Reaction mixtures were incubated for 1 h at 30°C. Nonspecific binding was determined in the presence of 10 μM GTPγS. Subsequently, bound and free [^{35}S]GTPγS were separated by filtration with GF/B filters under reduced pressure and the filter was washed with ice-cold buffer containing 50 mM Tris (pH 7.6), 5 mM $MgCl_2$ and 50 mM NaCl. Radioactivity in filters was determined by liquid scintillation counting. All experiments were performed in duplicate and repeated three times. Data were analyzed and values were determined with GraphPad Prism Software.

FRET Analysis

Fluorescence (Forsters) resonance energy transfer (FRET) analysis was used to determine the level of MOR dimerization by employing a modification of the flow cytometry method of Banning et al. (2010). The CHO cell line was transiently transfected with either rat MOR-CFP or MOR-YFP (molecular constructs a generous gift from Dr. Ping-Yee Law, University of Minnesota), or both to determine the energy transfer between MOR dimers. CHO cells were cultured in log phase and transfected with the 4D-Nucleofector (Lonza Group Ltd., Basel, Switzerland) using manufacturer's procedure for this cell line. Cells were excited in the flow cytometer with a 405 nm laser, and the CFP emission was detected with a standard 450 nm filter, while the FRET was detected with a 530 nm filter. Control samples were established with non-transfected CHO cells, CHO cells transfected with either MOR-YFP or MOR-CFP alone, and cell mixtures of CHO-YPF (single transfection) and CHO-CFP (single transfection) cells. The degree of FRET is measured by the degree of fluorescence intensity in the FRET cytometry gate using mean fluorescence intensity. Flow cytometry was carried out with the Becton-Dickinson Influx cytometer (BD Biosciences, San Jose, CA).

Data Analysis

The dose of morphine alone or O-1966 alone or in combination required to produce 50% maximum antinociceptive effect (ED50) during hotplate tests was derived using regression analysis (GraphPad Prism 5.0 software, Inc., La Jolla, CA).

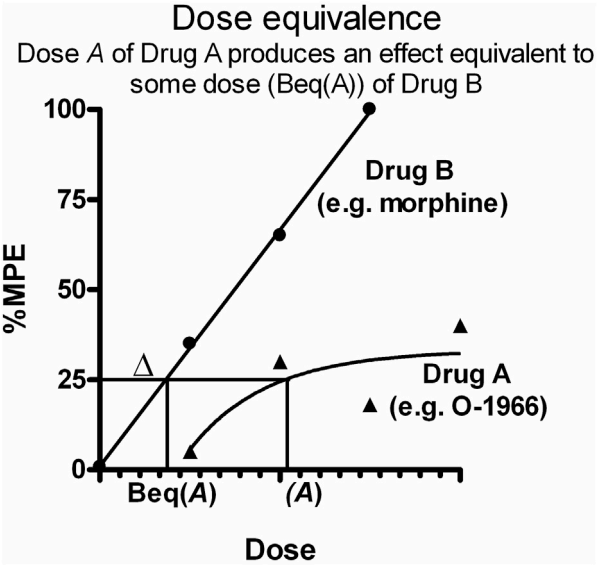


FIGURE 1 | Graphical representation of application of dose equivalence analysis to data from two drugs producing dose-response effects fit to different slopes. Each dose A of Drug A (e.g. O-1966) is equally effective to some dose of a more efficacious drug (Drug B, e.g. morphine). This equi-effective dose of Drug B is designated Beq **(A)**, or Δ. In the drug combination **(A,B)**, the administered Drug B dose B is increased by Δ, and the sum of the two doses (B+ Δ) allows the calculation of expected effects. Analysis proceeds comparing the expected effect with the observed effect.

when at least three data points were available on the linear portion of the dose-effect curve or by interpolation when only two data points (one above and one below 50%) were available. Acute studies were analyzed by comparing the expected effect with the observed effect using the principle of dose equivalence and application of a Student's t-test. This approach was taken instead of dose addition and isobolographic analysis as it was determined that morphine produced a linear dose response curve while the dose response for O-1966 was hyperbolic (Tallarida and Raffa 2010).

In dose equivalence analysis, the result of adding a given dose of Drug A (*a*) to a dose of Drug B (*b*) that produces a known effect level is predicted and then compared to the observed effect of the dose combination (*a, b*) (**Figure 1**). It is based on the principle that each dose *A* of Drug A (e.g. O-1966) is equally effective to some dose of a more efficacious drug (Drug B, e.g. morphine). As this equi-effective dose of Drug B is the equivalent dose in effect to dose *A,* it is designated *Beq (A),* or Δ. Therefore, in the combination (*A, B*), the administered Drug B dose *B* is increased by Δ, and the sum of the two doses (*B*+ Δ) allows the calculation of expected effects. Analysis proceeds comparing the expected effect with the observed effect. Student's *t*-test was used to compare the expected effect to the observed effect for all dose combinations in order to determine the nature of interaction between the morphine and O-1966 (additivity, sub-additivity, or synergy). For tolerance studies, doses producing a 50% reduction in nociception on the hotplate

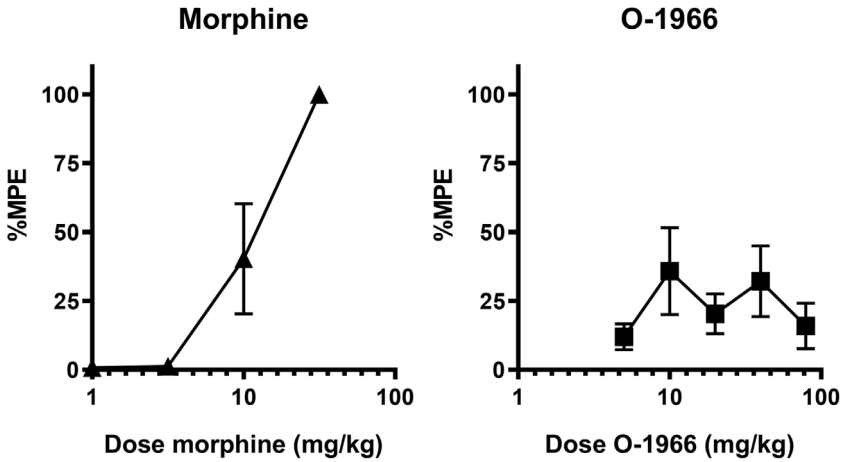


FIGURE 2 | Effect of morphine and O-1966 alone on antinociception as measured by withdrawal latency on a 54°C hotplate. X-axis: Cumulative dose of morphine (A), or O-1966 (B) in mg/kg. *Y*-axis: antinociception as percent maximum possible effect. Each data point represents the mean (±S.E.M.) from eight mice.

TABLE 1 | Predicted additive and actual observed ED50 values for simultaneous administration of O-1966, SR144528, and morphine combinations on acute antinociception on the hotplate.

Dose: O-1966(+SR144528)+Morphine	Effect additive	Effect observed Simultaneous administration
1.25 + 3.0	25.1	9.77
2.5 + 10	47.4	17.2
5.0 + 30	88.1	18.2
10 + 100	100	70.4
20 + 300	100	78

(ED50s) for morphine antinociception on Days 1 and 7 were calculated as the mean and SEM from individual animal ED50 calculations. Fold increases were determined by dividing Day 7 ED50s by Day 1 ED50s for each treatment group. Therefore, a relative potency of one suggests a lack of tolerance development (i.e. no shift in the morphine dose-effect curve). In contrast, a relative potency greater than one suggests that tolerance has developed (i.e. a rightward shift in the morphine dose-effect curve), and a quantitatively greater relative potency is indicative of increased tolerance development. Hyperalgesia was measured by comparing pre-drug baseline hotplate latencies between Day 1 and Day 7 using a Student's t-test. GTPγS binding data were analyzed by two-way ANOVA with order and concentration as factors. Results of FRET analysis were evaluated with one-way ANOVA.

RESULTS

Acute Morphine Antinociception

Cumulative dosing of morphine produced dose-dependent antinociception that was linearly related to dose with an ED50 value of 9.1 (1.6) (**Figure 2A**). In contrast, the CB2 agonist O-1966 showed limited efficacy and values that fit to the standard hyperbolic dose-effect function using nonlinear regression (**Figure 2B**). The two fitted curves allowed for the determination of the expected additive effect for each dose combination tested for comparison with the experimentally derived (observed) effect (**Table 1**).

For the combination experiments, a dose of 2.5 mg/kg O-1966 was selected to be tested in combination with the approximate ED50 dose of 10 mg/kg morphine to generate rational dose combinations for the prediction and experimental determination of effect. We selected this dose of O-1966 based on previously demonstrated robust effects from our laboratory of O-1966 at the 5.0 mg/kg dose on neuroprotection in several models. A full range of O-1966 + morphine dose combinations were explored based on this ratio of equi-effective doses. The results showed that when administered at the same time, the combination of morphine and O-1966 was subadditive, with statistical analysis showing a significant difference ($p < 0.05$) between the observed effects and predicted additive effects (**Figure 3A**; **Table 1**). Pretreatment with CB2 antagonist at the same dose as CB2 agonist showed that SR144528 attenuated the sub-additive interaction and restored the morphine dose-effect curve. The ED50 (sem) was determined to be 31.5 (5.68) for morphine + O-1966, and 11.8 (1.97) for morphine + O-1966 + SR144528. Parallel line analysis (Tallarida and Murray 1987) was used to determine that the three lines have slopes that are not significantly different (**Figure 3B**; **Table 1**).

In a separate group of mice, it was also determined that the acute antinociceptive interaction between morphine and O-1966 was dependent on the order of administration prior to

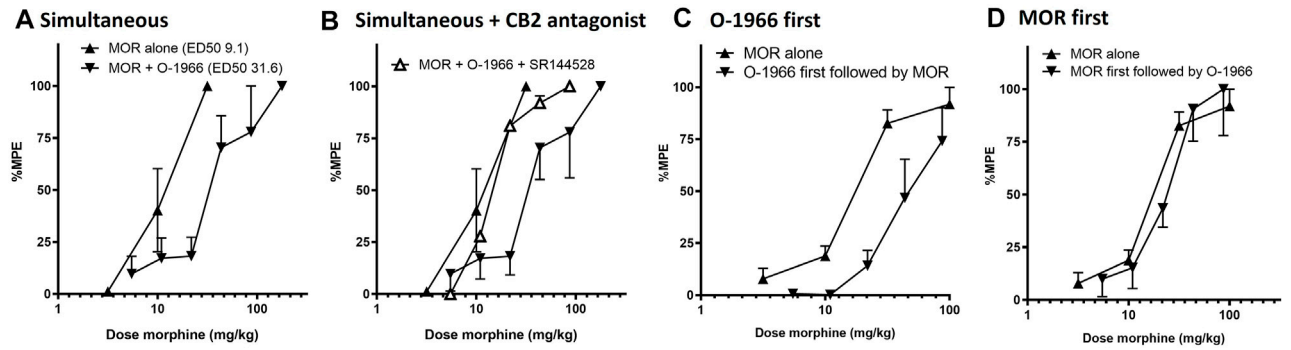


FIGURE 3 | Order effect of morphine and O-1966 in combination on antinociception as measured by withdrawal latency on a 54°C hotplate. Morphine and O-1966 were either administered simultaneously **(A)**, simultaneous with SR144528 **(B)**, O-1966 15 min prior to morphine **(C)**, or morphine 15 min prior to O-1966 **(D)**. X-axis: Cumulative dose of morphine in mg/kg. *Y*-axis: antinociception as percent maximum possible effect. Each data point represents the mean (±S.E.M.) from eight mice. See **Table 1** for doses of O-1966 and SR-144528 that corresponded to administered doses of morphine.

TABLE 2 | Effect of chronic dosing regimens on development of morphine antinociceptive tolerance.

Tolerance regimen	Day 1 ED50(sem)	Day 7 ED50(sem)	Fold shift
Saline	7.2 (1.9)	4.1 (0.6)	0.6
Cremophor vehicle	8.0 (2.9	12.2 (2.3)	1.5
Morphine 32	4.0 (1.3)	8.6 (3.4)	2.2
Morphine 100	7.1 (2.0)	27.8 (10.8)	3.9
O-1966 then Morphine 32	7.5 (1.7)	15.6 (8.2)	2.1
O-1966 then Morphine 100	6.4 (2.3)	42.0 (16.5	6.6
Morphine 100 then)-1966	6.6 (1.8)	12.6 (3.3)	1.9
O-1996	10.7 (2.4)	9.9 (2.6)	0.9

hotplate testing. Pretreatment with O-1966 15 min prior to morphine administration resulted in an approximate 2.5-fold shift in the morphine dose response curve (from an ED50 of 16.4 (1.1) to an ED50 of 43.8 (5.8) (**Figure 3C**), while no shift was observed when morphine was administered 15 min prior to O-1966 administration (ED50 20.8 (4.6)) (**Figure 3D**).

Morphine Antinociceptive Tolerance and Hyperalgesia

Chronic administration for 5 days with either saline or cremophor vehicle had no effect on morphine antinociception. Chronic administration of morphine produced a dose-dependent rightward shift in the morphine dose response curve, with twice daily administration of 100 mg/kg morphine leading to an approximate 4-fold shift in morphine's antinociceptive potency (**Table 2**; **Figure 4A,B**).

Pretreatment with 5.0 mg/kg O-1966 15 min prior to each morphine injection during the tolerance regimen led to a further rightward shift in the morphine dose response curve, with twice daily administration of O-1966 + morphine leading to an approximate 6.5-fold shift in morphine's antinociceptive

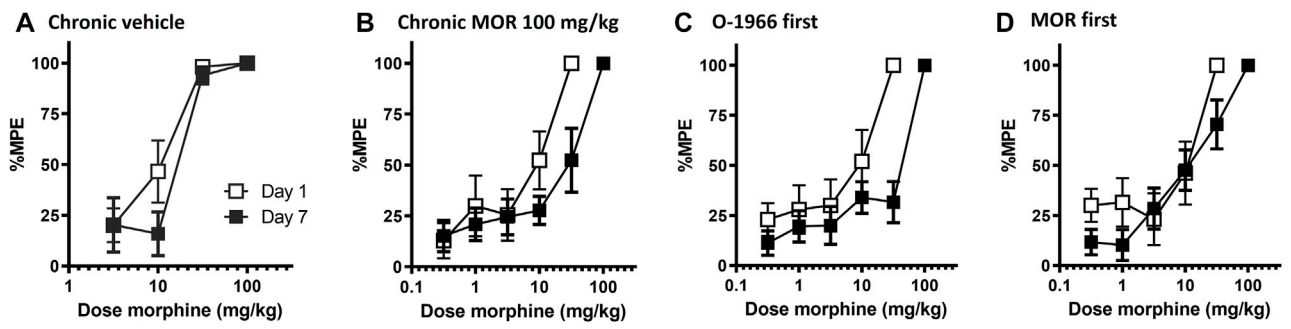


FIGURE 4 | Effect of chronic dosing regimens of vehicle **(A)**, morphine **(B)**, O-1966 (data shown in **Table 2**), and morphine + O-1966 **(C,D)** on development of morphine antinociceptive tolerance. *X*-axis: Cumulative dose of morphine in mg/kg. *Y*-axis: antinociception as percent maximum possible effect. Each data point represents the mean (±S.E.M.) from eight mice. Open squares represent morphine antinociceptive effect on Day 1, and closed squared represent morphine antinociceptive effect in the same mice on Day 7 following a 5 day chronic dosing regimen. Titles above the graphs describe agents administered during the 5 day dosing regimen. In groups that received morphine and O-1966 during the dosing regimen, drugs were given 15 min apart.

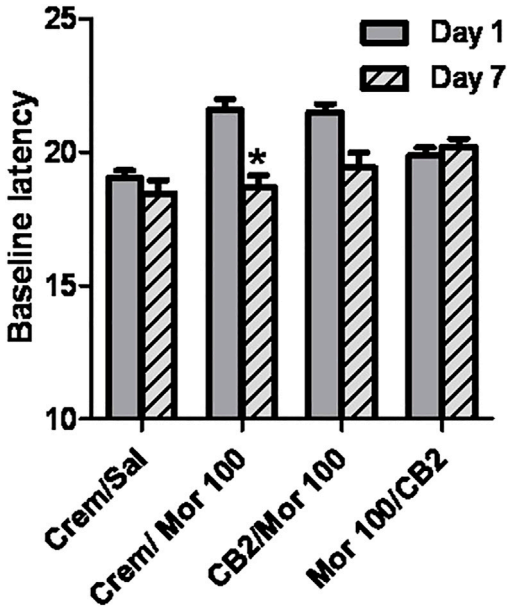


FIGURE 5 | Effect of chronic dosing regimens on development of morphine hyperalgesia. *X*-axis: Agents administered during the 5 day chronic dosing regimen. *Y*-axis: Baseline latency to lift, lick, or shuffle hindpaw(s) on a 54°C hotplate prior to morphine antinociceptive testing. Each bar represents the mean (±S.E.M.) from eight mice. Solid grey bars represent baselines on Day 1, and hatched grey and black bars represent baselines in the same mice on Day 7 following a 5 day chronic dosing regimen. In groups that received morphine and O-1966 during the dosing regimen, drugs were given 15 min apart.

potency (**Table 2**; **Figure 4C**). Conversely, when 5.0 mg/kg O-1966 was administered 15 min following each morphine injection during the tolerance regimen, the rightward shift in the morphine dose response curve was smaller than that seen following morphine alone treatment, producing an approximate 2-fold shift in morphine's antinociceptive potency (**Table 2**; **Figure 4D**). Chronic administration for 5 days with O-1966 alone had no effect on morphine antinociception (**Table 2**).

Morphine-Induced Hyperalgesia

The presence of hyperalgesia was determined by comparing withdrawal latencies at baseline on day 1 with those measured on day 7 following the 5-day dosing regimen. The only group that showed a significant decrease in thermal sensitivity on day 7 as compared with day 1 was the group that received Morphine 100 mg/kg alone, as measured by Student's t-test, $p < 0.05$ (**Figure 5**). No other treatment regimen produced a significant change in baseline sensitivity to the hotplate.

Displacement of [3H]DAMGO by O-1966

Competition binding with O-1966 and [^{3}H]DAMGO (2 nM) revealed that O-1966 does not have appreciable affinity for the CHO-rMOR. The K_i value for O-1966 was 3.04 μM (**Figure 6**).

[35S]GTPγS Binding in CHO Cell Membranes

In the O-1966 experiment (**Figure 7A**), two-way ANOVA revealed a significant effect of order of application [$F(1,16) = 18.19$, $p < 0.05$] and significant effect of O-1966 concentration [$F(3, 16) = 3.253$, $p < 0.05$ but no significant interaction [$F(3, 16)<1$, ns]. Bonferroni posttest revealed a significant difference between treatment groups at the 10 μM concentration of O-1966. In the SR144528 experiment (**Figure 7B**), two-way ANOVA revealed a significant effect of order of application [$F(1,16) = 7.178$, $p < 0.05$] but no significant effect of SR144528 concentration [$F(3, 16)<1$, ns] and no significant interaction [$F(3, 16)<1$, ns]. Bonferroni posttest revealed no significant difference between treatment groups at any concentration of SR144528. In the SR144528 + O-1966 experiment (**Figure 7C**), two-way ANOVA revealed a significant effect of order of application [$F(1,16) = 17.97$, $p < 0.05$] but no significant effect of SR144528 concentration [$F(3, 16)<1$, ns] and no significant interaction [$F(3, 16)<1$, ns]. Bonferroni posttest revealed a significant difference between treatment groups at the 0.1 μM concentration of SR144528 + 0.1 μM concentration of O-1966. A comparison of the effect of O-1966, SR144528, and SR144528+O-1966 pretreatments shows that O-1966 alone at the 10 μM concentration attenuates morphine-stimulated [35S]GTPγS binding, and that this attenuation is blocked by co-administration of SR144528 (**Figure 7D**).

MOR Dimerization

Experiments were carried out using FRET analysis to determine the impact of O-1966 treatment on MOR dimers. CHO cells were co-transfected with molecular constructs which express MOR-CFP and MOR-YFP and assessing the energy between the CFP and YFP

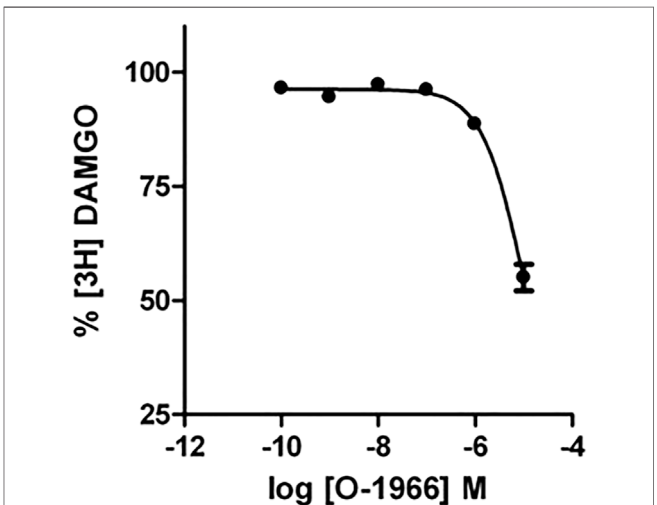


FIGURE 6 | Effect of increasing concentrations of O-1966 on [^{3}H] DAMGO binding. O-1966 has a low affinity for the rMOR. At a dose of 10 μM, O-1966 inhibited ~50% of radiolabled [^{3}H]DAMGO (2 nM) to rMOR. Lower doses (0.1 nM–1 μM) of O-1966 have no effect on [^{3}H]DAMGO binding to CHO-rMOR. Each data point represents the mean (±S.E.M.) from three independent experiments run in duplicate.

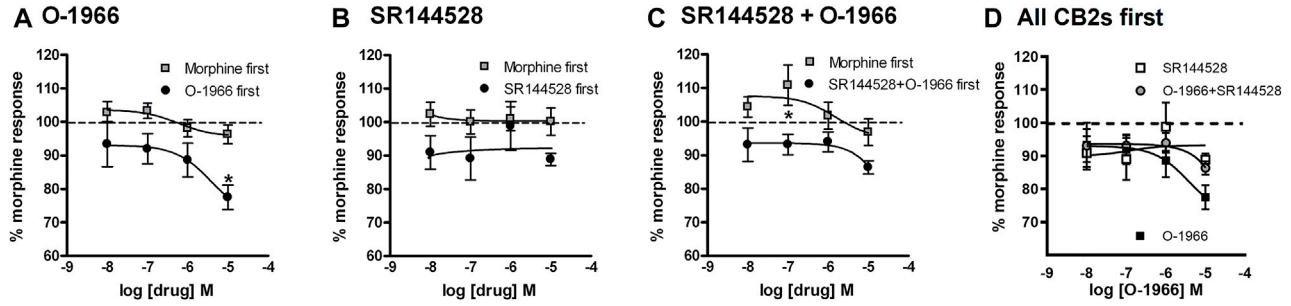


FIGURE 7 | Effect of increasing doses of O-1966 and SR144528 on morphine-stimulated GTPγS binding. O-1966 given before, but not after, morphine inhibits morphine response in [^{35}S]GTPγS binding assays. At the highest dose (10 μM) O-1966, there is a 20% reduction in the morphine response from baseline. Open squares indicate 10 min pretreatment with 0.5 μM morphine and closed squares indicate 10 min pretreatment with varying concentrations of either O-1966 **(A)**, SR144528 **(B)**, or a combination of SR144528 with O-1966 **(C)** (10 nM–10 μM). When the cannabinoid compounds are administered first, the co-administration of SR144528 and O-1966 indicates that the inhibitory effect of O-1966 can be blocked by SR144528 **(D)**. Each data point represents the mean (±S.E.M.) of three independent experiments run in duplicate (*) indicates a statistically significant ($p < 0.05$) difference from baseline morphine response.

TABLE 3 | FRET Analysis of morphine and O-1966 co-treated cells.

Group	FRET inhibition
Control	0 ± 0
morphine (10 min)+O-1966 (60 min)	17.7 ± 4.2
O-1966 (10 min)+morphine (60 min)	11.9 ± 3.8
morphine (60 min) + O-1966 (10 min)	16.3 ± 4.2
O-1966 (60 min) + morphine (10 min)	18.6 ± 2.9
Morphine	5.7 ± 1.4
O-1966	–0.5 ± 3.7

fluorescence partners. The results (**Table 3**) show that co-transfected cells treated with either morphine or O-1966, followed with either O-1966 or morphine, respectively, showed some reduction in the level of dimerization (based on inhibition of FRET). However, the data show that the pre-treatment with O-1966 did not significantly change the dimer status when compared to morphine pre-treatment. In no case was there a statistically significant difference between the morphine and O-1966 pretreatment groups.

DISCUSSION

The present results demonstrate modulation of morphine antinociception and antinociceptive tolerance by the CB2-selective agonist O-1966. Our results support the previous finding by Tumati et al. (2012) that CB2 receptor agonism attenuated the development of morphine hyperalgesia, and partially supported the findings by Zhang et al., 2016 and Lin et al., 2018 that co-administration of CB2 receptor agonists with morphine reduced development of antinociceptive tolerance in rodent models of cancer pain and chemotherapy-induced neuropathic pain respectively.

In contrast to our overall hypotheses, however, we observed that co-administration of the CB2-selective agonist attenuated acute morphine antinociception, while having more complex effects on the development of morphine tolerance, with all of these findings depending on the order of administration of O-1966 and morphine. The effect of O-1966 on acute morphine antinociception was dependent on O-1966 being administered prior to or simultaneous with morphine and was reversed by co-administration of the CB2 selective antagonist SR144528. In contrast, when morphine was administered prior to O-1966, O-1966 had no effect on morphine acute antinociception.

During the tolerance dosing regimen, chronic administration of morphine led to the induction of morphine tolerance as measured by the hotplate. Administration of O-1966 prior to each morphine injection during the chronic dosing regimen led to a significantly more pronounced tolerance than did morphine alone. Oppositely, when morphine was administered prior to O-1966 during the chronic dosing regimen, this combination led to the development of less tolerance than did chronic administration of morphine alone. Taken together, these results suggest that two distinct mechanisms of O-1966 action are mediating these opposing effects on the development of morphine antinociceptive tolerance.

These observations that pretreatment with O-1966 led to decreased morphine acute antinociception and increased morphine antinociceptive tolerance led us to speculate that O-1966 was directly affecting the function of the mu-opioid receptor, as it appears from these data that O-1966 is interfering with mu-opioid receptor activation acutely and mu-opioid receptor availability following the tolerance dosing regimen. We observed that O-1966 dose-dependently decreased [3H] DAMGO binding, but only at a high concentration, with a K_i value for O-1966 of 3.04 μM. These data suggest that O-1966 may be functioning as a negative allosteric modulator at the mu-opioid receptor, interfering with the orthostatic binding site. In the GTPγS binding assay, done in MOR-CHO cells absent of CB2 receptors, we found that administration of O-1966 decreased functional activation of the mu-opioid receptor by morphine. The observed interaction between O-1966 and functional activity was also shown to be dependent on order of administration, in

that application of O-1966 prior to morphine decreased GTPγS-activation, while application of morphine followed by O-1966 did not impact the ability of morphine to stimulate the G-protein. The effect of O-1966 pretreatment on morphine-stimulated GTPγS binding was also blocked by co-administration with the CB2 receptor antagonist SR144528, as was seen on the hotplate, again suggesting that O-1966, and well as SR144528, interactions with morphine are mediated at least in part by direct activity on mu-opioid receptors. These data suggest that we observe different pharmacological effects of O-1966 on morphine antinociception and tolerance based on order of administration based on whether the presence of O-1966 is interfering with morphine binding at the mu-opioid receptor.

There are other examples in the literature of cannabinoid compounds that can interact in a similar manner with the mu-opioid receptor (see Raffa and Ward 2012 for review). For example, the phytocannabinoids THC and cannabidiol, which share several structural similarities with O-1966, have also been reported as allosteric modulators at the mu and delta opioid receptors (Kathmann et al., 2006). Additional reports have linked CB1 selective antagonists with direct actions on mu-opioid receptors. For example, the CB1 selective antagonist SR141716 (AKA rimonabant) also significantly decreases both basal and DAMGO-stimulated GTPγS binding in MOR-CHO membranes and in mouse cortex and binds directly to MORs with low micromolar affinity (Cinar and Szucs 2009). Also, Seely et al. (2012) reported that SR141716 and the structurally similar CB1 receptor antagonist AM-251 bind with mid-nanomolar affinity to human mu-opioid receptors, antagonize morphine–induced G-protein activation in MOR-CHO cells, and attenuate morphine antinociception.

Our results suggest that the presence of the CB2 agonist O-1966 may alter the functional activity of morphine at the receptor, impacting both the acute antinociceptive effects of morphine as well as its ability to produce antinociceptive tolerance. To follow up on this line of thinking, we tested the hypothesis that our findings were a result of O-1966-mediated disruption of mu-opioid receptor homodimerization that might lead to less analgesic efficacy but increased mu-opioid receptor internalization. Studies from a number of laboratories have supported the notion that the mu opioid receptor forms both homodimers and heterodimers with other class A GPCRs, and the functional activity of these oligomers is the subject of ongoing research (Ferre et al., 2014; Moller et al., 2020). We considered the possibility that the pre-treatment with O-1966 might alter the physical status of mu-opioid receptor homodimerization. Indeed, binding pockets have been identified, that when occupied, can impact mu-opioid receptor homodimerization (Zheng et al., 2012), so perhaps O-1966 binding was interrupting this process. Our FRET results showed that co-transfected cells treated with either morphine or O-1966, followed with either O-1966 or morphine, respectively, showed some reduction in the level of dimerization, based on inhibition of FRET. However, the data show that the pre-treatment with O-1966 did not significantly change the dimer status when compared to morphine pre-treatment. In no case was there a statistically significant difference between the morphine and O-1966 pretreatment groups. Taken together, the mechanism of the O-1966 effect on the function of MOR is not clear at this time, but suggest that O-1966 functions as a negative allosteric modulator at the mu-opioid receptor, leading to attenuation of the acute antinociceptive effects of morphine, but additional experiments are needed to determine this and rule out a role for direct activation of CB2 receptors on this interaction.

As mentioned previously, we did observe that when O-1966 treatment followed daily morphine administration, this combination lessened the development of antinociceptive tolerance and hyperalgesia. This supported our initial hypothesis, which we formed based on other work showing interplay between CB2 receptors, inflammation, and morphine tolerance (e.g. Huang et al., 2012; Jin et al., 2012; Jun et al., 2013; Vacca et al., 2013). We did not test whether our morphine/O-1966 dosing regimes altered inflammation in the present study, but as previously mentioned we have extensively characterized the protective and anti-inflammatory effects of the CB2 receptor agonist O-1966 in several rodent models of CNS injury (Zhang et al., 2007; Adhikary et al., 2011; Elliott et al., 2011; Amenta et al., 2012; Ramirez et al., 2012; Ronca et al., 2015). Therefore based on the order effects of our data results suggest that when O-1966 is administered following morphine (and mu receptor signalling is not impacted), O-1966 is working through a CB2 receptor mediated anti-inflammatory mechanism to decrease the development of morphine tolerance. As mentioned in the methods section, the affinity of O-1966 for CB1 and CB2 cannabinoid receptors was reported previously to be 5055 ± 984 and 23 ± 2.1 nmol/L, and we have not observed any hallmark CB1 receptor activation effects of O-1966 throughout our experience with the compound.

Further studies must be undertaken to determine whether this attenuation, as well as the attenuation observed in morphine hyperalgesia, was associated with anti-inflammatory, glial-inhibitory effects of O-1966 in this assay. Lastly, given the identification of sex differences regarding opioid analgesia and analgesic tolerance, cannabinoid pharmacology, as well as neuroinflammation, further work should also be conducted in female rodent models.

In conclusion, results from the present experiments provide surprising evidence of interactions between the CB2 receptor selective agonist O-1966 and morphine that are likely mediated in part by direct binding activity of O-1966 on the mu-opioid receptor, a property shared by other cannabinoid ligands as well. This interaction results in decreased potency of morphine to produce acute thermal antinociceptive effects but can also lead to the potentiation of morphine antinociceptive tolerance, suggesting complex alterations in morphine signaling. However, O-1966 co-administration also blocked morphine hyperalgesia, and led to an attenuation of morphine tolerance when administration followed each morphine injection, perhaps due to well-documented anti-inflammatory effects of CB2 receptor agonism. Overall, these data demonstrate that like other cannabinoid ligands, CB2 receptor ligands can influence the antinociceptive effects of morphine, and

more work needs to be done to determine the clinical implications of these interactions, given the promise of CB2 receptor agonist pharmacotherapy for treatment of diseases and disorders associated with CNS injury that are often accompanied by opioid analgesia use.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Temple University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

Participated in research design: ZWR, RTa, L-Y L-C, TR, RTu, and SJW. Conducted experiments: SW, ZWR, KDM, SR, Ambrose Performed data analysis: ZWR, KDM, RT, TR, and SJW. Wrote or contributed to the writing of the manuscript: ZWR, KDM, TR, RT, and SJW.

FUNDING

This work was supported by RO1A1084065 (RFT, funded animals and supplies for *in vivo* studies), RO1DA040619 (TJR, funded staff and supplies for FRET), RO1DA049745 (TJR, funded staff and supplies for FRET), P30DA013429 (EMU, funded mu-opioid receptor binding assays) and T32DA007237-24 (EMU, funded trainees).

REFERENCES

Adhikary, S., Li, H., Heller, J., Skarica, M., Zhang, M., Ganea, D., et al. (2011). Modulation of Inflammatory Responses by a Cannabinoid-2-Selective Agonist After Spinal Cord Injury. *J. Neurotrauma.* 28 (12), 2417–2427. doi:10.1089/neu.2011.1853

Amenta, P. S., Jallo, J. I., Tuma, R. F., and Elliott, M. A. (2012). A Cannabinoid Type 2 Receptor Agonist Attenuates Blood-Brain Barrier Damage and Neurodegeneration in a Murine Model of Traumatic Brain Injury. *J. Neurosci. Res.* 90 (12), 2293–2305. doi:10.1002/jnr.23114

Banning, C., Votteler, J., Hoffmann, D., Koppensteiner, H., Warmer, M., Reimer, R., et al. (2010). A Flow Cytometry-Based FRET Assay to Identify and Analyse Protein-Protein Interactions in Living Cells. *Plos One* 5 (2), e9344. doi:10.1371/journal.pone.0009344

Beltramo, M., Bernardini, N., Bertorelli, R., Campanella, M., Nicolussi, E., Fredduzzi, S., et al. (2006). CB2 Receptor-Mediated Antihyperalgesia: Possible Direct Involvement of Neural Mechanisms. *Eur. J. Neurosci.* 23 (6), 1530–1538. doi:10.1111/j.1460-9568.2006.04684.x

Chen, C., Xue, J. C., Zhu, J., Chen, Y. W., Kunapuli, S., Kim de Riel, J., et al. (1995). Characterization of Irreversible Binding of Beta-Funaltrexamine to the Cloned Rat Mu Opioid Receptor. *J. Biol. Chem.* 270 (30), 17866–17870. doi:10.1074/jbc.270.30.17866

Cichewicz, D. L., Haller, V. L., and Welch, S. P. (2001). Changes in Opioid and Cannabinoid Receptor Protein Following Short-Term Combination Treatment with delta(9)-tetrahydrocannabinol and Morphine. *J. Pharmacol. Exp. Ther.* 297 (1), 121–127.

Cichewicz, D. L., and Welch, S. P. (2003). Modulation of Oral Morphine Antinociceptive Tolerance and Naloxone-Precipitated Withdrawal Signs by Oral Delta 9-tetrahydrocannabinol. *J. Pharmacol. Exp. Ther.* 305 (3), 812–817. doi:10.1124/jpet.102.046870

Cinar, R., and Szücs, M. (2009). CB1 Receptor-independent Actions of SR141716 on G-Protein Signaling: Coapplication with the Mu-Opioid Agonist Tyr-D-Ala-Gly-(NMe)Phe-Gly-Ol Unmasks Novel, Pertussis Toxin-Insensitive Opioid Signaling in Mu-Opioid Receptor-Chinese Hamster Ovary Cells. *J. Pharmacol. Exp. Ther.* 330 (2), 567–574. doi:10.1124/jpet.109.152710

Elliott, M. B., Tuma, R. F., Amenta, P. S., Barbe, M. F., and Jallo, J. I. (2011). Acute Effects of a Selective Cannabinoid-2 Receptor Agonist on Neuroinflammation in a Model of Traumatic Brain Injury. *J. Neurotrauma.* 28 (6), 973–981. doi:10.1089/neu.2010.1672

Ferré, S., Casadó, V., Devi, L., Filizola, M., Jockers, R., Lohse, M., et al. (2014). G Protein–Coupled Receptor Oligomerization Revisited: Functional and Pharmacological Perspectives. *Pharmacol. Rev.* 66, 413–434.

Finn, D. P., Beckett, S. R., Roe, C. H., Madjd, A., Fone, K. C., Kendall, D. A., et al. (2004). Effects of Coadministration of Cannabinoids and Morphine on Nociceptive Behaviour, Brain Monoamines and HPA axis Activity in a Rat Model of Persistent Pain. *Eur. J. Neurosci.* 19 (3), 678–686. doi:10.1111/j.0953-816x.2004.03177.x

Fischer, B. D., Ward, S. J., Henry, F. E., and Dykstra, L. A. (2010). Attenuation of Morphine Antinociceptive Tolerance by a CB(1) Receptor Agonist and an NMDA Receptor Antagonist: Interactive Effects. *Neuropharmacology* 58 (2), 544–550. doi:10.1016/j.neuropharm.2009.08.005

Grenald, S. A., Young, M. A., Wang, Y., Ossipov, M. H., Ibrahim, M. M., Largent-Milnes, T. M., et al. (2017). Synergistic Attenuation of Chronic Pain Using Mu Opioid and Cannabinoid Receptor 2 Agonists. *Neuropharmacology* 116, 59–70. doi:10.1016/j.neuropharm.2016.12.008

Guindon, J., and Hohmann, A. G. (2008). Cannabinoid CB2 Receptors: A Therapeutic Target for the Treatment of Inflammatory and Neuropathic Pain. *Br. J. Pharmacol.* 153 (2), 319–334. doi:10.1038/sj.bjp.0707531

Huang, Y. N., Tsai, R. Y., Lin, S. L., Chien, C. C., Cherng, C. H., Wu, C. T., et al. (2012). Amitriptyline Attenuates Astrocyte Activation and Morphine Tolerance in Rats: Role of the PSD-95/NR1/nNOS/PKCγ Signaling Pathway. *Behav. Brain Res.* 229 (2), 401–411. doi:10.1016/j.bbr.2012.01.044

Hutchinson, M. R., Bland, S. T., Johnson, K. W., Rice, K. C., Maier, S. F., and Watkins, L. R. (2007). Opioid-induced Glial Activation: Mechanisms of Activation and Implications for Opioid Analgesia, Dependence, and Reward. *ScientificWorldJournal* 7, 98–111. doi:10.1100/tsw.2007.230

Hutchinson, M. R., Shavit, Y., Grace, P. M., Rice, K. C., Maier, S. F., and Watkins, L. R. (2011). Exploring the Neuroimmunopharmacology of Opioids: An Integrative Review of Mechanisms of central Immune Signaling and Their Implications for Opioid Analgesia. *Pharmacol. Rev.* 63 (3), 772–810. doi:10.1124/pr.110.004135

Iyer, V., Slivicki, R. A., Thomaz, A. C., Crystal, J. D., Mackie, K., and Hohmann, A. G. (2020). The Cannabinoid CB2 Receptor Agonist LY2828360 Synergizes with Morphine to Suppress Neuropathic Nociception and Attenuates Morphine Reward and Physical Dependence. *Eur. J. Pharmacol.* 886, 173544. doi:10.1016/j.ejphar.2020.173544

Jin, H., Li, Y. H., Xu, J. S., Guo, G. Q., Chen, D. L., and Bo, Y. (2012). Lipoxin A4 Analog Attenuates Morphine Antinociceptive Tolerance, Withdrawal-Induced Hyperalgesia, and Glial Reaction and Cytokine Expression in the Spinal Cord of Rat. *Neuroscience* 208, 1–10. doi:10.1016/j.neuroscience.2012.02.009

Jun, I. G., Kim, S. H., Yoon, Y. I., and Park, J. Y. (2013). Intrathecal Lamotrigine Attenuates Antinociceptive Morphine Tolerance and Suppresses Spinal Glial Cell Activation in Morphine-Tolerant Rats. *J. Korean Med. Sci.* 28 (2), 300–307. doi:10.3346/jkms.2013.28.2.300

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 (5), 354–361. doi:10.1007/s00210-006-0033-x

Lin, X., Dhopeshwarkar, A. S., Huibregtse, M., Mackie, K., and Hohmann, A. G. (2018). Slowly Signaling G Protein-Biased CB2 Cannabinoid Receptor Agonist

LY2828360 Suppresses Neuropathic Pain with Sustained Efficacy and Attenuates Morphine Tolerance and Dependence. *Mol. Pharmacol.* 93 (2), 49–62. doi:10.1124/mol.117.109355

Maguire, D. R., and France, C. P. (2018). Antinociceptive Effects of Mixtures of Mu Opioid Receptor Agonists and Cannabinoid Receptor Agonists in Rats: Impact of Drug and Fixed-Dose Ratio. *Eur. J. Pharmacol.* 819, 217–224. doi:10.1016/j.ejphar.2017.11.038

Manzanares, J., Corchero, J., Romero, J., Fernández-Ruiz, J. J., Ramos, J. A., and Fuentes, J. A. (1999). Pharmacological and Biochemical Interactions between Opioids and Cannabinoids. *Trends Pharmacol. Sci.* 20 (7), 287–294. doi:10.1016/s0165-6147(99)01339-5

Möller, J., Isbilir, A., Sungkaworn, T., Osberg, B., Karathanasis, C., Sunkara, V., et al. (2020). Single-molecule Analysis Reveals Agonist-specific Dimer Formation of μ-opioid Receptors. *Nat. Chem. Biol.* 16(9), 946–954. doi:10.1038/s41589-020-0566-1

Raffa, R. B., and Ward, S. J. (2012). CB_1-independent Mechanisms of Δ^9-THCV, AM251 and SR141716 (Rimonabant). *J. Clin. Pharm. Ther.* 37 (3), 260–265. doi:10.1111/j.1365-2710.2011.01284.x

Rahn, E. J., Thakur, G. A., Wood, J. A., Zvonok, A. M., Makriyannis, A., and Hohmann, A. G. (2011). Pharmacological Characterization of AM1710, a Putative Cannabinoid CB2 Agonist from the Cannabilactone Class: Antinociception without central Nervous System Side-Effects. *Pharmacol. Biochem. Behav.* 98 (4), 493–502. doi:10.1016/j.pbb.2011.02.024

Ramirez, S. H., Haskó, J., Skuba, A., Fan, S., Dykstra, H., McCormick, R., et al. (2012). Activation of Cannabinoid Receptor 2 Attenuates Leukocyte-Endothelial Cell Interactions and Blood-Brain Barrier Dysfunction Under Inflammatory Conditions. *J. Neurosci.* 32 (12), 4004–4016. doi:10.1523/JNEUROSCI.4628-11.2012

Ronca, R. D., Myers, A. M., Ganea, D., Fan, S., Tuma, R. F., Walker, E. A., et al. (2015). A Selective Cannabinoid CB2 Agonist Attenuates Damage and Improves Memory Retention Following Stroke in Mice. *Life Sci.* 138, 72–77. doi:10.1016/j.lfs.2015.05.005

Seely, K. A., Brents, L. K., Franks, L. N., Rajasekaran, M., Zimmerman, S. M., Fantegrossi, W. E., et al. (2012). AM-251 and Rimonabant Act as Direct Antagonists at Mu-Opioid Receptors: Implications for Opioid/cannabinoid Interaction Studies. *Neuropharmacology* 63 (5), 905–915. doi:10.1016/j.neuropharm.2012.06.046

Smith, F. L., Cichewicz, D., Martin, Z. L., and Welch, S. P. (1998). The Enhancement of Morphine Antinociception in Mice by delta9-tetrahydrocannabinol. *Pharmacol. Biochem. Behav.* 60 (2), 559–566. doi:10.1016/s0091-3057(98)00012-4

Tallarida, R. J., and Murray, R. B. (1987). *Manual of Pharmacologic Calculations with Computer Programs*. Second edition. New York: Springer-Verlag.

Tallarida, R. J., and Raffa, R. B. (2010). The Application of Drug Dose Equivalence in the Quantitative Analysis of Receptor Occupation and Drug Combinations. *Pharmacol. Ther.* 127 (2), 165–174. doi:10.1016/j.pharmthera.2010.04.011

Tham, S. M., Angus, J. A., Tudor, E. M., and Wright, C. E. (2005). Synergistic and Additive Interactions of the Cannabinoid Agonist CP55,940 with Mu Opioid Receptor and Alpha2-Adrenoceptor Agonists in Acute Pain Models in Mice. *Br. J. Pharmacol.* 144 (6), 875–884. doi:10.1038/sj.bjp.0706045

Tumati, S., Largent-Milnes, T. M., Keresztes, A., Ren, J., Roeske, W. R., Vanderah, T. W., et al. (2012). Repeated Morphine Treatment-Mediated Hyperalgesia, Allodynia and Spinal Glial Activation Are Blocked by Co-administration of a Selective Cannabinoid Receptor Type-2 Agonist. *J. Neuroimmunol* 244 (1-2), 23–31. doi:10.1016/j.jneuroim.2011.12.021

Vacca, V., Marinelli, S., Luvisetto, S., and Pavone, F. (2013). Botulinum Toxin A Increases Analgesic Effects of Morphine, Counters Development of Morphine Tolerance and Modulates Glia Activation and μ Opioid Receptor Expression in Neuropathic Mice. *Brain Behav. Immun.* 32, 40–50. doi:10.1016/j.bbi.2013.01.088

Viganò, D., Rubino, T., and Parolaro, D. (2005). Molecular and Cellular Basis of Cannabinoid and Opioid Interactions. *Pharmacol. Biochem. Behav.* 81 (2), 360–368. doi:10.1016/j.pbb.2005.01.021

Wang, Y., Tang, K., Inan, S., Siebert, D., Holzgrabe, U., Lee, D. Y., et al. (2005). Comparison of Pharmacological Activities of Three Distinct Kappa Ligands (Salvinorin A, TRK-820 and 3FLB) on Kappa Opioid Receptors *In Vitro* and Their Antipruritic and Antinociceptive Activities *In Vivo*. *J. Pharmacol. Exp. Ther.* 312 (1), 220–230. doi:10.1124/jpet.104.073668

Wiley, J. L., Jefferson, R. G., Griffin, G., Liddle, J., Yu, S., Huffman, J. W., et al. (2002). Paradoxical Pharmacological Effects of Deoxy-Tetrahydrocannabinol Analogs Lacking High CB1 Receptor Affinity. *Pharmacology* 66 (2), 89–99. doi:10.1159/000065631

Williams, J. T., Ingram, S. L., Henderson, G., Chavkin, C., von Zastrow, M., Schulz, S., et al. (2013). Regulation of μ-opioid Receptors: Desensitization, Phosphorylation, Internalization, and Tolerance. *Pharmacol. Rev.* 65 (1), 223–254. doi:10.1124/pr.112.005942

Wotherspoon, G., Fox, A., McIntyre, P., Colley, S., Bevan, S., and Winter, J. (2005). Peripheral Nerve Injury Induces Cannabinoid Receptor 2 Protein Expression in Rat Sensory Neurons. *Neuroscience* 135 (1), 235–245. doi:10.1016/j.neuroscience.2005.06.009

Yuill, M. B., Hale, D. E., Guindon, J., and Morgan, D. J. (2017). Anti-nociceptive Interactions between Opioids and a Cannabinoid Receptor 2 Agonist in Inflammatory Pain. *Mol. Pain.* 13, 1744806917728227. doi:10.1177/1744806917728227

Zhang, J., Hoffert, C., Vu, H. K., 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 (12), 2750–2754. doi:10.1046/j.1460-9568.2003.02704.x

Zhang, M., Wang, K., Ma, M., Tian, S., Wei, N., and Wang, G. (2016). Low-Dose Cannabinoid Type 2 Receptor Agonist Attenuates Tolerance to Repeated Morphine Administration via Regulating μ-Opioid Receptor Expression in Walker 256 Tumor-Bearing Rats. *Anesth. Analg* 122 (Issue 4), 1031–1037. doi:10.1213/ANE.0000000000001129

Zhang, M., Martin, B. R., Adler, M. W., Razdan, R. K., Jallo, J. I., and Tuma, R. F. (2007). Cannabinoid CB(2) Receptor Activation Decreases Cerebral Infarction in a Mouse Focal Ischemia/Reperfusion Model. *J. Cereb. Blood Flow Metab.* 27 (7), 1387–1396. doi:10.1038/sj.jcbfm.9600447

Zheng, H., Pearsall, E. A., Hurst, D. P., Zhang, Y., Chu, J., Zhou, Y., et al. (2012). Palmitoylation and Membrane Cholesterol Stabilize μ-opioid Receptor Homodimerization and G Protein Coupling. *BMC Cel Biol.* 13, 6. doi:10.1186/1471-2121-13-6

Zhu, J., Luo, L. Y., Li, J. G., Chen, C., and Liu-Chen, L. Y. (1997). Activation of the Cloned Human Kappa Opioid Receptor by Agonists Enhances [35S]GTPgammaS Binding to Membranes: Determination of Potencies and Efficacies of Ligands. *J. Pharmacol. Exp. Ther.* 282 (2), 676–684.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Pharmacology

ORIGINAL RESEARCH
published: 27 April 2022
doi: 10.3389/fphar.2022.841766

Cannabinoid CB_2 Receptors Modulate Microglia Function and Amyloid Dynamics in a Mouse Model of Alzheimer's Disease

Samuel Ruiz de Martín Esteban[1†], *Irene Benito-Cuesta*[1†], *Itziar Terradillos*[2,3], *Ana M. Martínez-Relimpio*[1], *M. Andrea Arnanz*[1], *Gonzalo Ruiz-Pérez*[1], *Claudia Korn*[4], *Catarina Raposo*[4], *Roman C. Sarott*[5], *Matthias V. Westphal*[5], *Izaskun Elezgarai*[2,3], *Erick M. Carreira*[5], *Cecilia J. Hillard*[6], *Uwe Grether*[4], *Pedro Grandes*[2,3], *M. Teresa Grande*[1] *and Julián Romero*[1]*

[1]*Faculty of Experimental Sciences, Universidad Francisco de Vitoria, Pozuelo de Alarcón, Spain,* [2]*Department of Neurosciences, Faculty of Medicine and Nursing, University of the Basque Country UPV/EHU, Leioa, Spain,* [3]*Achucarro Basque Center for Neuroscience, Science Park of the University of the Basque Country UPV/EHU, Leioa, Spain,* [4]*Roche Pharma Research & Early Development, Roche Innovation Center Basel, F. Hoffmann-La Roche Ltd., Basel, Switzerland,* [5]*Laboratorium Für Organische Chemie, Eidgenössische Technische Hochschule Zürich, Zürich, Switzerland,* [6]*Department of Pharmacology and Toxicology, Neuroscience Research Center, Medical College of Wisconsin, Milwaukee, WI, United States*

The distribution and roles of the cannabinoid CB_2 receptor in the CNS are still a matter of debate. Recent data suggest that, in addition to its presence in microglial cells, the CB_2 receptor may be also expressed at low levels, yet biologically relevant, in other cell types such as neurons. It is accepted that the expression of CB_2 receptors in the CNS is low under physiological conditions and is significantly elevated in chronic neuroinflammatory states associated with neurodegenerative diseases such as Alzheimer's disease. By using a novel mouse model ($CB_2^{EGFP/f/f}$), we studied the distribution of cannabinoid CB_2 receptors in the 5xFAD mouse model of Alzheimer's disease (by generating 5xFAD/$CB_2^{EGFP/f/f}$ mice) and explored the roles of CB_2 receptors in microglial function. We used a novel selective and brain penetrant CB_2 receptor agonist (RO6866945) as well as mice lacking the CB_2 receptor (5xFAD/$CB_2^{-/-}$) for these studies. We found that CB_2 receptors are expressed in dystrophic neurite-associated microglia and that their modulation modifies the number and activity of microglial cells as well as the metabolism of the insoluble form of the amyloid peptide. These results support microglial CB_2 receptors as potential targets for the development of amyloid-modulating therapies.

Keywords: cannabinoids, CB_2 receptor, amyloid, Alzheimer's disease, microglia

Abbreviations: Aβ, Amyloid beta; AD, Alzheimer's Disease; ALS, Amyotrophic Lateral Sclerosis; APP, Amyloid Precursor Protein; BACE1, Beta-site Amyloid Precursor Protein Cleaving Enzyme 1; cAMP, Cyclic adenosine monophosphate; CNS, Central Nervous System; CREB, cAMP Response Element-Binding; CSFR1, Colony Stimulating Factor Receptor-1; EGFP, Enhanced Green Fluorescent Protein; ERK1/2, Extracellular Signal-Regulated Kinases ½; GABA-A, Gamma-Amino Butyric Acid Receptor Subunit Alpha-1; GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase; GPCRs, G-protein coupled receptors; HRP, Horseradish Peroxidase; Iba1, Ionized Calcium Binding Adapter Molecule 1; MAPK, Mitogen-Activated Protein Kinases; TREM2, Triggering Receptor Expressed On Myeloid Cells 2.

OPEN ACCESS

Edited by:
Maria Grazia Morgese, University of Foggia, Italy

Reviewed by:
Agnes Nadjar, Université de Bordeaux, France
Adriano Lama, University of Naples Federico II, Italy

***Correspondence:**
Julián Romero
J.romero.prof@ufv.es

[†]*These authors have contributed equally to this work*

Specialty section:
This article was submitted to Experimental Pharmacology and Drug Discovery, a section of the journal Frontiers in Pharmacology

Received: *22 December 2021*
Accepted: *30 March 2022*
Published: *27 April 2022*

Citation:
Ruiz de Martín Esteban S, Benito-Cuesta I, Terradillos I, Martínez-Relimpio AM, Arnanz MA, Ruiz-Pérez G, Korn C, Raposo C, Sarott RC, Westphal MV, Elezgarai I, Carreira EM, Hillard CJ, Grether U, Grandes P, Grande MT and Romero J (2022) Cannabinoid CB_2 Receptors Modulate Microglia Function and Amyloid Dynamics in a Mouse Model of Alzheimer's Disease. Front. Pharmacol. 13:841766. doi: 10.3389/fphar.2022.841766

INTRODUCTION

Cannabinoid receptors include two types of G-protein coupled receptors (GPCRs), CB_1 and CB_2, that exhibit profound differences in their distribution in the organism of mammals (Pertwee et al., 2010). While the CB_1 receptor is one of the most abundant GPCRs in the brain and its expression is constitutive in a wide variety of cells and tissues, the distribution of CB_2 receptors is restricted to specific types of cells (B-lymphocytes, natural killer cells, monocytes, etc) and tissues (spleen, Peyer's patches) and its brain expression is low under physiological conditions (for review, see Mechoulam and Parker, 2013). Importantly, the expression of cannabinoid CB_2 receptors is significantly increased under pathological conditions and, specifically, in the context of chronic neuroinflammation (Maresz et al., 2005; Mecha et al., 2016).

The pattern of expression of CB_2 receptors and its biological relevance in the CNS is still a matter of debate. It is currently accepted that microglial cells express CB_2 receptors under both normal and pathological conditions (Komorowska-Müller and Schmöle, 2020). Interestingly, although its presence in neuronal elements is believed to be low (if any), there are reports that CB_2 receptors contribute to functions ascribed to neurons, such as pain or reward (Zhang et al., 2014; Cabañero et al., 2020; He et al., 2021). CB_2 receptors also seem to play important roles in neurodegenerative conditions, although their precise contribution has not been elucidated yet due to conflicting results (Mecha et al., 2016; Galán-Ganga et al., 2021; Rodríguez-Cueto et al., 2021).

The amyloid hypothesis of Alzheimer's disease (AD) is currently the most widely accepted among the scientific community (Hampel et al., 2021). Together with hyperphosphorylated tau-enriched neurofibrillary tangles, neuritic plaques (primarily constituted by amyloid peptides and, specifically, beta amyloid 1-42, Aβ) are the main pathologic features of AD. Multiple deleterious consequences derive from the accumulation of both in the brain, including mitochondrial dysfunction, axonal degeneration, alterations in synaptic transmission and neuroinflammation (Hampel et al., 2021).

There is an urgent need for novel approaches for the treatment of this devastating disease. Efforts have been focused on tackling the neuroinflammatory process triggered by the presence of pathological forms of Aβ as it is presently thought that these peptides possess intrinsic pro-inflammatory properties that play a crucial role in the loss of neurons in specific areas of the AD brain. This process involves several types of cells (microglia, astrocytes) and mediators (cytokines, reactive oxygen species, lipids) that, acting in a concerted and time-dependent manner, expand the damage initiated by neuritic plaques and neurofibrillary tangles (see Hampel et al., 2021, for review).

Microglia seem to play a prominent role in this scenario. In the healthy brain, these cells of myeloid origin are continuously sensing their surrounding environment (Nimmerjahn et al., 2005). When an alteration takes place in the brain parenchyma, these cells become "activated", and shape their phenotype to cope with this alteration by modifying their structural properties, gene expression profile, ability to produce cytokines and other cell mediators, and phagocytic activity (becoming "damage-associated microglia", DAM; Deczkowska et al., 2018). Among other adaptations, microglia express cannabinoid CB_2 receptors in the context of AD (Mecha et al., 2016), and remarkably, in neuritic plaque-associated microglia (Benito et al., 2003).

In the present study, we analyzed the expression of cannabinoid CB_2 receptors in cortical areas of the brain of an AD mouse model (5xFAD/$CB_2^{EGFP/f/f}$) by electron microscopy. In addition, we explored the potential roles of this receptor through its activation with a selective agonist (RO6866945) and through its genetic deletion (5xFAD/$CB_2^{-/-}$).

MATERIALS AND METHODS

Mice and Treatment

Mice used in these experiments were described in our previous study (López et al., 2018) and were housed and bred in the animal facilities of Universidad Francisco de Vitoria (Pozuelo de Alarcón, Madrid, Spain). Experimental protocols met the European and Spanish regulations for protection of experimental animals (86/609/EEC and RD 1201/2005 and 53/2013) and were approved by the committee of Ethics for Animal Welfare of the Universidad Francisco de Vitoria and University of the Basque Country (M20/2015/093). Efforts were made to minimize the number and suffering of animals.

Mice co-expressing five familial Alzheimer's disease mutations (5xFAD) were purchased from Jackson Laboratories (Bar Harbor, ME, United States; Oakley et al., 2006) on the C57BL/6J background and were mated with $CB_2^{EGFP/f/f}$ and $CB_2^{-/-}$ mice and backcrossed for at least ten generations to generate 5xFAD/$CB_2^{EGFP/f/f}$ and 5xFAD/$CB_2^{-/-}$ mice.

Prior to the experiment, mice were homogenously distributed per group according to bodyweight. A stock solution of 90 mg/ml RO6866945 (Roche Pharma Research and Early Development, Roche Innovation Center Basel, Basel, Switzerland) in ethanol was conserved at −20°C, and diluted in vehicle solution [5% ethanol, 5% kolliphor (Sigma, C5135), 90% NaCl 0.9% (Braun, 857367)] the day of use. 6 months old 5xFAD/$CB_2^{EGFP/f/f}$ and 5xFAD/$CB_2^{-/-}$ male mice were treated (i.p.) with RO6866945 10 mg/kg, or vehicle (VEH) daily for 28 days. RO6866945 ((3*S*)-1-[5-*tert*-butyl-3-[(4-methyl-1,2,5-oxadiazol-3-yl)methyl] triazolo[4,5-d]pyrimidin-7-yl]pyrrolidin-3-ol; CAS Registry Number 1433360-72-5) was synthesized as described in US20130116236 A1 (Example 136) (Adam et al. (2013). Preparation of [1,2,3]triazolo[4,5-d]pyrimidine derivatives useful as cannabinoid receptor 2 agonists, *US20130116236 A1*). It is a highly potent CB_2 agonist across species (human CB_2 cAMP EC_{50} 0.2 nM, 104% efficacy; mouse CB_2 cAMP EC_{50} 0.2 nM, 101% efficacy) which does neither interact with the CB_1 receptor in the cAMP (human CB_1 cAMP EC_{50} > 10′000 nM) nor in the radioligand binding assay (human CB_1 Ki > 10′000 nM; Ouali Alami et al., 2018). RO6866945 exhibits an excellent early ADME profile including an oral bioavailability of 44% in mice and penetrates through the blood brain barrier.

Twenty-four hours before the end of the treatment, mice were intraperitoneally injected with 10 mg/kg methoxy-X04 (Tocris, 4920) in 15% DMSO, 15% kolliphor and 70% NaCl 0.9%. Then, mice were anaesthetised with 170 mg/kg ketamine (Richter Pharma, 580393.7) and 10.7 mg/kg xylazine (Calier, 572599.4) in NaCl 0.9%, and transcardially perfused with cold PBS pH 7.4. From each mouse, right cortex, hippocampi and cerebellum were dissected and stored at −80°C. Left cortex and the rest of the brain were immediately processed to isolate microglia for analysis by flow cytometry.

Preservation of Brain Tissue for Immunocytochemistry

Three male $CB_2^{EGFP/f/f}$ and three 5xFAD/$CB_2^{EGFP/f/f}$ mice were anaesthetized with ketamine/xylazine (100mg/10 mg/kg body weight, intraperitoneal injection) and subsequently perfused transcardially at room temperature (RT) with 4% formaldehyde (freshly depolymerized from paraformaldehyde), 0.2% picric acid and 0.1% glutaraldehyde in PBS 0.1 M (pH 7.4) for 10–15 min. The brains were then removed from the skull, post-fixed in the fixative solution for 1 week at 4°C and cut into 50 µm thick coronal sections using a vibratome.

Double Pre-Embedding Immunogold and Immunoperoxidase Method for Electron Microscopy

Our protocol previously published was used (Puente et al., 2019). Brain sections containing the subiculum were pre-incubated in a blocking solution of 10% bovine serum albumin (BSA), 0.02% saponin and 0.1% sodium azide in Tris-hydrogen chloride buffered saline (TBS 1X), for 30 min on a shaker at RT. Tissue was then incubated for 2 days at 4°C with both a rat monoclonal anti-GFP antibody (1:500, GF090R, Nacalai) and a rabbit polyclonal anti-Iba1 antibody (1:500, 019-19741, FUJIFILM Wako Pure Chemical Corporation) prepared in 10% BSA, 0.1% sodium azide and 0.004% saponin. After washes in 1% BSA/TBS, sections were incubated with 1.4 nm gold-conjugated goat anti-rat IgG antibody (Fab' fragment, 1:100; Nanoprobes Inc., Yaphank, NY, United States) and with biotinylated anti-rabbit IgG antibody (1:200; Biotin-SP-AffiniPure donkey anti-rabbit IgG) diluted in 1% BSA/TBS with 0.004% saponin on a shaker for 4 h at RT. They were washed in 1% BSA/TBS and then incubated with the avidin-biotin peroxidase complex (1:50; Elite, Vector Laboratories, Burlingame, CA, United States) for 1.5 h at RT. Sections were then washed in 1% BSA/TBS and kept in the same washing solution overnight at 4°C, postfixed with 1% glutaraldehyde in TBS for 12 min at RT and washed in double distilled water. Gold particles were silver-intensified with the HQ Silver kit (Nanoprobes Inc., Yaphank, NY, United States) in the dark for 12 min at RT. The biotinylated antibody was exposed to 0.05% diaminobenzidine (pH 7.4) with 0.01% hydrogen peroxide for 3.5 min at RT. Sections were incubated with 1% osmium tetroxide, pH 7.4, in the dark for 20 min, washed in PB 0.1 M, dehydrated and embedded in Epon 812 resin. 50 nm-thick sections were cut with an ultra-diamond knife (Diatome United States) and collected on nickel mesh grids. They were counterstained with 2.5% lead citrate for 20 min and examined with a transmission electron microscope (JEOL JEM 1400 Plus, Canada). Tissue was photographed using a Hamamatsu FLASH digital camera inserted in the electron microscope. Anatomical landmarks were taken to locate the subiculum region.

To ensure homogeneous labelling between all samples, only the first 1.5 µm from the section surface of each specimen was collected. Random electron micrographs were taken of the subicula. Areas of 3,524 μm^2 in $CB_2^{EGFP/f/f}$ and 4,078 μm^2 in 5xFAD/$CB_2^{EGFP/f/f}$ mice were examined to assess CB_2 receptors in Iba1-positive microglia. GFP gold particles were counted and differentiated between their localization in membrane (between 0 and 30 nm of the membrane) or cytosol (more than 30 nm). Minor contrast and brightness adjustments were made to the figures using ImageJ software (NIH; RRID: SCR_003070), Adobe Photoshop and Gimp.

Isolation of Microglial Cells and Flow Cytometry

Flow cytometry was employed to determine the ability of microglial cells to phagocytize Aβ (stained with methoxy-X04), and the levels of CB_2 with RO7246360 probe (compound 3b in Sarott et al., 2020). 6-month-old animals were injected i. p. with Methoxy-X04 (Tocris Bioscience) at 10 mg/kg body weight. 24 h after injection, animals were deeply anesthetized by i. p. administration of a mixture of ketamine (170 mg/kg) and xylazine (10.7 mg/kg) and transcardially perfused with cold PBS 1X, pH 7.4. Brains were dissected and enzymatically digested to facilitate microglia separation. The cell suspension was mechanically dissociated and filtered through a 70 µm-cell strainer. Microglial cells, isolated by percoll gradient (GE Healthcare), were washed with PBS 1X and blocked with 1% BSA/PBS 1X for 20 min. Cells were stained with CD11b-PE and CD45-APC antibodies and with RO7246360 fluorescent probe for 40 min. Samples were read on a MACSQuant Flow Cytometer and analysed with MACS Quantify software (Miltenyi Biotec).

Debris and aggregates were eliminated from analysis by forward and side scatter characteristics. Then microglia were identified as $CD11b^+$ $CD45^{lo}$. The CB_2 receptor expression was determined by the fluorescent signal of RO7246360 probe. Fluorescence signals were corrected by fluorescence minus one (FMO) control. For each hemisphere, approximately ten thousand CD11b + singlets were analysed.

Cyclic Adenosine Monophosphate Assay

Extracts from frozen brain cortices were obtained by homogenization in magnesium lysis buffer (MLB: 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM $MgCl_2$, 1 mM EDTA) containing 10% glycerol, and protease and phosphatase inhibitors (1 mM Na_3VO_4, 25 mM NaF and protease inhibitor cocktail; Roche) and were maintained at 4°C. Homogenates were centrifuged at 12000 g for 20 min at 4°C and supernatants were collected to determine their protein content by BCA protein assay (Pierce™ BCA protein assay kit, Thermo Scientific). Homogenates were used to measure cAMP levels

using an ELISA kit (cat.no. ab65355, Abcam) following the manufacturer's instructions. Standards and samples were plated in duplicate, and the absorbance was measured at 450 nm using a Varioskan Flash multifunction plate reader (Sunrise, Tecan).

$A\beta_{1-42}$ Peptide Quantification

Frozen mouse brain cortices were homogenized in four volumes (weight: volume) of TBS extracting buffer (140 mM NaCl, 3 mM KCl, 25 mM Tris, pH 7.4, 5 mM EDTA and protease inhibitor cocktail; Roche). Homogenates were centrifuged at 16,000 g for 20 min at 4°C. The supernatants were saved to quantify the soluble $A\beta_{1-42}$ peptide fraction and the pellets were again homogenized in four volumes (weight: volume) of 5M guanidine 50 mM Tris-HCl pH 8. The supernatants obtained after the centrifugation step were collected to quantify the insoluble $A\beta_{1-42}$ peptide fraction. An equal volume of PBS containing 1 mM serine protease inhibitor AEBSF (Sigma) was added to all samples and their protein content was determined by micro-BCA protein assay (Micro BCA™ protein assay kit, Thermo Scientific). Human $A\beta_{1-42}$ Ultrasensitive ELISA kit (cat.no. KHB3544 Invitrogen) was used for the quantification of soluble and insoluble fractions of $A\beta_{1-42}$ peptide following the instructions provided by the manufacturer. Standards and samples were plated in duplicate, and the absorbance was measured at 450 nm using a Varioskan Flash multifunction plate reader (Sunrise, Tecan).

Western Blotting

Extracts from frozen brain cortices were obtained following the procedure previously described for cAMP assay. Lysates (60 µg/lane) were separated by SDS-PAGE and transferred onto nitrocellulose membranes (BioRad) and PVDF membranes (BioRad, used for the transference of phosphorylated proteins). After blocking in 5% bovine serum albumin in TTBS (10 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween 20) membranes were incubated overnight at 4°C, as appropriate, with primary antibodies: anti-phospho-p38 MAPK (1:1000; Cell Signaling Technology, 4511T), anti-p38 MAPK (1:1000; Cell Signaling Technology, 8690T), anti-phospho-CREB (1:1000; Cell Signaling Technology, 9198S), anti-CREB (1:1000; Cell Signaling Technology, 9197T), anti-phospho-ERK1/2 MAPK (1:1000; Cell Signaling Technology, 9101S), anti-Erk1/2 MAPK (1:1000; Santa Cruz Biotechnology, sc514302), Anti-Iba1 (1:1000, FUJIFILM Wako Pure Chemical, 016-20001), Anti-APP N-terminus (1:1000, EMD Millipore, MAB348), Anti-APP C-terminus (1:2000, Sigma, A8717), anti-BACE1 (1:500, Abcam, ab 2077) and anti-GAPDH (1:1000; Abcam, ab8245). Membranes were incubated with corresponding horseradish peroxidase (HRP)-conjugated secondary antibody anti-mouse IgG-HRP (1:10000; Abcam, ab97046), anti-rabbit IgG-HRP (1: 5000; Cell Signaling Technology, 7074S) and were developed using a chemoluminiscent reagent (Western Lighting ECL Plus, PerkinElmer, NEL103001EA) in the appropriate equipment (ChemiDoc, Bio-Rad). GAPDH was used as an internal control. The relative quantity of protein levels in western blot was measured using ImageJ software (ImageJ; NIH).

Statistical Analyses

All statistical analyses were performed, and graphs were generated using GraphPad Prism v 9.0 (GraphPad). Graphs represent average values ± standard error of the mean. Normality of data distribution was determined with the Shapiro-Wilk or the D'Agostino-Pearson tests. For GFP labeling, data were analyzed by means of the Mann-Whitney U test. For the rest of determinations, data were analysed by means of two-way ANOVA, followed by Tukey's post-hoc tests. A p-value < 0.05 was considered as statistically significant. Only male animals were used in the experiments. The number of animals used for each experiment is reported in the figure legends.

RESULTS

Microglial Localization of the CB_2 Receptor in the Subiculum of $CB_2^{EGFP/f/f}$ and 5xFAD/$CB_2^{EGFP/f/f}$ by Electron Microscopy

The GFP/CB_2 labelling was localized in Iba-1 immunopositive microglial processes in both $CB_2^{EGFP/f/f}$ and 5xFAD/$CB_2^{EGFP/f/f}$ mice (**Figure 1**). GFP-positive microglial processes increased significantly in 5xFAD/$CB_2^{EGFP/f/f}$ (0.7126 ± 0.2311) relative to $CB_2^{EGFP/f/f}$ (0.1648 ± 0.07686, *p: 0.0176; **Figure 2**). Likewise, a significant increase in the proportion of GFP-positive microglial ramifications was seen in 5xFAD/$CB_2^{EGFP/f/f}$ (16.71 ± 3.664%) with respect to $CB_2^{EGFP/f/f}$ (5.430 ± 2.631%; $p = 0.0191$; **Figure 2**). Also, the total number of GFP particles per area of microglial ramifications was significantly greater in 5xFAD/$CB_2^{EGFP/f/f}$ (1.238 ± 0.2534) than in $CB_2^{EGFP/f/f}$ mice (0.6962 ± 0.4138; $p = 0.0467$; **Figure 2**), and the number of GFP particles in microglial branches per 100 μm^2 was statistically higher in 5xFAD/$CB_2^{EGFP/f/f}$ (0.8343 ± 0.2962) than in $CB_2^{EGFP/f/f}$ (0.1648 ± 0.07686; $p = 0.0176$; **Figure 2**). Noticeably in 5xFAD/$CB_2^{EGFP/f/f}$, the percentage of GFP immunoparticles localized in microglial membranes (77.22 ± 11.40%) was significantly higher than the proportion distributed in the cytosol (22.78 ± 11.40%; $p = 0.0106$; **Figure 2**). As to $CB_2^{EGFP/f/f}$, 100% of the GFP particles were found in microglial membranes.

RO6866945 is a Selective CB_2 Agonist *in vivo*

We then studied whether the chronic treatment with RO6866945 had an impact on the expression levels of cannabinoid CB_2 receptors. We used two different approaches: first, RT-PCR revealed no significant effects of the 28-days treatment with the agonist on CB_2 mRNA levels ($F(1,23) = 0.6509$, $p = 0.4280$) and confirmed the absence of CB_2 expression in samples from 5xFAD/$CB_2^{-/-}$ mice (**Figure 3A**; $F(1,23) = 437.6$, $p < 0.0001$). Second, we employed flow cytometry to quantify the binding of the selective fluorescent probe RO7246360 to the CB_2 receptor; we found no changes induced by the chronic exposure to the agonist [$F(1,22) = 0.02066$, $p = 0.8870$] and confirmed the

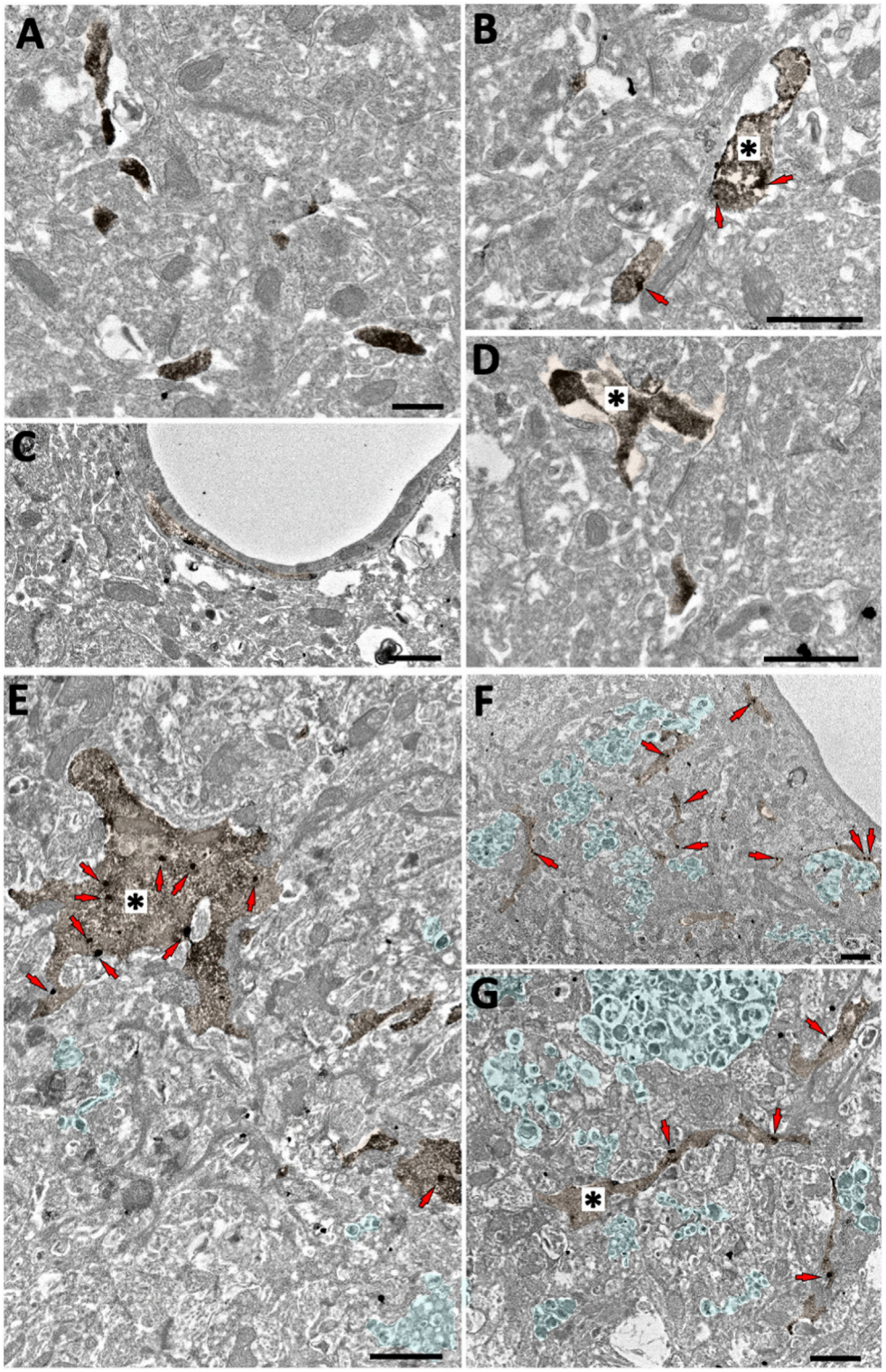


FIGURE 1 | Microglial GFP localization in the subiculum of $CB_2^{EGFP/f/f}$ and 5xFAD/$CB_2^{EGFP/f/f}$ mice. Double pre-embedding immunogold (GFP) and immunoperoxidase (Iba1) method for electron microscopy. GFP particles (red arrows) localize in Iba1-positive microglial elements (DAB immunodeposits, brown, *). In $CB_2^{EGFP/f/f}$ **(A–D)**, only GFP membrane localization is observed (arrows, **(B)**. In 5xFAD/$CB_2^{EGFP/f/f}$, GFP particles are found in both membranes and cytosol **(E–G)**. Notice dystrophic neurites (light green areas contoured by white dashed lines) in 5xFAD/$CB_2^{EGFP/f/f}$. Scale bars: 1 μm.

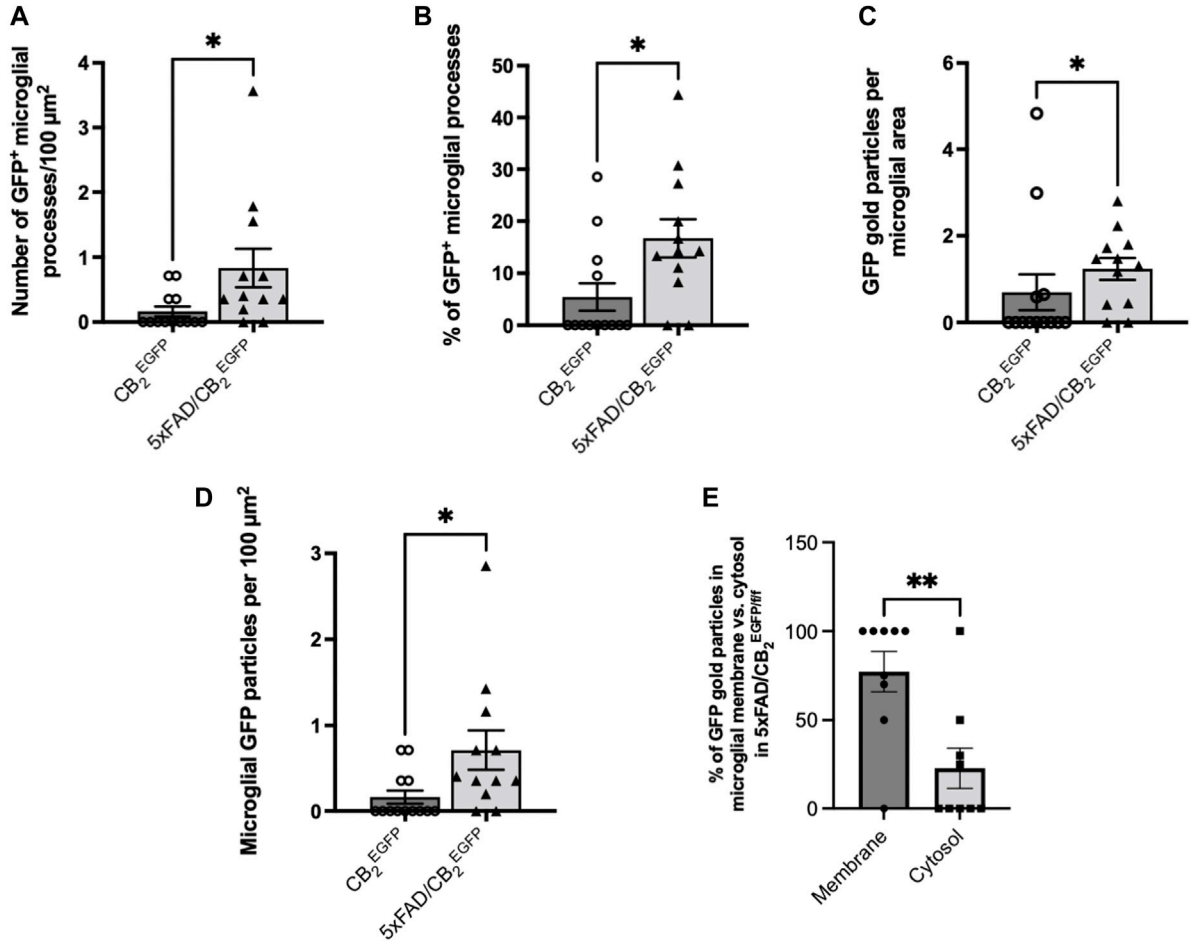


FIGURE 2 | Assessment of the microglial GFP/CB_2 localization in the subiculum of $CB_2^{EGFP/f/f}$ and 5xFAD/$CB_2^{EGFP/f/f}$ mice. **(A)** Number of microglial GFP-positive processes per 100 µm^2. **(B)** Percentage of GFP-positive microglial processes. **(C)** GFP gold particles per microglial area. **(D)** Microglial GFP particles per 100 µm^2. **(E)** Percentage of GFP particles in microglial membrane vs. cytosol in 5xFAD/$CB_2^{EGFP/f/f}$ mice. Mann-Whitney U test. *$<p0.05$; **$<p0.01$; ***$<p0.001$; ****$<p0.0001$. N = 3 mice per group. Data represent mean ± SEM.

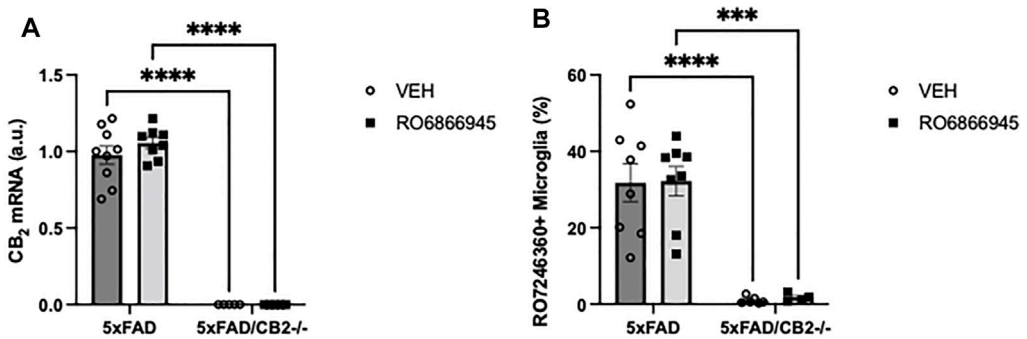


FIGURE 3 | The chronic exposure to the CB_2 selective agonist, RO6866945, did not modify the expression of cannabinoid CB_2 receptors. **(A)** mRNA levels of the cannabinoid CB_2 receptor did not vary after treatment with RO6866945 but were completely absent in 5xFAD/$CB_2^{-/-}$ mice. **(B)** Binding of the fluorescent probe RO7246360 to cannabinoid CB_2 receptors was used to quantify protein levels, revealing no changes after treatment with the agonist and the negligible levels of CB_2 protein in 5xFAD/$CB_2^{-/-}$ mice. Two-way ANOVA followed by Tukey's post-hoc test. **$<p0.01$; ****$<p0.0001$. N = 4–9 mice per group. Data represent mean ± SEM.

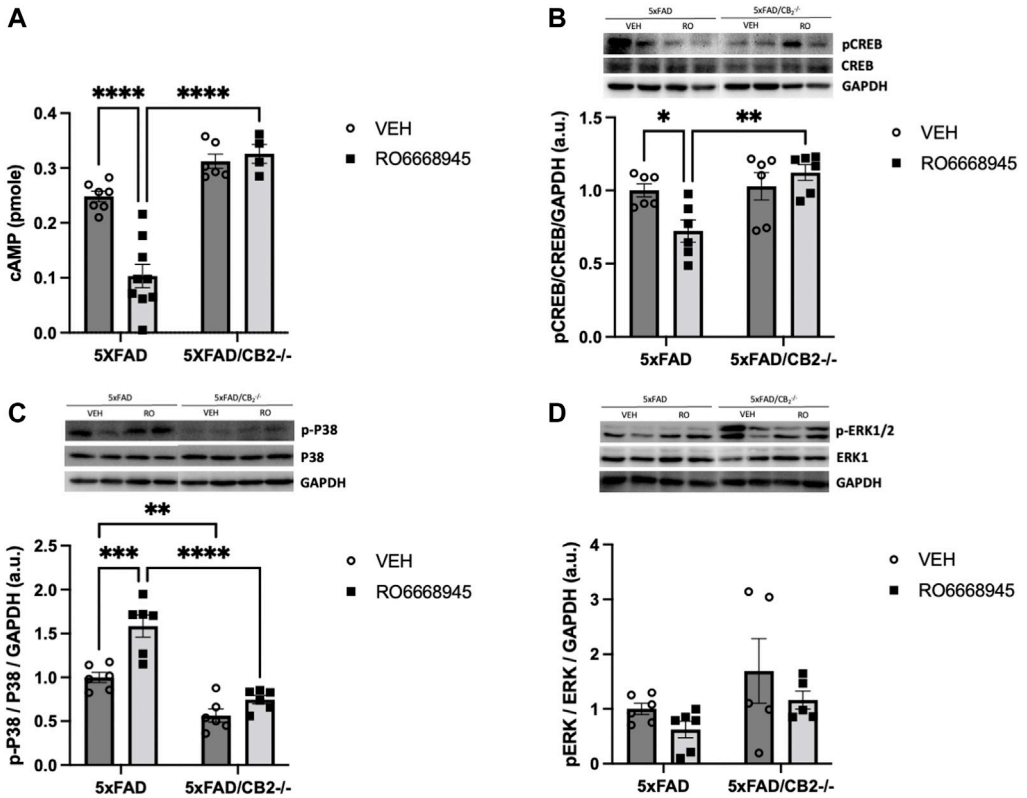


FIGURE 4 | Signaling cascades regulated by the activation and deletion of cannabinoid CB_2 receptors. **(A)** cAMP and p-CREB **(B)** levels were significantly decreased by the treatment with the CB_2 agonist, remaining unaltered in samples from CB_2-null mice. **(C)** p-p38MAPK levels were significantly elevated by the exposure to the agonist; in addition, samples from 5xFAD/$CB_2^{-/-}$ mice exhibited significantly lower levels. **(D)** p-ERK levels were not modified by the treatment with the CB_2 agonist or the genetic deletion of the receptor. Two-way ANOVA followed by Tukey's post-hoc test. *$<p0.05$; **$<p0.01$; ***$<p0.001$; ****$<p0.0001$. N = 4–9 mice per group. Data represent mean ± SEM.

absence of CB_2 protein in isolated microglia from 5xFAD/$CB_2^{-/-}$ mice (**Figure 3B**; $F(1,22) = 55.62$, $p < 0.0001$).

We next analyzed the signaling cascades affected by CB_2 activation or deletion (**Figure 4**). We found that the CB_2 agonist had a significant impact on cAMP levels [**Figure 4A**; $F(1,19) = 8.851$, $p = 0.0078$]. Post-hoc analysis revealed a decrease in cAMP in 5xFAD/$CB_2^{EGFP/f/f}$ mice as a consequence of the treatment ($p < 0.0001$) that was absent in 5xFAD/$CB_2^{-/-}$ mice ($p = 0.9802$). No differences due to the genotype were observed in vehicle-treated mice ($p = 0.2537$), although were significant between RO6866945-treated 5xFAD/$CB_2^{EGFP/f/f}$ vs. 5xFAD/$CB_2^{-/-}$ mice ($p < 0.0001$).

Regarding pCREB levels (**Figure 4B**), genotype had a significant effect [$F(1,20) = 9.370$, $p = 0.0062$]. Post-hoc analysis revealed that the agonist significantly decreased pCREB levels in 5xFAD/$CB_2^{EGFP/f/f}$ mice ($p = 0.0492$) but not in 5xFAD/$CB_2^{-/-}$ mice ($p = 0.7765$). No differences due to the genotype were observed in vehicle-treated mice ($p = 0.9917$), although were significant between RO6866945-treated 5xFAD/$CB_2^{EGFP/f/f}$ *vs.* 5xFAD/$CB_2^{-/-}$ mice ($p = 0.0033$).

p-p38MAPK levels (**Figure 4C**) were modified by the treatment with RO6866945 [$F(1,20) = 21.77$, $p = 0.0001$], by genotype [$F(1,20) = 60.28$, $p < 0.0001$] and by the interaction of both factors [$F(1,20) = 6.088$, $p = 0.0228$]. p-p38MAPK was increased in 5xFAD/$CB_2^{EGFP/f/f}$ mice as a consequence of CB_2 activation by the agonist ($p = 0.0003$) and exhibited significantly lower levels in samples from both vehicle- and RO6866945-treated CB_2-lacking mice ($p = 0.0064$ and $p < 0.0001$, respectively). These observations highlight the selectivity of RO6866945 as a CB_2-selective agonist and suggest a putative constitutive activation of p-38MAPK signaling cascade by CB_2 receptors in the context of AD.

Finally, p-ERK levels remained unaltered after treatment with the agonist [$F(1,18) = 2.377$, $p = 0.1405$] as well as in 5xFAD/$CB_2^{-/-}$ mice (**Figure 4D**; $F(1,18) = 4.339$, $p = 0.0518$).

CB_2-Lacking Mice Express Lower Levels of Iba1 and Exhibit Impaired Phagocytic Activity

As microglia are the main source of cannabinoid CB_2 receptors in the brain of 5xFAD/$CB_2^{EGFP/f/f}$ mice, we analyzed the putative changes triggered in these cells by the activation of the receptor and by its genetic deletion (**Figure 5**). We found no changes in Iba1+ microglia (**Figure 5A**; $F(1,20) = 0,7931$, $p = 0.3837$) nor in its phagocytic activity (measured by its ability to internalize

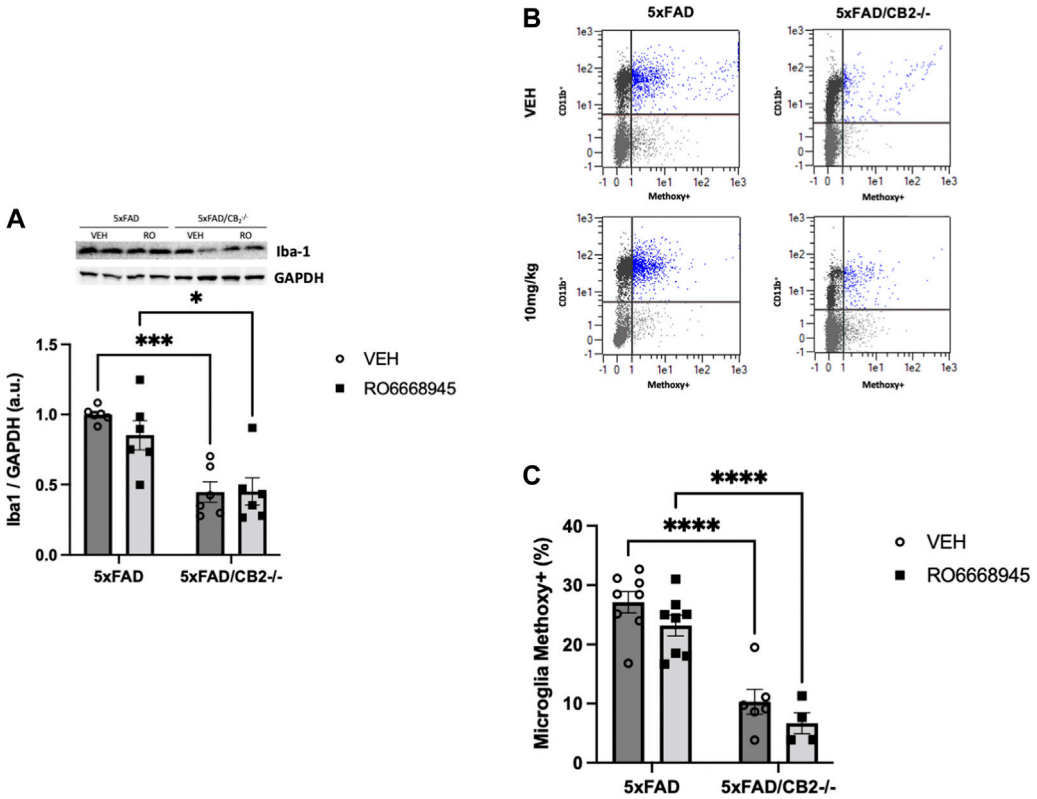


FIGURE 5 | Iba1+ microglia and phagocytic activity is decreased after genetic deletion of the cannabinoid CB_2 receptor. Analysis of cortices by western blot **(A)** and flow cytometry **(B,C)** revealed no changes associated to the treatment with the agonist together with a significant decrease in Iba1+ microglia **(A)**. Phagocytic activity **(B,C)** of microglia was significantly impaired in 5xFAD/$CB_2^{-/-}$ mice. Scatter plots of CD11b isolated microglia after intraperitoneal administration of methoxy-X04 are shown **(B)**. Phagocytic capacity was calculated as percentage of methoxy-X04+/CD11b+/CD45lo cells to CD11b+/CD45lo cells **(C)**. Two-way ANOVA followed by Tukey's post-hoc test. *$<p0.05$. **$<p0.01$; ***$<p0.001$; ****$<p0.0001$. N = 5–6 mice per group. Data represent mean ± SEM.

methoxy-X04-stained amyloid; **Figures 5B,C**; $F(1,22) = 3.602$, $p = 0.0709$) derived from CB_2 activation by the agonist. However, significant differences were evident between 5xFAD/$CB_2^{EGFP/f/f}$ and 5xFAD/$CB_2^{-/-}$ microglia; thus, we found a decrease in Iba1+ microglia abundance [**Figure 5A**; $F(1,20) = 34.95$, $p < 0.0001$] as well as an impairment in its phagocytic activity (**Figure 5B**; $F(1,22) = 69.96$, $p < 0.0001$).

The Activation as Well as the Genetic Deletion of CB_2 Receptors Modify Amyloid Metabolism *in vivo*

We next measured the impact of CB_2 modulation on Aβ levels. To that end, we quantified several amyloid-related peptides (APP, C83 and BACE1) as well as the soluble and insoluble forms of $Aβ_{1-42}$, the main component of neuritic plaques (**Figure 6**). Our data showed no changes in APP [$F(1,20) = 0.08911$, $p = 0.7684$], C83 [$F(1,20) = 0.1794$, $p = 0.6764$] or BACE1 [$F(1,20) = 3.026$, $p = 0.0973$] after treatment with RO6866945. CB_2 deletion induced significant differences in protein levels of BACE1 [$F(1,20) = 10.34$, $p = 0.0043$], but not in C83 ($F(1,20) = 1.705$, $p = 0.2065$) and APP [$F(1,20) = 2.468$, $p = 0.1319$].

Levels of soluble amyloid were unaltered after treatment with the CB_2 agonist [$F(1,14) = 0.2681$, $p = 0.6127$] or genetic inactivation of the receptor [$F(1,14) = 0.1408$, $p = 0.1731$; **Figure 6D**]. Cortical amounts of insoluble amyloid, however, were significantly modified by both (**Figure 6E**). Thus, the treatment with RO6866945 led to a significant increase in insoluble amyloid levels [$F(1,17) = 17.88$, $p = 0.0006$] in 5xFAD/$CB_2^{EGFP/f/f}$ mice, while 5xFAD/$CB_2^{-/-}$ mice exhibited decreased levels of this peptide [$F(1,17) = 209.3$, $p < 0.0001$].

DISCUSSION

In the present manuscript we report a significant role of cannabinoid CB_2 receptors in microglial functions and in the metabolism of Aβ in an animal model of Alzheimer's disease (5xFAD). Specifically, we found that the absence of CB_2 receptors decrease the total number of microglial cells as well as their ability to phagocytose Aβ and have a modulatory role in the accumulation of the insoluble form of this pathogenic peptide. Furthermore, our data suggest that microglial CB_2 receptors may be constitutively activated in the context of AD, as indicated by p38 phosphorylation state.

Our present data confirm the increased expression of cannabinoid CB_2 receptors in plaque-associated microglia (Benito et al., 2007a). By using electronic microscopy, we have observed that the presence of EGFP (expressed under the control

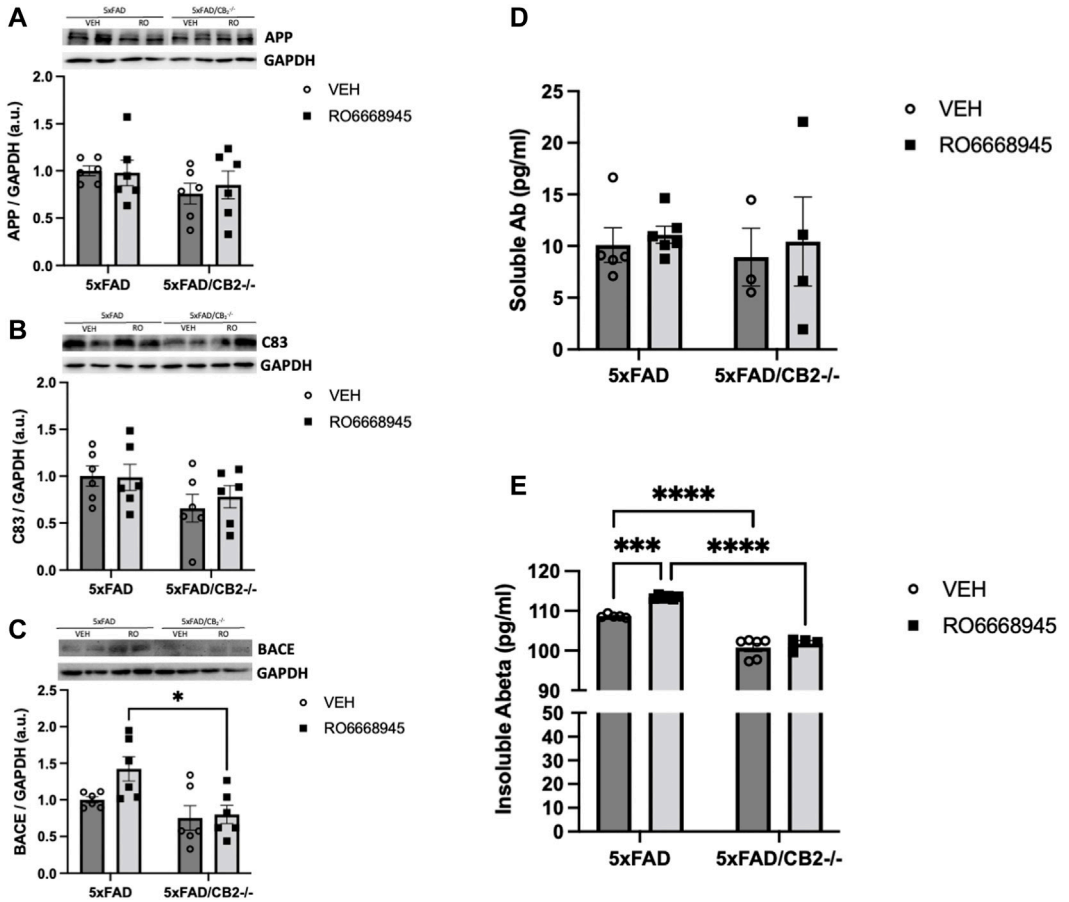


FIGURE 6 | Cannabinoid CB_2 receptors modulate amyloid dynamics *in vivo*. No changes were evident after treatment in APP **(A)**, C83 **(B)** or BACE1 **(C)**, as measured by western blot. Soluble amyloid levels **(D)** remained also unaltered, while those of insoluble amyloid **(E)** were significantly increased by the exposure to the CB_2 agonist and decreased in 5xFAD/$CB_2^{-/-}$ mice. Two-way ANOVA followed by Tukey's post-hoc test. *$<p0.05$; ****$<p0.0001$. N = 4-6 mice per group. Data represent mean ± SEM.

of the *Cnr2* promoter region) was enhanced specifically in these cells, in 5xFAD/$CB_2^{EGFP/f/f}$ mice, while in controls remained low or undetectable in microglial cells as well as in other cell types (such as neurons or astrocytes). These observations match with previous data obtained from our group (Benito et al., 2007b; López et al., 2018) and from others (Savonenko et al., 2015; Spangenberg et al., 2019) and confirm the selective expression of CB_2 receptors in activated microglial cells in the context of the chronic neuroinflammation triggered by amyloid accumulation.

These findings allow us to assume that the changes in signal transduction cascades observed after treatment with the CB_2 agonist, RO6866945, or after the genetic deletion of the receptor are mostly derived from the modulation of microglial cells, although the contribution of other receptor's populations located in different types of cells cannot be completely ruled out. It might be possible that, even when expressed at very low levels, CB_2 receptors could modulate the activity of neurons and/or astrocytes, as has been reported by other authors (Onaivi et al., 2008; Espejo-Porras et al., 2019). In our hands, however, microglial CB_2 receptors must play a major role in the observed changes in cAMP, CREB and p38MAPK signaling cascades. Interestingly, p38MAPK regulation might be under the tonic influence of CB_2 receptors, as its activity was significantly reduced in CB_2-lacking mice.

These data match with those recently reported by Reusch et al. (Reusch et al., 2022) regarding microglial phagocytosis and signaling cascade (p38MAPK) profiles. By using cultures of BV-2 and primary microglia cells, these authors found that CB_2 receptors are necessary for TLR-mediated activation, as shown by gene transcription, morphological and functional (LPS/IFN-γ, CpG and Polyl:C stimulation) analysis and that p38MPK signaling was directly involved in the CB_2-mediated regulation of TLR function. Thus, primary neonatal microglia from $CB_2^{-/-}$ exhibited a dysregulation of this intracellular route at the transcriptional level that was especially evident after challenge with LPS/IFN-γ and Polyl:C, with a significant reduction in the phosphorylation level of p38. Furthermore, the significant decrease in the phagocytic activity of CB_2-lacking microglia we herein report may be also associated to the loss of TLR function, as these receptors are well-known for their critical role in the uptake and clearance of amyloid by these cells (Tahara et al., 2006). Finally, our present observations match well with our

previously published study in which a decrease in methoxy-X04+ plaques in 5xFAD/$CB_2^{-/-}$ mice was found (López et al., 2018).

As a limitation of the present studies, only male mice were employed. The question on putative sex differences in the 5xFAD model has been recently addressed by Forner et al. (Forner et al., 2021). These authors performed a comprehensive analysis of pathology-associated changes in male and female 5xFAD mice and found that female mice develop the disease at an earlier age, exhibit more significant weight loss, higher levels of insoluble Aβ and improved motor performance in the rotarod test than their males counterparts. A trend to increased microgliosis was also observed.

The current view on the pathogenesis of AD indicates that the accumulation of Aβ is one of the main hallmarks of this disease, together with the formation of tau-enriched neurofibrillary tangles (Querfurth and LaFerla, 2010). Both factors contribute to a significant loss of active synaptic connections in the cortex and hippocampus, triggering the well-known symptoms of this disease, such as memory loss, cognitive decline, etc. The animal model that we have employed in the present studies exhibits an enhanced amyloidogenic status, leading to the production of increased amounts of Aβ at early stages of the mouse's lifespan and to the formation of neuritic plaques as early as 3 months of age (Oakley et al., 2006).

Though still controversial, the role of microglia in the formation and accumulation of amyloid-enriched neuritic plaques seems very relevant (Song and Colonna, 2018). It is thought that, in the context of AD and as the presence of increased species of Aβ extends in time, activated microglia become a relevant contributor to neuronal damage mainly by secreting elevated amounts of cytokines, ROS, and other mediators, and by losing their ability to phagocytose and degrade these pathological peptides (Song and Colonna, 2018). Importantly, microglia are thought to perform a "shielding" task by effectively surrounding neuritic plaques and thus preventing the expansion of the damage in the brain parenchyma (Condello et al., 2015). The complex role of microglia has been recently highlighted by recent reports showing that *in vivo* depletion of microglia (for instance, by the administration of antagonists of colony stimulating factor receptor-1, CSFR1, to mice) significantly alters plaque dynamics in the mouse brain. Spangenberg et al. (2019) and, very recently, Casali et al. (2020) have shown that microglia depletion prevents the formation of Aβ-enriched neuritic plaques and that microglia restoration favors its compact structure (Spangenberg et al., 2019; Casali et al., 2020). Our observations that CB_2-deficient AD mice exhibit a decreased phagocytic activity combined with a decrease in cortical insoluble amyloid levels are suggestive of a role of CB_2 receptors in plaque dynamics, in which their activation could contribute to a compaction of amyloid plaques while their deletion could lead to a more diffuse appearance. A similar effect has been described in TREM2-lacking mice, suggesting an impairment in microglial function (Wang et al., 2016).

Finally, it is important to note that other authors have reported conflicting data associated to the genetic deletion of cannabinoid CB_2 receptors, different to those reported here. Koppel et al. (2014) used J20 APP mice to study the effects of CB_2 genetic inactivation and found increased levels of soluble amyloid and plaques as well as enhanced plaque-associated microgliosis. In line with these data, Aso et al. (2016) also found significant increases in Aβ1-40 as well as in amyloid deposition in the APP/PS1 mouse model of AD. Wu et al. (2017) reported a stimulatory effect of a CB_2 agonist on amyloid clearance combined with decreased microgliosis in the hippocampus of APP/PS1 mice. Conversely, Schmöle et al. (2015); Schmöle et al. (2018) found decreased microgliosis and amyloid levels as a consequence of CB_2 deletion in APP/PS1 mice. Most of these studies also revealed no CB_2-mediated effects on spatial memory. This variability regarding the effects of cannabinoid CB_2 receptors may be partially explained by the variety of AD mouse models employed in these studies but may be also suggestive of the subtle and limited effects of modulating the activity of these receptors, as well as may reflect putative adaptive responses in constitutive knock-out models.

CONCLUSION

We have confirmed (by immunoelectron microscopy) microglia as the main source of cannabinoid CB_2 receptors in the 5xFAD/$CB_2^{EGFP/f/f}$ mouse model of AD. In addition, we have found that these receptors regulate the ability of these cells to phagocytose amyloid peptides *in vivo* and, probably in direct relation with this, in the composition of amyloid species in the brain. These data thus suggest a role for microglial cannabinoid CB_2 receptors in the initiation, maintenance and removal of plaques and open new venues for the microglia-based therapeutic approaches in AD.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Universidad Francisco de Vitoria.

AUTHOR CONTRIBUTIONS

PG, MTG, and JR designed the experiments; SRdME, IB-C, IT, AMM-R, MAA, GR-P, and IE carried out the experiments; CK, CJH, CR, RCS, MVW, EMC, and UG provided materials. PG, UG, and JR wrote the manuscript. SRdME and IB-C contributed equally to this work. All authors revised the final version of the manuscript.

FUNDING

The present work has been supported by a grant from Ministerio de Ciencia e Innovación (ref PID2019-108992RB-I00 and ref PID2019-107548RB-I00) to JR and PG, respectively, by the Basque Government (ref IT1230-19) to PG, and the Research and Education Component of the Advancing a Healthier Wisconsin Endowment at the Medical College of Wisconsin to CJH.

REFERENCES

Aso, E., Andrés-Benito, P., Carmona, M., Maldonado, R., and Ferrer, I. (2016). Cannabinoid Receptor 2 Participates in Amyloid-β Processing in a Mouse Model of Alzheimer's Disease but Plays a Minor Role in the Therapeutic Properties of a Cannabis-Based Medicine. *J. Alzheimers Dis.* 51, 489–500. doi:10.3233/JAD-150913

Benito, C., Núñez, E., Pazos, M. R., Tolón, R. M., and Romero, J. (2007a). The Endocannabinoid System and Alzheimer's Disease. *Mol. Neurobiol.* 36, 75–81. doi:10.1007/s12035-007-8006-8

Benito, C., Núñez, E., Tolón, R. M., Carrier, E. J., Rábano, A., Hillard, C. J., et al. (2003). Cannabinoid CB2 Receptors and Fatty Acid Amide Hydrolase Are Selectively Overexpressed in Neuritic Plaque-Associated Glia in Alzheimer's Disease Brains. *J. Neurosci.* 23, 11136–11141. doi:10.1523/jneurosci.23-35-11136.2003

Benito, C., Romero, J. P., Tolón, R. M., Clemente, D., Docagne, F., Hillard, C. J., et al. (2007b). Cannabinoid CB1 and CB2 Receptors and Fatty Acid Amide Hydrolase are Specific Markers of Plaque Cell Subtypes in Human Multiple Sclerosis. *J. Neurosci.* 27, 2396–2402. doi:10.1523/JNEUROSCI.4814-06.2007

Cabañero, D., Ramírez-López, A., Drews, E., Schmöle, A., Otte, D. M., Wawrzczak-Bargiela, A., et al. (2020). Protective Role of Neuronal and Lymphoid Cannabinoid CB2 Receptors in Neuropathic Pain. *Elife* 9, e55582. doi:10.7554/eLife.55582

Casali, B. T., MacPherson, K. P., Reed-Geaghan, E. G., and Landreth, G. E. (2020). Microglia Depletion Rapidly and Reversibly Alters Amyloid Pathology by Modification of Plaque Compaction and Morphologies. *Neurobiol. Dis.* 142, 104956. doi:10.1016/j.nbd.2020.104956

Condello, C., Yuan, P., Schain, A., and Grutzendler, J. (2015). Microglia Constitute a Barrier that Prevents Neurotoxic Protofibrillar Aβ42 Hotspots Around Plaques. *Nat. Commun.* 6, 6176. doi:10.1038/ncomms7176

Deczkowska, A., Keren-Shaul, H., Weiner, A., Colonna, M., Schwartz, M., and Amit, I. (2018). Disease-Associated Microglia: A Universal Immune Sensor of Neurodegeneration. *Cell* 173, 1073–1081. doi:10.1016/j.cell.2018.05.003

Espejo-Porras, F., García-Toscano, L., Rodríguez-Cueto, C., Santos-García, I., de Lago, E., and Fernandez-Ruiz, J. (2019). Targeting Glial Cannabinoid CB2 Receptors to Delay the Progression of the Pathological Phenotype in TDP-43 (A315T) Transgenic Mice, a Model of Amyotrophic Lateral Sclerosis. *Br. J. Pharmacol.* 176, 1585–1600. doi:10.1111/bph.14216

Forner, S., Kawauchi, S., Balderrama-Gutierrez, G., Kramár, E. A., Matheos, D. P., Phan, J., et al. (2021). Systematic Phenotyping and Characterization of the 5xFAD Mouse Model of Alzheimer's Disease. *Sci. Data* 8, 270. doi:10.1038/s41597-021-01054-y

Galán-Ganga, M., Rodríguez-Cueto, C., Merchán-Rubira, J., Hernández, F., Ávila, J., Posada-Ayala, M., et al. (2021). Cannabinoid Receptor CB2 Ablation Protects against TAU Induced Neurodegeneration. *Acta Neuropathol. Commun.* 9, 90. doi:10.1186/s40478-021-01196-5

Hampel, H., Hardy, J., Blennow, K., Chen, C., Perry, G., Kim, S. H., et al. (2021). The Amyloid-β Pathway in Alzheimer's Disease. *Mol. Psychiatry* 26, 5481–5503. doi:10.1038/s41380-021-01249-0

He, X. H., Galaj, E., Bi, G. H., He, Y., Hempel, B., Wang, Y. L., et al. (2021). β-Caryophyllene, an FDA-Approved Food Additive, Inhibits Methamphetamine-Taking and Methamphetamine-Seeking Behaviors Possibly via CB2 and Non-CB2 Receptor Mechanisms. *Front. Pharmacol.* 12, 722476. doi:10.3389/fphar.2021.722476

Komorowska-Müller, J. A., and Schmöle, A.-C. (2020). CB2 Receptor in Microglia: The Guardian of Self-Control. *Int. J. Mol. Sci.* 22, 19. doi:10.3390/ijms22010019

Koppel, J., Vingtdeux, V., Marambaud, P., d'Abramo, C., Jimenez, H., Stauber, M., et al. (2014). CB2 Receptor Deficiency Increases Amyloid Pathology and Alters Tau Processing in a Transgenic Mouse Model of Alzheimer's Disease. *Mol. Med.* 20, 29–36. doi:10.2119/molmed.2013.00140.revised

López, A., Aparicio, N., Pazos, M. R., Grande, M. T., Barreda-Manso, M. A., Benito-Cuesta, I., et al. (2018). Cannabinoid CB2 Receptors in the Mouse Brain: Relevance for Alzheimer's Disease. *J. Neuroinflammation* 15, 158. doi:10.1186/s12974-018-1174-9

Maresz, K., Carrier, E. J., Ponomarev, E. D., Hillard, C. J., and Dittel, B. N. (2005). Modulation of the Cannabinoid CB2 Receptor in Microglial Cells in Response to Inflammatory Stimuli. *J. Neurochem.* 95, 437–445. doi:10.1111/j.1471-4159.2005.03380.x

Mecha, M., Carrillo-Salinas, F. J., Feliú, A., Mestre, L., and Guaza, C. (2016). Microglia Activation States and Cannabinoid System: Therapeutic Implications. *Pharmacol. Ther.* 166, 40–55. doi:10.1016/j.pharmthera.2016.06.011

Mechoulam, R., and Parker, L. A. (2013). The Endocannabinoid System and the Brain. *Annu. Rev. Psychol.* 64, 21–47. doi:10.1146/annurev-psych-113011-143739

Nimmerjahn, A., Kirchhoff, F., and Helmchen, F. (2005). Resting Microglial Cells Are Highly Dynamic Surveillants of Brain Parenchyma *In Vivo*. *Science* 308, 1314–1318. doi:10.1126/science.1110647

Oakley, H., Cole, S. L., Logan, S., Maus, E., Shao, P., Craft, J., et al. (2006). Intraneuronal Beta-Amyloid Aggregates, Neurodegeneration, and Neuron Loss in Transgenic Mice with Five Familial Alzheimer's Disease Mutations: Potential Factors in Amyloid Plaque Formation. *J. Neurosci.* 26, 10129–10140. doi:10.1523/JNEUROSCI.1202-06.2006

Onaivi, E. S., Ishiguro, H., Gong, J. P., Patel, S., Meozzi, P. A., Myers, L., et al. (2008). Functional Expression of Brain Neuronal CB2 Cannabinoid Receptors are Involved in the Effects of Drugs of Abuse and in Depression. *Ann. N. Y. Acad. Sci.* 1139, 434–449. doi:10.1196/annals.1432.036

Ouali Alami, N., Schurr, C., Olde Heuvel, F., Tang, L., Li, Q., Tasdogan, A., et al. (2018). NF-κB Activation in Astrocytes Drives a Stage-Specific Beneficial Neuroimmunological Response in ALS. *EMBO J.* 37 (16), e98697. doi:10.15252/embj.201798697

Pertwee, R. G., Howlett, A. C., Abood, M. E., Alexander, S. P., Di Marzo, V., Elphick, M. R., et al. (2010). International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid Receptors and Their Ligands: beyond CB_1 and CB_2. *Pharmacol. Rev.* 62, 588–631. doi:10.1124/pr.110.003004

Puente, N., Río, I. B., Achicallende, S., Nahirney, P. C., and Grandes, P. (2019). High-Resolution Immunoelectron Microscopy Techniques for Revealing Distinct Subcellular Type 1 Cannabinoid Receptor Domains in Brain. *Bio Protoc.* 9, e3145. doi:10.21769/BioProtoc.3145

Querfurth, H. W., and LaFerla, F. M. (2010). Alzheimer's Disease. *N. Engl. J. Med.* 362, 329–344. doi:10.1056/NEJMra0909142

Reusch, N., Ravichandran, K. A., Olabiyi, B. F., Komorowska-Müller, J. A., Hansen, J. N., Ulas, T., et al. (2022). Cannabinoid Receptor 2 Is Necessary to Induce Toll-like Receptor-Mediated Microglial Activation. *Glia* 70, 71–88. doi:10.1002/glia.24089

Rodríguez-Cueto, C., Gómez-Almería, M., García Toscano, L., Romero, J., Hillard, C. J., Lago, E., et al. (2021). Inactivation of the CB 2 Receptor Accelerated the Neuropathological Deterioration in TDP-43 Transgenic Mice, a Model of Amyotrophic Lateral Sclerosis. *Brain Pathol.* 31, e12972. doi:10.1111/bpa.12972

Sarott, R. C., Westphal, M. V., Pfaff, P., Korn, C., Sykes, D. A., Gazzi, T., et al. (2020). Development of High-Specificity Fluorescent Probes to Enable Cannabinoid Type 2 Receptor Studies in Living Cells. *J. Am. Chem. Soc.* 142, 16953–16964. doi:10.1021/jacs.0c05587

Savonenko, A. V., Melnikova, T., Wang, Y., Ravert, H., Gao, Y., Koppel, J., et al. (2015). Cannabinoid CB2 Receptors in a Mouse Model of Aβ Amyloidosis: Immunohistochemical Analysis and Suitability as a PET Biomarker of Neuroinflammation. *PLoS One* 10, e0129618. doi:10.1371/journal.pone.0129618

Schmöle, A. C., Lundt, R., Ternes, S., Albayram, Ö., Ulas, T., Schultze, J. L., et al. (2015). Cannabinoid Receptor 2 Deficiency Results in Reduced Neuroinflammation in an Alzheimer's Disease Mouse Model. *Neurobiol. Aging* 36, 710–719. doi:10.1016/j.neurobiolaging.2014.09.019

Schmöle, A. C., Lundt, R., Toporowski, G., Hansen, J. N., Beins, E., Halle, A., et al. (2018). Cannabinoid Receptor 2-Deficiency Ameliorates Disease Symptoms in a Mouse Model with Alzheimer's Disease-Like Pathology. *J. Alzheimers. Dis.* 64, 379–392. doi:10.3233/JAD-180230

Song, W. M., and Colonna, M. (2018). The Identity and Function of Microglia in Neurodegeneration. *Nat. Immunol.* 19, 1048–1058. doi:10.1038/s41590-018-0212-1

Spangenberg, E., Severson, P. L., Hohsfield, L. A., Crapser, J., Zhang, J., Burton, E. A., et al. (2019). Sustained Microglial Depletion with CSF1R Inhibitor Impairs Parenchymal Plaque Development in an Alzheimer's Disease Model. *Nat. Commun.* 10, 3758. doi:10.1038/s41467-019-11674-z

Tahara, K., Kim, H. D., Jin, J. J., Maxwell, J. A., Li, L., and Fukuchi, K. (2006). Role of Toll-Like Receptor Signalling in Abeta Uptake and Clearance. *Brain* 129, 3006–3019. doi:10.1093/brain/awl249

Wang, Y., Ulland, T. K., Ulrich, J. D., Song, W., Tzaferis, J. A., Hole, J. T., et al. (2016). TREM2-Mediated Early Microglial Response Limits Diffusion and Toxicity of Amyloid Plaques. *J. Exp. Med.* 213, 667–675. doi:10.1084/jem.20151948

Wu, J., Hocevar, M., Foss, J. F., Bie, B., and Naguib, M. (2017). Activation of CB2 Receptor System Restores Cognitive Capacity and Hippocampal Sox2 Expression in a Transgenic Mouse Model of Alzheimer's Disease. *Eur. J. Pharmacol.* 811, 12–20. doi:10.1016/j.ejphar.2017.05.044

Zhang, H. Y., Gao, M., Liu, Q. R., Bi, G. H., Li, X., Yang, H. J., et al. (2014). Cannabinoid CB2 Receptors Modulate Midbrain Dopamine Neuronal Activity and Dopamine-Related Behavior in Mice. *Proc. Natl. Acad. Sci. U. S. A.* 111, E5007–E5015. doi:10.1073/pnas.1413210111

Conflict of Interest: Authors CK, CR and UG were employed by the company F. Hoffmann-La Roche Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership