Advances in Experimental Medicine and Biology 1162

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Recent Advances in Cannabinoid Physiology and Pathology

Springer

Advances in Experimental Medicine and Biology

Volume 1162

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ISSN 0065-2598 ISSN 2214-8019 (electronic)
Advances in Experimental Medicine and Biology
ISBN 978-3-030-21736-5 ISBN 978-3-030-21737-2 (eBook)
https://doi.org/10.1007/978-3-030-21737-2

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Introduction

The physiological effects of cannabis use represent one of the most rapidly growing, yet controversial, areas of research. Although recreational and therapeutic use of marijuana has been practiced by mankind for centuries, scientific advancement of the field is being fueled by recent discoveries in cannabinoid chemistry and pharmacodynamics. Inspired by these findings, this book examines current topics in cannabinoid research and medicinal use at different levels of resolution.

Many recent discoveries in cannabinoid research would not be possible without a thorough understanding of endocannabinoid system component distribution and function in living organisms. Thus, the book begins with an introduction to the topic of endocannabinoid system and the role of this system in ontogenesis. The "ABCs" of cannabinoids would not be complete without a detailed overview of the interaction of phyto- and endocannabinoids and synthetic cannabinoids with their protein targets. Such understanding arises from the growing number of high-resolution structural data that depict cannabinoid-protein interactions at the atomic resolution. Stemming from the progress in modern crystallography and rapidly emerging cryogenic electron microscopy, high-resolution structural data are reviewed in a separate chapter of this book.

Several chapters within this book capitalize on cannabinoid-protein interactions by focusing on the prevalent health disorders, such as cancer and cardiovascular disease. These chapters describe in detail the role of endocannabinoid system and the potential use of cannabinoid-related compounds to combat these pathologies. However, cannabinoids have the potential for abuse and dependence. The book addresses this topic with two chapters. One presents a discussion of a cross talk between the endocannabinoid system and neuronal circuits that enable alcohol use disorders. The theme of drug abuse is further developed in a chapter that reviews current knowledge on candidate genes that may drive marijuana use and dependence.

The book concludes at the most integrative level with a chapter that considers cannabinoids as lead compounds in the development of pharmacotherapies against pain, epilepsy, and neurodegenerative disorders.

The unique feature of this book is that the content is presented by researchers and clinical scientists at different stages of their careers. While some chapters are contributed by well-recognized researchers, others are prepared by young investigators emerging in the dynamic field of cannabinoid research and medicinal use. These diverse contributions reflect rapid growth, diversity,

and many promising pharmacological leads in the field of cannabinoid research. I hope that the book will spark the reader's interest, enthusiasm, and commitment toward advancing knowledge of cannabinoid-related physiology and pathology.

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About the Editor

Anna N. Bukiya, Ph.D., is an associate professor at the Department of Pharmacology, College of Medicine, The University of Tennessee Health Science Center and a member of Biophysical Society, American Society for Pharmacology and Experimental Therapeutics, Research Society on Alcoholism, and International Drug Abuse Research Society. Her scientific interests lie in the field of lipid modulation of ionic currents and cerebral artery sensitivity to drugs of abuse starting from in utero development and spanning into the adulthood. These studies employ an array of computational, biochemical, electrophysiological, and integrative approaches.

1 Endocannabinoid System Components: Overview and Tissue Distribution

Neal Joshi and Emmanuel S. Onaivi

Abstract

Marijuana/cannabinoid research has been transformed into mainstream science during the last half-century. Evidence based research and remarkable biotechnological advances demonstrate that phytocannabinoids and endocannabinoid (eCBs) acting on cannabinoid receptors (CBRs) regulate various aspects of human physiological, behavioral, immunological and metabolic functions. The distribution and function of the components of the endocannabinoid system (ECS) in the central nervous system (CNS) and immune processes have garnished significant research focus with major milestones. With these advances in biotechnology, rapid extension of the ECS research in the periphery has gained momentum. In this chapter, we review the components and tissue distribution of this previously unknown but ubiquitous and complex ECS that is involved in almost all aspects of mammalian physiology and pathology.

Keywords

Cannabis · Cannabinoids · Endocannabinoids · Cannabinoid receptors

Abbreviations

2-AG	2-arachidonyl glycerol
CBRs	cannabinoid receptors
CNS	central nervous system
eCB	endocannabinoids
ECS	endocannabinoid system
ENS	enteric nervous system
ER	endoplasmic reticulum
GABA	gamma-aminobutyric acid
GI	gastrointestinal
mtCBR	mitochondrial cannabinoid receptor
NAc	nucleus accumbens
p-CREB	phosphor-cAMP response element-binding protein
PPARs	peroxisome proliferator-activated receptors
THC	tetrahydrocannabinol
TRPV1	transient receptor potential vanilloid type 1
CNR	cannabinoid receptor gene
GPCR	G-protein coupled receptor

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A. N. Bukiya (ed.), *Recent Advances in Cannabinoid Physiology and Pathology*, Advances in Experimental Medicine and Biology 1162, https://doi.org/10.1007/978-3-030-21737-2_1

1.1 Introduction

This chapter covers the progress in elucidating the elements and tissue distribution of this previously unknown but ubiquitous and complex endocannabinoid system (ECS) that is involved in almost all aspects of mammalian physiology and pathology. Humans have used cannabis/marijuana for millennia and its contentious history from widespread use to prohibition and now back to the global rising trends, with interest in cannabinoid medication, and recreational use. This is largely due to the discovery of the psychoactive constituent Δ^1 tetrahydrocannabinol (Δ^1-THC) and the elements of the ECS [1, 2]. The ECS consists of genes encoding CBRs, endocannabinoids (eCBs), (Fig. 1.1), and the enzymes involved in the synthesis and degradation of the eCBs. Although cannabis use remains, one of the most widely used drugs in the world, research on the molecular and cellular basis of the physiological effects lagged behind that of other natural products such as tobacco, cocaine, and opium because of lack of specific tools and decades of irrational prejudice [3, 4]. Now remarkable advances in cannabinoid research and the discovery of the endocannabinoid system has transformed marijuana – cannabinoid research into mainstream science with significant implications in human health and disease [4]. With more evidence, based research there is a growing transformation of cannabis industry and business into Main Street.

Cannabinoids and many other diverse compounds are constituents in cannabis, and endocannabinoids (eCBs) are the endogenous marijuana-like substances found in animals and humans, that play significant roles not only in mammalian reproduction and growth of the newborn but also in most biological systems [1–4]. eCBs such as anandamide was the first eCB discovered [5], and it remains the most widely studied, yet anandamide acts only as a partial agonist at CBRs (Table 1.1). 2-arachidonyl glycerol (2-

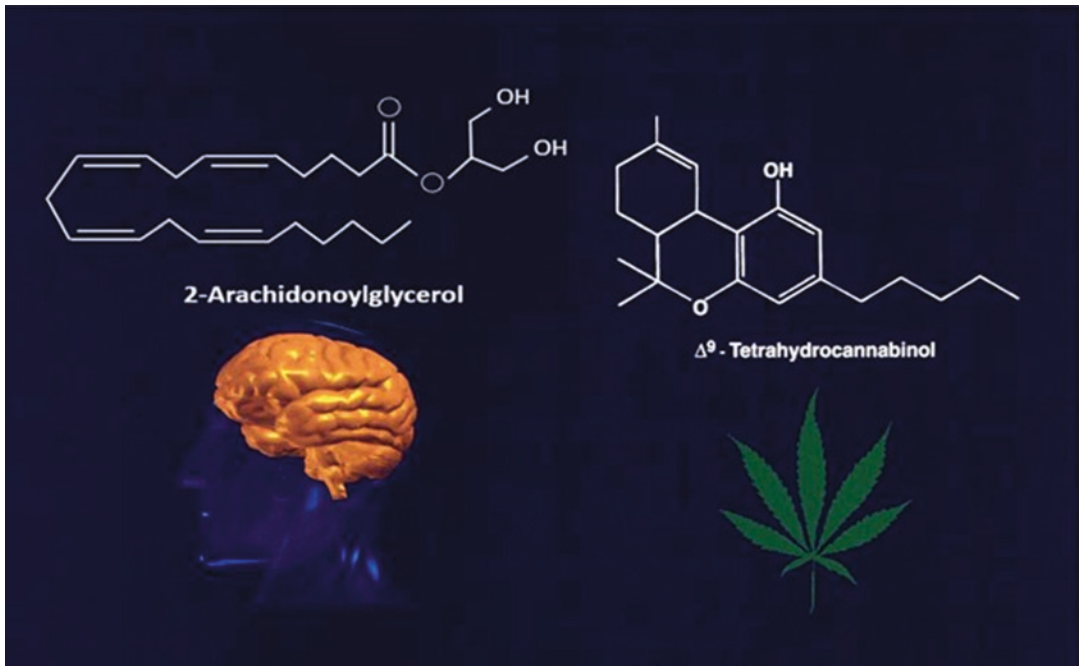

Fig. 1.1 Structure of endocannabinoid (eCB) 2-arachidonly glycerol (2-AG) produced in the body. There are many other eCBs including anandamide that was the first eCB discovered. The cannabis plant produces cannabinoids and Δ^9-tetrahydrocannabinol (Δ^9–THC) is the major psychoactive ingredient in cannabis along with over 100 cannabinoids. The plant also produces terpenes and flavonoids that are of growing research interest for therapeutic applications

Table 1.1 Cannabinoid receptor sub-types

	CB1R	CB2R
Gene name	*CNR1*	*CNR2*
Chromosomal location	6q14-q15	1p34-p35
Endocannabinoids		
Partial agonists	Anandamide	Anandamide
Full agonist	2-Arachidonylglycerol	2-Arachidonylglycerol
Peripheral distribution	Yes	Yes
CNS distribution	Yes	Yes
CBR-subtypes	CB1, CB1A…CB1n	CB2A, CB2B, CB2C, CB2D…CB2n

CB1n and CB2n refers to cannabinoid receptors/subtypes that remain to be discovered

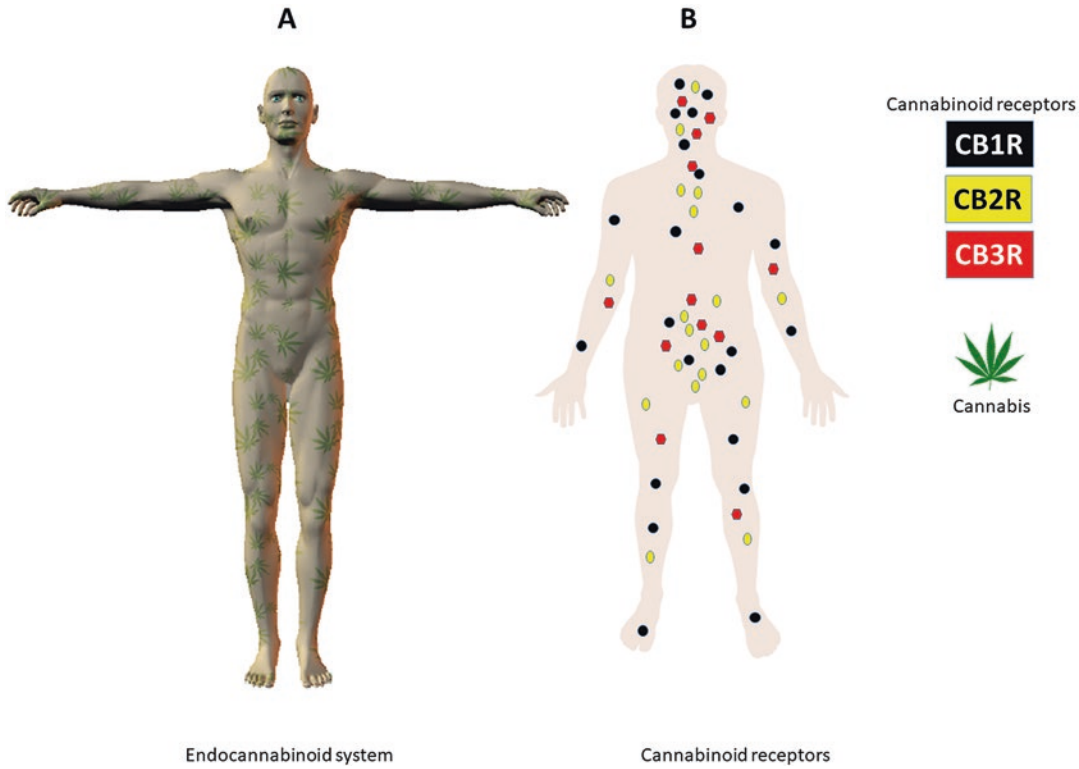

Fig. 1.2 The Endocannabinoid system: (**a**) depicts the distribution of endogenous cannabis-like compounds-the endocannabinoids throughout the body and (**b**): depicts the distribution of the CB1R, CB2R and TRPV1 that has been proposed as a putative CB3R. (See text on functional distribution of cannabinoid receptors

AG) is the most abundant eCB in brain, and recognized as the full agonist at the CBRs, since anandamide cannot be an endogenous natural ligand and act as a partial agonist at CBRs receptors [6]. Therefore, anandamide's role is increasingly recognized as full agonist at the transient receptor potential cation channel subfamily V member 1, (TRPV1) also called vanilloid receptor 1 (VR1), that some have suggested to be classified as CB3R (Fig. 1.2). One of the two major eCBs, 2-AG, serves as a retrograde messenger at various types of synapses throughout the brain. Upon postsynaptic activation, 2-AG is released immediately after de novo synthesis, activates presynaptic CB1Rs, and inhibits classical neurotransmitter release [7, 8]. When CB1R

activation is combined with some other factors such as presynaptic activity, the suppression is converted to a long-lasting form. Whereas 2-AG primarily transmits a rapid, transient, point-to-point retrograde signal, the other eCB, anandamide, may function as a relatively slow retrograde or non-retrograde signal or as a full agonist of the vanilloid receptor. Thus, persistent CB1R activity limits neurotransmitter release at various synapses throughout the brain. Therefore, the ECS can be up- or down-regulated by a variety of physiological and environmental factors including stress, which might be clinically important and in the aging brain [8].

New understanding in the science of cannabis botany along with medical and biotechnological advances demonstrate that phytocannabinoids and eCBs acting on CBRs are important regulators of various aspects of physiological, behavioral, immunological and metabolic functions. Countries and states are slowly advancing legalization of medical and recreational cannabis, which merits much needed research into uncovering the potential medicinal/nonmedicinal effects that it may have. The Current State of evidence on the health effects of cannabis and cannabinoids provides support for the legitimate study, regulation, and prescription of therapeutic cannabinoids [9].

1.2 ECS – Cannabinoid Receptor Distribution Patterns in Humans and Animal Models

There is an explosion of new knowledge, affirming the traditional millennia use of cannabis for the antiemetic, analgesic, anti-inflammatory, anti-oxidant, and anti-proliferative properties that is fueling the global trend and catalyzing the wave of cannabis programs worldwide. Significant progress and advances in our understanding of the cannabis plant and the ECS is providing new frontiers and opportunities in cannabis and endocannabinoid system research. The availability of human and draft genome of the cannabis plant will aid in the development of therapeutic cannabis strains with tailored profiles in medicine and breeding of cannabis cultivar. CBRs are widely expressed throughout the body in the periphery, organs, glands, tissues, immune cells, as well as the CNS with a wide range of biological effects (Fig. 1.2). It is important to note that emerging evidence on the localization of the two well characterized CBRs suggests that CB1Rs and CB2Rs may act together, competitively, or in opposite direction, in some cases due to dimerization, to modulate their physiological effects. This interplay between CB1Rs, CB2Rs and perhaps CB3Rs may be dependent on the location and distribution of the receptors as we suggested [8]. Therefore, a precise mapping and localization at the subcellular, cellular, tissue and regional distribution of CBRs is vital in the determination of their functional roles. Opposing roles of CB1Rs and CB2Rs in glucose homeostasis in the regulation of glycaemia, and brain stimulation reward paradigm [10] have been reported. Another example is that the ECS play a significant role in the regulation of cardiovascular system functioning, and both CB1Rs and CB2Rs influence myocardial contractility, heart rate and blood pressure [11]. Stimulation of CB1Rs results in the weakening of cardiomyocyte contractility, while activation of the CB2Rs increases the force of cardiac muscle cell contraction [12]. Specifically, CB2Rs have been shown to elicit cardioprotective effects against injury caused by ischemia-reperfusion [13].

1.2.1 Cellular Localization of Cannabinoid Receptors (CBRs)

In addition to the classical notion of the localization of CBRs on plasma membranes, the presence of CBRs in intracellular compartments in endoplasmic reticulum (ER), endosomes, lysosomes, mitochochondria (mt) and nuclei have been demonstrated [14]. As mitochondria are key organelles providing energy and many other vital functions to the cell, the impact of CBR activation in the mitochondria is an area of research focus. Some studies have described cannabinoids

and mtCBRs affect mitochondria function and regulation in peripheral and central organs of the body providing support that mtCBRs modulate bioenergetic cellular processes [15]. At the subcellular level, mtCB1Rs are in dendrites and axon terminals, making their localization different from the pool localized at the plasma membranes that are mainly at presynaptic terminals. The cannabinoid link between mitochondria and memory showed that mtCB1Rs regulate memory processes via modulation of mitochondrial energy metabolism [16]. Thus acute cannabinoid intoxication that induces amnesia in humans and animals is associated with altering mitochondrial energetic activity. We have reported on the subcellular distribution of CB2Rs in neuronal, endothelial and glia cells in the cortex, hippocampus and substantia nigra using immunohistochemical electron microscopy [7, 17, 18]. In the studies we found that in neurons, CB2R immunoreactivity was present in the cell body as well as in large and medium sized dendrites. The CB2R labeling was associated with the rough ER, Golgi apparatus and dendrites in these brain regions. In the substantia nigra, some unmyelinated axons were immunoreactive for CB2Rs providing ultrastructural evidence that CB2Rs are mainly postsynaptic in the CA1 area of the hippocampus and the substantia nigra. However, CB1Rs in the hippocampus have been shown to be mainly presynaptic and located mostly in GABAergic neurons, whereas CB2Rs in the same area have a postsynaptic localization. Therefore, the results from our studies does not exclude presynaptic localization in some brain regions, just like CB1Rs that are not exclusively presynaptic with some postsynaptic distribution reported [19]. One of the physiological roles of CB1Rs in the nervous system is to mediate the eCB retrograde signaling involved in the regulation of synaptic transmission.

Differential expression patterns of CBRs have been reported in B cells, T cells, monocytes and dendritic cells and exert distinct biological functions [20]. Deep fascia is the dense connective layer that surround muscles, bones, nerves and blood vessels, and the expression of CBRs in fibroblast of human fascial tissue implicates targeting the ECS in fascial pathology [21]. CBRs are present in the enteric nervous system (ENS), and eCBs are synthesized and released on demand by enteric neurons and other cell types present in the gut wall [22]. Thus, cannabinoid signaling in the ENS suggest a role of the ECS in the modulation of enteric neural circuit activity, synaptic transmission and mitochondrial trafficking, and as possible target in gut function and disorders [22]. More research will uncover the therapeutic potential of components of the ECS that are expressed throughout the body in health and disease.

1.2.2 Functional Distribution of ECS – Cannabinoid Receptors (CBRs)

The distribution and function of the components of the endocannabinoid system in the CNS and immune processes have garnished significant research focus with major milestones. With advances in biotechnology, rapid extension of the endocannabinoid system in the periphery has gained momentum [23–25]. Now there is evidence for presence of the ECS, and the key roles it plays in the regulation of biological processes on many different cell types of the integumentary system (skin and associated structures). The functional localization of the ECS and cannabinoid signaling in the multiple cellular compartments and appendages of the skin including, cutaneous nerve fibers bundles, macrophages, epidermal keratinocytes, hair follicles, sebaceous and sweat glands, melanocytes, mast cells, and fibroblasts provides implications for anti-inflammatory, anti-tumor actions, and future research targets for cannabinoid ligands in number of skin pathologies [23–25]. The skin is the largest organ of the body with active and complex structures that is involved in maintenance of homeostasis, and provides first line defense against injury, inflammation and infection [26]. Therefore, the discovery that ECS plays a role in healthy and skin disorders such as atopic dermatitis, psoriasis, scleroderma, pruritus and skin cancer indicates a therapeutic potential of cannabinoids in dermatology that warrants clinical

exploration [26–31]. It is important to note that other putative CBRs, like transient receptor potential channels (TRPs) and peroxisome proliferator-activated receptors (PPARs) are modulated by phytocannabinoids and ligands for TRPs and PPARs. It has therefore been suggested that combined targeting of several end-points seems critical in providing better chances of therapeutic success instead of the one-disease-one-drug target [26].

The distribution of CBRs and eCB signaling at other peripheral organ systems and tissues has also been recognized as key mediators of many aspects of human pathophysiology for potential therapeutic exploitation of ECS-based medicines for peripheral diseases [24]. In the cardiovascular system, ECS-CBRs are present in cardiovascular tissues and a dysregulation of the ECS is involved in cardiometabolic disease. CB1Rs, CB2Rs (CBR3Rs) or TRPV in the gastrointestinal (GI) tract, are located on intestinal epithelium, enteric nerves, and enteroendocrine cells mediate the actions of eCBs by reducing neurotransmitter release, and influences GI function. eCBs have been identified in immune cells such as monocytes, macrophages, basophils, lymphocytes, and dendritic cells and are believed to be enzymatically produced and released "on demand" in a similar fashion as the eicosanoids. Low levels of ECS-CBRs are present in various types of liver cells, including hepatocytes, stellate cells and vascular endothelial cells with CB1Rs and CB2Rs associated with fat accumulation and reduction respectively [24]. ECS-CBRs are localized and involved in the control of energy metabolism by skeletal muscle and formation of new muscle fibers, whereas eCB signaling regulates bone elongation and bone remodeling. Many earlier studies have provided evidence for the presence and localization of ECS-CB2Rs in immune cells as the CB2Rs were previously thought to be predominantly expressed in immune cells in the periphery and were referred to as peripheral CB2Rs [7]. As discussed below others and we have demonstrated the expression of CB2Rs in neuronal, glial and endothelial cells beyond immune-cannabinoid activity [7, 24]. With the abundant distribution of the ECS-CBRs in other organs, such as the respiratory tract, sensory, urinary and reproductive systems we are witnessing a deeper understanding of previously unknown function of the ECS. It has also been discovered that the ECS is involved in regulating oogenesis, embryo oviduct transport, blastocyst implantation, placental development and pregnancy outcomes and in sperm survival, motility, capacitation and acrosome reaction. Men who reported smoking marijuana had, on average, "significantly higher" sperm concentrations than men who did not smoke and differences in the ECS of sperm from fertile and infertile men have been reported [32]. Conceivably, plasma and tissue eCBs may represent reliable diagnostic markers of reproduction and pregnancy outcomes, and in the functional state of human sperm. The impact, presence and function of the ECS in acrosome reaction, implantation, breast milk and development of the newborn, provides basis for the evaluation of the components of the ECS as potential targets in health and disease.

1.2.3 Overview of Mammalian Diversification and Expression of CBRs

CB1Rs are now regarded as one of the most abundant G- protein coupled receptors (GPCRs) in the mammalian brain and have been extensively studied for their role in the physiology of pain, behavior, anxiety, depression, nausea, substance abuse disorders and neurodegenerative disorders. CB1Rs are expressed in many brain regions, but sparsely distributed in the pons and medulla oblongata, that are responsible for cardiovascular and respiratory functions, hence there is no cannabis overdosing resulting in respiratory depression or cardiovascular failure that is associated with current opioid epidemic. This is because the opioid receptors are abundant in the pons and medulla oblongata and over dosing on opioids are associated with respiratory depression. CB1Rs have been shown to be expressed in projection neurons in the striatum that are involved in the corticostriatal circuits of rats [33]. CB1R pharmacology has been explored more

than CB2R because of the increasing importance of cannabis and cannabinoids in medicine. In the rat spinal cord model, CB1R expression has been shown which may highlight the importance of analgesics by targeting the components of the ECS [34]. CB1R expression has been shown in the adult mouse forebrain, which indicates the importance in cognition, memory, and learning. CB1R mRNA is expressed in GABAergic interneurons in the forebrain and in low levels in the hippocampus, amygdala, and entorhinal cortex [35]. Furthermore, it was shown that CB1R mRNA is expressed in human brain nucleus accumbens, striatum, globus pallidus, and substantia nigra, many of which are affected in Parkinson's disease [36]. CB1R isoforms have been shown to be expressed in pancreatic β-cells and hepatocytes in humans [37].

On the other hand, CB2Rs are not exclusively peripheral cannabinoid CB2Rs as previously thought and overwhelming scientific data indicate that just like CB1Rs, CB2Rs are distributed in normal brain and peripheral tissues. It has become clear that while the expression of CB2Rs in the brain is much less than CB1Rs [7, 38], CB2R expression is induced during inflammation. Recent studies suggest that cannabinoids may produce different pharmacological actions in experimental species, suggesting that cannabinoid effects in one species cannot be directly extrapolated to another species [39]. We hypothesized that specie differences in CB1R and CB2R expression, protein structure and function may contribute to different pharmacological actions produced by cannabinoids in different species. Using quantitative RT-PCR, we found species-specific differential expression of CB1R and CB2R isoforms in brain regions and peripheral tissues. Human, rhesus monkey and rat Cnr2 genes encode 360 amino acids while mouse Cnr2 gene encodes 347 amino acids with a premature stop codon at its C-terminus. Based on these findings, we predicted that different promoters, epigenetic signatures, exons and/or different sequences in 5′-UTR and 3′-UTR of different isoforms may alter CB1R/CB2R receptor expression in different tissues, brain regions and/or different cellular types, and therefore, contribute to different CB1R/CB2R receptor responses and signaling in different species. Computer modeling of the 3-D structures found significant species differences in receptor structures such as opposite charged amino acid residues located in the vicinities of putative ligand binding sites [40, 41]. It is not surprising that different species display different pharmacological responses to the same ligands suggesting significant species differences in cannabinoid receptor structures and functions. There are also different CNR2 transcript isoforms depending on the species that display significant differences in gene structures and brain expression patterns from mouse to humans. Human CNR2 (hCB2R) and mouse Cnr2 (mCB2R) genes transcribe two isoforms—hCB2A and hCB2B, and mCB2A and mCB2B, respectively, while rat Cnr2 (rCB2R) gene transcribes at least four isoforms—rCB2A, rCB2B, rCB2C, and rCB2D. Human hCB2A and hCB2B transcripts are enriched in testis and spleen, respectively. Rat and mouse CB2A and CB2B transcripts are both enriched in spleen. Mouse brain expresses mCB2AR and mCB2BR isoforms with mRNA level of mCB2AR, higher than that of mCB2BR in several brain regions. Mouse CB2R truncates 13 amino acids in the carboxyl-terminal motif containing autophosphorylation sites (Ser 352) that is involved in cellular internalization. The cloning and pharmacological characterization of other species [42–44] including the dog CB2R (dCB2R) have been described, with similar 360 amino acid sequence with hCB2R [45]. The dCB2R shares between 76 and 82% homology with rat, mouse, human and chimpanzee CB2Rs [45]. The effects of cannabinoids from one species to another may not be the same because of the differences and divergence of CB2Rs across species [42–45].

1.3 Distribution and Variation of Sub-types of CBR Genes

Others and we have reported that the human CB1R have a number of splice variants, which may in part account for the myriad behavioral effects of smoking marijuana. Up to five isoforms

including the canonical/long and short isoforms are known to be produced by alternative splicing of the CNR1 transcript [46]. Some effects of marijuana and other cannabinoids may include actions at CB2Rs that have received much less attention than CB1Rs. However, others and we have now identified and characterized glial and neuronal CB2Rs in the brain. Nonetheless, many features of the CNR2 gene structure, regulation, and variation remain poorly characterized compared to the CNR1. In humans, the CNR2 gene is reported to consist of a single translated exon flanked by 5 and 3 untranslated regions and a single untranslated exon [47]. Most regions of the CNR2 gene are highly conserved, but the human has glutamine at position 63 instead of arginine [47, 48] and another SNP H316Y has been reported and linked to autoimmune disorders [47, 48]. Our studies provide the first evidence for a role of CB2Rs in depression, schizophrenia, and substance abuse [49–54]. Others and we have identified splice variants of the human CB1Rs and CB2Rs but they have thus far been poorly characterized for functional specificity apart from the broad roles associated with CB1R and CB2R subtypes. Alternative splicing of RNAs appears to be more common than previously thought in people, and can generate a variety of proteins, with most genes producing at least two variants. The characterization of CBR variants will add validity to the functional evidence for the existence of multiple cannabinoid receptor subtypes. New knowledge on cannabinoid receptor post-transcriptional and posttranslational modifications, such as alternate splicing and perhaps RNA editing, may indicate formation of multiple proteins that could unravel specific mechanisms associated with numerous behavioral and physiological effects of marijuana use. The cloning and sequencing of CNR1 gene from 62 species has also been reported [55] and awaits full characterization. As predicted here, the identification and characterization of these putative CBR isozymes and different elements of the ECS may reveal novel targets for medication development. However, the limitless signaling capabilities and the endless complexity of the cannabinoid system require a continuous intensive investigation. Specific genetic variants and polymorphisms in multiple genes including variations in the ECS genes have been associated with neuropsychiatric and other pathophysiology of human diseases [56].

The CNR2 genomic structure and CB2R subtype specificity has been poorly defined. However, many features of the CNR2 gene structure, regulation and variation are beginning to emerge with the discovery and identification of CB2Rs in mammalian CNS [7, 57, 58]. This prior poor definition could be related to the previously held view that CNR2 gene and CB2Rs were not expressed in neurons in brain but mainly in immune cells. It was therefore less investigated for CNS roles except for the association with brain cells of macrophage lineage. Our most striking discovery about Cnr2 genomic structure is the species- and tissue- specific expression patterns and differences between CB2R genes in human, rat and mouse [46, 57]. We found a novel human CB2A and CB2B isoform [46, 57]. This may be why others were not able to detect CB2Rs in the brain, particularly in neurons, which had been controversial [59–61], but now the issue of neuronal CB2R expression has been largely resolved [38, 43, 54, 58, 62]. It has been demonstrated that CB2Rs are expressed in hippocampal principal cells and modulate neuronal function both *in vitro* and *in vivo*. These studies contribute to the understanding not only of cell type specific functional roles of CB2Rs but also providing insights into the molecular and behavioral effects associated with the modulation of CB2Rs. Therefore, our discovery of functional neuronal CB2Rs has successfully challenged the dogma that CB2Rs are peripheral CBRs and that they are not expressed in neurons [38, 39, 42–44, 49–54].

1.4 Overview of Sex Differences in CB1 and CB2 Cannabinoid Receptors

While more focused sex-specific effects of cannabinoids are covered in this book, we provide here an overview of sex differences in CBR distribution. Sexual dimorphism in the ECS gives insight into disproportionate incidences of neuropsychiatric, neurodegenerative and other health

disorders between males and females. Sex differences may also contribute to normal physiological differences such as working memory, development, and hormonal regulation. Many potential mechanistic differences may explain differences in males and females such as CBR up/downregulation due to direct or indirect changes in the availability of functional components of the elements of ECS. Although animal studies show interaction between the ECS and sex hormones, as well as sex difference of higher brain CB1R in males, human in vivo studies of sex differences have yielded inconsistent results. In this era of cannabis medicine targeting the ECS in a number of pathological conditions, it is important to characterize sex differences in the distribution of the components of ECS. In our earlier studies, we determined the expression of CBRs and their gene transcripts in human blood cells. We showed that human CBRs and their gene transcripts could be analyzed in blood samples when combined with polymerase chain reaction. The results also demonstrated that the expression of CBRs was dependent on gender and ethnic background [63]. The implication, physiological and therapeutic implication of sex and ethnic difference in human CBR distribution is slowly emerging with advances in pharmacogenomics [64].

With the increased cannabis use in both men and women, along with the sex-dependent chromosomal and gonadal hormonal effects on the ECS, the question of the "telescoping" effect and whether the therapeutic efficacy of cannabis and cannabinoids differs as a function of sex needs further research. A recent study showed sex differences in CB1R availability in humans using positron emission tomography with a CB1R tracer, [^{18}F] FMPEP-d2. Males had on average 41% higher CB1R signal compared to females with an even larger localized difference in the posterior cingulate, which encompass the retrosplenial cortex and parahippocampal cortex [65]. Another study looked at sex differences in human ageing hearts and found that in younger men, CB2R immunoreactivity was higher than women, and CB1R immunoreactivity was lower than women [66]. The physiology of ageing is complex, and more work needs to be done to elucidate lifespan differences between men and women and how other organs change with age in terms of endocannabinoid functionality. It has been noted that men have a decreased rate and severity of a subset of cannabis withdrawal symptoms compared to women, which may have to do with sexual dimorphisms in the ECS. Females were more likely to experience violent outbursts, nausea, and irritability compared to males. In terms of average severity of individual withdrawal symptoms, there were no differences between males and females. The people that reported experiencing restlessness, anxiety, and increased aggression during the final attempt to quit marijuana, females had more severe ratings compared to males [67].

Preclinical studies also show sexual dimorphism in terms of THC exposure and assessing downstream signaling of CB1Rs. In adult female rats treated with THC using autoradiographic binding studies, it was shown that they had significant downregulation of CB1Rs in the nucleus accumbens (NAc), amygdala, and ventral tegmental area compared to males [68, 69]. In support of this, downstream signaling was also decreased using CP-55, 940-stimulated [^{35}S] GTPγS binding assay in the same brain regions. Differences in these areas may contribute to sexual dimorphisms seen in neurodegenerative diseases such as Parkinson's disease. Effects of THC on the forced swim test were also tested; showing that in females there was significantly more time immobile compared to males, which may be due to a more depressive phenotype. The ECS may partly explain why depression is more prevalent in females than males. In addition, they showed that pCREB levels in females were significantly reduced in the prefrontal cortex, and hippocampus, while there was an increase in the NAc compared to males. These areas are involved in depressive-like behaviors, which further bolsters potential reasons for sexual dimorphism in depression [68]. Many more studies with humans must be done both antemortem and postmortem in clinical depression, anxiety, and neurodegenerative diseases to elucidate the role that ECS plays in sexual dimorphism and pathophysiology.

1.5 Conclusions

The endocannabinoid system, a previously unknown but with ubiquitous signaling pathways is expressed throughout the human body and distributed in almost all tissues. Major advances in cannabinoid research leading to the discovery of the ECS include the identification of specific genes coding for the different components, and the localization of CBR genes in human chromosome 6 and 1 encoding CB1Rs and CB2Rs respectively. CB1Rs are now regarded as one of the most abundant GPCRs in the mammalian brain, and have been extensively studied in the brain and periphery. However, the neuronal functional expression of CB2Rs have been less investigated for CNS function as they were thought to be predominantly expressed in immune cells and were referred to as peripheral CB2Rs. The localization of brain CB2Rs, when compared to that of CB1Rs may be an indication of other putative functional roles of CB2Rs in the CNS. Therefore, both CB1Rs and CB2RS seem likely to work independently and/or cooperatively in differing neuronal populations to regulate important physiological activities in mammalian physiology. The determination of the crystal structures of CB1R and CB2R reveals a yin-yang relationship and functional profile of CB2R antagonism versus CB1R agonism and the availability of cannabis genome will aid the development of therapeutic cannabis strains with tailored cannabinoid profiles in medicine and breeding of cannabis cultivar. The Current State of evidence on the health effects of cannabis and cannabinoids provides support for the legitimate study, regulation, and prescription of therapeutic cannabinoids

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2 Physiology of the Endocannabinoid System During Development

Anna N. Bukiya

Abstract

The endocannabinoid (eCB) system comprises endogenously produced cannabinoids (CBs), enzymes of their production and degradation, and CB-sensing receptors and transporters. The eCB system plays a critical role in virtually all stages of animal development. Studies on eCB system components and their physiological role have gained increasing attention with the rising legalization and medical use of marijuana products. The latter represent exogenous interventions that target the eCB system. This chapter summarizes knowledge in the field of CB contribution to gametogenesis, fertilization, embryo implantation, fetal development, birth, and adolescence-equivalent periods of ontogenesis. The material is complemented by the overview of data from our laboratory documenting the functional presence of the eCB system within cerebral arteries of baboons at different stages of development.

Keywords

Cannabis · Cannabinoid · Baboon · Nonhuman primate · Fetal artery · Cerebral artery

Abbreviations

ABHD4	α/β-hydrolase domain 4
AEA	anandamide
CB	cannabinoid
COX-2	cyclooxygenase-2
CP55,940	(-)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol
DAGL	diacylglycerol lipase
eCB	endocannabinoid (system)
ERK	extracellular-signal-regulated kinase
FAAH	fatty acid amide hydrolase
GABA	gamma aminobutyric acid
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
MAGL	monoacylglycerol lipase
NAE	N-acylethanolamine
NAPE	N-acylphosphatidylethanolamine
NAPE-PLD	N-acylphosphatidylethanolamine-specific phospholipase D
PCR	polymerase chain reaction
THC	Δ^9-tetrahydrocannabinol
TRP	transient receptor potential (protein, channel)
VGAT	vesicular GABA transporter
2-AG	2-arachidonoylglycerol.

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A. N. Bukiya (ed.), *Recent Advances in Cannabinoid Physiology and Pathology*, Advances in Experimental Medicine and Biology 1162, https://doi.org/10.1007/978-3-030-21737-2_2

2.1 Introduction

Humans have been consuming cannabis in the form of herbs since ancient times [1]. Although adverse reactions and high doses of cannabinoid preparations may trigger dysphoria [2], low-to-moderate consumption confers analgesic, anxiolytic, and antiemetic properties [3–6]. The modern understanding of mechanisms that stand behind physiological effects of cannabis consumption started to emerge in the middle of twentieth century with the isolation and characterization of the main psychoactive substance in *Cannabis sativa* plant – Δ^9-tetrahydrocannabinol (THC) [1]. These findings were followed by the discovery of the first cannabinoid receptor (CB1), also by structure elucidation and isolation of endogenously produced cannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG) [1]. Although cannabis preparations currently are used primarily for recreational purposes, they are also consumed for medical reasons: to reduce nausea and vomiting, relieve symptoms associated with neurological disorders, reduce intraocular pressure in glaucoma patients, and as an analgesic remedy [1, 7]. Moreover, CB-based preparations have been increasingly recognized as having therapeutic potential for treatment of numerous pathological conditions, including depression, epilepsy, anxiety-related disorders, and obesity [8–12].

The endocannabinoid (eCB) system is comprised of endogenously produced CBs, their receptors, as well as eCB synthesis, degradation enzymes, and transporting molecules [13]. The eCB system plays a crucial role at all stages of human early development, stemming from the influence over gametogenesis and embryo implantation, spreading into control of nervous system development, peripheral organogenesis, and finishing with postnatal development [14]. Thus, studies of the eCB system help to delineate fundamentals of development and pinpoint potential sites of pharmacological interventions against prevalent developmental disorders. In addition, the understanding of mechanisms that govern eCB-mediated control over ontogenesis will help to expand our knowledge on the consequences of prenatal exposure to marijuana. The latter is reported to affect 3.9–7% of pregnancies [15], reaching even higher numbers (13% of meconium samples) within high-risk populations [16].

Remarkably, there is no acute toxicity to the major psychoactive substance in marijuana – THC [2]. Yet, THC consumption leads to a plethora of physiological and psychological effects [2, 17, 18], reflecting the complexity of the eCB system and its susceptibility to exogenous interventions. Widening legalization of marijuana use, development of synthetic approaches to obtain a "transgenic pot," and the growing number of pharmaceutical agents that target eCB signaling are reasons that call for comprehensive understanding of the side effects and risks associated with modifications of eCB function.

This chapter summarizes knowledge in the field of CB contribution into gametogenesis, fertilization, embryo implantation, fetal development, birth, and adolescence-equivalent periods of ontogenesis. The literature overview in each section starts with the data on rodents and then gradually shifts to humans. The material is complemented by data from our laboratory documenting the functional presence of the eCB system within cerebral arteries of baboons at different stages of development.

2.2 Brief Overview of eCB System: From Genes to Products

Upon demand, eCBs are synthesized *de novo* using hydrolyzed lipid precursors from cellular membrane [14]. The two most widely studied endogenously produced CBs are AEA and 2-AG. AEA belongs to the N-acylethanolamine (NAE) family of lipid mediators that represent CB-related compounds. For instance, N-palmitoylethanolamine is able to activate cannabinoid receptors [19, 20]. The physiological role of NAEs is being actively investigated [21–23]. AEA synthesis originates from the rate-limiting step of the N-acylation of phosphatidylethanolamine rendering N-acylphosphatidylethanolamine (NAPE) [24]. One of the major pathways in AEA synthesis is

mediated by N-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD) that hydrolyses NAPE into AEA [24]. Other pathways engage phosphatase PTPN22 [25], α,β-hydrolase domain 4 (ABHD4) [26], and glycerophosphodiesterase (GDE1) [27].

The major synthetic pathway of 2-AG is presented as a two-step reaction. The first step is initiated upon phospholipase C activation and results in the generation of diacylglycerol from phosphatidylinositol. The second step, resulting in 2-AG release, is carried out by a membrane-bound diacylglycerol lipase (DAGLα/β) [24, 28].

Cell types that are most actively involved with eCB production are still under investigation, but likely represent quite an exhaustive catalog [29]. Upon production and release, eCBs are quickly degraded by hydrolytic enzymes. Fatty acid amide hydrolase (FAAH) largely enables AEA degradation, with additional contribution from other enzymes such as cyclooxygenase-2 (COX-2) [30–33]. 2-AG is largely processed by the monoacylglycerol lipase (MAGL) with additional contribution from α,β-hydrolases 6 and 12 [34, 35]. The relative contribution of these enzymes into 2-AG catabolism is tissue/cell specific [34, 35].

CB receptors are presented by canonical receptors of type 1 (CB1) and 2 (CB2) that are Gi/o protein-coupled receptors exhibiting constitutive activity [36–41].

CB1 receptors are widely expressed in numerous neuronal populations [42], within glia [43, 44], and are also found in peripheral organs such as adrenal gland, heart, lung, prostate, uterus, ovary, testis, bone marrow, thymus, and tonsils [45, 46]. Presence of a "pre-nervous" eCB system operating via transient receptor potential (TRP) protein orthologs has been proposed in invertebrate echinoderms (sea urchin, starfish) [47]. However, mammalian CB1 receptor ortholog-coding genes are absent in commonly studied nonvertebrate species such as *Drosophila melanogaster* and *Caenorhabditis elegans* [48]. To some extent, the lack of a CB1 receptor ortholog in invertebrate species poses limitations on the choice of animal models for CB-related studies. However, human CB1 ortholog-coding genes are described in a variety of vertebrates including fish, amphibians, avian species, and nonhuman primates [48–50].

Although CB2 receptors have been detected in glia [51, 52] and in specific areas of the brain [52–55], they are also attributed as CB receptors of predominantly peripheral tissue locations that play a central role in immunity [42, 45, 46, 56].

Stimulation of CB receptors results in a number of signaling events, including inhibition of adenylate cyclase, activation of mitogen-activated protein kinase (MAPK) signaling, and modulation of ion channels' activity [36, 37, 57–65]. For instance, CBs target calcium, potassium, and TRP channel families [66, 67], either directly or via secondary signaling events. Besides canonical CB receptors, G protein-coupled receptors GPR55 and GPR119 are advanced as eCB sensors [68].

One must recognize that the eCB system is under constant control from various physiological stimuli, such as hormonal levels. For example, progesterone and estrogen regulate AEA level and expression of FAAH [69]. Also, FAAH in human lymphocytes is upregulated by progesterone [70]. Neither AEA nor THC modifies the level of follicle stimulating hormone; however, AEA decreases while THC increases growth hormone production [71]. In rats, prolonged glucocorticoid treatment decreases CB1 receptor density in the hippocampus [72].

ECB controls a wide range of physiological processes that include, but are not limited to, energy metabolism, inflammation, cardiovascular function, etc. [11, 12]. Thus, it comes as no surprise that a dysfunctional eCB system frequently underlies common human pathologies. The search for mechanisms of prevalent disorders, and studies of novel medications, both feed interest in eCB system functions, including its role in ontogenesis.

2.3 Cannabinoids in Fertility and Gametogenesis

ECB system components are present in virtually any reproductive tissue/organ [13, 73]. Early work described the ability of AEA to diminish sperm fertilizing capacity in sea urchins by inhib-

iting the acrosome reaction [74]. The CB2 receptor is detected in mouse Sertoli cells [75]. In human oocytes, both CB1 and CB2 receptors have been detected by means of reverse transcription polymerase chain reaction (PCR) and Western blotting [76].

The selective activation of the CB2 receptor induces progression of spermatogonia towards meiosis, and thus plays a critical role in spermatogenesis [77]. Notably, a mouse study reports a high amount of 2-AG in the caput (head) of the epididymis [78]. In this compartment, sperm cells are immobile or do not possess consistent motility. However, in the epididymis tail, 2-AG amount is lower, and such a gradient in 2-AG levels is believed to empower caudal spermatozoa with physiologically necessary motility via a CB1 receptor-mediated mechanism that involves the CB1 receptor on the sperm cell membrane [78]. In humans, reduction in viability characteristics and acrosome reaction of mature sperm is documented in response to AEA and is mediated by the CB1 receptor [69, 79].

Plasma levels of AEA fluctuate with the female cycle. The highest levels are observed during ovulation and follicular phase, while the lowest are reported during luteal phase [80–82]. ECB activity exerts an effect on hormonal production, as reported for AEA- and THC-driven downregulation of luteinizing hormone and prolactin levels in ovariectomized rats [71]. THC, however, has been found to inhibit ovulation by suppressing the plasma levels of follicle-stimulating hormone and luteinizing hormone in rats when animals were exposed to THC on the day of proestrus [83].

Clinical observations in human population concur on the risks of CB exposure during periconception [84, 85]. In particular, heavy use of marijuana and cannabis-derived psychoactive products is associated with decreased female fertility, loss of pregnancy, and embryotoxicity [86, 87]. Chronic marijuana use also decreases male fertility in humans and in animal models due to reduced testosterone production, sperm mobility and viability [88–90]. Linkage between the phenomenology of CB's effect on fertility and molecular players that enable such action represents an area of active investigation [84, 85].

2.4 Embryonic Development

Zebrafish *Danio rerio* has been emerging as an important model for studying the role of eCB and consequences of environmental CB exposure in development [91, 92]. Use of this model allows tracing of eCB system gene expression using qPCR in zebrafish embryos throughout 1–120 h post-fertilization. Analysis reveals diverse patterns of eCB system gene expression [91]. For example, low levels of *cnr1, gpr55a,* and *abhd6* expression are detected throughout development [91]. In contrast, expression levels of *cnr2, cnrip1a* and *dagl* family are relatively high [91]. Expression levels of *naaa1a* and *abhd12* are progressively decreased, while expression of *ptgs2a* and *mgll* is increased throughout development [91].

A whole-mount *in situ* hybridization study on chick embryos detects the presence of CB1 gene expression within the first-appeared neurons of the central nervous system (within hindbrain, as early as stage 10 in the chick), followed by appearance within the peripheral nervous system (ophthalmic trigeminal placode) at stage 11 [93]. After these early milestones, CB1 expression is detected in other neuronal populations, such as within vestibuloacoustic, epibranchial ganglions and dorsal root ganglia. Notably, CB1 expression is not uniquely present in neurons. For example, CB1 expression is detected in the ventral forebrain that does not produce neurons in early development [93]. In addition, CB1 expression is detected in the mesoderm. Although neurons are showing CB1 expression very early on, the expression disappears at later stages of chick development [93]. Expression of CB2, TRPV1 and GPR55 is not apparent in chick embryonic central nervous system during development [94]. At early stages of nervous system establishment, eCB system is believed to play a critical role in axonal growth and formation of synaptic connections. Indeed, treatment of chick embryo central nervous system explants with CB1 receptor antagonist AM251 results in defective axonal growth and fasciculation [94]. With regards to other component of the eCB system, DAGL α and β isoforms are widely expressed throughout

the embryonic chicken brain, while MAGL is only expressed at later stages [94].

A combination of qPCR, mass spectroscopy, immunohistochemistry and Western blotting detects the presence of eCB system components in chick and mouse embryos at stages 9–11 and E8.75, respectively [41]. These time points correspond to the pre-neuronal phase of early development [95] and represent post-coital days 22–26 of human gestation [41]. An additional study utilizing an agonist-stimulated [^{35}S]GTPγS binding assay documents G protein activation in the brain primordium of chick embryos at stages 9 to 11 in presence of CB1 receptor agonist CP-55,940 [41]. This activation vanishes following pretreatment of samples with the inverse agonist of CB1 receptor SR141716A [41].

In mammals, a balanced eCB system is crucial for successful early pregnancy. At this stage, synchronization of embryo development with uterine receptivity for implantation is central for maintaining viable pregnancy. The eCB system has been shown to play a critical role at all stages of such synchronization, starting from the development of the preimplantation embryo, its movement through oviduct, throughout implantation and placenta development. AEA synthesis and degradation enzymes NAPE-PLD and FAAH, respectively, are expressed at the two-cell stage of embryonic development [69, 96]. While the CB1 receptor is detected from the four-cell stage, CB2 is found as early as the one-cell stage of embryonic development [69, 96]. Systematic studies point at the CB1 receptor and CB1-mediated signaling as critical players in early development. In the mouse model, only CB1 receptor is present in the maternal oviduct and uterus, while both CB1 and CB2 receptors are detected in preimplantation embryo [86, 87, 97]. Cross-talk between maternal and embryonic CB systems is at the center of successful early pregnancy and development. Indeed, CB1, CB2, or double knock-out mice show asynchronous embryo development during early pregnancy [86, 87, 97]. Remarkably, the development is rescued when knock-out females are mated with wild-type males, pointing at the ability of heterozygous embryos to navigate the proper timing of development and disregard the abnormal maternal CB knock-out environment [86, 87, 97].

Movement of the preimplantation embryo through the maternal oviduct ensures a path to implantation. CB1 knock-out mice show 40% pregnancy loss at this stage [86, 87, 97]. Unlike embryo development, movement along the oviduct cannot be restored by mating CB1 knock-out females with wild type males [86, 87, 97]. Thus, at this stage, maternal factors gain critical weight over embryonic characteristics of the CB system. It is noted that embryos that fail to move to implantation site, still retain their quality, as they could be implanted into pseudopregnant recipient uteri [86, 87, 97]. Implantation of normal embryos into pseudopregnant recipient uteri of CB1 knock-out mice also renders non-developing pregnancies [86, 87, 97]. This finding provides independent verification of the critical role of *maternal* CB characteristics in controlling embryo movement through the oviduct.

Mechanistic studies reveal a closely coordinated cross-talk between CB1-mediated and adrenergic signaling in control of oviduct motility. Loss of CB1 function increases noradrenaline release from adrenergic nerve terminals and increases smooth muscle contractility via alpha-adrenergic receptor (α-AR) preventing embryo movement. In contrast, CB1 receptor stimulation relaxes oviduct muscle and promotes embryo movement [97]. Consistent with observations in animal models, reduced CB1 expression in Fallopian tubes is detected during ectopic pregnancy in humans [98].

Last but not least, CB tone is central for embryo implantation. This process is tightly controlled by hormonal release of estrogens and progesterone. While progesterone primes the uterus for implantation, estradiol and its metabolite 4-hydroxy-17β-estradiol, produced in the uterus, mediate blastocyst activation for implantation [87, 99]. It has been well established that lower levels of CB1 receptors in the blastocyst and a decrease in uterine AEA levels are crucial for sustaining the "window" of uterine receptivity [86, 87, 97–100]. In the blastocyst, CB1 expression is lower during the activated state when compared to the dormant blastocyst [101]. In

uterus, higher levels of AEA, NAPE-PLD activity, and NAPE-PLD mRNA are detected in nonreceptive uterine states compared to receptive uteri [102, 103]. Of note, AEA levels in the uterus, similar to other organs (see below), are dramatically lower than those of 2-AG [13].

Studies in animal models are closely correlated with findings in humans: elevated plasma AEA levels are detected in women with nonviable pregnancies, while lower AEA is associated with positive pregnancy outcome [82, 104–106]. Moreover, decreased FAAH activity is detected in peripheral lymphocytes of women with pregnancy failure [82, 104, 105]. Low activity of placental FAAH, together with elevated CB1 levels, characterize human miscarriages, while higher FAAH activity is detected in placentas during normal pregnancies [107].

Notably, NAPE-PLD has been shown to play a critical role in maintaining uterine AEA levels [103] despite the fact that NAPE-PLD knock-out mice are characterized by near-normal AEA levels [108]. In the uterus, the implanting blastocyst exerts an inhibitory effect on NAPE-PLD activity [103], ensuring coordination in CB signaling between embryonic and maternal sites. NAPE-PLD has also been identified in human placental tissue and is believed to mediate AEA production in the placenta [105].

Further on in the process, successful implantation relies on the differentiation and invasion of a trophoblast originating from the blastocyst trophectoderm during early stages of pregnancy [109]. Low levels of AEA promote trophoblast growth, while elevated AEA inhibits the development of embryos [14]. Exploration of potential mechanisms that underlie the dual role of AEA in early pregnancy suggests two distinct pathways. In the murine model, low concentrations of AEA activate blastocyst extracellular-signal-regulated kinase (ERK) signaling and promote implantation [87, 99]. In the murine model and sheep pregnancy, higher levels of AEA inhibit calcium mobilization, induce cell apoptosis, and inhibit blastocyst cell proliferation thereby precluding successful implantation [87, 99, 110]. In a rat model, CB1 receptor activation results in ceramide release and p38 MAPK-mediated mitochondrial stress, leading to production of reactive oxygen species and apoptosis of uterine decidual cells [111]. MAPK triggers the COX-2 oxidative metabolism of AEA. Such metabolic pathways result in the formation of prostaglandin-like compounds termed “prostamides” [112]. The COX-2 metabolic pathway for AEA is competing with the conventional FAAH pathway [113]. In addition to COX-2-mediated oxidative metabolism resulting in oxidative stress, AEA-induced apoptotic effect is also associated with NF-kB activation [112].

When compared to CB1, the role of the CB2 receptor in early stages of pregnancy is less established. However, CB2 receptor transcript has been identified in both placenta and trophoblasts [114]. During hematopoietic differentiation of murine embryonic stem cell-derived embryoid bodies, CB1 and CB2 antagonists (AM251 and AM630, respectively) induce stem cell death and inhibition of cannabinoid agonist-induced chemotaxis [115].

A study on rat species utilizing RT-PCR, Western blot, and immunohistochemistry documents the presence of CB1 and CB2 receptors, TRPV1 transcripts, and protein products of corresponding encoding genes in rat mesometrial decidua [116]. While transcripts and protein products for CB1, CB2, and TRPV1 decrease throughout pregnancy overall, the CB1 protein amount shows a remarkable spike during day 12 of rat pregnancy. The spike is not detected in the CB1 transcript [116]. This fact underscores the importance of post-transcriptional regulatory mechanisms in maintaining optimal levels of key players within the eCB system.

In addition to direct targeting of eCB receptors within embryonic and maternal tissues, the eCB system has been proposed as exerting a modulatory effect on early pregnancy outcome via immunity [24]. Indeed, eCB system components are present in immune cells [117]; interestingly, immune response has been put forth as an important player in pregnancy initiation and maintenance [118, 119]. Possible cross-talk between the eCB system, immune cells, and reproductive success resulting in a formation of an “endocannabinoid-immune-reproductive axis” requires multifaceted experimental validation [24].

2.5 Midgestation

AEA and 2-AG are produced throughout the prenatal period, but their amounts are not constant [43, 120]. AEA levels in rat brain remain low throughout perinatal period, but then gradually increase as animals reach adulthood [120]. In contrast, changes in 2-AG level show a different time course. They remain relatively constant with the exception of a significant spike at day 21 of rat gestation [43, 120]. This is a full-term pregnancy in rats and in terms of brain development milestones, corresponds to approximately 23 weeks of pregnancy in humans [121].

The CB1 receptor is critical for placental development, as *Cnr* knock-out mice have smaller placentas and higher resorption rate at mid gestation when compared to their wild-type (CB1 receptor-containing) counterparts [122]. With regards to fetal tissue, CB1 receptor transcript is detected in rat embryo neural tube structures at E11 [114]. CB2 receptor messenger RNA is detected in rat embryonic liver as early as at E13 [114].

The occurrence and functional characterization of the eCB system has been actively studied within the developing nervous system [55, 123]. Establishment of critical structural components and connectivity within neuronal networks relies on several critical events, such as neuronal progenitor cell proliferation and differentiation, neuronal migration to target regions, and formation of synapses. All aforementioned events are connected with the functionality of eCB system components at various levels of resolution as shown in different experimental models [124].

Immunofluorescence labeling of rat fetuses detects the presence of CB1 receptors in E12.5-16.5 in migrating post-mitotic neurons during corticogenesis [125, 126]. Moreover, prenatal exposure to CB1 receptor agonist WIN 55,212-2 (0.75 mg/kg) via daily subcutaneous (s.c.) delivery to pregnant dams results in significant increases in the number of post-mitotic neurons [126]. However, this increase is not accompanied by a corresponding increase in *gamma*-aminobutyric acid (GABA)-positive immunostaining. In contrast, WIN 55,212-2 exposure increases immunofluorescence associated with T-box transcription factor Tbr2 that is characteristic of progenitor cells destined for glutamatergic development [126]. In contrast, the marker of post-mitotic glutamatergic neurons Tbr1 responds with transient decreases at E12.5 and E14.5 in brain samples from WIN 55,212-2-exposed fetuses [126]. Thus, the CB1 receptor plays a critical role in neuronal migration and corticogenesis. Variations in fetal CB1 receptor activity and functioning of the eCB system result in a complex reshaping of neuronal development, thus affecting formation of neuronal networks [127]. Studies in knock-out mouse lines demonstrate that lack of CB1 and CB2 receptors leads to impairment of neural progenitor cell proliferation [128, 129]. Mice lacking the CB1 receptor are characterized by diminished cortical progenitor cell proliferation and astrogliogenesis [128, 130]. Notably, work on cultured cell lines indicates that CB1 receptor activation can induce either neurite growth or retraction, depending on the CB1 receptor activation-triggered downstream signaling pathway [55, 131, 132]. Despite the fact that experimental probing of the CB1 receptor impact on the direction of neuronal development renders somewhat conflicting results, the overall picture seems to support positive correlations between CB1 receptor activation and neuronal cell proliferation and migration [124]. Yet, diminished CB1 receptor activity would likely favor cell differentiation, formation of a neuronal phenotype, and synaptogenesis [124].

Analysis of the CB1 receptor expression pattern in human fetuses during midgestation (17–22 weeks) reveals region-specific presence of CB1 receptor mRNA: while CB1 receptor expression is high in limbic structures, only moderate levels are detected in cerebral cortex, thalamus, medial/ventral striatum, and subventricular zone [133]. This expression profile remains unaltered by cannabis exposure *in utero* [133]. However, maternal cannabis use is associated with a significant decrease in dopamine receptor subtype 2 (D2) mRNA in the amygdala of male, but not female fetuses [133, 134]. D2 mRNA decrease is also detected in striatum; however,

dopamine receptor subtype 1 (D1) expression remains unchanged [133, 134]. In addition, prenatal rat THC exposure leads to fluctuations in brain mRNA levels of the enzyme tyrosine hydroxylase that represents a rate-limiting step in dopamine synthesis [135]. This effect exhibits marked sexual dimorphism, the latter being characteristic of alterations in dopaminergic system by prenatal exposure to cannabinoids [43].

With regards to the opioid system, prenatal cannabis exposure is associated with increased mu opioid receptor expression in amygdala [133]. Yet, proenkephalin RNA levels are decreased in fetal striatum from cannabis-using mothers, while prodynorphin levels remain unchanged [133].

In addition to alterations in dopaminergic and opioid systems, prenatal and postnatal exposure to THC in a rat model (from gestational day 5 to postnatal day 20) is reported to result in a decreased immunoreactivity against the GluR1 subunit in Bergmann glial cells and the GluR2/3 subunit in Purkinje neurons when evaluated at postnatal day 20 [136]. These changes persist after THC withdrawal at postnatal days 30 and 70 [136]. Moreover, the expression of glial (GLAST) and neuronal (EAAC1) glutamate transporters in astroglial cells and Purkinje neurons, respectively, is decreased in THC-exposed rat offspring compared to saline-treated controls [137].

The eCB system in the developing brain also represents a target for a drug of abuse other than cannabis itself – alcohol [138, 139]. Data from our laboratory show alcohol-induced decreases in blood velocity in the fetal middle cerebral artery during baboon maternal alcohol intoxication during second trimester-equivalent of human pregnancy [50, 140]. This drop is consistent with fetal cerebral artery dilation, and the latter is replicated using *in vitro* pressurized arteries from fetal baboons [50]. Notably, alcohol-induced (63 mg/dL ethyl alcohol) dilation of fetal cerebral arteries is blocked in the presence of AM251 in a mixture with AM630 (Fig. 2.1) [50]. The fact that an alcohol effect *in vitro* is sensitive to CB receptor block, and is mimicking the *in vivo* scenario, strongly suggests an active eCB system within fetal cerebral arteries of nonhuman primates.

In human brain, the CB1 receptor is immune-detected in the cortical plate as early as gestational week 9 [141]. It is notable that in the case of brain malformation, CB1 receptors are still present in dysplastic neurons [141]. By the second trimester (20 weeks of human gestation), the CB1 receptor mRNA is spiked within hippocampal CA region and basal nuclear group of the amygdaloid complex [142]. Notably, the adult brain, cerebral cortex, caudate nucleus, putamen and cerebellar cortex are also characterized by high mRNA levels of CB1, in addition to high levels in hippocampus and amygdala [142]. Thus, CB1 receptor occurrence is brain region-specific.

With regards to the relative distribution of CB1 and CB2 receptors across cell types, a differential expression pattern is documented at early stages of development. For example, while CB1 receptors are traced to astrocytes, CB2 receptors are present in microglia [141].

Prenatal marijuana use in humans does not alter fetal growth rates, evident from the analysis of human fetuses aborted at midgestation (17–22 weeks of pregnancy) [143]. However, there is a significant reduction in fetal foot length and body weight in the group of marijuana-exposed fetuses when compared to controls. Moreover, fetal foot length growth is inversely correlated with the amount and frequency of marijuana use reported by the mothers [143]. The consequences of maternal marijuana use are long-lasting. For instance, prenatal marijuana exposure significantly correlates with the age of onset and frequency of marijuana use among 14-year-old teens [144]. Overall, the early appearance, wide distribution, differential expression pattern, and physiological function of eCB system components support the hypothesis of a critical role of eCB signaling in physiology and pathology during midgestation.

2.6 Neonatal and Postnatal Development

At birth, eCB system components are widely distributed in maternal and fetal tissues [14, 49, 145]. While AEA remains low during normal

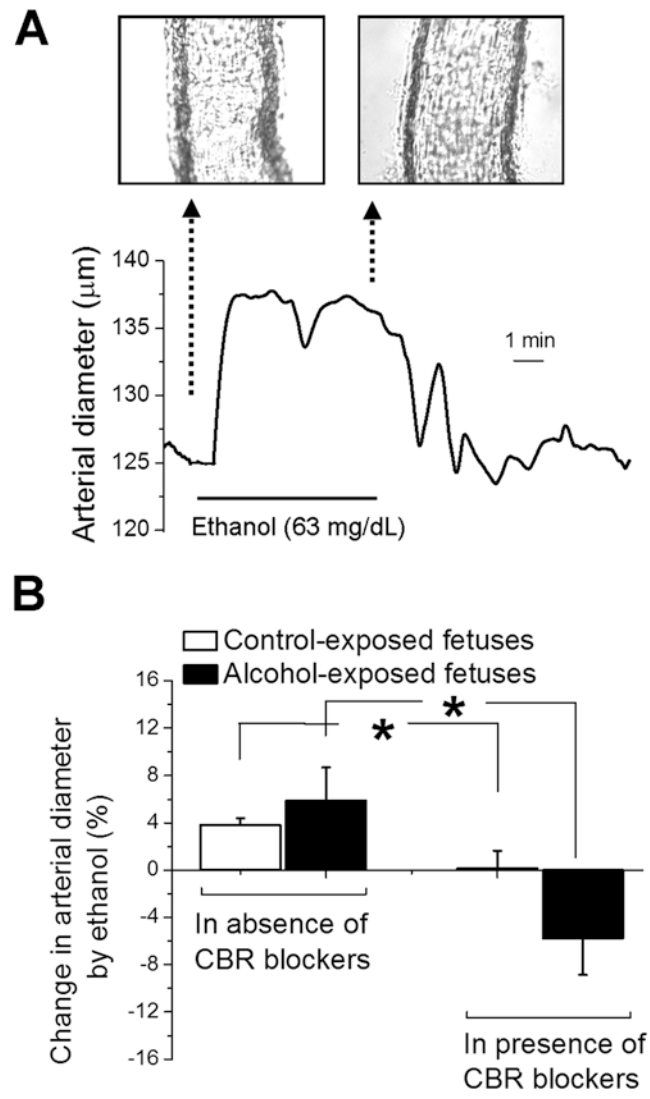

Fig. 2.1 Ethanol-induced dilation of fetal cerebral arteries is mediated by CB receptors. (**a**) Original diameter trace showing fetal cerebral artery dilation in response to 63 mg/dL ethanol. (**b**) Averaged data showing diminished ethanol-induced dilation of fetal cerebral arteries in presence of CB1 and CB2 receptor blockers AM251 and AM630, respectively. ∗Statistically significant difference ($P < 0.05$ by t-test). With modifications from [50]

pregnancy [82], its level increases dramatically as labor approaches [14]. Upon delivery, eCBs continue to play a central role in maternal-fetal interaction, as 2-AG is present in maternal milk [13]. 2-AG in milk exceeds that of AEA by 100–1000-fold and serves as a critical contributor into the initiation of milk suckling [146, 147]. Indeed, injections of CB1 receptor antagonist SR141716 (5–20 mg/kg s.c.) into newborn but not older mouse pups drastically reduces milk ingestion and pup growth [146]. This effect is not specific to the particular antagonist, as it has been replicated by another CB receptor antagonist VCHSR [13]. Interestingly, when CB receptor antagonist-treated mouse pups are introduced to a dish with a milk/cream mixture, they are able to lick and ingest this food [13]. Thus, CB1 receptor block is specifically altering suckling behavior, presumably via alterations in synaptogenesis required for neuromuscular coupling within tongue tissue [13].

Around early postnatal development (postnatal day 5), a peak in rat brain 2-AG level is observed when compared to prenatal and adult 2-AG content [13, 148]. In contrast to the bell-shaped ontogeny of 2-AG, AEA levels in rat brain progressively increase from birth into adulthood [13, 148]. Levels of 2-AG detected in

rat brain are much higher than those of AEA in rat brain: 2000–8000 pmol/g of tissue versus 3–6 pmol/g of tissue, respectively [43, 120]. However, regional patterns of 2-AG and AEA ontogeny do not always follow net levels, this variability being further complicated by a gender-specific component [42].

With regard to CB receptors, rodent and human brain CB1 receptor levels in fetal and juvenile tissues are generally higher than in adulthood [42, 120, 149, 150]. Yet, CB1 receptor distribution also shows region-specific variability with the predominant location within fetal white matter [148, 149], while CB1 expression in adulthood is predominantly located within grey matter [42, 148].

In a study utilizing C57BL/6 mouse strain, it was shown that the amount of CB1 receptor and its co-localization with GABA and glutamatergic synapses in the visual cortex is modulated by developmental plasticity and by visual input [151]. In particular, immunostaining against CB1 receptor reveals differential distribution of this protein across various layers of mouse visual cortex. The highest intensity of anti CB1 receptor staining is detected in layers II/III and VI. Moreover, CB1 receptor co-localization with presynaptic GABA transporter is detected by vesicular GABA transporter (VGAT)-positive staining and with vesicular glutamate transporter (VGluT)-positive staining. The former is attributed to localization of CB1 receptor within nerve terminals of inhibitory neurons, while the latter is associated with excitatory neurotransmission [151]. Dark rearing of mouse pups up to P30 results in the overall decrease of CB1 receptor-associated staining, decrease in co-localization of CB1 receptor with VGluT in deep layer of visual cortex, but produces an increase in co-localization of CB1 receptor with VGAT in this layer [151]. Notably, naturally uneven distribution of immunostaining signal among visual cortex layers remains unaltered by rearing conditions. Moreover, dark rearing until P50 does not modify the overall level of CB1 receptors, suggesting that visual input only exerts a modulatory role in the developmentally-programmed trajectory of CB1 receptor amounts in the deep layer of visual cortex [151]. At postnatal day 100, the overall amount of CB1 receptor in mouse primary visual cortex as detected by Western blotting, is significantly higher than the amount at an earlier postnatal age (P20) [151].

Targeting of the eCB system by administration of five daily AEA injections (20 mg/kg s.c.) to newborn mice from day 6 of age does not result in any effects on open field performance of the progeny until 4 weeks of age [152]. However, from 40 days of age, the offsprings from AEA-treated dams are characterized by a decrease in open field activity, catalepsy, and hypothermia [152]. It is noteworthy that a fully functional eCB system does not seem to emerge until adulthood, as acute challenge of mouse pups with AEA (20 mg/kg i.p.) does not produce anticipated analgesia and motor depression [152]. This outcome could be explained by the fact that the effects of CB challenge require complex interplay between several components of the eCB system that are only reaching their final levels and patterns of distribution in adulthood [42, 43].

THC exposure during the perinatal period (2.5–5 mg/kg per os, from gestational day 15 to postnatal day 9) results in an increased rate of vocalization in 12-day-old rat pups [153, 154]. However, acute treatment of 11–13-day-old rat pup with CB receptor agonist (-)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl) phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol (CP 55,940) shows a dose-dependent decrease in ultrasonic vocalizations, with a 1000-μg/kg CP55,940 causing an almost complete shutdown of vocalized calls [155]. CB receptor antagonist SR 141716A (20 mg/kg) reverses this effect [155]. Thus, there are critical windows of vulnerability to eCB system targeting that enable differential outcomes of eCB challenge on physiology/behavior.

In addition to nearly immediate effects, targeting of eCB system during *in utero* development has long-lasting consequences on developmental trajectories [156–159]. For example, treatment of rats with cannabinoid receptor agonist HU-210 (25 μg/kg throughout gestation and lactation) results in decreased corticosterone levels in adult male progeny [160].

Altered gene expression in progeny born to THC-treated rat dams has been described for 112 brain genes [161], including elevated pre-proenkephalin mRNA expression in the nucleus accumbens and central and medial amygdala [162], as well as modifications of cortical genes related to glutamatergic system and also to noradrenergic transmission [163]. Alterations in gene expression are associated with a decrease in the cortical extracellular levels of both neurotransmitters [163]. Within the serotoninergic system, perinatal THC exposure (5 mg/kg body weight from gestational day 5 through postnatal day 24) leads to a reduced serotonin level in rat brain samples [164].

Prenatal exposure to CB1 receptor agonist WIN55,212-2 (0.5 mg/kg s.c.) disrupts memory retention in 40- and 80-day-old rat progeny [165]. This memory impairment is correlated with alterations of hippocampal long-term potentiation (LTP) and glutamate release. LTP in hippocampal CA3-CA1 synapses decays faster in brain slices of progeny that was prenatally exposed to WIN55,212-2 when compared to a control group [165]. The effect is specific to a particular parameter of LTP, as post-tetanic and short-term potentiation is similar in WIN55,212-2-exposed and control groups [165]. *In vivo* microdialysis studies detect a decrease in basal and potassium-induced glutamate levels in the cerebral cortex of adolescent and adult rats born to WIN55,212-2-treated dams (0.5 mg/kg s.c.) when compared to a vehicle-treated group [166]. This decrease is reported to be associated with a WIN55,212-2-triggered increase in glutamate uptake through overexpression of GLT1 and EAAC1 glutamate transporter subtypes, as demonstrated in rat frontal cerebral cortex [167]. Moreover, while WIN55,212-2 treatment (0.1 mg/kg i.p.) increases dialysate glutamate levels in adult rats, blockade of the CB1 receptors with the selective antagonist SR141716A only ablates the WIN55,212-2-induced increase of glutamate in a control group of rats, but not in rats that were prenatally exposed to WIN55,212-2 [166]. It is noteworthy that, while prenatal stimulation of eCB system does not generally result in severe fetal malformations or exert apparent toxicity, prenatal exposure to WIN55,212-2 is reported to be associated with impaired neuronal growth/neurite branching [168]. In synthesis, *in utero* exposure to exogenous cannabinoids reshapes the eCB system and its communication with major neurotransmission systems [163, 168, 169].

Outside the central nervous system, consequences of exposure to cannabinoids during the perinatal period include long-term alterations in cytochrome P-450 levels [170], enkephalin and norepinephrine sensitivity in vas deferens [171], and neurochemical response to stress [43, 172].

Behavioral consequences of prenatal THC exposure may last into adulthood. Following THC exposure (2 mg/kg twice daily, s.c. gestational days 1-22, postnatal days 2–10), adult (90-day-old) male progeny exhibits decreased time in the inner part of the open field and increased investigation time in the test of social interaction [173]. Moreover, rats that were exposed to THC (0.15 mg/kg, from gestational day 5 to postnatal day 2) exhibit higher heroin-seeking activity at postnatal day 62 [162]. Likewise, daily oral THC administration (5 mg/kg, starting from gestational day 5 throughout postnatal day 24 at weaning) modifies rat brain mu opioid receptor density in a region- and gender-specific manner and facilitates morphine self-administration behavior [162, 174].

Alterations in the eCB system during neonatal and perinatal periods can be achieved not only by exposure to cannabinoids, but also by common stressors such as maternal deprivation. For example, maternal deprivation of neonatal rat pups leads to increased 2-AG content in male hippocampus [175]. Maternal deprivation for 24 h at postnatal day 9 induces a significant increase in DAGLα but not DAGLβ levels upon immunostaining of hippocampi from rat male and female progeny (13-day-old pups) [176]. Maternal deprivation also decreases CB1 receptor expression while CB2 receptor levels are increased [177]. The former phenomenon (decreased CB1 receptor expression and lack of CB1 receptor function) in turn increases progeny's susceptibility to stress [178]. MAGL protein and mRNA levels are decreased in deprived males [176]. A similar paradigm of maternal deprivation (24 h,

postnatal days 9–10) renders increased expression of eCB system component-coding genes in frontal cortex and hippocampus of adolescent (postnatal day 46) male and female rat progeny, respectively [179]. Sexual dimorphisms observed within eCB system distribution and function has been extensively discussed in recent literature [180–184]. Based on studies showing a link between stress and the eCB system, it has been proposed that cannabinoids serve as modulators of the hypothalamic-pituitary-adrenal axis; this modulatory effect may be critical in shaping brain maturation during development [185].

The role of nutrition as another environmental factor that shapes eCB system development has been studied in a model of variable postnatal nutrition in cross-fostering mouse dams [186]. In this study, mouse pups are assigned to new mothers upon birth, and different pup-to-mother ratios are used to regulate nutritional intake (3, 6, or 10 pups per mother). Conceivably, groups with 3 pups per mother show higher growth measures when compared to 6 and 10 pups/mother groups [186]. ECB system components evaluated at postnatal day 50 show progressive decreases in FAAH and MAGL gene expression in liver as pup-to-mother ratio was increased [186]. Visceral adipose tissue does not render significant changes in FAAH gene expression level as a function of early postnatal nutrition, yet MAGL expression level is decreased with increased pup-to-mother ratio. Moreover, expression of NAPE-PLD and DAGLα in visceral adipose tissue is also progressively decreased as pup-to-mother ratio is increased [186]. This study promotes the peripheral eCB system as a sensor of early postnatal nutrition. Conceivably, maternal high-fat diet (≈29% of calories from fat) in a rat model results in profound modification of the eCB system protein levels of progeny [187, 188]. In particular, male offspring of mothers subjected to high-fat diet exhibit significant increase in hypothalamus CB1 receptor protein level, while females show increased CB2 receptor protein level in this brain region when evaluated at birth [188]. In brown fat tissue, a maternal high-fat diet results in increased FAAH level in male and increased CB2 receptor protein level in female progeny, respectively [188]. However, unlike the hypothalamic CB1 receptor, the brown fat tissue CB1 receptor is decreased in male progeny from mothers fed a high-fat diet [188]. These findings reiterate the tissue- and gender-specific nature of the eCB system, showing sensitivity to modulation by exogenous interventions.

In addition to nutrition, alcohol exposure in rodent models emerges as another critical modulator of eCB system function during perinatal/early postnatal development. Indeed, ethyl alcohol treatment of C57BL/6J mice at postnatal day 7 (2.5 g/kg s.c. twice) increases AEA levels [189]. Unlike AEA, 2-AG level remains unchanged due to alcohol-induced up-regulation of both DAGLβ and MAGL activities [190]. Alcohol treatment also results in up-regulated CB1 receptor protein expression in the cortex and hippocampus [189]. Moreover, such alcohol treatment triggers neurodegeneration that is absent in CB1 receptor knock-out mice [189]. These findings reinforce the concept of a cross-talk between the eCB system and the molecular targets of another drug of abuse, alcohol [138, 139].

Data from nonhuman primates are consistent with reports on predominant abundance of 2-AG over AEA: mass spectroscopy analyses of baboon samples from our laboratory show ≈30 times higher 2-AG levels when compared to AEA in the blood circulation of near-term fetuses and their corresponding mothers (Fig. 2.2). Similarly, the relative abundance of 2-AG in baboon cerebral arteries is higher than AEA in both mothers and near-term fetuses (Fig. 2.2). Data from our laboratory also present evidence of dynamic changes in CB1 receptor function within baboon cerebral arteries during development. In particular, application of AM251 (1 μM) to *in vitro*-pressurized branches of middle cerebral arteries, harvested from fetal baboons at the end of second trimester equivalent of human pregnancy, renders artery constriction (Fig. 2.3) [50]. However, identical pharmacological probing results in artery dilation in near-term fetal and their maternal cerebral artery segments (Fig. 2.3).

With regards to other nutritional interventions, maternal high-fat diet (12% fat) during preg-

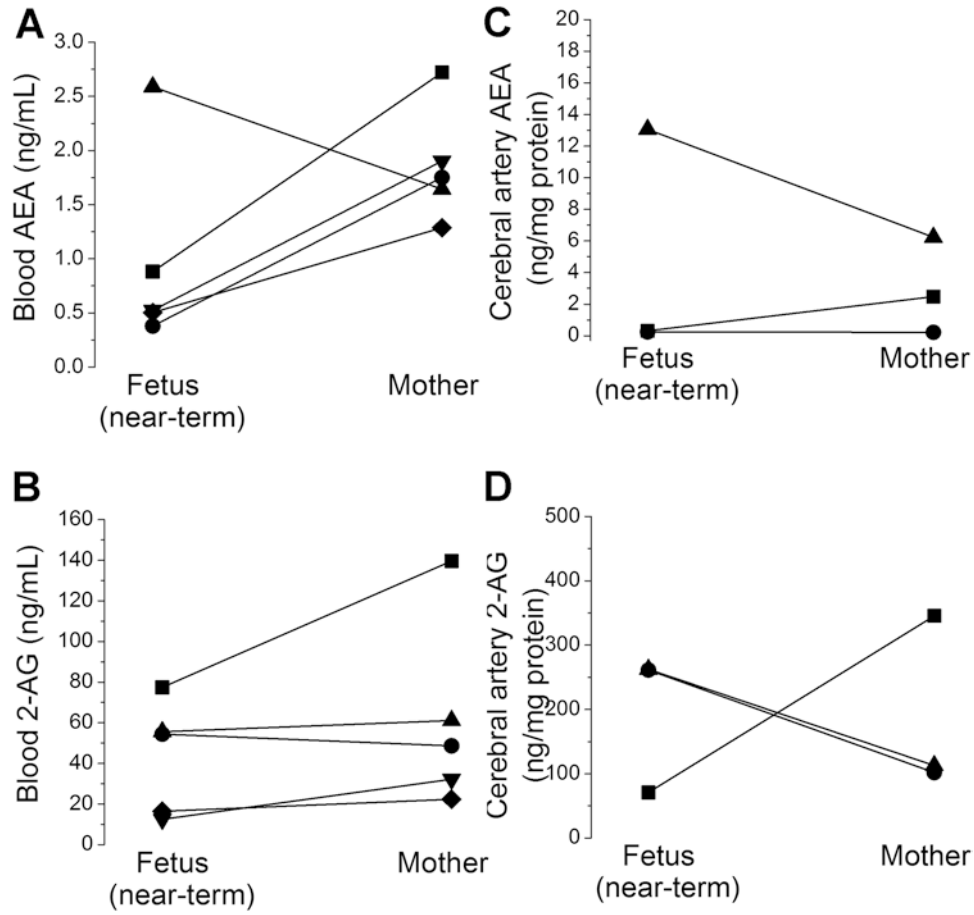

Fig. 2.2 Blood and tissue eCB levels in baboon (*Papio spp.*). (**a**) AEA levels in circulating blood of near-term fetal baboons and their corresponding mothers. Here and in B-D, data from a given fetus-mother pair are connected by a solid line. Different symbols correspond to data-points from separate fetus-mother pairs. (**b**) Circulating 2-AG levels in near-term fetal baboons and their corresponding mothers. (**c**) AEA levels in cerebral artery lysates of near-term fetal baboons and their corresponding mothers. Here and in D, the eCB reading within each sample was normalized to protein amount. (**d**) 2-AG levels in cerebral artery lysates of near-term fetal baboons and their corresponding mothers

nancy leads to decreased fetal baboon circulating 2-AG levels near–term, independent of fetal gender [191]. Interestingly, maternal baboon circulating 2-AG levels are increased by high-fat diet. In addition to modification of fetal circulating 2-AG level, fetal hepatic CB2 receptor, FAAH, and COX-2 expression values are lower in fetuses of both genders from the high-fat group. Within this group, DAGLα expression is selectively decreased in male fetuses [191].

In humans, a qPCR study on post-mortem samples from the middle frontal gyrus area of the donors between 39 days and 49 years of age unveils complex developmental trajectories for critical players within the eCB system [192]. In particular, expression of the CB1 receptor is progressively decreasing from infancy into adulthood with a slight local peak at toddler age. A similar profile is followed by MAGL [192]. In sharp contrast, NAPE-PLD, FAAH, and ABHD6 are progressively increased from infancy into

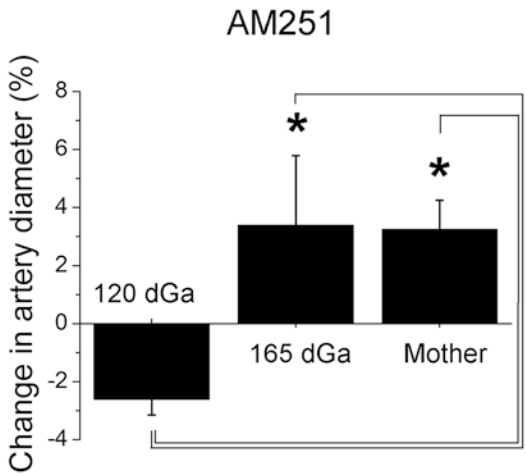

Fig. 2.3 Averaged Changes in cerebral artery diameter were assessed by probing of *in vitro* pressurized branches of fetal and maternal middle cerebral arteries harvested from baboons (*Papio spp.*). Effect of AM251 (1 μM) is presented as a percent change in artery diameter from pre-AM251 level. ∗Statistically significant difference ($P < 0.05$ by one-way ANOVA with Tukey post-test). dGa: days of gestational age

adulthood. Finally, DAGLα mRNA shows a bell-shaped pattern with the peak around school age [192].

Consequences of *in utero* cannabis exposure on developmental trajectories in humans have been studied in several longitudinal cohort studies, and are found to be long-lasting. Several early reports describe increased tremors, startle response, and poor visual responsiveness of cannabis-exposed infants [193]. These characteristics are apparent in the absence of effect on morphometric (growth) parameters, such as weight and head circumference [194]. Notably, characteristics of infants born to cannabis users disappear by 30 days of age [193], demonstrating a remarkable plasticity that allows the development of compensatory measures in response to cannabis-driven alterations in physiological processes. Later in childhood, however, prenatal cannabis exposure negatively reflects on attention processes [195] and cognitive performance within executive function [154, 196]. *In utero* CB exposure leads to more aggressive behavior and attention problems in 18-month-old girls [17]. Moreover, maternal light-to-moderate marijuana use during pregnancy is associated with deficits in Wide Range Achievement Test-Revised reading and spelling scores and a lower rating on the teachers' evaluations of the children's performance in 10-year-olds [197]. Functional magnetic resonance imaging on eighteen-to-twenty two-year-old adults that were prenatally exposed to cannabis demonstrates alteration in visuospatial working memory processing [198]. Increased impulsive behavior has also been reported as a consequence of prenatal cannabis exposure [133, 199]. Yet, maternal marijuana use does not affect growth parameters of the progeny in puberty [200].

2.7 Adolescence

Although exact age limits of the adolescent period are poorly defined, adolescence usually refers to as a period of pubertal maturation [201]. Adolescence is a period of active brain development, representing the transition between childhood and adulthood [201, 202]. It is also often characterized by cannabis use [202, 203]. Considering that eCB system controls several fundamental processes of neuronal and glial development, such as cell proliferation, migration, and differentiation [1, 204, 205], alterations in the eCB system during adolescence are expected to impact neuronal maturation.

Studies in rat species describe a peak of CB1 receptor expression in prefrontal cortex, limbic, striatal, and midbrain areas during adolescence (postnatal days 25–29 in rats), this peak later declines to adult levels [185, 206, 207]. Consistent with this peak in CB1 receptor expression, studies in rodent models show that the adolescent brain is particularly vulnerable to CB stimulation when compared to adults. Rats that were exposed to THC (1.5 mg/kg i.p. every third day) at postnatal days 28 (early adolescence) to 49 (late adolescence) show profound alterations in the endocannabinoid levels in prefrontal cortex and nucleus accumbens regions [208]. Adolescent rats that were repeatedly exposed to THC (5 mg/kg i.p. starting from postnatal day 28) show less vocalization during the THC administration pro-

cedure when compared to adults (starting from postnatal day 60) [209]. This result suggests that THC is less aversive to adolescent rats. Also, after THC withdrawal, THC-exposed adolescent rats exhibit impaired object recognition memory. Proteomics analysis of hippocampal samples detects significant changes in 27 proteins following THC exposure in adolescence, compared to only 10 proteins in adults. The former are represented by oxidative stress/mitochondrial and cytoskeletal targets [209]. This finding confirms the greater vulnerability of the adolescent brain to cannabis exposure compared to the adult brain. Similarly, synthetic cannabinoid agonist WIN 55,212-2 (1.2 mg/kg i.p.) treatment of pubertal rats results in poorer recognition memory when compared to identical treatment of adult rats [210]. Also, working memory impairment and a significant decrease in social interaction is reported in female rats in response to CB receptor agonist CP 55,940 administered daily for 21 days at 150, 200, and 300 μg/kg i.p. for 3, 8 and 10 days, respectively [211]. In addition, in a study on rats using eCB system stimulation with CP 55,940, it is concluded that chronic CB exposure leads to long-term memory impairments and increased anxiety, irrespective of the age at which drug exposure occurs (either at the perinatal period, adolescence, or young adulthood) [212].

In a different experimental paradigm, adolescent male rats were administered AEA hydrolysis inhibitor URB597 (0, 0.1, or 0.5 mg/kg/day at postnatal days 38–43) [213]. Following this treatment, a decrease in CB1 receptor is detected in caudate-putamen, nucleus accumbens, ventral tegmental area, and hippocampus, while an opposite effect is observed in the locus coeruleus [213]. Similar treatment with FAAH inhibitor URB597 (0.3 mg/kg i.p.) reverts depressive-like symptoms induced by adolescent exposure to THC in female rats [214]. Moreover, MAGL inhibitor JZL 184 ameliorates a deficit in presynaptic long-term plasticity triggered by exposure of adolescent mice to WIN 55,212-2 [215]. These findings demonstrate the possibility of persistent attenuation in AEA and 2-AG levels as an underlying cause of neuronal deficits associated with adolescent THC exposure.

In addition to alterations within the eCB system, adolescent exposure to CB stimulation interferes with sensitivity to other drugs of abuse. For example, exposure of adolescent rats to THC (1.5 mg/kg i.p., every third day during postnatal days 28–49) results in increased sensitivity to opiates and heroin self-administration in adulthood (postnatal days 57 and 102) [216]. Mu opioid receptor GTP-coupling is potentiated in mesolimbic and nigrostriatal brainstem regions in THC-exposed animals, with mu opioid receptor function in the nucleus accumbens shell being specifically correlated with heroin intake [216]. Thus, the consequences of eCB alteration during adolescence are likely region-specific.

There is also a gender-specific component in responses of the adolescent brain to eCB stimulation. THC administration to rats twice a day (2.5 mg/kg at postnatal days 35–37, 5 mg/kg at postnatal days 38–41, and 10 mg/kg at postnatal days 42–45, i.p) results in significant decreases of CB1 receptor level and CB1/G-protein coupling in the amygdala, ventral tegmental area, and nucleus accumbens in females [217]. However, males only exhibit these alterations in the amygdala and hippocampus. Additional neuronal consequences of adolescence THC exposure include dendritic atrophy and decreases in markers of neuroplasticity [218, 219]. At the behavioral level, females present behavioral despair in a forced swim test, which is accompanied by anhedonia in a sucrose preference test [217]. Males only present anhedonia [217].

Ontogeny of CB1 receptor expression in humans somewhat differs from that of rats. In particular, a gradual increase in CB1 expression in the human brain towards adulthood is reported [149]. The function of these receptors is successfully assessed by [^{35}S]GTPγS autoradiography. Moreover, high levels of CB receptors are detected during prenatal development in fiber-enriched areas, these areas being devoid of CB receptor signal in adulthood [149]. Several other reports on human dorsolateral prefrontal cortex samples also fail to detect a rodent-characteristic peak in brain CB1 receptor level in adolescence [192, 220]. While species-specific expression pattern of the CB1 receptor and, perhaps, eCB

system function, should be considered as a primary cause for such discrepancy, it has been proposed that such inconsistency between reports might reflect overall instability of the developing eCB system [42].

Computer-assisted attention testing that addresses visual scanning, alertness, divided attention, flexibility, and working memory in humans detects a significant impairment in visual scanning reaction times in early-onset (before age 16) cannabis users but not in late-onset (after age 16) users [221]. This outcome suggests that the brain in early adolescence is particularly vulnerable to alterations in the eCB system upon exposure to exogenous cannabis. Adolescent cannabis use has also been suggested to exert a modulatory effect over anxiety-related behaviors and depression [154]. In the latter case, a link between adolescent cannabinoid exposure and serotonergic hypoactivity has been proposed [222].

2.8 Concluding Remarks

Ample data from invertebrate and vertebrate species, including humans, document the complex roles of the eCB system in development. Gender, timing, and pharmacological routes of eCB challenge are all-important in establishing the final trajectory of eCB ontogenesis and its role in physiology and pathology.

Continuous growth in proposed CB-based pharmacological remedies and increasing THC content in recreational cannabis preparations [223, 224] call for concerns over incomplete understanding of eCB function. Despite the fact that the eCB system represents an attractive therapeutics target for various conditions that represent developmental pathology, the major difficulty in developing eCB-targeting pharmacotherapy arises from the complexity of the eCB system. We are far from finalizing complete characterization of all eCB components, therefore the process of fully characterizing the eCB system continues.

Acknowledgements The author is thankful to Dr. Dejian Ma and Dr. Wei Li (Dept. Pharmaceutical Sciences, University of Tennessee Health Science Center) for mass spectroscopy quantifications of cannabinoid levels in baboon blood and tissue samples. The author also extends gratitude to Dr. Syed Ali (US Food and Drug Administration) for critical reading of the manuscript and Dr. Richard Redfearn (Office of Scientific Writing, Office of Research, University of Tennessee Health Science Center) for editorial assistance. This work was supported by the National Institutes of Health grant number R21 AA022433 [ANB].

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3 Cannabinoid Interactions with Proteins: Insights from Structural Studies

Anna N. Bukiya and Alex M. Dopico

Abstract

Cannabinoids have been widely used for recreational and medicinal purposes. The increasing legalization of cannabinoid use and the growing success in Medicinal Chemistry of cannabinoids have fueled recent interest in cannabinoid-sensing sites in receptor proteins. Here, we review structural data from high-resolution cryo-EM and crystallography studies that depict phytocannabinoid, endocannabinoid, and synthetic cannabinoid molecules bound to various proteins. The latter include antigen-binding fragment (Fab), cellular retinol binding protein 2 (CRBP2), fatty acid-binding protein 5 (FABP5), peroxisome proliferator-activated receptor γ (PPAR γ), and cannabinoid receptor types 1 and 2 (CB1 and CB2). Cannabinoid-protein complexes reveal the complex design of cannabinoid binding sites that are usually presented by conventional ligand-binding pockets on respective proteins. However, subtle differences in cannabinoid interaction with amino acids within the binding pocket often result in diverse consequences for protein function. The rapid increase in available structural data on cannabinoid-protein interactions will ultimately direct drug design efforts toward rendering highly potent cannabinoid-related pharmacotherapies that are devoid of side effects.

Keywords

Lipid-protein interactions · Tetrahydrocannabinol · Anandamide · 2-arachidonoylglycerol · Cannabinoid receptor agonist · Cannabinoid receptor antagonist · G protein-coupled receptor

Abbreviations

2-AG	2-arachidonoylglycerol
AEA	anandamide
CB1	cannabinoid receptor type 1
CB2	cannabinoid receptor type 2
CRBP2	cellular retinol binding protein 2
cryo-EM	cryogenic electron microscopy
ECL	extracellular loop
FABP	fatty acid-binding protein
GPCR	G protein-coupled receptor
ICL	intracellular loop
NMR	nuclear magnetic resonance
PDB	protein data bank
PPAR	peroxisome proliferator-activated receptor
THC	delta9-tetrahydrocannabinol
TM	transmembrane

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A. N. Bukiya (ed.), *Recent Advances in Cannabinoid Physiology and Pathology*, Advances in Experimental Medicine and Biology 1162, https://doi.org/10.1007/978-3-030-21737-2_3

3.1 Introduction

Cannabinoids and related compounds constitute a family of structurally diverse chemicals. Currently, all family members can be grouped into three major subclasses: phytocannabinoids, which are extracted from plants; endogenous cannabinoids (endocannabinoids), which are produced by other live organisms, and synthetic cannabinoids that possess some or all the physiological properties of the natural phyto- and/or endocannabinoids. The chemical diversity within each group (Fig. 3.1) is continuously growing, prompting newer classifications of cannabinoids based on their chemical identity [1]. Such growth can be partially attributed to the better resolution of modern detection techniques. Other contributors, however, are the "black market" demand for illicit compounds with psychoactive properties and the increasing development of cannabinoid preparations for therapeutic use. This development, in turn, is largely driven by the fact that while some cannabinoids, such as the naturally-occurring phytocannabinoid delta9-tetrahydrocannabinol (THC) are well-tolerated, many others (e.g., THC's synthetic counterpart, MDMB-Fubinaca) have a very slim safety margin and pose substantial risk of overdose. The growing number of newly developed synthetic cannabimimetics of high potency further adds to the safety concerns and legal complexities of regulating the market for cannabinoid-related compounds.

The diverse chemical and pharmacological profile of cannabinoid-related compounds calls for studies on the molecular features and physicochemical forces that enable functional interactions of cannabinoids and related compounds with their protein targets. In this regard, substantial advances have been made in the technological approaches utilized to accurately study and thus predict affinities of the cannabinoid family members to cannabinoid-sensing proteins. Such advances include exhaustive structure-activity relationship studies on cannabinoid binding to canonical cannabinoid receptors of type 1 (CB1) and type 2 (CB2) [reviewed by 2–3], the development of robust, low-cost *in silico* prediction tools for cannabinoid affinity to CB1 receptors based on a quantitative structure-activity relationship model [4], and the ligand-assisted protein structure (LAPS) paradigm for characterizing cannabimimetic-binding domains [5]. The task of studying cannabinoid interactions with protein targets, however, is becoming increasingly difficult as novel protein targets, not always structurally-related, of cannabinoid action are continuing to unveil. Indeed, in addition to their biological effects driven from interactions with canonical CB1 and CB2 receptors, cannabinoids exert physiological effects via transient receptor potential (TRP) channels [6] and G protein-coupled orphan receptors (such as GPR55) [7, 8], among many others [reviewed by 8–9]. Thus, it is not surprising that in recent years, a growing number of studies have focused on elucidating cannabinoid-protein complexes at high resolution with proteins other than canonical cannabinoid receptors.

In this chapter, we describe the different structures of cannabinoid ligands in complex with their respective receptor proteins available in the Protein Data Bank (PDB; https://www.rcsb.org, retrieved on February 1, 2019). The PDB was searched using the following terms: cannabinoid, anandamide (AEA), tetrahydrocannabinol, 2-arachidonoylglycerol (2-AG), tetrahydrocannabinolic acid, cannabidiolic acid, cannabidiol, cannabinol, cannabigerol, cannabichromene, tetrahydrocannabivarin, and cannabidivarin. A summary of the search results is presented in Table 3.1. Below, we discuss the search results in an attempt to navigate the molecular maps that describe cannabinoid binding to cannabinoid-sensing proteins.

3.2 High-Resolution Structures of Cannabinoid-Protein Complexes

3.2.1 Phytocannabinoids

The *Cannabis* plant consists of over 400 chemical compounds, of which THC is the principal psychoactive substance, and more than 60 are phyto-

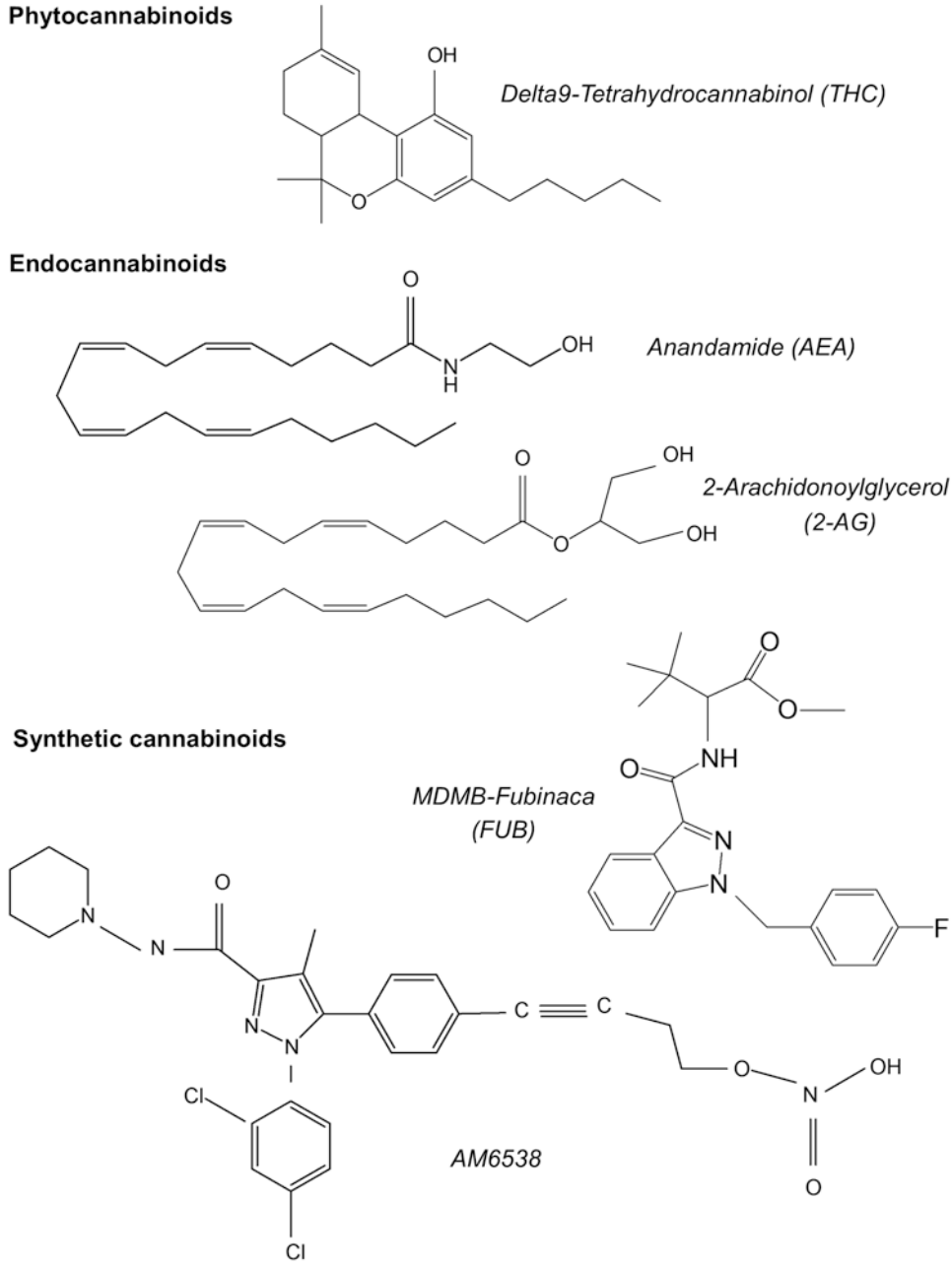

Fig. 3.1 Chemical diversity within cannabinoid family of ligands. Examples of phyto-, endo-, and synthetic cannabinoid chemical structures are presented

cannabinoids with a diverse functional profile [10]. The crystal structure of THC in complex with a protein emerged early on [11]. In this work, the structures of free versus THC-bound antigen-binding (Fab) protein fragment from mouse spleen cells were examined. The crystallized Fab structure follows the immunoglobulin architecture with the antigen-binding domain being located in the complementarity-determining region. The bound THC is detected in a narrow cavity between the variable domains of the light (*L*) and heavy (*H*) chains (Fig. 3.2) [11]. The authors noted a lack of

Table 3.1 Summary of cannabinoid-protein complexes

Ligand	Protein	PDB ID	Resolution	Method	Pocket	References
Phytocannabinoids						
THC	Murine Fab	3LS4	2.00 Å	X-ray crystallography	Q89*L*, I94*L*, L96 *L*; V33*H*, V35*H*, L37*H*, W47*H*, S50*H*, Y58*H*, T96*H*, V99*H*, A100*H*, W103*H*	[11]
Endocannabinoids						
AEA	Murine FABP5	4AZP	2.10 Å	X-ray crystallography	Y22, L26, L32, A36, P41, V60, A78, V118, R129, Y131	[18]
AEA	Human FABP5	4AZR	2.95 Å	X-ray crystallography	F19, Y22, M23, M35, P41, C43, R109, C120, Y131	[18]
2-AG	Murine FABP5	4AZQ	2.00 Å	X-ray crystallography	F19, L32, C43, T56, A78, R109, R129, Y131	[18]
2-AG	Human CRBP2	6BTH	1.35 Å	X-ray crystallography	F16, Y19, M20, L23, I25, T29, A33, L36, Q38, K40, T51, T53, S55, F57, R58, N59, Y60, V62, F64, E72, L77, Q108, W106, L117, L119	[20]
Synthetic cannabinoids						
FUB	Human CB1-Gi complex	6N4B	3.00 Å	Cryo-EM	F170, S173, F174, F177, H178, L193, T197, F200, F268, Y275, L276, W279, W356, L359, M363, S383	[41]
Ajulemic acid	Human PPARγ	2OM9	2.80 Å	X-ray crystallography	F264, K265, H266, I281, F282, G284, C285, V339, I341, S342, M348, L353, L365	[26]
AM841	Human CB1	5XR8	2.95 Å	X-ray crystallography	F108, F170, S173, F174, F177, H178, F189, K192, L193, V196, T197, F200, I267, F268, P269, I271, Y275, L276, W279, W356, L359, M363, F379, A380, S383, C386	[45]
AM11642	Human CB1	5XRA	2.80 Å	X-ray crystallography	F108, F170, S173, F174, F177, H178, F189, K192, L193, V196, T197, F200, I267, F268, P269, I271, Y275, L276, W279, W356, L359, M363, F379, A380, S383, C386	[45]
AM6538	Human CB1	5TGZ	2.80 Å	X-ray crystallography	F102, M103, I105, I119, S123, G166, S167, F170, F174, L193, V196, T197, F268, W356, L359, M363, F379, A380, S383, M384, C386, L387	[35]
Taranabant	Human CB1	5 U09	2.60 Å	X-ray crystallography	F102, M103, D104, I105, F108, I119, S123, I169, F170, F174, L183, V196, F268, W279, W356, L359, M363, F379, A380, S383, M384, L387	[36]
AM10257	Human CB2	5ZTY	2.80 Å	X-ray crystallography	F87, F91, F94, H95, L107, V113, T114, F117, S165, F183, I186, W194, W258	[47]

2-AG 2-arachidonoylglycerol, *AEA* anandamide, *CB1* cannabinoid receptor type 1, *CB2* cannabinoid receptor type 2, *CRBP2* cellular retinoid binding protein 2, *cryo-EM* cryogenic electron microscopy, *Fab* antigen-binding (fragment), *FABP* fatty acid-binding protein, *FUB* MDMB-Fubinaca, *H* heavy chain location, *L* light chain location, *PDB* protein data bank, *THC* delta9-tetrahydrocannabinol

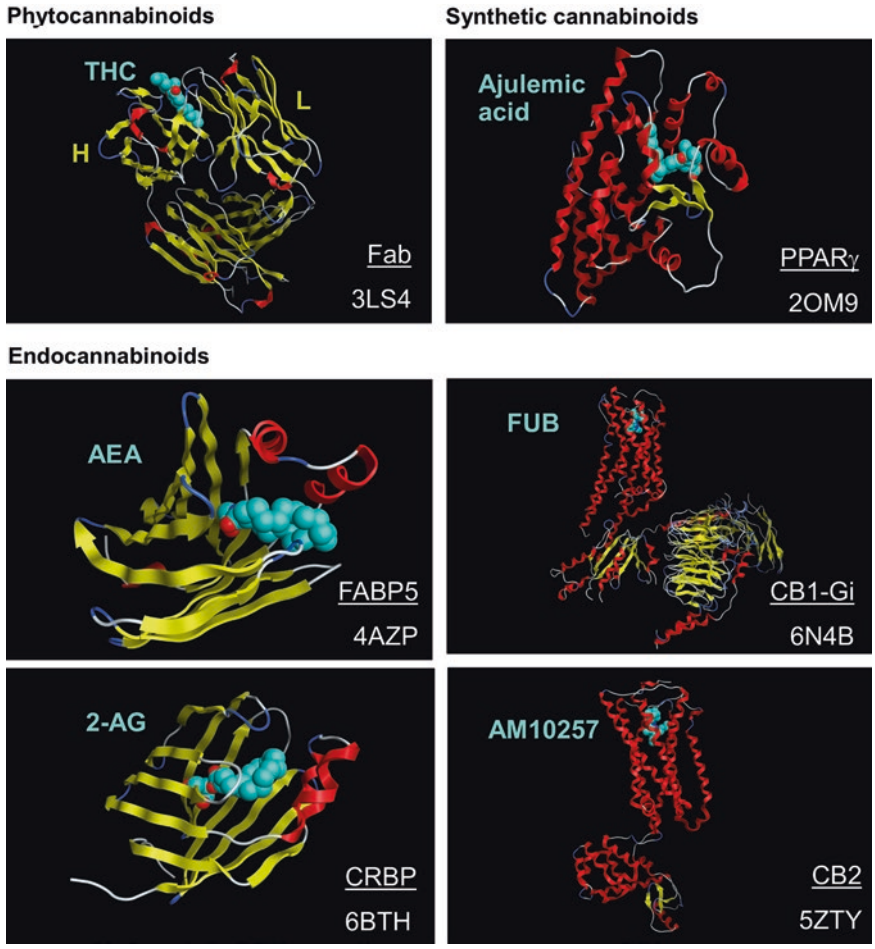

Fig. 3.2 Cannabinoid binding to various proteins depicted by high-resolution structural studies. *2-AG* 2-arachidonoylglycerol, *AEA* anandamide, *CB1* cannabinoid receptor type 1, *CB2* cannabinoid receptor type 2, *CRBP2* cellular retinol binding protein 2, *FABP* fatty acid-binding protein, *FUB* MDMB-Fubinaca, *PPAR* peroxisome proliferator-activated receptor, *THC* delta9-tetrahydrocannabinol. Protein data bank accession codes are listed at bottom corner of each panel

global structural rearrangements upon THC binding to the Fab protein. Yet, the ligand-recognizing cavity is larger in the THC-bound structure when compared to ligand-free Fab. This finding supports the notion that THC-recognizing protein domains are malleable to some extent. This flexibility in the binding pocket may facilitate shape complementarity between cannabinoids and their respective sensing proteins. This important property of cannabinoid-binding protein sites is further supported by structural data from a variety of other proteins (see below).

With regards to Fab, two oxygen atoms within the THC structure form hydrogen bonds with S50*H* and T96*H* (*"H"* referring to heavy chain as opposed to light one) while the remaining amino acids within the binding cavity are mostly presented by aliphatic residues (Table 3.1). When compared with THC, its hydroxyl and carboxyl metabolites form additional hydrogen bonds with S52*H* and R53*H*, likely rendering an improved binding to the Fab fragment.

3.2.2 Endocannabinoids

AEA and 2-AG are major endocannabinoids with largely similar chemical structures (Fig. 3.1), yet

quite different functional activities [12]. Crystallographic studies on AEA and 2-AG complexes with proteins have been focused on the binding of these endocannabinoids to transporting proteins.

The fatty acid-binding protein (FABP) belongs to a group of small intracellular proteins that recognize a variety of lipid ligands, such as fatty acids, their amides, xenobiotics and exogenously introduced cannabinoids [13–16]. FABPs are capable of transporting endocannabinoids to cellular domains where cannabinoid hydrolysis occurs. Therefore, FABP inhibitors increase endocannabinoid levels [17]. Binding of AEA and 2-AG to FABP5, the epidermal isoform of FABS, has been reported in a crystallographic study [18]. Consistent with the overall structure of FABPs, FABP5 is basically a ten-stranded beta-barrel with a three-dimensional architecture that resembles a clamshell [13]. The barrel consists of two orthogonal beta-sheets [18]; while one side of the beta-sheet is capped by a helix-loop-helix structure, the other is capped with an amino-terminal peptide. Thus, lipid ligand binding occurs inside the barrel, with the large binding pocket being able to accommodate lengthy hydrocarbon chains [19]. Upon AEA binding (Kd $\approx$ 1.3 μM), the murine FABP5 overall conformation does not undergo apparent changes (Fig. 3.2). AEA resides in a conventional substrate binding pocket within FABP5, forming a number of hydrophobic interactions with respective amino acids (Table 3.1). In addition, the AEA hydroxyl moiety engages into a hydrogen bonding with Y131, and also forms a water-mediated hydrogen bond with R129 [18].

The chemical and three-dimensional structures of AEA closely resemble the features of 2-AG. Thus, 2-AG binding to murine FABP5 is nearly identical to AEA. However, while 2-AG resides within the same conventional lipid binding region, the 2-AG molecule fits deeper inside the binding pocket [18]. As a result, in addition to the AEA-characteristic hydrogen bonding with R129 and Y131, both hydroxyl groups of 2-AG form three additional hydrogen bonds with the FABP5 amino acids C43, T56, and R109.

In contrast to its mouse counterpart, human FABP5 exists in two forms: monomeric and dimeric. The latter is achieved by a domain swapping between two FABP molecules. Such domain swapping allows deeper entry of AEA into the binding pocket. Thus, in human FABP5 AEA binding resembles that of 2-AG on murine isoform. Human FABP5 interacts with AEA via hydrogen bonding with R109, as the AEA oxygen tip is no longer able to hydrogen-bond with Y131. Overall, however, the interaction map of AEA with the human FABP5 binding pocket remains similar to AEA binding to the murine isoform (Table 3.1).

The ability of transporter proteins to fit into conventional lipid-carrying cavities is not a unique feature of endocannabinoid interactions with FABP5. Indeed, a similar fitting of the endocannabinoid molecule into the lipid-transporting cavity has been reported for 2-AG bound to cellular retinoid binding protein 2 (CRBP2) (Fig. 3.2) [20]. CRBP2 belongs to a group of carrier proteins for members of the vitamin A (retinol) family [21, 22]. 2-AG occupies a central cavity of CRBP2 with hydrogen bonds formed between 2-AG and K40, T51, and Q108. The remaining interior surface of the binding pocket is lined with both aliphatic and polar amino acids (Table 3.1).

3.2.3 Synthetic Cannabinoids

Synthetic cannabinoids constitute the most diverse groups of cannabinoids in regard to chemical structure and functional profile (Fig. 3.1) [23–25]. Ajulemic acid (CT-3, or IP-751, or 1′,1′-dimethylheptyl-delta^{8}-tetrahydrocannabinol-11-oic acid) is a synthetic THC analog [26] known to exert physiological THC-like action via the peroxisome proliferator-activated receptor PPARγ, and thus seems to be free of psychotropic effects [27–29].

PPARs are members of the nuclear receptor family of ligand-activated transcription factors. The PPARγ isotype is involved in a range of physiological processes, including fat cell differentiation, glucose and lipid homeostasis, aging,

and inflammation [30–32]. The PPARγ ligand-binding cavity is provided by a large (≈1300 Å^3) hydrophobic region [33, 34]. Approximately a third of the T-shaped ligand-binding cavity in the PPARγ protein is occupied by ajulemic acid (Fig. 3.2) [26]. The amino acid partners supporting binding of ajulemic acid are mostly aliphatic, with the exception of hydrogen bond-forming amino acids K265, H266, and S342 (Table 3.1). Notably, in PPARα, F264 is substituted by an alanine, eliminating the hydrophobic interaction with ajulemic acid that occurs with the PPARγ isotype. Moreover, either substitution of the phenylalanine with the larger tryptophane or substitution of G284 with polar cysteine or arginine hinders the entrance of ajulemic acid into the ligand-binding cavity.

Several structural studies on synthetic cannabinoids have been performed on the principal cannabinoid receptor CB1. CB1 belongs to the superfamily of G protein-coupled receptors. CB1 contains seven transmembrane (1–7) helixes connected by three extracellular loops (ECL1-3) and three intracellular ones (ICL1-3) [35, 36]. Of note, this receptor possesses rather peculiar pharmacological properties, exhibiting constitutive activity in the absence of an agonist [9]. Moreover, there are allosteric points of modulation in addition to the orthosteric binding site for cannabinoids; the latter is located within the transmembrane (TM) helix bundle [5, 37–40].

3.2.3.1 CB1 Receptor Agonists

A recent study revealed details of CB1 receptor interaction with methyl N-{1-[(4-fluorophenyl) methyl]-1H-indazole-3-carbonyl}-3-methyl-L-valinate (MDMB-Fubinaca, or FUB) (Fig. 3.1), a full agonist, illicit cannabinoid [41]. FUB is representative of fubinacas, a class of synthetic constituents of "Spice" drugs that confer a "zombielike" behavior to its users [42]. FUB is classified as an ultrapotent synthetic agonist of the CB1 receptor with a binding constant Ki of 98.5 pM [43]. In a cryogenic electron microscopy (cryo-EM) map, FUB resides in an orthosteric binding pocket comprised of amino acids located in transmembrane (TM) helixes 2, 3, 5 and 7 of the human CB1 receptor (Fig. 3.2). This site overlaps with the binding site for agonist AM-11542 [41] and somewhat overlaps with the site for antagonist AM-6538 binding [35]. The unusually high potency of FUB has been proposed to emerge from the strong aromatic interactions between FUB indazole ring and CB1 receptor amino acid residues F200$^{3.36}$ and W356$^{6.48}$ (Fig. 3.3 and Table 3.1) [41] with the superscripts indicating the generic numbering of GPCRs according to the Ballesteros-Weinstein Scheme [44]. Strong aromatic interactions are not the sole contributor to the unprecedented potency of FUB; rigidity of the chemical structure has been proposed to play an additional role [41]. Indeed, when compared to the full agonist FUB, the CB1 receptor partial agonist THC has wider conformational diversity, which likely dampens THC ability to interact with the CB1 receptor with high affinity [41].

The X-ray crystallographic study of the CB1 receptor in complex with AM841 and AM11542 provided further details on agonist-CB1 receptor interactions within the orthosteric site. Both AM841 and AM11542 are full agonists of CB1, as determined from their ability to inhibit forskolin-induced accumulation of cyclic AMP [45]. Both ligands bind to CB1 in a similar manner, that is, triggering large structural rearrangements of the receptor, thus rendering a large contact area between the CB1 receptor and G protein. The interactions between the agonists under study and the CB1 receptor protein mostly involve hydrophobic and aromatic amino acids (Table 3.1).

3.2.3.2 CB1 Receptor Antagonists

Insights into the binding of CB1 receptor antagonists emerged from a study that used the cannabinoid receptor antagonist AM6538 to stabilize CB1 during crystallization [35]. AM6538 exhibits functional antagonism over the CB1 receptor activators THC and CP55,940 [35]. The reported affinity of AM6538 for the CB1 receptor is high, with a $K_i \approx 5.1$ nM [35]. Molecular map of AM6538 binding to CB1 receptor includes a set of hydrophobic interactions (Table 3.1) within the orthosteric binding area with all CB1 receptor helices with the exception of helix 4. Notably, the

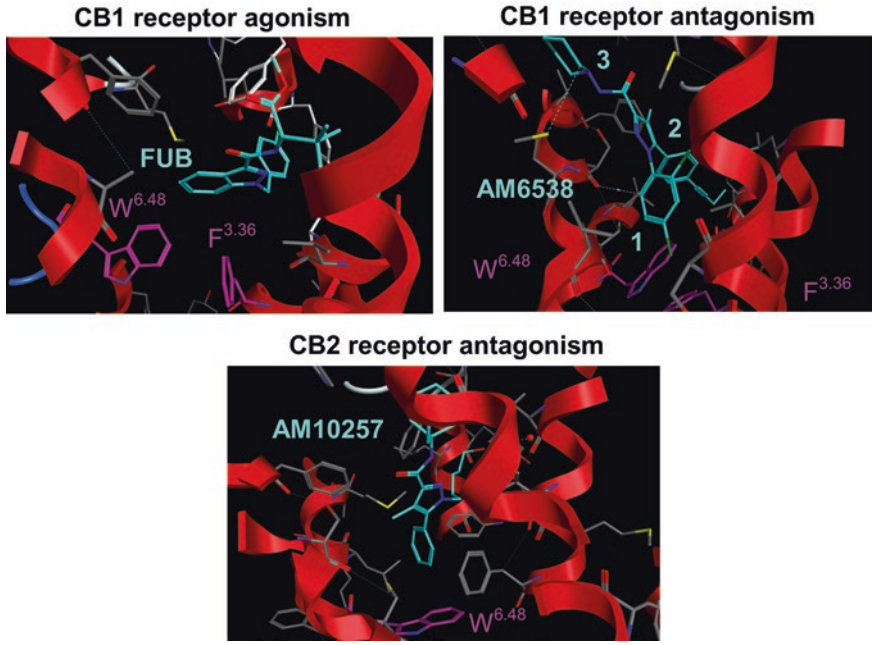

Fig. 3.3 Nuances of cannabinoid interactions with the orthosteric binding sites in CB1 and CB2 receptors result in functional agonism or antagonism. Binding site-forming amino acids are depicted in grey, with $W^{6.48}$ and $F^{3.36}$ highlighted in pink. CB1 receptor agonism and antagonism, and CB2 receptor antagonism are depicted in the crystal structure of FUB with the CB1 receptor (PDB ID 6N4B) [41], the structure of AM6538 with CB1 receptor (PDB ID 5TGZ) [35], and the structure of AM10257 with CB2 receptor (PDB ID 5ZTY) [47]. In the AM6538 structure, the chemical "Arms" are sequentially numbered from 1 to 3 [35]

N-terminal region of the receptor forms a plug that covers the binding area. The binding pocket itself has a rather complex shape: the "Gap", "Side pocket" and "Long channel" regions of the receptor are accommodating three "arms" of the AM6538 molecule that are provided by distinct chemical moieties (Fig. 3.3). Overall, it appears that the AM6538 antagonist literally plugs several intra-protein cavities, hindering receptor activation. Indeed, in the AM6538-bound state, $F200^{3.36}$ points away from the ligand binding pocket and forms an aromatic stacking interaction with $W356^{6.48}$, likely stabilizing the CB1 protein in inactive state [45]. This stacking interaction does not seem to accompany the binding of the full agonists AM11542 and AM841. Moreover, when compared to agonists, antagonist binding is unable to provide inward motions of $F170^{2.57}$ and $F174^{2.61}$, which results in reduced volume (384 $Å^3$) of the cannabinoid agonist-binding cavity when compared to that of antagonists (822 $Å^3$) [45].

In a separate study, the CB1 receptor inverse agonist taranabant (K_i = 0.13 nM) was found to confer thermostability to the CB1 protein and was co-crystallized as a ligand [36]. Taranabant-CB1 receptor complex represents the CB1 receptor in an inactive conformation, with an ionic lock formed between $R412^{3.50}$ and $D338^{6.30}$ [36]. Taranabant resides in an orthosteric binding site shielded from solvent by the N-terminus of the CB1 protein. Conceivable with the position within the CB1 receptor TM area, the cannabinoid orthosteric binding site is lined by hydrophobic amino acids. Only a few amino acids that form interactions with taranabant are polar (Table 3.1). Similar to the antagonist AM6538, taranabant is proposed to block and plug the orthosteric site to prevent CB1 interactions with cannabinoid agonists [36].

3.2.3.3 CB2 Receptor Antagonist

The human CB2 receptor has a 44% sequence identity with the human CB1 protein [46].

Interest in high-resolution imaging of the CB2 receptor and bound ligands stems from a medicinal need to develop CB receptor modulators with high selectivity toward specific CB receptor types. While high-resolution structure of CB2 receptor in complex with agonist remains to be solved, the CB2 protein has been recently crystallized with a bound antagonist [47]. Overall, the CB2 receptor architecture closely follows the CB1 structural profile, with a seven TM helix bundle, several ECL and ICL regions, and an amphipathic helix 8. The CB2 receptor complex with the antagonist was obtained using AM10257 (K_i for the CB2 protein ≈0.08 nM) [47]. This compound has been newly developed as a CB2 receptor-selective antagonist using the CB1 receptor antagonist SR141716A as a starting point [47]. AM10257 resides in the area assigned as an orthosteric binding site in the CB2 receptor (Fig. 3.2). However, there are remarkable differences in antagonist binding between the CB1 and CB2 proteins. First, the N-terminal region does not participate in binding of AM10257 to CB2 protein. This feature differs from the CB1 complexes with the CB1 antagonists AM6538 and taranabant, in which the N-terminal area forms a V-loop to cover the orthosteric binding pocket [35, 36]. Second, the overall antagonist binding pocket in the CB2 receptor is much smaller than the antagonist binding area in the CB1 protein [35, 36, 47]. The smaller pocket for antagonist binding on the CB2 protein resembles the small pocket for agonist binding in CB1. This finding could explain why CB2 antagonists may possess partial agonist properties toward CB1 [5, 47]. Third, the CB2- antagonist complex shows a unique interaction map between the antagonist molecule and CB2 amino acids that participate in ligand recognition and retention. While the overall population of antagonist-interacting amino acids is presented by a usual set of hydrophobic and aromatic amino acids (Table 3.1), AM10257 directly confines $W258^{6.48}$, eliminating the need for $F200^{3.36}$ participation in locking the receptor in an inactive state (Fig. 3.3) [47]. The aforementioned differences between antagonist binding on CB2 versus CB1 proteins ensure antagonist selectivity toward a particular CB receptor type.

3.3 Concluding Remarks and Future Directions

Without a doubt, we are facing an unprecedented increase in the number of high-resolution structures that describe interactions of cannabinoids with their protein targets. Despite the chemical diversity of cannabinoids and the diversity of the targeted proteins, a common theme emerges, that is, the ability of cannabinoids to occupy conventional binding pockets. These pockets include a complementarity region in Fab, lipid-binding cavities in transport proteins, and an orthosteric site in canonical cannabinoid receptors. High-resolution studies are able to capture the intricate details of cannabinoid-protein interactions and thus, provide clues into the long-recognized selectivity of cannabinoid receptor antagonists, the differential potency of cannabinoid compounds, and their ability to exert differential functional effects upon interaction with closely related receptor proteins. In the case of the CB1 receptors, the wide array of functional consequences of ligand binding may arise from the structural plasticity of the receptor. Indeed, ligand binding cavities dramatically vary in size upon agonist versus antagonist binding. Such plasticity allows accommodation of ligands with quite diverse chemical characteristics [45] and likely explains the long-standing enigma of obtaining cannabis-like functional effects from chemically diverse cannabimimetics [48].

Direct translation of the results obtained from the high-resolution structural studies to the *in vivo* scenario, however, should be exerted with caution. The well-recognized limitations of high-resolution structural work include alterations of amino acid sequence to stabilize the protein, such alterations being able to modify ligand binding [5, 47, 49]. In addition, well-resolved structures usually represent a limited number of protein conformations, which may differ from the conformational library of the unmodified protein in its native environment. Last, with regards to transmembrane proteins such as cannabinoid receptors, the influence of membrane bilayer lipid composition on protein conformation and function may also play a critical role [50–53].

Cross-validation of high-resolution structural data using other techniques, such as computational modeling and point mutagenesis, among others, complemented with functional *in vivo* studies, is necessary to aid therapeutic efforts to understand cannabinoid-related physiology. Moreover, such validation will reinforce the foundation for pharmaceutical efforts to further diversify the synthetic family of modulators that target cannabinoid-sensing proteins of relevance to human pathology. In this regard, there is a need for a diverse family of modulators, starting from potent and safe agonists, and extending to antagonists that provide controlled or even irreversible binding to cannabinoid-sensing proteins in order to achieve a functional knock-out of over-reactive receptors.

Acknowledgements This work was supported by NIH R21 AA022433 (ANB). The authors extend their gratitude Dr. Avia Rosenhouse-Dantsker (University of Illinois at Chicago) for critical reading of the manuscript and Office of Research (The University of Tennessee Health Science Center) for editorial assistance.

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Cannabinoid Signaling in Cancer

4

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Abstract

The family of chemical structures that interact with a cannabinoid receptor are broadly termed cannabinoids. Traditionally known for their psychotropic effects and their use as palliative medicine in cancer, cannabinoids are very versatile and are known to interact with several orphan receptors besides cannabinoid receptors (CBR) in the body. Recent studies have shown that several key pathways involved in cell growth, differentiation and, even metabolism and apoptosis crosstalk with cannabinoid signaling. Several of these pathways including AKT, EGFR, and mTOR are known to contribute to tumor development and metastasis, and cannabinoids may reverse their effects, thereby by inducing apoptosis, autophagy and modulating the immune system. In this book chapter, we explore how cannabinoids regulate diverse signaling mechanisms in cancer and immune cells within the tumor microenvironment and whether they impart a therapeutic effect. We also provide some important insight into the role of cannabinoids in cellular and whole body metabolism in the context of tumor inhibition. Finally, we highlight recent and ongoing clinical trials that include cannabinoids as a therapeutic strategy and several combinational approaches towards novel therapeutic opportunities in several invasive cancer conditions.

Keywords

Cannabinoids · Tumor microenvironment · Signaling · Metabolism

Abbreviations

2-AG	2-arachindonoylglycerol
AEA	anandamide
AMPK	5′ AMP-activated protein kinase
CBD	cannabinoids
CBR	cannabinoid receptor
COX-2	cyclooxygenase-2
CXCL	C-X-C motif chemokine ligand
EGFR	epidermal growth factor receptor
EMT	epithelial to mesenchymal transition
E:R	endoplasmic reticulum
mTOR	mammalian target of rapamycin

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A. N. Bukiya (ed.), *Recent Advances in Cannabinoid Physiology and Pathology*,
Advances in Experimental Medicine and Biology 1162, https://doi.org/10.1007/978-3-030-21737-2_4

PPAR	peroxisome proliferator-activated receptors
ROS	reactive oxygen species
TAMs	tumor associated macrophages
THC	Δ9-tetrahydrocannabinol
TME	tumor microenvironment
TRPV2	transient receptor potential cation channel subfamily V member 2.

4.1 Introduction

Cannabinoids have typically been assumed to originate from the plant *Cannabis*, however, broadly speaking cannabinoids are the group of chemical structures that mainly act in the body through cannabinoid receptors (CB); CB1 (Central receptor) and CB2 (Peripheral receptor) [1, 2]. They can be divided into different groups based on their source of origin as plant derived cannabinoids (phytocannabinoids), endogenously produced cannabinoids (endocannabinoids) and chemically produced synthetic cannabinoids. They all represent a broad range of ligands that interact with the CB receptors termed cannabinoids.

Amongst the several phytocannabinoids, Δ9-tetrahydrocannabinol (THC) is the main psychoactive compound. These compounds are responsible for many physiological effects such as euphoria, pain relief and anti-inflammatory activities [3].

Endogenous ligands like anandamide (AEA) and arachindonoylglycerol (2-AG) interact with CB as part of the endocannabinoid system [4]. Majority of the CB are expressed in neural tissues as CB1 receptor, and are known to modulate the central nervous system. CB2 receptors are predominantly expressed in immune cells and thus can modulate both the innate and adaptive immune systems [5–8] Interestingly, cannabinoids bind not only to classical receptors (CB1 and CB2), but also to certain orphan receptors and ion channels like transient receptor potential vanilloid-2 (TRVP2) and peroxisome proliferator-activated receptors (PPAR) [9] (Table 4.1).

4.2 Cannabinoids and Cancer Signaling

Several studies have suggested that cannabidiol and THC directly inhibit cancer cells growth by activation of diverse signaling pathways associated with apoptosis, proliferation, angiogenesis and metastasis [10, 11]. A schematic representation of these pathways is presented in Fig. 4.1 [12]. Previously, it has been reported that THC mediates its pro-apoptotic effect in tumor cells by increased synthesis of the proapoptotic sphingolipid ceramides [13]. In glioma cells, ceramide-dependent upregulation of the stress protein p8 induced apoptosis via the upregulation of the endoplasmic reticulum (ER) stress related gene Activating Transcription Factor *4 (*ATF-4), C/EBP homologous protein (CHOP) and Tribbles homolog 3 (TRB3) [14]. It was also found the ceramide can induce apoptosis in leukemic cells by regulation of p38 MAPK signaling. Experimental studies also revealed that THC causes apoptosis in leukemia T cells by down-regulation of Raf-1/mitogen-activated protein kinase/ERK kinase pathway and thus, leads to translocation of BCL2 Associated Agonist of Cell Death (BAD) to mitochondria [15]. On the other hand, it can activate apoptosis in colorectal cancer cells by inhibition of RAS-MAPK/ERK and PI3K-AKT survival signaling cascades accompanied by activation of the pro-apoptotic BAD [16].

Most interestingly, THC promotes autophagy mediated apoptosis by inducing ceramide accumulation via Tribbles homolog 3 dependent inhibition of the AKT/mTORC1 complex axis in human glioma [4] and in hepatocellular carcinoma [17] cells. The combined administration of THC and temozolomide was also found to exert a strong anti-tumoral effect *in-vivo* in in glioma mouse model [18]. THC treatment was also reported to inhibit the proliferation of breast cancer cells by activating the CB2 receptors with subsequent arrest of cell cycle in G2-M phase via downregulation of Cyclin-Dependent Kinase 1 (CDC2) protein [19] or modulation of JunD (a member of the AP-1 transcription factor family)

Table 4.1 Role of cannabinoids in different physiological processes

Cannabinoids	Target receptor	Effect
Anandamide (AEA)	CB1	Analgesic, antiemetic, appetite stimulant, tumour growth inhibitor
2-arachidonoyl-glycerol (2-AG)	CB1/CB2 agonist	Analgesic, antiemetic, appetite stimulant, tumour growth inhibitor
Palmitoyl-ethanolamide (PEA)	CB2 agonist	Neuromodulatory and immunomodulatory
Docosatetraenyl ethanolamide	CB1 agonist	Neuromodulatory and immunomodulatory
Homo-γ-linoenylethanolamide	CB1 agonist	Neuromodulatory and immunomodulatory
Oleamide	CB1 agonist	Neuromodulatory and immunomodulatory
Δ9-tetrahydrocannabinol (Δ9-THC)	CB1/CB2 agonist	Analgesic, antiemetic, appetite stimulant tumour growth inhibitor
Δ8-tetrahydrocannabinol (Δ8-THC)	CB1/CB2 agonist	Anti-tumor agent, inhibitors of mitochondrial O_2 consumption in human sperm, antiemetic, appetite stimulant
Cannabidiol (CBD)	CB1 agonist	Anti-tumor agent, attenuate catalepsy, immunosuppressive, inflammatory or anti-inflammatory agent (depends upon used concentration of drug), antipsychotics
Cannabigerol (CBG)	CB1/CB2 agonist	multiple sclerosis, antiemetic, anti-inflammatory agent, treatment for neurological disorder
Cannabichromene (CBC)	CB2 selective agonist	anti-inflammatory agent, treatment for neurological disorder, hypomotility, antinociception, catalepsy, and hypothermia
Tetrahydrocannabivarin (THCV)	CB1 antagonist and partial CB2 agonist	Hepatic ischaemia, anti-inflammatory
HU-210	CB1/CB2 Nonselective agonist	Analgesic, multiple sclerosis, neuroprotective
CP-55,940	CB1/CB2 Nonselective agonist	Anti-cancer agent, Analgesic, antiemetic, appetite stimulant
R-(+)-WIN 55,212-2	CB1/CB2 Nonselective agonist	Analgesic, antiemetic, appetite stimulant, tumour growth inhibitor, multiple sclerosis
JWH-015	CB2 selective agonist	Anti-tumor, anti-inflammatory, antiemetic
JWH-133	CB2 selective agonist	Neurological disorders, Anti-cancer
JWH-139	CB2 selective agonist	Analgesic, antiemetic, appetite stimulant tumour growth inhibitor
HU-308	CB2 selective agonist	Tumour growth inhibitor (in glioma, skin carcinoma, lymphoma
CP55940	CB/CB2 agonist	Analgesic, antiemetic, appetite stimulant, tumour growth inhibitor, multiple sclerosis
R-(+)-methanandamide	CB1 agonist	Analgesic, antiemetic, appetite stimulant tumour growth inhibitor
AM251	CB1antagonist	Metabolic syndrome
AM281	CB1antagonist	Improves recognition loss induced by naloxone in morphine withdrawal mice, various pharmacological property

[20] It also upregulated several PPAR dependent signaling pathways in cancer cells [21].

Additionally, further study confirmed that cannabidiol inhibited cancer cell viability and proliferation, which was reversed *in-vitro* in the presence of blockers of either CB2, Transient Receptor Potential Vanilloid 1 (TRPV1) or melastatin-related transient receptor potential (TRPM), cyclooxygenase-2 (COX-2) or PPAR and in tumor derived primary culture from a patient with non-small cell lung cancer in presence of PPAR antagonists [22, 23]. Our research group demonstrated that cannabinoid mediate its anti-proliferative effects in highly aggressive

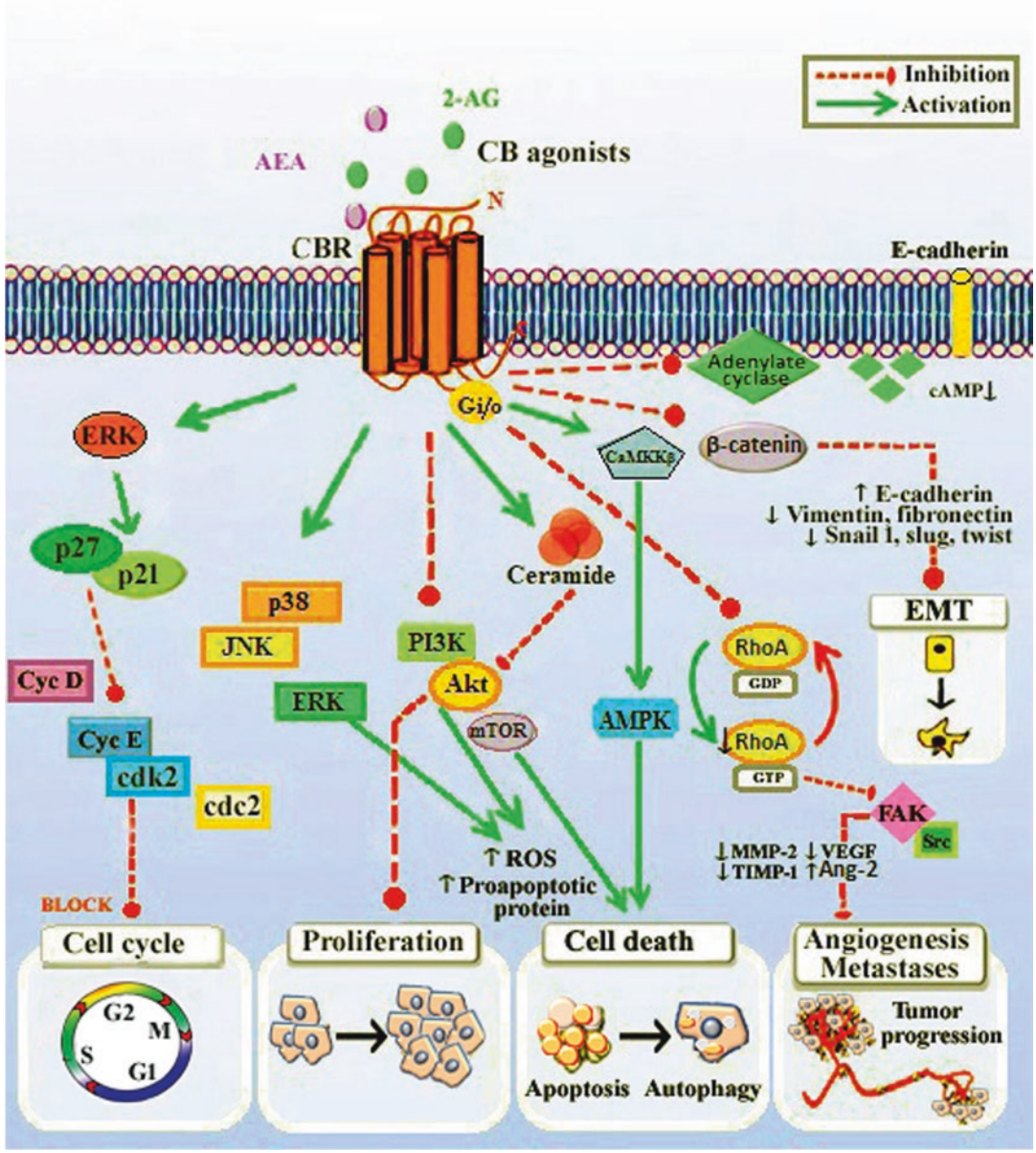

Fig. 4.1 Schematic representation of the main signaling cascades highlighting the downstream CB receptor activation by cannabinoids which impact all the hallmark processes of cancer such as proliferation, apoptosis, migration, invasion, angiogenesis and EMT (epithelial-mesenchymal transition)

human breast cancer cells in part, by inhibition of epidermal growth factor (EGF), NF-kB, extracellular signal–regulated kinases/AKT (ERK/AKT) and matrix metalloproteinase 2 and 9 signaling pathways [24]. Cannabinoid also reduces angiogenesis in both tumor and endothelial cells. Experimental evidence suggested that cannabinoid treatment also suppresses the expressions of pro-angiogenic gene (COX-2) and decreases the secretion of prostaglandin E2 (PGE2) [25]. Furthermore, it is reported that anandamide showed decreased phosphorylation of focal adhesion-associated protein kinases, which are

components of cell adhesion machinery, and influence their migration [26].

Furthermore, the treatment of THC also inhibits the growth of Lewis lung adenocarcinoma via inhibition of DNA synthesis [27]. It has also been found that THC suppresses the growth and metastasis of A549 and SW-1573 (human lung cancer cell lines) both *in-vitro* and *in-vivo* by inhibition of epidermal growth factor-induced phosphorylation of ERK1/2, c-Jun-NH2-kinase1/2 and Akt [27, 28]. Recently, research studies from our group also revealed that CB2-specific synthetic cannabinoids, JWH-015 inhibits CXCL-12 induced migration and invasion by suppressing the phosphorylation of ERK and C-X-C chemokine receptor type 4 (CXCR4) polymerization [29]. It was also reported that the treatment of cannabinoids induces apoptosis in different malignant immune cells (Jurkat and EL-4) in lymphomas and leukemia's [30] via mitochondria mediated ROS pathway and activation of different caspases [31].

4.3 Cannabinoids and the Immune System

Presently, many advanced therapeutic approaches have been developed to treat different cancers which mainly include surgery, radiation and chemotherapy, endocrine therapy, or targeted therapy. Although, these therapies have decreased breast cancer specific mortality, they have also shown dramatic failures due to the emergence of drug resistance, relapse, multi-organ metastasis and subsequently death [32, 33]. Recently, it has been reported that the tumor microenvironment (TME) plays an essential role in regulating the stemness and drug resistance of cancer cells. TME play important roles in tumor initiation, development, invasion, and metastasis. TME is basically comprised of cancer cells, endothelial cells, fibroblasts and different types of immune cells known as tumor associated macrophages (TAMs).

TAMS have been shown to secrete different types of growth factors which can regulate TME and thus support cancer growth and subsequent metastasis [34]. Moreover, it has also been reported that M2 macrophages, which can secrete a diverse array of essential growth factors, can promote invasion and metastasis of cancer cells into multiple organs [34]. Recently, research findings have shown that the *in-vivo* treatment of Cannabidiol inhibits the recruitment of total macrophages and especially, M2 macrophage populations in tumor stroma as well as in lung metastatic nodules [24]. In this study, Zhu et al. demonstrated that that the *in-vitro* treatment of 4T1.2 cells with cannabinoid inhibited the secretion of specific cytokines such as CCL3 and GM-CSF in its condition medium (CM) as compared to CM of vehicle control. The CM harvested from cannabinoid treated 4T1.2 cells also significantly reduced the migration of mouse monocytic cells, RAW 264.7, comparatively to CM collected from vehicle control [24].

Furthermore, research findings also showed that the cannabinoid treatment inhibited the M2 macrophages induced epithelial mesenchymal transition (EMT) in non-small cell lung cancer (NSCLC) cells via downregulation of EGFR signaling cascade [35–37]. It has been reported that TAM can secrete EGF like ligands which can activate EFGR pathway and thus can cause increased EMT in cancer cells [38]. In addition, it has been shown that the treatment of JWH-015 inhibits the EMT induction by suppressing the activation of EGFR signaling in NSCLC cells both *in-vitro* and *in-vivo* systems. The treatment of JHW-015 also reduced the expression of proliferative marker (Ki67), angiogenic marker (CD-31), EMT markers (N-Cadherin, Snail and Slug) and also inhibited the infiltration of CD11b/F4/80/CD206 M2 macrophages into tumor. Investigation of these interactions and signaling has led to novel insights in the cannabinoids-mediated modulation of TME in cancer [39].

It has also been reported that the treatment of cannabinoids induces the conversion of T helper 1 cell (Th1) to T helper 2 cell (Th2) subpopulations by activating the expression of interleukin (IL), IL-10, and TGF-β and also decreases the production of TH1 cytokines (IL-2, IL-12 and Interferon-γ) [40] [41]. On the other hand, IL-10 and TGF-β play significant roles in mediating the

THC induced suppression of anti-tumor immunity, and abrogation of either cytokine alone is sufficient to reverse the detrimental effect of THC. The study suggests that THC promotes tumorigenicity and limits immunogenicity *in-vivo* by upregulating the potent immune inhibitory cytokines [42].

THC has also been reported to modulate the activity of different immune cells such as macrophages, NK cells and T lymphocytes. THC and other cannabinoid agonists may exert their immune modulating effects through the disruption of Th1 to Th2 conversion [43]. THC mediates these effects by inhibiting the production of type-1 cytokines and promoting type-2 cytokine production by lymphocytes [44]). The synthetic analogues of cannabinoid were also reported to suppress the proliferation of T cells by inhibiting the production of IFN-γ. So overall, several studies show that cannabinoid and its different synthetic analogues can modulate host immunity and thus, it can regulate tumor growth and metastasis in different human malignancies [45].

4.4 Cannabinoids in Cellular Metabolism

Aberration of cellular metabolism is a hallmark feature of solid tumors as well as leukemic cancers [46–48]. For several decades, cancer associated metabolism has been defined in context of the Warburg effect, which suggests that highly proliferative cancer cells are entirely dependent on glycolysis rather than the mitochondria driven oxidative phosphorylation for their energetics. Since Warburg's initial observations, research has questioned the dogma of the Warburg effect and helped to establish the significant contributions of metabolic reprogramming in mitochondrial function, and cellular energetics in cancer cell survival, metastasis, and even drug resistance [49–51]. Moreover, recent experimental and epidemiological research has also implicated whole body metabolism and changes induced by factors such as high fat diet, particularly obesity, in the development of a pro-tumor microenvironment [52] [53]. This has led to a greater interest in targeting cellular metabolic pathways, such as the 5′ AMP-activated protein kinase (AMPK), protein kinase C (PKC), and mammalian target of rapamycin (mTOR) pathways [54–56], Although PKC inhibitors have been successfully tested in the experimental setting, the efficacy of these inhibitors as monotherapy against cancer has been limited to B-cell lymphoma malignancy [57] Similarly, targeting other individual pathways, including mTOR, AKT and AMPK have had limited success in eliciting anti-tumor activity. Another product of cellular metabolism, particularly in cancer cells is reactive oxygen species (ROS). ROS are thought to further contribute to inflammatory pathways and damage cellular macromolecules and nucleotides, particularly DNA, thus potentially perpetuating cancer survival and metastasis [58].

Meanwhile, growing body of research in cannabinoids indicates a close mechanistic link between cannabinoids and metabolism. Cannabinoids have primarily been investigated as palliative therapy for individuals with advanced cancer. In this section, we hope to provide an overview of current literature linking cannabinoids and their anti-tumor activity mediated through metabolism and metabolic pathways, thereby shedding light on the potential of cannabinoids as a therapy against cancer.

For several decades, the link between metabolic syndrome and obesity, and cancer has sparked interest in whole body metabolism in patients with cancer. Fatty acid oxidation by tumor cells is often linked with various cancers, including prostate cancer, breast cancer, pancreatic cancer, etc. [59–61]. Fatty acid oxidation, mediated through the mitochondria, is a highly energetic process linked with high ROS generation. Experimental studies have shown that an inhibition of mitochondrial metabolism and a switch to glycolytic energy generation in tumors is linked with better prognosis as well as drug response [62]. Mitochondrial uncoupling is also critically important in inducing programmed cell death, thus making this shift from mitochondrial respiration to glycolysis in tumor cells a key therapeutic target [63].

It has also been observed that patients with cancer undergo greater loss of lean mass rather than fat mass, which in turn is linked with poor outcome and quality of life. Lean mass loss in patients with cancer can be regulated by protein nutritional support, however, the course of therapies often leads to loss of appetite, which greatly impact nutrition in these patients [64]. This is partially benefitted by cannabinoids, as cannabinoids enhance appetite through Ghrelin receptor interaction. Ghrelin receptor, a receptor for the anabolic hormone Ghrelin, is expressed is all vital organs. It is known to modulate appetite, fat accumulation and energy expenditure. Moreover, the synthetic cannabinoids HU210, impacts cellular energy metabolism via Gherlin receptor interaction [65].

In spite of its central role in nutrient sensing and metabolic regulation, AMPK appears to have both pro-tumor and anti-tumor effects. On one hand, AMPK promotes this metabolic plasticity through promotion of fatty acid oxidation, while on the other hand AMPK is closely linked with tumor suppressors p53 and tuberous sclerosis complex (TSC2) [55]. While a thorough investigation of AMPK subunits and variants involved in various solid tumors and leukemia has never been performed, several studies indicate reduced AMPK activity in lung cancer, colorectal cancer, breast cancer, ovarian cancer, hepatic cancer, etc. [1, 60, 66–69]. Interestingly, AMPK activation in cancer models, including hepatoma, has been shown to inhibit PPAR-gamma and PCG-1alpha leading to a decrease in fatty acid oxidation [70] The effect of the mitochondrial inhibitor, metformin, in patients with breast cancer is also shown to be mediated by AMPK [71].

Although not as extensively investigated in different models and cancers, synthetic cannabinoids arachidonoyl cyclopropamide (APCA) and GW405833 have been shown to inhibit mitochondrial metabolism and induce AMPK-dependent autophagy in pancreatic cancer cells [72]. Cannabinoid receptor cross-talk with AMPK is well documented in several tissues and is linked with reduction in mitochondrial biogenesis, thus disrupting mitochondrial metabolism [73]. Another systemic effect of Cannabinoids on metabolism as well as their anti-tumor activity may be exerted through the insulin signaling pathway. The key factors downstream of insulin-insulin receptor interaction such as AKT, mitogen activated protein kinase kinase 1/2 (MEK1/2) and ERK are known to contribute to cell proliferation, motility, and cancer cell survival. Cannabinoids have been shown to induce hepatic insulin resistance and multiple studies report that Cannabinoids inhibit insulin receptor signaling in pancreatic beta cells showing direct interaction between the CB1 receptor and insulin signaling [74, 75]. This is an entirely new and therapeutically sound avenue to alter crucial cell survival pathways with minimal toxicity to healthy cells.

Cancer cells have high energy needs to maintain proliferation and migration. Cannabinoids are known to inhibit mitochondrial energetics leading to autophagy [76] In pancreatic cancer cells, in combination with Gemcitabine, APCA is known to induce ROS-mediated autophagy, once again suggesting the possible role of mitochondrial electron transport chain uncoupling in response to Cannabinoids, thus directly affecting cancer cell death [77].

Cannabinoid may also potentially induce anti-tumor activity via immune cell, particularly macrophages. It has been observed that the activation of CB1 by ACEA in macrophages, which modulates ROS production, is dependent on the phosphorylation of p38-mitogen-activated protein kinase (p38-MAPK). This is known to lead to tumor necrosis factor-α and monocyte chemoattractant protein-1 expression, thus enhancing a pro-inflammatory phenotype [78]. Nevertheless, the direct effects of Cannabinoids on macrophage phenotype and function have not been thoroughly tested.

Finally, it is important to note that in the present epidemic of metabolic diseases and obesity that drive various cancers, phytocannabinoids, particularly THC, act in a manner similar to metformin. Metformin is a mild inhibitor of complex 1 of the mitochondria, and therefore is thought to play an important role in metabolic reprogramming. While long term use of metformin has been linked with risk of cardiomyopathy, the effect of chronic use of phytocannabinoids and synthetic

cannabinoids on systemic health, while predictable based on several studies, may need to be performed specifically in the context of cancer survivor cohorts [79].

4.5 Recent Advances of Cannabinoids in Clinical Trials

The use and understanding of mechanisms of cannabinoids in context of tumors are almost completely limited to preclinical studies. Nevertheless, its lower toxicity led it to the first clinical application of THC on humans, conducted on nine terminal patients with recurrent glioblastoma and resistant to standard chemotherapy [85]. THC was administered intratumorally and dose was determined to be safe and without any psychoactive effects [80]. Furthermore, this study also confirmed the antiproliferative action and induction of apoptosis induced by THC, however, further studies are needed to determine the correct dosage or any potent systemic interaction [80]. There have been some clinical trials currently ongoing or recently completed using combinatorial treatments of nabiximols and temozolomide in patients with recurrent glioblastoma (NCT01812603, NCT01812616). Another clinical study was conducted using Cannabidoil as a single regimen on different solid tumor patients (NCT02255292). In addition, many recent clinical studies also underscore the promising therapeutic potential of one of the synthetic cannabinoids, dexanabinol, in patients with different solid tumors or brain cancer, compared with other healthy subjects (NCT01489826, NCT01654497, and NCT02054754).

4.6 Concluding Remarks

In summary, cannabinoid modulates the tumor growth and metastasis in different human malignancies by regulating different signaling cascades linked with proliferation, survival, angiogenesis and metastatic spread of cancer cells. It can also regulate the TME by regulating different types of immune cells associated with pro and anti-tumor immunity. Cannabinoid may induce ROS generation and lower mitochondrial activity in cancer cells, leading to autophagy and cell death. Cannabinoid also cross-talks with cellular metabolism via AMPK and mTOR, subsequently enhancing cancer cell death. Overall, application of cannabinoid will have high translational significance and impact for developing novel immune and metabolic-based therapies directed against different metastatic cancers with minimal to low side effects.

Acknowledgements This work is supported in part by the Department of Defense Level II Breakthrough Award and Pelotonia IDEA Award.

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Cannabinoids and Cardiovascular System

5

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Abstract

Cannabinoids influence cardiovascular variables in health and disease via multiple mechanisms. The chapter covers the impact of cannabinoids on cardiovascular function in physiology and pathology and presents a critical analysis of the proposed signalling pathways governing regulation of cardiovascular function by endogenously produced and exogenous cannabinoids. We know that endocannabinoid system is overactivated under pathological conditions and plays both a protective compensatory role, such as in some forms of hypertension, atherosclerosis and other inflammatory conditions, and a pathophysiological role, such as in disease states associated with excessive hypotension. This chapter focuses on the mechanisms affecting hemodynamics and vasomotor effects of cannabinoids in health and disease states, highlighting mismatches between some studies. The chapter will first review the effects of marijuana smoking on cardiovascular system and then describe the impact of exogenous cannabinoids on cardiovascular parameters in humans and experimental animals. This will be followed by analysis of the impact of cannabinoids on reactivity of isolated vessels. The article critically reviews current knowledge on cannabinoid induction of vascular relaxation by cannabinoid receptor-dependent and –independent mechanisms and dysregulation of vascular endocannabinoid signaling in disease states.

Keywords

Cannabis · Endocacannabinoids · Cannabinoid receptors · Endothelial cells · Vascular

Abbreviations

2-AG	2-Arachidonoylglycerol
ACPA	arachidonylcyclopropylamide
BK_{Ca}	large conductance calcium-activated potassium channel, KCa1.1
CB1	cannabinoid receptor type 1
CB2	cannabinoid receptor type 2
CBe	endothelial cannabinoid receptor
CGRP	calcitonin gene-related peptide
COX	cyclooxygenase, prostaglandin-endoperoxide synthase

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A. N. Bukiya (ed.), *Recent Advances in Cannabinoid Physiology and Pathology*,
Advances in Experimental Medicine and Biology 1162, https://doi.org/10.1007/978-3-030-21737-2_5

DOC salt hypertension	deoxycorticosterone acetate-induced hypertension
EDHF	endothelium-derived hyperpolarizing factor
FAAH	fatty acid amide hydrolase
IK_{Ca}	intermediate conductance calcium-activated potassium channel, KCa3.1
K_{ATP}	ATP-sensitive potassium channel
NAGly	N-arachidonoyl glycine
NCX	Na^+-Ca^{2+} exchanger
NO	nitric oxide
PPAR	peroxisome proliferator-activated receptor
SHR	spontaneously hypertensive rats
TASK	TWIK-related acid-sensitive potassium channel
THC	Δ^9-tetrahydrocannabinol
TRPA	transient receptor potential cation channel subfamily A (ankyrin)
TRPV	transient receptor potential cation channel subfamily V (vanniloid)

5.1 Introduction

Cannabinoids influence the function of many organs and systems and apart from the well-known neurobehavioral and analgesic effects, cannabinoids exert a profound effect on cardiovascular, immune, digestive, reproductive function, and influence cell fate, body temperature, bone formation and other aspects of human physiology. Key cardiovascular parameters such as blood pressure, vasomotor control, cardiac contractility, vascular inflammation, preconditioning and angiogenesis are controlled by cannabinoids. Changes in the levels of circulatory cannabinoids and cannabinoid receptor expression in the vasculature as well as the associated perturbations in cannabinoid signalling have been detected under a number of pathophysiological conditions including obesity, diabetes, advanced liver cirrhosis, cardiotoxicity, circulatory shock, atherosclerosis and hypertension [1–8]. Consequently, the endocannabinoid system is widely accepted to represent an attractive therapeutic target to tackle a range of abnormalities including cardiovascular disorders [9–11].

Because of psychotopic and pain-relieving effects, the members of the plant family Cannabaceae have a long history of cultivation and human use both for recreational and medical purposes, rooting through several thousand years [12]. Recent boosted recreational marijuana abuse and accessibility of a growing number of synthetic psychoactive cannabinoids with greatly increased potencies as compared to that of Δ^9-tetrahydrocannabinol (THC), the main psychoactive constituent of marijuana, are coincided with the reported serious cardiovascular events such as myocardial infarction, cardiomyopathy, arrhythmias and stroke with documented fatalities even among young and relatively healthy men [13–15]. While the mechanisms for these events are still not entirely clear, these observations emphasize a casual association between recreational cannabis abuse and cardiovascular abnormalities, calling for a need to advance our understanding of the fundamental mechanisms so severely affecting cardiovascular function by cannabis. Besides the principal phytocannabinoid THC, which binds to cannabinoid receptors and determines psychoactive properties of the plant, cannabis contains an extensive number of non-psychoactive phytocannabinoids with low affinity binding at cannabinoid receptors type 1, CB1, and type 2, CB2, such as cannabidiol, cannabinol, cannabidivarin and cannabigerol and others. Because of beneficial effects in a range of disorders and a lack of psychoactivity, therapeu-

tic effects of these compounds and their mechanisms of action is a subject of intense research [10].

The discovery of THC, the main psychoactive phytocannabinoid contained in Cannabis plant [16], was the first major step in the recognition of the role of the endocannabinoid system in health and disease. Cannabinoid research received a strong impetus following the identification and characterization of CB1 [17] and CB2 receptors [18, 19]. The latter was initially identified as peripherally restricted receptor expressed by immune cells. Subsequent studies have shown that the CB2 receptors are also distributed in vascular cells [20, 21] and central and peripheral nervous system [22–25].

The endogenous ligands for cannabinoid receptors, anandamide and 2-arachidonoyl glycerol (2-AG) were detected initially in the brain [26, 27] and gut [28]. Subsequent studies showed that endocannabinoids are synthetized at the plasma membranes of virtually all cell types including vascular [29–32], cardiac cells [33], monocytes and platelets [34]. Cardiovascular pathologies, such as coronary circulatory dysfunction, myocardial infarction, hypertension, atherosclerosis and diseases accompanied by vascular dysfunction, such as diabetes, obesity and cirrhosis, are associated with alterations in cannabinoid signaling and increased plasma levels of 2-AG and anandamide [1, 5, 34–38].

Under normal conditions, CB1 receptors have been detected in different vascular beds, including endothelial cells from rat mesentery [37, 39, 40], rat [2, 32] and human aorta [41], human hepatic artery endothelial cells [42], rat aortic smooth muscle cells [43], pointing at engagement of the endocannabnoid system in regulation of vascular function. Indeed, endocannabinoids and their synthetic analogues exert hypotensive and cardiodepressant effects, control cardiac contractile reactions [1, 11, 44]. The chapter will review the impact of cannabinoids on cardiovascular function.

5.2 Effects of Cannabinoids on Cardiovascular Parameters

Highly diverse actions of cannabinoids are mediated via surprisingly wide number of targets, spanning from classical G protein-coupled cannabinoid receptors, non-CB1/CB2 targets, including G-protein coupled receptors GPR18, GPR35, GPR55 and GPR119 [45–50], and a broad number of ion transport systems [51–54]. The reactions of cardiovascular variables in response to cannabinoids depend on several factors and besides the type of predominantly stimulated cannabinoid receptors, there are many other determinants of the reaction, such as direct targeting of ion channels and transporters located in plasmalemma, engagement in the response of non-CB1/CB2 receptors, intracellular ion channels [55, 56] or intracellularly located receptors for cannabinoids [57–59]. Via stimulation of specific cannabinoid receptors, cannabinoids may attenuate or intensify cardiovascular pathological states and, accordingly, play a protective or pathophysiological role.

The molecular mechanisms underlying diverse effect of cannabinoids and their synthetic analogues on vascular function, although progressively unveiled in the last two decades, are not yet entirely clear. In vivo effects of cannabinoids involve sites of action in the central [60, 61] and the peripheral nervous system [62, 63] as well as both cannabinoid receptor-dependent and –independent targets located on cardiac myocytes [64–66], vascular smooth muscle [40, 67–71] and endothelial cells [6, 40, 52, 53, 72–75].

Apart from regulation of vasoactivity, cannabinoids influence cardiac performance and modulate ischemia-reperfusion injury [8, 76–78], endothelial [79], and smooth muscle cell migration [71], angiogenesis [80, 81], vascular wall inflammation and atherogenesis [3, 82]. We will briefly review the literature describing the impact of smoked cannabis and intravenous THC, as well as central and peripheral regulation of cardiovascular parameters by cannabinoids.

5.2.1 Effects of Marijuana Smoking on Cardiovascular Parameters

The cardiovascular effects elicited by marijuana smoking largely depend on chemical composition of the plant, specifically, the THC content, the dose inhaled and the smoking method. The impact of cannabinoids on cardiovascular parameters has been studies since early 1970s. The studies were mostly directed on examining the effect of THC as the principle active ingredient of Cannabis sativa [83, 84]. From the very beginning of investigation of the effects of constituents of cannabis on cardiovascular parameters, the differences in the effects of THC and cannabidiol both on heart rate and some psychological reactions were noticed [85]. Early reports on the effects of cannabis on humans were focused mainly on psychotropic effects and pointed at pharmacological difference between oral injestion and inhalation of smoke of cannabis products [86]. Smoking cannabis was shown to lead to a potent bronchodilation of human airways [87] and an immediate increase in heart rate up to 90 beats per minute that may last more than 1 h and an increase in limb blood flow [88, 89]. These responses were not observed after administration of propranolol, a beta-adrenergic blocker, pointing for beta-adrenergic stimulation [89]. However, repeated users within several days or weeks develop a tolerance to the initial effects and experience bradycardia and hypotension. Most of these studies conclude that THC alters autonomic control of the cardiovascular system resulting in parasympathetic dominance. Early [90, 91] and more recent [92] studies have shown that marijuana smoking is associated with increased cerebral blood flow. Systematic reviews of the reported cases indicate that marijuana smoking is linked to increased likelihood of development of severe cardiovascular events including atrial fibrillation, enhanced left ventricular systolic function, transient loss of consciousness and a fall, ventricular arrhythmias, coronary artery disease, severe stroke development, peripheral arteritis [15, 93–95].

5.2.2 Effects of Cannabinoids on Blood Pressure and Heart Rate

5.2.2.1 Human Studies

In healthy men volunteers, 30 mg of THC received orally increased the heart rate and blood pressure [85, 96]), while cannabidiol (15–60 mg) produced no effect when administered alone and blocked the effect of THC when the drugs were administered together [85]. Acute administration of cannabidiol at higher dose (600 mg) was reported to reduce resting blood pressure and the blood pressure increase elicited by exercise and mental stress [97]. The uncovered differences in the impacts of THC and cannabidiol on heart rate and blood pressure in early 1970s [85, 96, 98] initiated an extensive research in this field aiming to identify the mechanisms underlying the impact of cannabinoids on cardiovascular system. While acute administration of THC generally results in an increase in blood pressure and heart rate, repeated administration of THC decreases blood pressure and heart rate. In conscious humans, an acute intravenous administration of THC in the dose of 25 μg/kg elicits tachycardia without significant alterations in systolic and diastolic blood pressures [99]. Prior beta adrenergic blockade partially inhibited this response. Acute oral or intravenous administration of THC at higher dose (0.2–0.3 mg/kg) resulted in an increase in both heart rate and blood pressure in healthy volunteers [96, 100]. The reactions, however, switched to the opposite when THC was injested for a prolonged time [101]. These data point at complex mechanisms involved in bidirectional regulation of cardiovascular parameters by cannabinoids.

5.2.2.2 Animal Studies

The hemodynamic effects of cannabinoids in conscious rats are quite different from those observed in anesthetized rats [102, 103]. In anaesthetized rats, the most prominent response to anandamide and THC infusion is a long-lasting hypotension and bradycardia, generally ascribed as phase 3 of the triphasic response [104–106]. The long-lasting hypotension and bradycardia evoked by anandamide infusion to

anesthetized rats is preceded by an immediate brief drop in blood pressure and heart rate ascribed as phase 1, which was followed by a brief (30–60 s) pressor response and tachycardia, ascribed as phase 2. The long lasting depressor effect of anandamide, but not the two initial transient phases, is inhibited by rimonabant [105, 107] and after transaction of the cervical spinal cord or blockade of alfa-adrenergic receptors [103, 104, 107], suggesting that the depressor response is due to CB1 receptor-mediated inhibition of norepinephrine release from peripheral sympathetic nerve terminals in the heart and vasculature and subsequent inhibition of catecholamine release [108]. It was shown also that in anaesthetized rats, cannabidiol and its synthetic analogue O-1918, which have low affinity to CB1 and CB2 receptors, elicit a prolonged decrease in blood pressure, heart rate and mesenteric and renal blood flow, masking/reducing the similar cardiovascular effects of anandamide that are normally observed in the absence of cannabidiol and O-1918 [109]. The authors attributed the hypotensive effects of anandamide in anesthetized rats to stimulation of the third type cannabinoid receptor sensitive to O-1918 [109]. In the earlier study of Malinowska et al [110], the anandamide-evoked decrease in heart rate and blood pressure in anaesthetized rats (phase 3) has been attributed to stimulation of the CB1 and TRPV1 receptors.

The phases 1 and 2 are absent in TRPV1-defficient mice [106, 107, 111], pointing for the involvement of TRPV1 receptor. A TRPV1 agonist capsaicin was shown to be more potent than methanandamide and anandamide at eliciting an immediate short-lasting decrease in heart rate and blood pressure that is inhibited by a selective TRPV1 antagonist capsazepine [110], suggesting that a short-lived depressor effect of anandamide is evoked by the Bezold-Jarisch reflex [112]. A brief pressor response (phase 2) is enhanced after alfa-receptor blockade or cervical cord transaction [104].

Unlike the responses elicited by THC and anandamide, the hypotension and bradycardia elicited by 2-AG is insensitive to rimonabant and is preserved in CB1 knock- out mice [113]. However, cardiovascular effects of 2-AG were found to be masked by a rapid degradation of the endocannabinoid by a monoacylglycerol lipase with generation of arachidonic acid. A metabolically stable 2-AG analogue 2-AG ether was found to elicit hypotension that is sensitive to rimonabant and absent in CB1 knock-out mice [113], suggesting the CB1 receptor-mediated signalling, possibly through sympathetic nerves innervating the resistance vessels.

In contrast to anaesthetized rats, in conscious rats, intravenous administrations of anandamide, its stable analogue methanandamide, THC and WIN55212-2 fail to produce a prolonged hypotension, but result in a brief pressor response, that was potentiated by rimonabant [105], indicating that the CB1 receptor-dependent signaling attenuates the pressor response. Consistent with the idea that stimulation of CB1 receptor results in vasodilation, intravenous administration of a synthetic CB1 and CB2 receptor agonist WIN55512-2 to pithed, conscious rabbits in which the sympathetic outflow was continuously stimulated electrically was shown to decrease blood pressure and heart rate [114] and the effect was antagonized by rimonabant.

In other study performed on conscious rats, anand amide (75–1250 μg/kg) elicited a short-lived increase in arterial blood pressure associated with vasoconstriction in renal, mesenteric and hindquarters vascular beds [115]. When anandamide was administered at the higher dose (2.5 mg/kg) to conscious rats, a pressor response was preceded by a transient fall in arterial blood pressure. After high dose of anandamide, the hindquarters vasoconstriction was followed by vasodilation. Intriguingly, in conscious rats, none of the hemodynamic responses to anandamide were found to be influenced by antagonism of CB1 receptors with AM251 [115], suggesting that the anandamide-evoked responses may not involve CB1 receptors. In contrast, in the presence of beta2 adrenoceptor antagonist ICI 118551 the hindquarters vasodilation was inhibited and the pressor response prolonged. Similar to anandamide, WIN55212-2 and HU-210 evoked a pressor response associated with renal and mesenteric vasoconstriction and hindquarters vasodi-

lation that was antagonized by AM251 and the beta2 receptor antagonist ICI 118551 [116], a finding consistent with the involvement of beta2 adrenoreceptors in the CB1 receptor-mediated hindquarters vasodilation. Notably, AM251 [116] has no noticeable effects on resting hemodynamics and blood pressure, suggesting negligible role of CB1 receptor-dependent signaling in cardiohemodynamics under normal conditions.

Conclusively, studies on anesthetized and conscious rats demonstrate complexity of hemodynamic effects of cannabinoids. While in anesthetiized rats, cannabinoids evoke a triphasic response, the most prominent of which is a sustained vasodilation, in conscious rats, cannabinoids evoke dose-dependent brief pressor response. In the absence of anesthetics, the only vascular bed that shows vasodilation is the hindquarters.

5.3 Effects of Cannabinoids on Reactivity of Isolated Vessels

The mechanisms of action of cannabinoids and cannabinoid-like substances on vascular cells have been widely studied in isolated vessel preparations with the use of wire myography. In a great number of isolated pre-contracted vascular preparations, cannabinoids produce vasodilation of varying degree, however, constriction responses have also been reported [117], emphasizing complex vascular cannabinoid pharmacology. While it is established that both CB1 and CB2 are distributed in both endothelial and vascular smooth muscle cells [21, 32, 40, 41], a link between stimulation of vascular cannabinoid receptors and vasodilation remains controversial, with the prevailing conclusions that the relaxation of healthy arteries may not require stimulation of vascular cannabinoid receptors. In fact, the mechanisms affecting vasomotor activity elicited by topically applied cannabinoids independently of CB1 and CB2 receptor stimulation are extremely versatile and seem to be predominantly responsible for both endothelium-dependent and -independent relaxation in a vast number of vascular beds.

Numerous studies indicate that the mechanisms of cannabinoid-induced vasodilation vary between species, vessel type and have regional differences [70, 118–120]. Conclusions of different research groups on the involvement of CB1 receptor in the responses of isolated vessels to anandamide and participation of endothelium-dependent mechanisms in these responses sometimes are controversial even with regard to the same vascular bed, adding some confusion into the topic [119, 121]. Thus, in isolated rat mesenteric artery, the relaxation to anandamide has been identified as endothelium- and CB1 receptor-independent [121, 122], whereas other groups showed endothelium-dependency of the response in the same artery with [119] or without [123] CB1 receptor involvement. It is possible that the choice of different constricting agents, unspecific CB1 antagonists and the method of de-endothelization influenced the results obtained.

Anandamide at 30 μM was shown to relax U-46619-pre-contracted rat aortic rings by 22% in endothelium-independent manner [124]. The relaxation was unaffected by rimonabant, AM251 and capsaicin, but was reduced to 13% in pertussis toxin-pre-treated preparations, allowing the authors to suggest the involvement of yet unidentified non-CB1/CB2 cannabinoid receptor located on smooth muscle cells [124]. In other study [125], anandamide (100 μM) gradually relaxed rat aortic rings pre-contracracted with phenylephrine by 51% within 7–10 min before reaching a plateau and the relaxation was reduced to 20% following removal of the endothelium. The relaxation was insensitive to pre-treatment with rimonabant and the CB2 antagonist SR144528, but was inhibited by O-1918, allowing the authors to suggest the involvement of unidentified non-CB1/CB2 cannabinoid receptor located on endothelial cells [125]. In rat aortic rings pre-contracted with the combined presence of U-46619 and methoxamine, the endothelium-dependent relaxation to anandamide was shown to be insensitive to CB1 and CB2 receptor antagonists, but inhibited by peroxisome proliferator-activated receptor (PPAR) gamma antagonist

GW9662 [59], suggesting that the relaxation is mediated by stimulation of nuclear PPAR gamma receptor. In phenylephrine-pre-contracted aortic rings isolated from normotensive sham-operated rats subjected to excision of the left renal artery without clipping, the maximal relaxation to 30 μM anandamide amounted 4% only [20]. As could be seen from these studies, the proposed mechanisms governing the anandamide-evoked relaxation of the same vascular preparations principally differ between different research groups. The reasons for the discrepancies are unclear but might be related to variations in wire myography protocols or the constricting agent used (a thromboxane mimetic U-46619 vs. phenylephrine).

Obviously, varying outcomes of the studies as for the requirement for the given cannabinoid receptor in the vasodilation to cannabinoids are unlikely to be solely explained by intrinsic variations in the cannabinoid receptor expression between the vascular beds. A part of the problem is that the pharmacology and molecular modes of action of cannabinoid receptor agonists and antagonists developed and widely used in wire myography studies as selective have not been clearly defined. Mounting number of studies points for additional, cannabinoid-receptor-independent effects and targets, such as PPAR, TRPV4 and large conductance calcium-activated potassium (BK_{Ca}) channels. Employment of highly selective cannabinoid receptor agonists and antagonists with precise molecular mechanisms of action is essential to delineate possible role of cannabinoid receptors in vascular effects of cannabinoids in health and disease. Another possible contributor into variable outcomes of the studies is the choice of technique to capture the mechanisms of vascular cannabinoid signalling and clarify whether the response requires specific receptor. Electrophysiological studies on isolated endothelial and smooth muscle cells and intact vascular preparations allowed to identify a large number ion-transporting systems targeted by cannabinoids and cannabinoid-like substances, including BK_{Ca} channels [72, 126, 127], intermediate conductance calcium-activated potassium (IK_{Ca}) channels [53], voltage-gated Ca^{2+} channels of L and T type [128–130], TRPV4 [74], TRPA1 [131, 132], and the TASK subfamily of two pore domain K^+ channels responsible for background K^+ currents [133–136], Na^+-Ca^{2+} exchanger (NCX) [65, 127, 137, 138] and Na^+-K^+- ATPase [52, 139, 140]. All these players are present in the vasculature, determining the membrane potential of endothelial and smooth muscle cells, the release of variety of endothelium-derived vasoactive substances and contractile responses to cannabinoids.

5.3.1 Endothelium-Independent Relaxation to Cannabinoids

The earliest study describing the effect of locally applied endocannabinoids on vascular reactivity showed that in anesthetized rabbits, topically applied anandamide and THC dilate cerebral arterioles [141]. The dilation was suggested to be mediated by the release of endogenous arachidonic acid. The study initiated an intense research into the effects and the mechanisms of action of cannabinoids on blood vessels. In experiments performed on pre-contracted rat superior mesenteric arterial bed, anandamide was found to induce endothelium-independent relaxation that was suppressed by the CB1 receptor inhibitor rimonabant [39]. Relaxations to carbachol and Ca^{2+} ionophore A23187 were also sensitive to rimonabant, suggesting that carbachol and anandamide share the mechanisms of action and that anandamide is an endothelium-derived hyperpolarizing factor (EDHF), an entity responsible for endothelium-dependent relaxation under conditions of inhibition of nitric oxide (NO) synthesis. This mechanism was also proposed to govern the relaxation in rat coronary artery [142]. Since these observations, much attention has been given to investigation of the mechanisms of vasoactive effects of anandamide and other cannabinoids. However, in the rat isolated perfused mesenteric vascular bed, HU-210, WIN55212-2 and THC failed to cause vasodilation and even produced constriction [143]. The possibility that anandamide acts as a mediator of NO-independent vasodilation to endothelium-dependent vasodilators was extensively evaluated in late 1990s in

different vascular beds, including isolated rat mesenteric arteries [70, 118, 144, 145], rat hepatic [146] and rat coronary arteries [147], guinea-pig basilar artery [148] and in anaesthetized rabbits [149] with general conclusion that anandamide is not EDHF in these vascular beds.

5.3.1.1 Potential Role of Cannabinoid Receptors in Vasodilation

The involvement of cannabinoid receptors in the vasodilation to anandamide and other cannabinoids in different vascular beds has been most extensively investigated using wire myography approach with the help of pharmacological modulators of cannabinoid receptors. Less often, pressure myography is used. In a typical wire myography protocol, the excised vessels are cut into approximately 2 mm-long segments and the rings are attached to hooks mounted in a Mulvany-Halpern wire myograph [150] and then properly stretched to achieve a largest contractile response to a submaximal dose of one of the contractile agents such as phenylephrine, methoxamine or U-46619, a stable thromboxane A2 receptor agonist. Following stabilization of the tone, the substances of interest are applied and the vasorelaxation is computed as the ratio to the imposed contraction. In wire myography, tension is measured under isometric conditions. The limitation of the method is that optimum resting tension is not the same as physiological and the attachment of vessel ring leads to nonphysiological geometry and loading [151, 152]. It should be noted, that in the absence of imposed contraction, i.e. in the absence of stimulated Ca^{2+} entry into smooth muscle cells, most of the cannabinoids fail to cause a significant changes in the baseline tension and THC elicits a significant contraction [124].

While the majority of studies concluded that neither CB1, nor CB2 receptors are involved in endothelium-dependent or –independent vasodilation to cannabinoids in healthy arteries [69, 70, 122, 123, 144, 153–155], some limited number of reports point for engagement of CB1 receptors [37, 43, 67, 119, 156–158] or CB2 receptors [37, 40] in the vasodilation. In many early and recent vascular myography studies, rimonabant and structurally very close compound AM251 have been the most widely employed CB1 receptor antagonists. Rimonabant was first described by Sanofi Aventis as a selective and orally active inverse CB1 receptor agonist with CB1 affinity in low nanomolar range [159]. It should be noted that although the low nM concentrations of rimonabant and AM251 are required to block CB1 receptors, these compounds are frequently used in low micromolar (1–3 μM) concentrations in assessing the role of CB1 receptor in the vasodilation [124, 125, 158, 160, 161]. At these concentrations, these CB1 antagonists display a number of CB1 receptor-independent effects. Thus, at 1 μM rimonabant inhibits cannabinoid-induced hypotension and mesenteric vasodilation via a target distinct from CB1 receptor, as the effect is observed in CB1 and CB1/CB2 knockout mice [123]. The unspecific effects of rimonabant include inhibition of myo-endothelial gap junctions [162], endothelium-dependent relaxation to carbachol, acetylcholine, bradykinin, Ca^{2+} ionophor A23187 and ionomycin [39, 142, 143, 157, 162], direct suppressive action on the BK_{Ca} channel function [163] and Ca^{2+} entry mechanism [164]. In the isolated quinea pig carotid artery, rimonabant at concentrations 0.1–10 μM hyperpolarizes vascular smooth muscle cells by up to 10 mV and significantly inhibits the smooth muscle cell hyperpolarization evoked by acetylcholine [70, 118]. Rimonabant at 10 μM was shown to strongly attenuate the relaxation of cannulated pre-contracted rat mesenteric artery induced by levcromacalim, an opener of ATP-sensitive K^+ (K_{ATP}) channel [122]. Similar to rimonabant, AM251 was found to have a number of non-specific effects [54, 131, 163, 165–167]. Interestingly, the activation of TRPV1 channels by anandamide was reported to be antagonized by rimonabant and AM251, although at concentrations higher than those required for CB1 antagonism. Collectively, the wealth of data points for non-specific effects of widely used CB1 receptor antagonists rimonabant and AM251, warranting considerations in interpretation of the relevant data. Cannabinoid receptor-independent targets for CB1 antagonists rimonabant and AM251 and for the cannabidiol

Table 5.1 Cannabinoid receptor-independent targets for rimonabant, AM251 and O-1918 in the vasculature

CB antagonists	Site of action	Action	References
Rimonabant	Myo-endothelial gap junctions	Inhibition	[162]
Rimonabant	BKCa	Inhibition	[163]
Rimonabant	Unidentified Ca^{2+} entry	Inhibition	[164]
Rimonabant	Resting MP of SMC	Hyperpolarization	[70]
Rimonabant	Ach-evoked SMC hyperpolarization	Inhibition	[118]
Rimonabant	KATP opener-evoked SMC hyperpolarization	Inhibition	[122]
AM251	TRPA1	Activation	[131]
AM251	BKCa	Inhibition	[163]
O-1918	BKCa	Inhibition	[163]
O-1918	NCX	Inhibition	[127]

analogue O-1918 widely used as a "selective" antagonist of "endothelial cannabinoid receptor" are listed in Table 5.1.

Vasoactive properties of anandamide are most frequently studied among other cannabinoids. There is a general consensus that this endocannabinoid elicits a relaxation in a vast number of isolated pre-contracted vascular preparations with both endothelium-independent and –dependent mechanisms. The results published are somewhat controversial even when the studies from the same vascular bed are compared.

Anandamide was shown to elicit endothelium-independent relaxation of pre-contracted rat small mesenteric artery [39, 121, 143, 145, 157, 168, 169], rat coronary artery [168], rat aorta [124]. In another study performed on isolated rat mesentery pre-contracted with phenylephrine, de-endothelization slightly but significantly reduced the relaxation to anandamide [143]. Removal of the endothelium fails to inhibit the relaxation of pre-contracted rat gastric arteries in response to the stable anandamide analoque methanandamide [69] and the anandamide-evoked smooth muscle cell hyperpolarization in the rat small mesenteric artery [118]. In other study performed on isolated rat small mesenteric artery, the hyperpolarization to anandamide was endothelium-dependent and sensitive to a selective inhibitor of ATP-sensitive K^+ (K_{ATP}) channel glibenclamide, but not rimonabant [70].

In a number of isolated pre-contracted vascular preparations, including those from human [170] and rat pulmonary arteries [171], rabbit [162] and human mesenteric arteries [172], bovine [153] and sheep coronary artery [173], anandamide produces endothelium-dependent relaxation. In contrast, in isolated porcine coronary artery with or without endothelium, anandamide (30 μM) did not modify the tension and the membrane potential of smooth muscle cells [70].

Reports on the effect of 2-AG on reactivity of isolated vessels point for engagement of both endothelium-dependent [174] and –independent mechanisms [117, 174, 175]. 2-AG can dually modulate the contractile reactions, as both the relaxing and constricting responses have been described. Consistent with the described hypotensive effect [113, 176], 2-AG was shown to cause relaxation of isolated vascular preparations [30, 67, 174, 175, 177]. In isolated pre-contracted bovine coronary arteries, the relaxation to 2-AG depends on the intact endothelium and is blocked by inhibition of phospholipase C, FAAH, cyclooxygenase (COX) and cytoxhrome C450 [30]. In contrast, in rabbit pre-contracted mesenteric arteries, the relaxation to 2-AG is endothelium-independent but sensitive to rimonabant [175]. In mesenteric arteries isolated from patients undergoing surgical treatment of bowel carcinoma and inflammatory bowel disorders, the relaxation to 2-AG is endothelium-independent and is insensitive to the antagonists of CB1 and CB2 receptors AM251 (100 nM) and AM-630 (100 nM) [177]. De-sensitization of TRPV1 channels and FAAH inhibition failed to affect the dilation that was reduced by the COX-1 inhibitors [177].

In isolated rat aorta, 2-AG is ineffective at influencing the basal tone, but in pre-contracted rings, the endocannabinoid induces a biphasic response consisting of a transient relaxation followed by a sustained constriction [117], the responses being unaffected by endothelial denudation and inhibition of both types of cannabinoid receptors. Unlike in human mesenteric artery, in rat aorta, pretreatment with COX-1 inhibitor indomethacin failed to inhibit the weak relaxation to 2-AG, however, abolished the contraction phase both in endothelium-intact and denuded rings.

Experimental data derived from the study of the effect of THC, a CB1 agonist, on vascular contractility, also fails to support the engagement of CB1 receptor in the dilator response. In pre-contracted rat superior mesenteric artery, 3 μM THC elicited a marginal relaxation amounting 4% only. The relaxation was enhanced to 16% in the presence of the COX inhibitor indomethacin [120], pointing that the stimulated release of vasoconstrictor prostanoids masks the vasodilation. De-endothelization and 100 nM rimonabant had no effect on the relaxation. Higher concentrations of THC (10–100 μM) elicit a rimonabant-sensitive vasoconstriction that was converted to a weak relaxation following de-endothelization [120]. In third order branches of mesenteric artery, however, THC was shown to elicit endothelium-independent vasorelaxation, sensitive to charybdotoxin, a dual inhibitor of BK_{Ca} and IK_{Ca} channels, and apamin, a selective blocker of small conductance Ca^{2+} -activated K^+ channel, but insensitive to antagonists of CB1 and TRPV1 receptors. In these arteries, THC was shown to inhibit the contractile response elicited by Ca^{2+} re-addition, indicating for inhibition of Ca^{2+} influx into smooth muscle cells [120].

5.3.1.2 The Role of TRPV1 Channels

Vasorelaxation to anandamide may involve stimulation of TRPV1 receptor as first demonstrated in isolated rat hepatic and mesenteric arteries and guinea pig basilar arteries [155]. The relaxation was shown to be abolished after treatment with capsazepin, a TRPV1 antagonist, and involves calcitonin gene-related peptide released from perivascular sensory nerves [155]. In that study, rimonabant (0.3 μM) failed to inhibit the vasodilator effect of anandamide. Neither 2-AG, nor synthetic CB1 and CB2 agonists were able to mimic the effect of anandamide [155], strongly indicating that neither of the known cannabinoid receptors mediate the relaxation of these arterial beds. However, the anandamide-induced vasodilation was abolished by capsazepine, a selective TRPV1 antagonist. The vasodilation was not reproduced following TRPV1 desensitization by pretreatment with capsaicin, a selective TRPV1 agonist. Following this original observation, several other studies confirmed, at least partial engagement of this mechanism in the dilation to anandamide [119, 121, 122]. However, no evidences for TRPV1 role in vasodilation to anandamide were obtained in the rat pulmonary [171] and rat coronary [168] arteries. The role of TRPV1 in relaxation to anandamide was further confirmed in pre-contracted small mesenteric artery, where anandamide was shown to produce endothelium-independent relaxation that was reduced following capsaicin pre-treatment [119, 121]. In the study of Ho and Hiley [121], the relaxation to anandamide, although was sensitive to 3 μM rimonabant, was unaffected by AM251 (3 μM) or CB2 receptor inhibitor SR144528, suggesting that perivascular TRPV1, but not CB1, plays a major role in the relaxation. In contrast, in the study of O'Sullivan et al. [119] the relaxation of small mesenteric arteries to anandamide was partially endothelium-dependent and reduced by both rimonabant (100 nM) and AM251 (100 nM), implicating activation of CB1 receptors. The only difference between the studies is that in the study of Ho and Hiley [121] the arteries were pre-contracted with α1-adrenergic receptor agonist methoxamine, while in the study of O'Sullivan [119], U-46619, a thromboxane A_2 receptor agonist, was used as a constrictor. Similarly, in isolated rat gastric arteries, methanandamide, a metabolically stable analogue of anandamide, induces endothelium-independent relaxation [69] insensitive to AM251 and the CB2 receptor antagonists AM-630 and SR144528 when applied either alone or in combination. The authors found that the addition of exogenous

CGRP relaxed pre-contracted arteries and capsaicin- and capsazepine pre-treatment only slightly inhibited the relaxation to methanandamide. Ca^{2+} -induced vasodilation was found to be inhibited in the presence of methanandamide, suggesting that the endocannabinoid induces smooth muscle relaxation by CB1 and CB2 receptor-independent inhibition of Ca^{2+} entry [69]. In rat mesenteric and gastric arteries, methanandamide and anandamide induce a slowly developing smooth muscle cell hyperpolarization that is reproduced by exogenous CGRP and abolished by capsazepine.

The expression of the mRNA coding for the TRPV1 receptor was detected also in endothelial cells from rat mesenteric artery, where anandamide at nanomolar concentrations was shown to elicit an acute release of NO secondary to stimulation of endothelial TRPV1 [178].

5.3.1.3 The Role of KCa Channels in Endothelium-Independent Vasodilation

Several lines of evidences point for involvement of Ca^{2+} -dependent K^+ (KCa) channels in endothelium-independent relaxation to cannabinoids occurred independently on cannabinoid receptors. In isolated pre-contracted segments of rat superior mesenteric artery, the relaxation to anandamide is associated with the smooth muscle cell hyperpolarization due to activation of BK_{Ca} and, likely, IK_{Ca} channels [144]. The relaxation was found to be endothelium- and CB1 receptor-independent, as rimonabant even at 5 μM failed to modify the relaxation, and HU-210, a selective CB1 receptor agonist, and WIN55212-2, a non-selective cannabinoid receptor agonist, failed to reproduce the effect of anandamide [144]. This observation is in line with electrophysiological studies performed on isolated rat mesenteric artery, where anandamide-evoked smooth muscle cell hyperpolarization is unaffected by 1 μM rimonabant [70, 118]. In addition, neither the synthetic CB1 receptor agonists HU-210 and WIN55212-2, nor palmitoylethanolamide, a CB2 receptor agonist, affected the membrane potential of the smooth muscle cells [70, 118]. In smooth muscle cells of the main mesenteric artery [118], guinea pig carotid artery and porcine coronary artery [70], anandamide at up to 30 μM failed to produce shifts in the membrane potential.

In isolated pre-contracted rat aortic rings, arachidonylcyclopropylamide (ACPA), a selective CB1 receptor agonist, elicits a weak relaxation (~20%) occurred independently of the presence of endothelium [43]. The relaxation was reduced to 9% in the presence of iberiotoxin, a selective BK_{Ca} inhibitor. In rat superior mesenteric artery, the relaxation to 30 μM ACPA was reduced by endothelial denudation from 18% to 8% [40]. In rings with intact endothelium, iberiotoxin diminished the relaxation to 9%, while in endothelium-free rings, the BK_{Ca} inhibitor fully inhibited the relaxation [40].

5.3.1.4 Inhibition of Ca^{2+} Entry as a Mechanism of Relaxation to Cannabinoids

In order to observe the relaxation to cannabinoids, they are administered to pre-contracted arterial segments, i.e under conditions of pre-stimulated Ca^{2+} entry into smooth muscle cells. Clearly, any interference with any of the Ca^{2+} entry pathways would decrease the tone. In a number of vascular myography studies an inhibition of Ca^{2+}-induced relaxation by cannabinoids and their analogues was observed, indicating that one of the mechanisms of endothelium-independent vasorelaxation to these compounds is an inhibition of Ca^{2+} entry into smooth muscle cells [69, 121, 157, 179]. These observations are supported by electrophysiological studies that identified anandamide as a direct inhibitor of NCX [65, 127], and voltage gated Ca^{2+} channel of L- and T- type [180, 181]. Noteworthy, both NCX and L- type Ca^2 channels are crucial determinant of the contraction imposed by norepinephrine and phenylephrine [182, 183] and targeting these systems by cannabinoids should be considered in assessing the mechanisms of vasodilation elicited by cannabinoids.

5.3.2 Endothelium-Dependent Relaxation to Cannabinoids

Endothelium-dependent component of relaxation to anandamide and some other endogenous lipid signaling molecules belonging to N-acyl amino acids, such as N-arachidonoyl glycine, NAGly, [184, 185] and N-arachidonoyl L-serine [186], has been reported in a number of vascular beds with the dominated but yet unproven hypothesis that a novel endothelial G-protein coupled cannabinoid receptor, the so called endothelial cannabinoid receptor (CBe) also referred in literature as abnormal cannabidiol or endothelial atypical cannabinoid receptor, underlies the dilation [109, 123, 143, 154, 161, 172, 187]. Despite some inconsistencies in the proposed signalling mechanisms reviewed earlier [188], the observed sensitivity of cannabinoid-evoked vasodilation to high (micromolar) concentration of CB1 receptor blockers rimonabant and AM251, and the cannabidiol analogue O-1918 was explained by a possibility that these compounds selectively target CBe receptor that remained to be identified [73, 109, 119, 123, 143, 160, 170, 172, 184, 189–191]. Stimulation of CBe was postulated to be coupled to EDHF [119] and NO [184] release, leading to a delayed hypotension in anesthetized rats [109].

The "endothelial cannabinoid receptor" hypothesis has been challenged by recent electrophysiological demonstrations that cannabinoids, cannabinoid-like substances and synthetic analogues at concentrations that are commonly considered to be CBe specific, bidirectionally efficiently affect the BK_{Ca} activity in cell-free patches excised from native cells and cells heterologously expressing BK_{Ca} α, α+β1 or α+β4 subunits [72, 126, 163, 192–195], the effect being dependent on the plasma membrane cholesterol content [126, 194]. The topic of lipid- and cannabinoid-induced regulation of BK_{Ca} channel function is covered in a review of Bukiya and Dopico [196]. Cholesterol, an essential component of eucariotic plasma membranes, plays an important role in regulation of the activity of a number of membrane proteins [197–199], including TRP [200, 201], BK_{Ca} channels [202, 203] and cannabinoid signaling [204]. The latter is achieved via direct interaction between cholesterol and anandamide molecules by the establishment of a hydrogen bond and via regulation of interaction between anandamide and CB1 receptor [205, 206]. Consistent with view that direct cannabinoid-BK_{Ca} targeting is responsible for the relaxation, the hyperpolarizing effect of NAGly, a proposed ligand for GPR18 and a candidate for CBe [189, 207], was shown to be fully intact in endothelial cells following intracellular dialysis with GPR18 antibody [163]. A number of studies have provided evidences against the GPR18 involvement in the action of NAGly [127, 208–210], prompting a reconsideration of the concept of existence of a third type cannabinoid receptor required for endothelium-dependent vasodilation to cannabinoids.

Apart of direct targeting BK_{Ca} channels, NCX, TRPV1, V4, PPAR gamma and voltage-dependent Ca^{2+} channels, vasodilation to cannabinoids may also require cannabinoid interaction with vascular CB1 and CB2 receptors. In human mesenteric arteries collected during surgical operations from patients suffering from bowel carcinoma and inflammatory bowel disease, the CB1 receptor was concluded to mediate endothelium-dependent vasodilation to anandamide, with no evidence for engagement of CB2 receptor [172]. Cannabidiol, a phytocannabinoid wih low affinity to CB1 and CB2 receptors that exhibits high potency as an antagonist of both CB_1 and CB_2 receptors (Thomas et al. [244]), was shown to induce endothelium-dependent vasodilation of human mesenteric arteries sensitive to blockers of CB1 receptor and TRPV1 channels [172]. A synthetic CB1 receptor agonist CP55,940 produced a greater relaxation compared to that evoked by anandamide. In endothelium-intact rings obtained from rat superior mesenteric arteries, a highly potent CB1 receptor agonist ACPA (EC_{50} = 2.2 nM) used at extremely high concentration 30 μM was shown to relax pre-contracted arteries by 18% in NO-dependent manner [40] and the relaxation was partially BK_{Ca}-dependent. Endothelial denudation reduced the vasodilator effect of ACPA to 7.7%. In this study, a CB2 receptor agonist JWH-133 (30 μM) relaxed pre-

Table 5.2 Summary of the proposed cannabinoid receptor-dependent and -independent sites governing relaxation to cannabinoids

Target	Ligand	Cell type	Vascular bed	References
CB1R	AEA	SMC	RMA	[190]
CB1R	AEA, CP55,940, HU-210	SMC	RMA	[157]
CB1R	ACPA	SMC	RA	[43]
CB1R	AEA	EC, SMC	RSMA	[120]
CB1R	AEA, ACEA	EC, SMC	RMA	[37]
CB1R	ACPA	EC	RMA	[40]
CB1R	2-AG	SMC	RPA	[67]
CB2R	AEA, JWH-015	EC, SMC	RMA	[37]
CB2R	JWH-133	SMC	RMA	[40]
TRPV1	AEA	SMC	RSMA, RMA, RHA, GPBA	[122, 123, 146]
TRPV4	2-AG	EC	RSMA, RA	[75]
Cav1.2	ACPA	SMC	RA	[43]
Cav1.2	AEA	CM		[180]
Cav3.1/3.2	AEA	Cloned channels		[54]
NCX	AEA, NAGly, LPI	EC, EA.hy926	MA	[128, 137]
NCX	AEA	CM		[180]
BKCa	ACPA	SMC	RMA	[40]
BKCa	AEA, NAGly	EC, EA.hy926	MA	[6, 128, 163, 194]
BKCa	NAS	Transfection model		[4]
IKCa	LPI	EA.hy926		[53]
Na-K ATPase	LPI	EA.hy926		[52]
TASK-1	AEA	SMC	RMA, RPA, HPA	[135, 136]
PPAR gamma	AEA	AEA	RA	[59]

CM cardiac myocytes, *EC* endothelial cells, *HPA* human pulmonary artery, *PPAR* peroxisome proliferator-activated receptor, *RA* rat aorta, *RMA* rat mesenteric artery, *RSMA* rat small mesenteric artery, *RabPA* rabbit pulmonary artery, *SMC* smooth muscle cells, *RPA* rat pulmonary atery, *MA* mouse aorta

contracted mesenteric arteries by 14% in NO-dependent manner. Similar results were obtained in another study performed in isolated endothelium-intact rat mesenteric arteries [37], where anandamide, ACPA and the CB2 agonist JWH-015 elicited concentration-dependent vasodilation. Blockade of TRPV1 channels partially decreased the relaxation, while the blockade of CGRP receptors completely inhibited the relaxation to anandamide. Pre-treatment of vascular preparations with AM251 and the CB2 antagonist AM630, only slightly decreased the relaxation responses to anandamide allowing to conclude that CB1, CB2 and TRPV1 mediate the relaxation to anandamide [37]. The conclusion of the two aforementioned studies on involvement of both CB1 and CB2 receptors in the vasorelaxation of rat mesenteric artery to cannabinoids contrasts with that derived by others [70, 118, 125, 143, 144]. Electrophysiological studies addressing the effect of CB2 receptor agonists on electrical responses of vascular cells are quite limited. In healthy arteries, CB2 receptor agonists failed to elicit measurable shifts in membrane potential either in smooth muscle [70, 118] or endothelial cells [6]. The action potential-driven endocannabinoid release in hyppocampal pyramidal cells is accompanied by a long-lasting CB2-dependent hyperpolarization [25]. Table 5.2 summarizes the key findings on the cannabinoid receptor-dependent and -independent targets governing relaxation to cannabinoids.

5.4 Dysregulation of Vascular Endocannabinoid Signaling in Disease States

Circulating levels of endocannabinoids and cannabinoid receptor expression are altered in disease states. Under various pathophysiological conditions accompanied by vascular abnormalities, the vasodilator potencies of cannabinoid receptor agonists differ from those observed in healthy arteries. Cannabinoids may exert both the beneficial and deleterious effects on cardiovascular system [38, 82]. The majority of beneficial effects of cannabinoids have been ascribed to CB2 receptor stimulation. Signalling via CB1 and CB2 receptors differentially affects vascular inflammation. Stimulation of vascular and cardiac CB1 receptors contributes to pathophysiology of various cardiovascular diseases via promotion of oxidative and nitrosative stress, activation of mitogen-activated protein kinase and cell demise [66, 211–214]. In contrast, a wealth of experimental data indicate that stimulation of CB2 receptors displays cardioprotective effect [215–218], reduces cerebral ischemic injury [219, 220], limits inflammation, oxidative/nitrosative stress, cell demise [221], progression of atherosclerosis [3, 214, 222–224], prevents nephrotoxicity [225]. An increased expression level of CB2 receptors in the cardiovascular system under pathophysiological conditions, such as inflammation and tissue injury, is considered to represent a compensatory protective mechanism [216, 226–228]. In the vasculature, CB2 receptor protein expression was shown to be up-regulated under some pathophysiological conditions, including atherosclerosis, inflammatory insults and DOCA-salt hypertension model [21, 226, 229–231].

In mesenteric arteries excised from young obese rats, anandamide produced an attenuated endothelium-dependent relaxation [37]. The reduction was accompanied by a decreased CB1, CB2, but not TRPV1, protein expression level. In this model, the relaxation responses to acetylcholine and CB1 and CB2 receptor agonists ACPA and JWH-015 were also decreased. The responses were, however, restored following pre-incubation of the arteries with FAAH inhibitor URB597, indicating that an increased anandamide degradation is responsible for attenuation of the relaxant responses.

Overactivation of cannabinoid system contributes to an increased vasodilation and hypotension. Thus, in patients with cirrhosis, the plasma anandamide and the CB1 receptor expression levels in vascular endothelial cells are elevated [42, 232]. The vasodilator state in chronic liver cirrhosis and hypotension in advanced cirrhosis is determined by activation of vascular endothelial cells cannabinoid CB1 receptors by endogenous cannabinoids and is reversed by the rimonabant treatment [42]. In cirrhotic mesenteric vessels, an increased relaxation to anandamide is mediated by an enhanced signalling via CB1 receptors, perivascular TRPV1 channels and vascular KCa channels [233, 234]. However, while in cirrhotic liver tonic CB1 stimulation plays a pathophysiological role determining chronic vasodilator state, in spontaneously hypertensive rats, chronic CB1 activation seems to represent a part of protective mechanism directed to reduction of blood pressure. In normotensive rats, CB1 receptor antagonism had no effect on blood pressure and other hemodynamic parameters. However, in spontaneously hypertensive rats (SHR), the expression of CB1 receptor is increased in heart and aortic endothelium as compared with Wistar-Kyoto rats, and rimonabant elicited a further increase in blood pressure and myocardial contraction with no change in heart rate [2], pointing at protective effect of tonic CB1 receptor activation. As intracerebroventricular microinjection of rimonabant did not influence blood pressure, it is highly expected that the effect of intravenous rimonabant is mediated by peripheral mechanisms [2]. Similar hypertensive effects were observed with AM251 when administered to either hypertensive salt-sensitive Dahl rats maintained on high salt diet, or to rats with angiotensin II–induced hypertension. Elevation of the endogenous anandamide level by FAAH inhibitor URB597 had no detectable hemodynamic effect in control rats, however, decreased arterial blood pressure in hypertensive rats [2, 235]. The hypotensive effects of WIN55212-2

are described in conscious rats with several forms of experimental hypertension, including SHR rats and Wistar rats made acutely hypertensive by infusion of angiotensin II and arginine vasopressin [235, 236], but not in transgenic hypertensive rats [102] Consistent with the upregulated role of endocannabinoid system in blood pressure regulation in hypertension, anandamide dose-dependently decrease the mean arterial blood pressure in conscious hypertensive, but not normotensive, rats [235]. While in normotensive rats WIN55212-2 elevates blood pressure, in acutely hypertensive rats WIN55212-2 produces hypotensive effect that was attenuated by AM251 [235]. In conscious hypertensive transgenic rats, however, the pressure and vasoconstrictor effect of WIN55212-2 are little affected [102].

In hypertension, alterations in cannabinoid signalling are model-specific. Thus, while in SHR both cardiac and plasma levels of anandamide and 2-AG are decreased, in DOCA-salt model, the endocannabinoid levels are elevated [231]. In both models, the CB1 receptor expression is higher in the heart and aortic endothelium [2, 33, 231]. However, higher CB2 receptor expression was detected only in DOCA-salt model [231]. Injection of THC (1.5 mg/kg) to rats with experimental renal hypertension elicited a significant decrease in blood pressure and heart rate [237]. The reaction developed within 15 min and in 24 h the parameters returned to the initial levels. However, daily THC injections for 3–5 weeks did not produce any difference in the heart rate and the systolic blood pressure between the control and hypertensive groups.

Numerous beneficial cardiovascular effects have been reported for in vivo cannabidiol treatment in a number of disorders. It was shown that cannabidiol treatment improves endothelium-dependent relaxation in mesentery of diabetic fatty rats and leads to improvement of serum biomarkers [238], prevents cerebral infarction [239], is cerebroprotective via cannabinoid receptor-independent pathway [240, 241], attenuates cardiac dysfunction and vascular inflammation, reduces infarct size, oxidative stress and inflammatory pathway in diabetic cardiomyopathy [242, 243].

5.5 Concluding Remarks

Cannabinoids, through central and local mechanisms, affect key cardiovascular parameters in health and disease, such as heart rate, blood pressure, vascular and cardiac contractility and inflammation. Studies over the last decades demonstrated that endocannabinoid system is overactivated under pathological conditions and plays both a pathophysiological role, such as in disease states associated with excessive hypotension, and a protective compensatory role, such as in some forms of hypertension and inflammatory conditions. Mechanisms of local regulation of vascular reactivity by cannabinoids include modulation of a number of ion transporting systems. This modulation may be accomplished either in cannabinoid receptor-dependent or –independent mechanisms. A main emphasis on the local regulation of cardiovascular function by endocannabinoids has been devoted to examining the role of specific type of cannabinoid receptors in vasodilation. Ironically, cannabinoid receptor antagonists of first generation designed and widely used in functional myography assays as selective were later found to be of low selectivity, displaying off-target effects on a number of ion transporting systems. Consequently, the conclusions yielded are at times speculative and controversial, often overlooking receptor-independent effects of cannabinoids and cannabinoid receptor blockers. To advance our understanding of cannabinoid actions on the vasculature, more information is essentially needed on the impact of selective cannabinoid receptor stimulation in disease states and the mechanisms of direct action of cannabinoids on the function of endothelial and smooth muscle cell ion channels and how these effects are translated into mechanotransduction, regulation of inflammation, angiogenesi, etc. Similar to steroids, general anesthetics and alcohols, cannabinoids modulate the function of a number of ion channels. An important still unanswered question is whether the given effect of cannabinoids requires direct interactions between cannabinoid molecules with specific channel protein subunit or the effect is indirect, due to change in lipid composition of the plasma membrane and chang-

ing the physical parameters of plasmalemma. Taking into account the vital role of potassium and TRP channels in physiology and pathophysiology of cardiovascular system, better insights into the intrinsic mechanisms of modulation of the channel function by cannabinoids, would not only advance our basic knowledge of local modulation of vascular function by cannabinoids, but pave the way for development of new selective cannabinoid receptor and ion channel modulators and their therapeutic application.

Acknowledgements The author gratefully acknowledges financial support from the Austrian Science Fund, FWF, grant # P27238-B27.

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Endocannabinoid System and Alcohol Abuse Disorders

6

Balapal S. Basavarajappa

Abstract

Δ^9-tetrahydrocannabinol (Δ^9-THC), the primary active component in *Cannabis sativa* preparations such as hashish and marijuana, signals by binding to cell surface receptors. Two types of receptors have been cloned and characterized as cannabinoid (CB) receptors. CB1 receptors (CB1R) are ubiquitously present in the central nervous system (CNS) and are present in both inhibitory interneurons and excitatory neurons at the presynaptic terminal. CB2 receptors (CB2R) are demonstrated in microglial cells, astrocytes, and several neuron subpopulations and are present in both pre- and postsynaptic terminals. The majority of studies on these receptors have been conducted in the past two and half decades after the identification of the molecular constituents of the endocannabinoid (eCB) system that started with the characterization of CB1R. Subsequently, the seminal discovery was made, which suggested that alcohol (ethanol) alters the eCB system, thus establishing the contribution of the eCB system in the motivation to consume ethanol. Several preclinical studies have provided evidence that CB1R significantly contributes to the motivational and reinforcing properties of ethanol and that the chronic consumption of ethanol alters eCB transmitters and CB1R expression in the brain nuclei associated with addiction pathways. Additionally, recent seminal studies have further established the role of the eCB system in the development of ethanol-induced developmental disorders, such as fetal alcohol spectrum disorders (FASD). These results are augmented by *in vitro* and ex vivo studies, showing that acute and chronic treatment with ethanol produces physiologically relevant alterations in the function of the eCB system during development and in the adult stage. This chapter provides a current and comprehensive review of the literature concerning the role of the eCB system in alcohol abuse disorders (AUD).

Keywords

Marijuana · Cannabinoid receptors · FASD · Synaptic plasticity · Learning and memory · CREB

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A. N. Bukiya (ed.), *Recent Advances in Cannabinoid Physiology and Pathology*,
Advances in Experimental Medicine and Biology 1162, https://doi.org/10.1007/978-3-030-21737-2_6

Abbreviations

Δ^9-THC	Δ^9-tetrahydrocannabinol
2-AG	2-arachidonylglycerol
$5HT_3$	serotonin type 3
AA	arachidonic acid
ABHD4	abhydrolase domain 4
AC	adenylate cyclase
AEA	arachidonoyl ethanolamine, or anandamide
AMPAR	amino-3-hydroxy-5-methyl--4-isoxazolepropionic acid receptor
Arc	activity-regulated cytoskeleton-associated protein
AUD	alcohol use disorders
BLA	basolateral amygdala
CaMKIV	calcium/calmodulin-dependent protein kinase IV
CB	cannabinoid
CB1R	CB1 receptors
CB2R	CB2 receptors
CBD	cannabidiol (CBD)
CDK5	a cyclin dependent kinase 5
CeA	central nucleus
CHO	chinese hamster ovary
c-JNK	c-Jun N-terminal kinase
CREB	cAMP-response-element binding protein
DAGL	Diacylglycerol lipases
eCB	endocannabinoid
EPSP	excitatory postsynaptic potential
ERK1/2	extracellular signal-regulated kinase 1/2
ERP	evoked related potentials
FAAH	fatty acid amidohydrolase
FAK	focal adhesion kinase
FASD	fetal alcohol spectrum disorders
GABA	γ-aminobutyric acid
GDE1	glycerophosphodiesterase
GPCR	G-protein coupled receptor
IP3	inositol 1,4,5-triphosphate
IPSC	inhibitory postsynaptic currents
KO	knock-out
LTP	long-term potentiation
MAGL	monoacylglycerol lipase
MAPK	mitogen-activated protein kinase
MeCP2	methyl-CpG-binding protein 2
MetAEA	R(+)-methanandamide
MLC	mantle cell lymphoma
msP	marchigian sardinian alcohol-preferring
NAc	nucleus accumbens
NAPE-PLD	N-acylphosphatidylethanolamine-specific phospholipase D
NMDA	*N*-methyl-D-aspartate
PFC	prefrontal cortex
PKA	protein kinase A
PLC	phospholipase C
PPARs	peroxisome proliferator-activated receptors
PTPN22	phosphatase
Rac1	Ras-related C3 botulinum toxin substrate 1
SNP	single nucleotide polymorphism
TRPV1	transient receptor potential vanilloid 1
VTA	ventral tegmental area
WT	wild type

6.1 Introduction

The knowledge of endogenous cannabinoids (eCBs) as an essential neuromodulatory system appeared more than two decades ago. However, the earliest anthropological evidence of cannabis use comes from the oldest known Neolithic culture in China. It was cultivated and used by humans in the production of hemp for ropes and textiles and as an intoxicant used for recreational and religious purposes [1]. Cannabis has been used in various cultures for centuries [For review see [2]]. The appreciation of eCBs was, however, delayed until the isolation and characterization of the psychoactive components of cannabis, such as cannabidiol (CBD) in 1940 by the Nobel laureate Lord Alan Todd [3] and Roger Adams [4] and Δ^9-tetrahydrocannabinol (Δ^9-THC) in 1964 by Raphael Mechoulam and Yechiel Gaoni [5]. Later, it was discovered that Δ^9-THC (Fig. 6.1) was mainly responsible for the psychotropic effects of *Cannabis* plant preparations [6, 7]. The Δ^9-THC binding site was identified after almost two decades of synthetic chemistry studies after

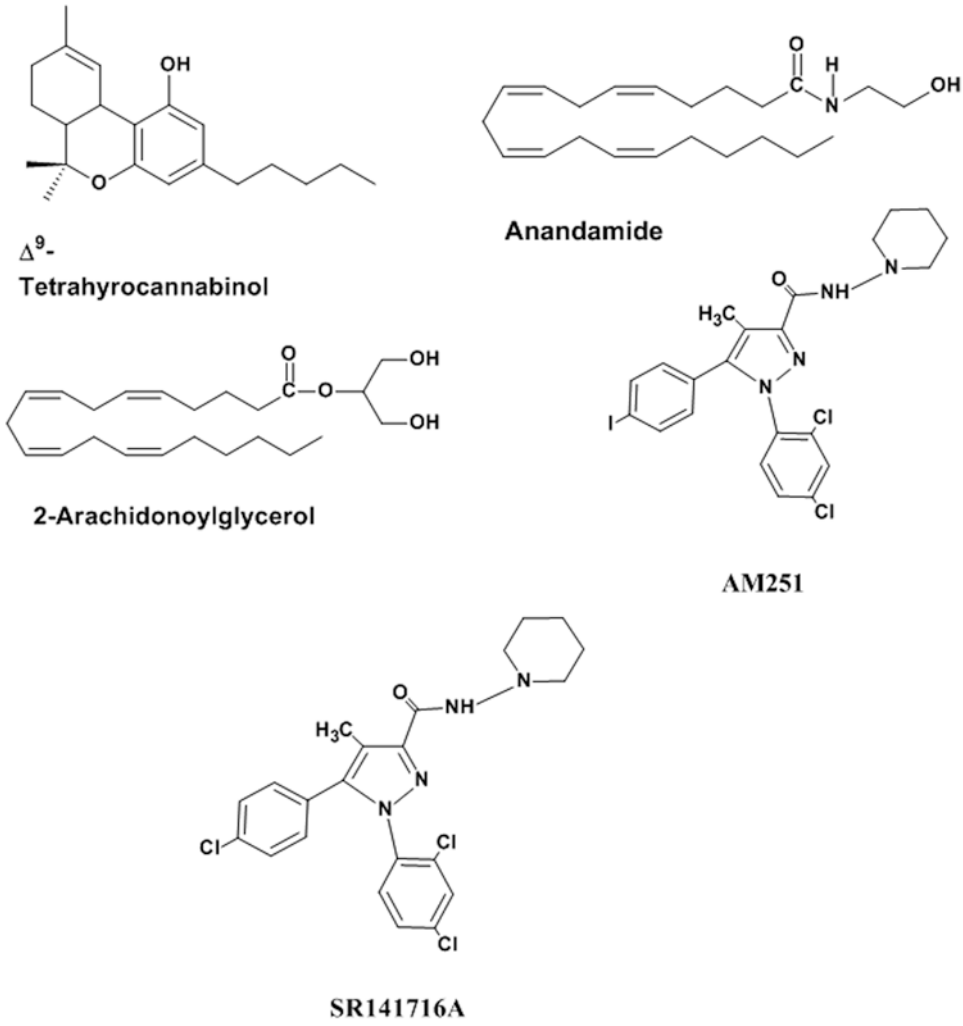

Fig. 6.1 The molecular structure of THC, AEA, 2-AG and CB1R antagonists

Δ^9-THC discovery. In 1990, an "orphan" G-protein coupled receptor (GPCR) was characterized as the first cannabinoid receptor type 1 (CB1R) in the brain [8], and, three years later, the cloning of a second peripheral receptor for cannabinoids (CBs) [9] (CB2R) was performed. The isolation of the first endogenous ligand for CB1R was reported in 1992, with the characterization of arachidonoyl ethanolamine, or anandamide (AEA) [10]. The 2-arachidonylglycerol (2-AG), as the second endogenous ligand for CB1R, was discovered in 1995 [11].

6.2 The Endocannabinoid System

The eCB system is now recognized as the most widely distributed system in the brain and in peripheral tissues. Its function is highly specific and localized [12, 13]. The eCB system has been described as one of the most widespread and versatile signaling systems ever identified (for review, see Refs [12, 14, 15]). The eCB system consists of lipid molecules (eCBs) that bind to and activate cannabinoid receptors (CB1R and CB2R). These lipid molecules are synthesized

from phospholipid precursors [16–21] and are released from cells following stimulation in a non-vesicular manner to act in a paracrine fashion [16, 18–22]. Therefore, the eCB system includes CB1R and CB2R, their endogenous lipid ligands (AEA and 2-AG) and eCBs synthesizing and degrading enzymes [13, 15]. The structures of endogenous and synthetic CB1R-specific agonists are shown in Fig. 6.1. Additional eCBs, such as oleamide, virodhamine, N-arachidonoylglycine and noladin ether, were also identified in specific brain tissues [15]. AEA is synthesized by several pathways (see review for details [2]). Importantly, there is strong evidence of calcium dependence in both of these synthesis steps, which may underlie the requirement for postsynaptic Ca^{2+} in specific forms of depolarization-induced synaptic plasticity (for details see [23]). The biosynthesis of AEA from membrane phospholipid precursors is catalyzed by several enzymes. The most well-studied of these is N-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD; [24, 25]), but others include glycerophosphodiesterase (GDE1) [26], abhydrolase domain 4 (ABHD4) [27] and phosphatase PTPN22 [28] (Fig. 6.2a). AEA is degraded by the fatty acid amidohydrolase (FAAH) enzyme. The biosynthesis of 2-AG occurs via two possible routes in neurons (see recent review [29]). Diacylglycerol lipases (DAGL)-α and β both contribute, to a large extent, to the regulation of steady-state levels of 2-AG in the brain and other tissues (Fig. 6.2b). The degradation of 2-AG is carried out by the monoacylglycerol lipase (MAGL) enzyme. The eCB system functions in many physiological processes and behaviors, such as neural development, immune function, metabolism and energy homeostasis, pain, emotional state, arousal, sleep, stress reactivity, synaptic plasticity, learning, and reward processing of many drugs of abuse, including alcohol [30–38]. CBs elicit their function principally via CB1Rs, which are chiefly confined within the CNS, and CB2Rs, which are widely expressed in peripheral systems and in the CNS in lesser concentrations. Although not detailed in this section, peroxisome proliferator-activated receptors (PPARs) and transient receptor potential vanilloid 1 (TRPV1), as well as other receptors, were also shown to mediate some functions of eCBs [39]. AEA was shown to affect gene expression via the activation of both TRPV1 and PPAR-α/PPAR-γ receptors [40, 41]. AEA and 2-AG have precise and exclusive affinities to different eCB receptors. 2-AG binds to CB1R and CB2R with a high-efficacy, whereas AEA binds with a low-efficacy to both receptors. Both CB1Rs and CB2Rs are negatively coupled to GPCRs; once they are activated, they primarily couple to Gi/Go proteins, leading to the inhibition or activation of numerous intracellular signaling cascades and to many different cellular functions [32, 40–43]. CB1Rs are considered the most abundant metabotropic receptor in the brain [44] and are highly expressed in the cortex, basal ganglia, hippocampus and cerebellum regions [45]. Subcellularly, CB1Rs are present at the presynaptic terminals [267]. Therefore, the CB1R is often referred to as the *"brain cannabinoid receptor."* CB1R densities are similar to those of γ-aminobutyric acid (GABA)- and glutamate-gated ion channel levels [46]. Several CB1R-specific antagonists have been developed and are shown in Fig. 6.1. The presence of CB2R in the brain has been identified in distinct locations of the CNS in many animal species, including humans, in moderate levels [47, 48] and are restricted to microglia and vascular elements [49]. However, the specific functions of this receptor in the CNS are emerging slowly [47, 50–55]. Recent strong evidence suggests the presence of CB2R mRNA in neuronal cells of the hippocampus [51] and dopamine-expressing neurons in the ventral tegmental area (VTA) [54, 55]. CB2R-mediated modulation of cell type-specific synaptic plasticity was shown in the hippocampus [52, 56, 57]. Furthermore, increased CB2R levels in neurons were detected under pathological conditions [48, 58].

6.3 CB1R Signaling Mechanisms

CB1R activation facilitates its interaction with GTP-binding proteins, resulting in guanosine diphosphate/guanosine triphosphate exchange

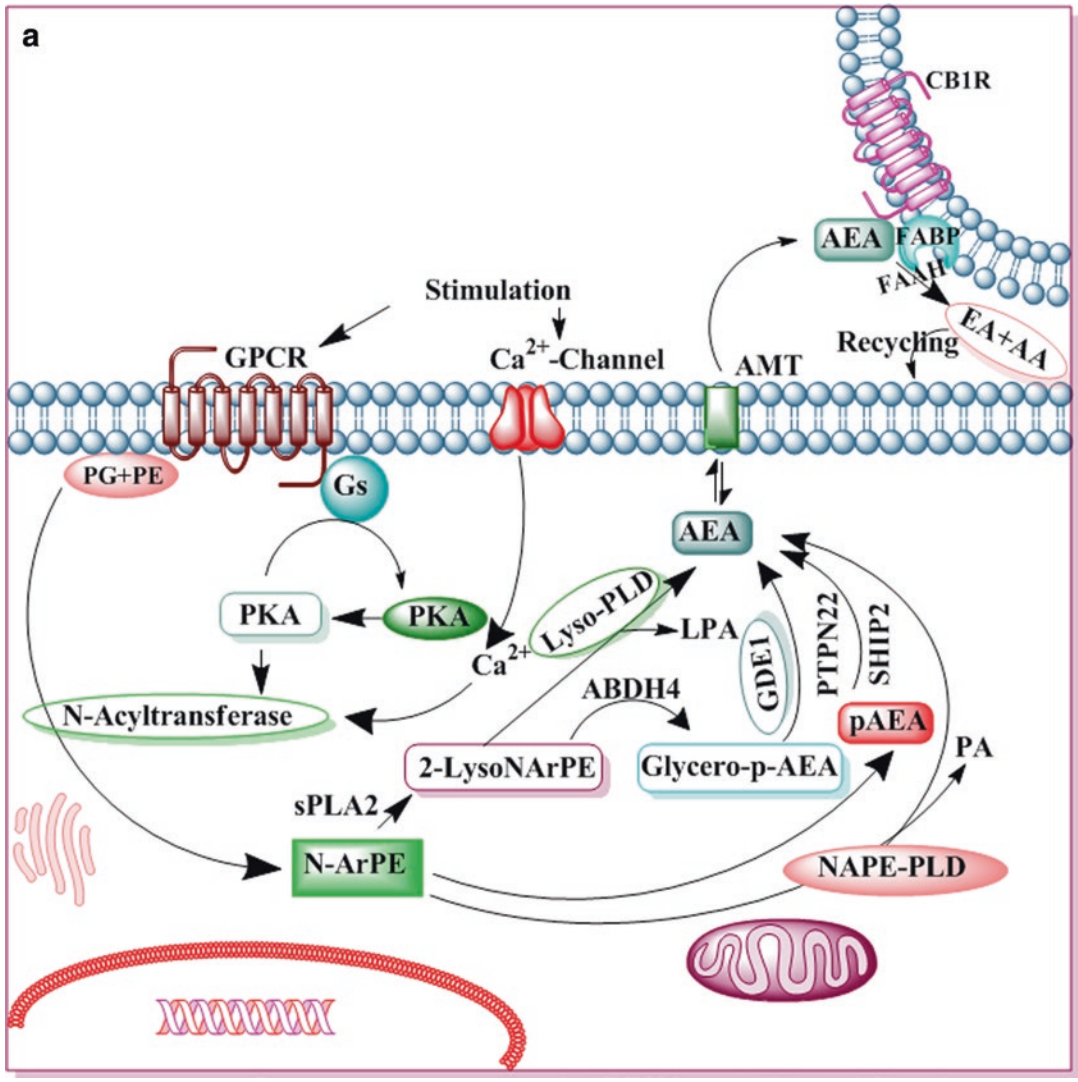

Fig. 6.2 AEA and 2-AG metabolic pathways. (**a**) Stimulation of AC and PKA potentiates N-acyltransferase (Ca^{2+}-dependent transacylase, CDTA) and results in N-arachidonoyl phosphatidylethanolamine (N-ArPE) formation as an intermediate. This N-ArPE is then hydrolyzed by a phospholipase D (PLD)-like enzyme to yield anandamide (AEA). Another pathway regulates the conversion of NAPE into 2-lysol-NAPEs by the activity of secretory PLA_2 (sPLA2). 2-Lysol-NAPEs by the action of selective lysophospholipase D (lyso-PLD) [270] is then converted into *N*-acyl-ethanolamides, including AEA. 2-Lysol-NAPEs, through the action of abhydrolase domain 4 (ABHD4) [27], are turned into glycero-p-AEA, which is then converted by glycerol phosphodiesterase (GDE1) [26] into AEA. AEA is inactivated by reuptake [271, 272] via an uncharacterized membrane transport molecule, the 'AEA membrane transporter' (AMT) [21, 271, 273–277], and subsequently undergoes intracellular enzymatic degradation. FAAH metabolizes AEA to arachidonic acid (AA) and ethanolamine, leading to rapid clearance of AEA from extracellular compartments [278, 279]. (**b**) Intracellular Ca^{2+} initiates 2-AG biosynthesis by activating the formation of diacylglycerol (DAG) [11, 280] in the membrane by stimulating the phosphatidylinositol-phospholipase C (PI-PLC) pathway. 2-AG is also the product of DAG-lipase (DAGL) acting on DAG [281, 282]. The second route involves the hydrolysis of phosphatidylinositol (PI) by phospholipase A1 (PLA_1) and the hydrolysis of the resultant lyso-PI by a specific lyso-PLC [11]. 2-AG is also synthesized through the conversion of 2-arachidonyl lysophosphatidic acid (LPA) by phosphatase [283]. Like AEA, 2-AG is inactivated by reuptake [271, 272] via the AMT [21, 271, 273–277] and is subsequently degraded [16, 278, 284] by monoacylglycerol lipase (MAGL)

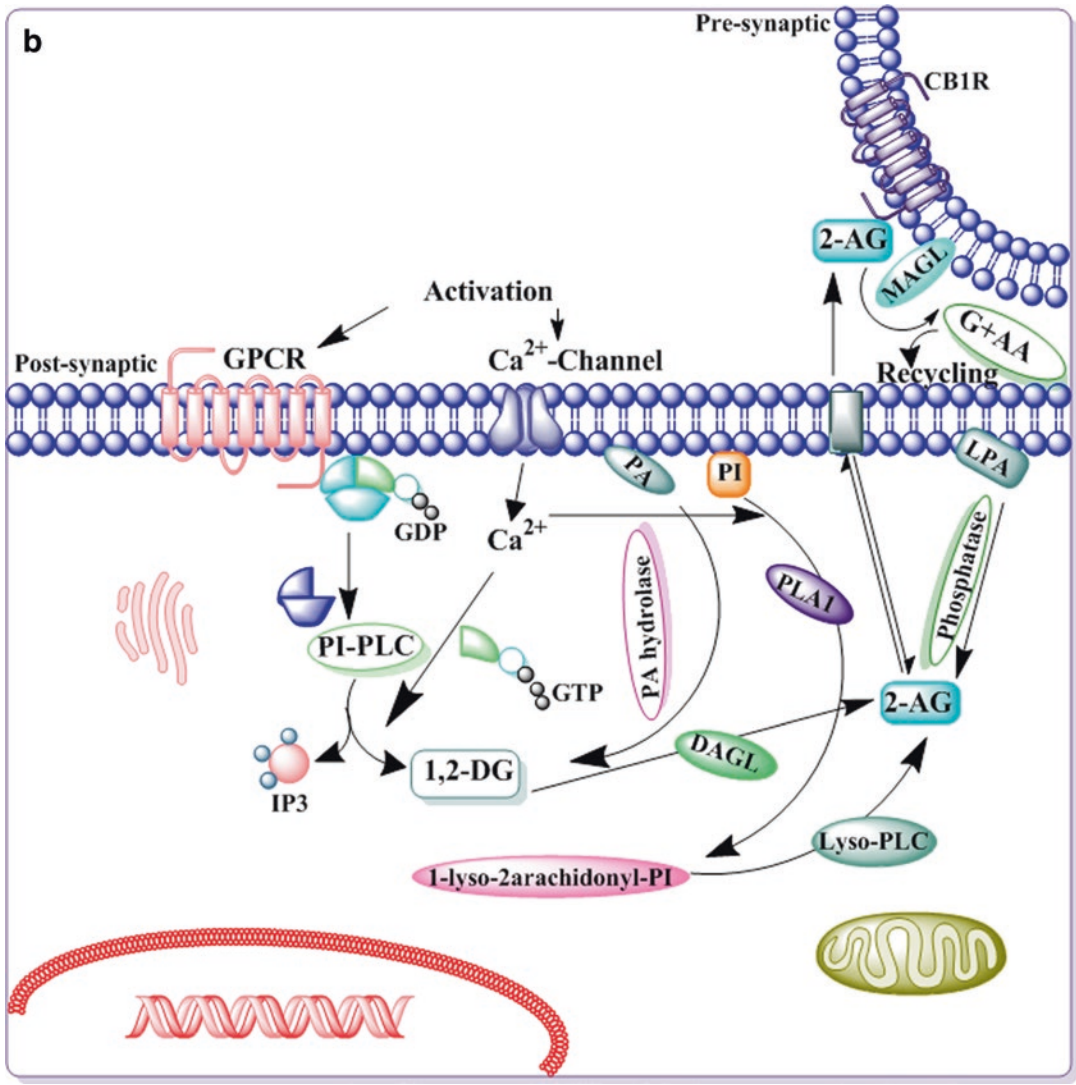

Fig. 6.2 (continued)

and dissociation of the α and βγ subunit proteins. Furthermore, the dissociated α and βγ subunit proteins further regulate the activity of multiple effector proteins to elicit biological functions. The affinity of CB1Rs to the G_i or G_o protein varies, as determined by several receptor agonists and receptor agonist-stimulated GTPγS binding studies [59, 60]. The CB1R differs from many other GPCR-G proteins because it is constitutively active due to precoupling with G-proteins [61]. Figure 6.3 depicts a diagram of the cannabinoid (CB)-mediated signal transduction pathway. CB1R activation by R(+)-methanandamide (MetAEA) and ECs in N18TG2 cells promoted the inhibition of adenylate cyclase (AC) activity [62–64]. In some cases, upregulation of AC activity was reported without $G_{i/o}$ coupling (pertussis toxin-sensitive), likely through activation of G_s proteins [65]. Furthermore, *in vitro* expression of specific isoforms of AC (I, III, V, VI, or VIII) with coexpression of CB1R is associated with the suppression of cyclic AMP accumulation. However, the expression of the AC isoforms II, IV, or VII with coexpression of CB1R is related to stimula-

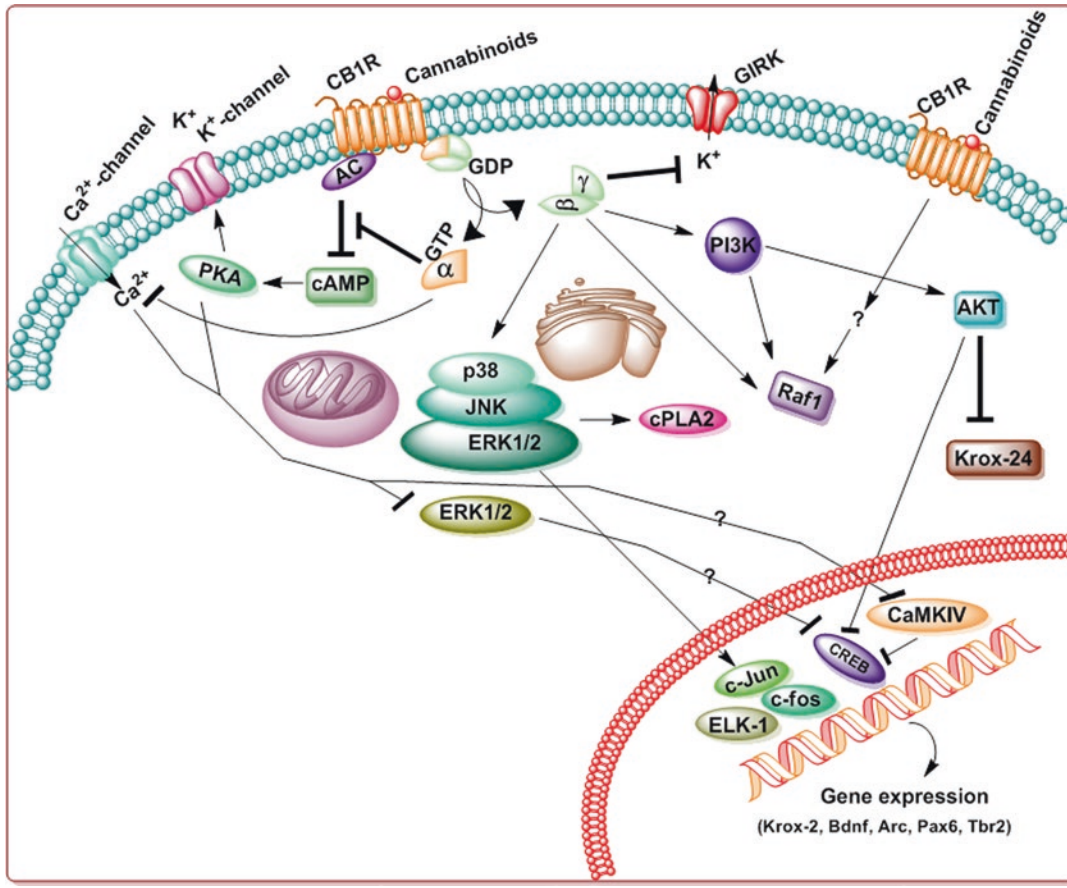

Fig. 6.3 CB1R signaling pathway. Δ^9-THC and other CBs elicit their effects by binding to CB1Rs. CB1Rs are a seven-transmembrane domain, and G protein-coupled receptors are located in the cell membrane. The Ca^{2+} channels inhibited by activation of CB1Rs include N-, P/Q-and L-type channels. The actions on Ca^{2+} channels and adenylyl cyclase (AC) are thought to be mediated by the G protein α subunits, while GIRK and PI3K activation is regulated by the βγ subunits. The βγ complex further activates the p38/JNK/ERK1/2 pathways, followed by phosphorylation of several downstream targets, such as cPLA2, ELK-1, c-fos, c-jun and CREB, leading to the expression of target genes, such as krox-24 and BDNF. PI3K mediated AKT inhibition of CREB activation. Inhibition of AC activity and the subsequent decrease in cAMP reduces the activation of cAMP-dependent protein kinase (PKA), which results in decreased K^+ channel phosphorylation. Inhibition of ERK1/2 activation followed by inhibition of CaMKIV and CREB phosphorylation was also found in certain conditions, leading to inhibition of Arc expression. Stimulatory effects are shown by the → sign and inhibitory effects are shown by the ⊥ sign

tion of cAMP accumulation [66]. Further characterization of the mechanism by which activation of the CB1R can lead to accumulation of Gα-GTPβγ heterotrimers, a mechanism that has been proposed for other GPCRs [67, 68], would enhance our understanding of CB1R signal transduction. It is also important to characterize the downstream effectors and signaling cascades of these heterotrimer (Gα, Gβγ and Gα-GTPβγ) proteins.

Exogenous CBs have been shown to inhibit N-type voltage-gated calcium channels in several neuronal cells using intracellular Ca^{2+} analysis and whole-cell voltage clamp techniques [69–72]. Exogenous CBs acting on CB1R have also been shown to inhibit L-type Ca^{2+} channels in the arterial smooth muscle cells of the cat brain, which express CB1R mRNA and proteins [73] and were blocked by pertussis toxin and CB1R antagonist, SR [73]. The stimulation of CB1Rs

leads to the activation of A-type and inwardly rectifying potassium channels, possibly through AC/$G_{i/o}$ proteins [74], which leads to the inhibition of cAMP-dependent protein kinase A (PKA). CB activation of CB1R has been shown to regulate potassium current (outward/inward) through PKA-mediated phosphorylation of potassium channels [75]. The activation of CB1R by CBs inhibits N-type and P/Q-type calcium channels and D-type potassium channels [64, 76]. Furthermore, CBs can close sodium channels, but whether this effect is receptor-mediated has yet to be determined. In rat hippocampal CA1 pyramidal neurons, CB1Rs are inversely coupled to M-type potassium channels [77]. CB1Rs may also mobilize arachidonic acid and block the serotonin type 3 ($5HT_3$) receptor ion channels [78]. Under specific conditions, CB1Rs also activate AC [79] and reduce the outward potassium (K) current via G_s proteins, possibly through arachidonic acid-mediated stimulation of protein kinase C (PKC) [80]. CB1Rs have also been reported to activate phospholipase C (PLC) through G proteins in COS-7 cells co-transfected with CB1Rs and Gα subunits [81]. The activation of CB1Rs increases *N*-methyl-D-aspartate (NMDA)-mediated calcium release from inositol 1,4,5-triphosphate (IP3)-gated intracellular stores in cultured neurons [82]. The activation of CB1Rs by CB agonists evokes a rapid, transient increase in intracellular free Ca^{2+} in N18TG2 and NG108-15 cells [83–85].

Furthermore, CBs have also been shown to influence neuritogenesis, synaptogenesis, and axonal growth. However, the molecular mechanisms involved in these processes have not been well characterized. The regulation of cellular growth is typically associated with the activation of tyrosine kinase receptors. However, studies indicate that GPCRs can activate the mitogen-activated protein kinase (MAPK) pathway and thereby induce cellular growth. After the first observation of activation of the MAPK cascade by AEA [86], several studies using both eCBs and CBs investigated this pathway in both *in vivo* and *in vitro* models. Enhanced activation of extracellular signal-regulated kinase 1/2 (ERK1/2) (p42/p44) was observed in nonneuronal U373MG astrocytoma cells, and host cells expressing recombinant CB1Rs were mediated by CB1R and the $G_{i/o}$ protein [87] in a short time window. Similarly, activation of the $G_{i/o}$ protein via CB1R by Δ^9-THC and HU-210 activated p42/p44 MAPK in C6 glioma and primary astrocyte cultures [88, 89]. In WI-38 fibroblasts, AEA promoted tyrosine phosphorylation and the activity of ERK1/2 via CB1R and $G_{i/o}$ [86]. In some cells, CB1R-mediated activation of MAPK involved the PI3 kinase pathway [86, 87]. AEA and CBs (CP 55,940, CP and WIN 55,212-2, WIN) increased the phosphorylation of FAK+ 6,7, a neural isoform of focal adhesion kinase (FAK), in hippocampal slices and cultured neurons [90]. Δ^9-THC and eCBs stimulated tyrosine phosphorylation of the Tyr-397 residue in the hippocampus, which is crucial for FAK activation [91]. The activation of CB1Rs by CB treatment enhanced the phosphorylation of p130 Cas, a protein associated with FAK in the hippocampus. Further, eCBs increased the association of Fyn but not Src with FAK+6,7. These effects were mediated via inhibition of the cAMP pathway. CB1R-stimulated FAK autophosphorylation was shown to function upstream of Src family kinases [91]. CB1R-mediated regulation of the MAPK pathway may play a role in the eCB-induced regulation of cell migration, neurite remodeling, and synaptic plasticity. Δ^9-THC promoted phosphorylation of Raf-1 and subsequent translocation to the membrane in cortical astrocytes [88]. The CB1R-mediated release of the βγ subunits leads to the activation of PI3K, resulting in tyrosine phosphorylation and the activation of Raf-1 as well as the resulting MAPK phosphorylation. Experiments using Chinese hamster ovary (CHO) cells expressing recombinant CB1Rs [92] and human vascular endothelial cells expressing endogenous CB1Rs [93] have demonstrated the activation of p38 MAPK through CB1R stimulation. Furthermore, in CHO cells expressing recombinant CB1Rs, the activation of CB1R by Δ^9-THC induced activation of the c-Jun N-terminal kinase (JNK1 and JNK2) [92]. The pathway for JNK activation involves CB1R-coupled $G_{i/o}$ protein, PI3K and Ras [92].

CB1R-mediated activation of MAPK stimulates the Na^+/H^+ exchange in CHO cells that stably express CB1R [94]. Furthermore, eCB-stimulated activation of MAPK activity was shown to promote the phosphorylation of cytoplasmic phospholipase A2 (cPLA2), followed by the generation of arachidonic acid (AA) and the resulting synthesis of prostaglandin E2 in WI-38 cells [86]. MAPK activation by CBs was shown to induce immediate early gene expression (k*rox*-24) in U373MG human astrocytoma cells [95]. Δ^9-THC induced the expression of *krox*-24, BDNF and *c-Fos* in the mouse hippocampus [96]. The CB1R- and MEK-ERK-mediated activation of *krox*-24 is negatively regulated by the PI3K-AKT pathway in Neuro2a cells [97]. Additionally, eCB/CB1R/PKA-mediated MAPK activation results in the inhibition of prolactin and nerve growth factor receptor (Trk) synthesis [98]. CB1R agonists induce the expression of c-Fos and c-Jun in the brain [99–101]; whether CB1R-activated MAPK mediates this activity is not known. Δ^9-THC-induced phosphorylation of the transcription factor Elk-1 is mediated by MAPK/ERK [102]. Intracerebroventricular injection of eCBs induced an increase in c-Fos protein in rat brains with a similar distribution to that of CB1Rs [103]. Δ^9-THC and HU-210 increased glucose metabolism and glycogen synthesis in C6 glioma and astrocyte cultures [89]. In CHO cells expressing recombinant CB1Rs or U373MG astrocytoma cells, stimulation of the CB1R activated protein kinase B/AKT (isoform IB) occurs via $G_{i/o}$ and PI3K signaling [104]. In adult animals, acute activation of CB1R by synthetic CBs failed to alter ERK1/2 activation but impaired activation of CaMKIV and CREB in a CB1R-dependent manner, resulting in long-term potentiation (LTP) and learning and memory deficits [32]. In another study, the acute inhibition of FAAH not only enhanced endogenous AEA levels in the hippocampus but also increased ERK1/2 activation and inhibited the activation of calcium/calmodulin-dependent protein kinase IV (CaMKIV) and cAMP-response-element binding protein (CREB) [31]. These AEA/CB1R signaling events lead to LTP and learning and memory deficits in a CB1R-dependent manner [31]. In several studies, CB1R knockout (KO) mice exhibited enhanced CREB activation [31, 32, 105] and activity-regulated cytoskeleton-associated protein (Arc) expression [105], increased LTP and several enhanced behavioral phenotypes, including those involved in learning and memory [31, 32, 105–113].

In other studies, CB1R activation has been associated with the generation of ceramide [114]. This widespread lipid second messenger is known to play a significant role in the control of cell fate in the CNS. Studies have shown that CB-dependent ceramide generation occurs by a G protein-independent process and involves two different metabolic pathways: sphingomyelin hydrolysis and *de novo* ceramide synthesis. Ceramide, in turn, mediates CB-induced apoptosis, as shown by *in vitro* and *in vivo* studies. CB1R activation by CBs induces apoptosis via the accumulation of ceramide, phosphorylation of p38, depolarization of the mitochondrial membrane and activation of caspase in both mantle cell lymphoma (MCL) and primary MCL cell lines but not in normal B-cells [115]. The CB1R of astrocytes has been shown to be coupled to sphingomyelin hydrolysis through the adapter protein (FAN) factor associated with neutral sphingomyelinase activation [116].

6.4 The Function of eCBs in Alcohol Use and Abuse Disorders

The first proof of the interaction of ethanol and marijuana use was observed in a study in which participants with heavy marijuana use exhibited less intoxication from ethanol and presented less ethanol-induced neuropsychological deficiencies than those without heavy marijuana use [117]. However, this study lacked data from non-cannabis-using control subjects. The findings indicated that a prior history of heavy marijuana use might result in cross-tolerance to acute ethanol effects. Additionally, the synergetic interaction between acute ethanol and marijuana use was found in rodents in a study in which coadministration of ethanol with Δ^9-THC enhanced

sleep time compared to that observed after administration of either drug alone [118, 119]. It was shown that, in a double-blinded placebo controlled study, ethanol and marijuana interacted synergistically to cause cognitive, psychomotor [120] and attention performance deficits [121, 122] when coadministered [120]. A study demonstrated significantly impaired learning in rats that were chronically administered ethanol or marijuana for six months after ceasing ethanol and marijuana administration for one month before training [123]. These studies indicate that, while ethanol and cannabis have distinct specific effects, these two drugs cause similar cognitive functional defects after both acute and chronic administration. Consistent with these historical reports, a more recent study established that a single dose of ethanol exhibits tolerance to a subsequent dose of marijuana. Furthermore, pretreatment with a single dose of marijuana facilitates acute ethanol effects [124]. Later, studies suggested that acute ethanol effects were CB1R-dependent [125]. Another study also demonstrated similar cross-tolerance in mice exposed to chronic ethanol continuously for ten days. Mice exhibited significantly reduced sensitivity to CB-induced hypomotility, hypothermia, and antinociception through reduced CB1Rs in the periaqueductal gray, hypothalamus, and ventral tegmental regions [126]. As mentioned earlier, most of the neurophysiological consequences of intoxication with CBs are exerted through the eCB system to modulate neurotransmission primarily at the glutamatergic and GABAergic synapses. However, the molecular substrates by which ethanol interacts are many and differ significantly in terms of the neurochemical processes. However, increasing evidence from biochemical, genetic, electrophysiological, and behavioral studies performed over the past two decades suggests that the eCB system contributes significantly to acute ethanol effects and participates in the underlying neuropathology leading to alcohol abuse and dependence disorders (Table 6.1).

Despite the early burst of research regarding the synergistic interactions between marijuana and ethanol, there was a noticeable lack of studies evaluating the interaction between ethanol and CB substances until the 1990s. The first reports to suggest ethanol as a modulator of eCB biosynthetic enzymes were established by our laboratory and indicated that chronic ethanol exposure caused specific upregulation of a Ca^{2+}-dependent, arachidonic acid-specific isoform of PLA_2 in mice brains [127, 128]. Shortly after that, we found that chronic ethanol exposure via vapor reduced CB1R receptor numbers and function in the mouse brain [129, 130]. Further, AEA and 2-AG levels were enhanced via Ca^{2+}-mediated activation of PLA_2, followed by NAPE-PLD in cultured cells exposed to chronic ethanol [19, 20]. During this time, studies have demonstrated that CB1R agonists [131] and antagonists [132–135] could enhance or inhibit ethanol consumption, respectively, and suggested that alcohol consumption could be regulated via CB1R. These seminal studies and progress on the biochemistry and physiology of the eCB system have contributed significantly to the firmly established role of the eCB system in regulating the reinforcing properties of ethanol and the pathology of alcohol dependence.

Short-term incubation of forebrain synaptosomes [136] or hippocampal neurons [137] with ethanol suggested an increase in intracellular Ca^{2+} via release from intracellular stores. Consistent with these findings, several studies have indicated that the effects of acute ethanol are mediated in part by the release of eCBs from neural tissue and their consequent actions on neurotransmission. It was shown that the exposure of hippocampal cultures to acute ethanol (approximately 200 mg/dL) was sufficient to enhance both AEA and 2-AG in a Ca^{2+}-dependent manner and was shown to inhibit presynaptic glutamate release [138]. In another study, chronic pretreatment with WIN rescued the acute ethanol-induced spontaneous firing rate in the basolateral amygdala (BLA) [139] and ventral tegmental area (VTA) [140] projection neurons. Similar findings were also reported from evoked activity in nucleus accumbens (NAc) neurons [140]. These findings together suggest that the rewarding properties of ethanol may be reduced following

Table 6.1 Acute ethanol effects on the EC system

Measure	Ethanol exposure	System	Species	Genetic background	Method of analysis	Brain region	Effect	References
AEA	50 mM ethanol, 30 min	In vitro	Mus	C57BL/6J	Cell and medium extract	HC	↑	[138]
	8% v/v ethanol in liquid diet, 24 h access	Ex vivo	Rattus	Sprague	Tissue content	AMG	↓	[142]
						CPu	↓	
						HyTh	↓	
						PFC	None	
	4 g/kg ethanol, i.p. injection	Ex vivo	Rattus	Wistar	Tissue content	Cereb	↓	[141]
						HC	↓	
						Nac	↓	
					Microdialysis	Nac	None	
	10 % (w/v) ethanol, self-administration (30 min)	In vivo	Rattus	Wistar	Microdialysis	NAc	None	[168]
	Oral red wine; 28 g ethanol, 20–45 min	In vivo	Homo	–	Plasma	–	None	[286]
2-AG	50 mM ethanol, 30 min	In vitro	Mus	C57BL/6J	Cell and medium extract	HC	↑	[138]
	8% v/v ethanol in liquid diet, 24 h access	Ex vivo	Rattus	Sprague	Tissue content	AMG	None	[142]
						CPu	None	
						HyTh	None	
						PFC	↓	
	10 % (w/v) ethanol, self-administration (30 min)	In vivo	Rattus	Wistar	Microdialysis	NAc	↑	[168]
	Oral red wine; 28 g ethanol, 20-45 min	In vivo	Homo	–	Plasma	–	None	[286]
CB1R	50 mM ethanol, 30–60 min	In vitro	Mus	C57BL/6J	Protein	HC	None	[138]
	8% v/v ethanol in liquid diet, 24 h access	Ex vivo	Rattus	Sprague	Protein	AMG	↓	[142]
						CPu	None	
						HyTh	None	
						PFC	↓	
	4 g/kg ethanol, i.p. injection	Ex vivo	Rattus	Wistar	mRNA	Cereb	None	[141]
						HC	None	
FAAH	8% v/v ethanol in liquid diet, 24 h access	Ex vivo	Rattus	Sprague	Activity/ protein	AMG	None	[143]
						CPu	None	
						HyTh	Activity (↓); protein (↑)	
						PFC	Activity ↓; protein (none)	
	4 g/kg ethanol, i.p. injection	Ex vivo	Rattus	Wistar	Activity/ mRNA	Cereb	None	[141]
						HC	Activity (↓); mRNA (none)	
						NAc	None	

2-AG 2-arachidonyl glycerol, *AEA* anandamide, *AMG* amygdala, *CB1R* cannabinoid receptor 1, *Cereb* cerebellum, *CPu* caudate putamen, *FAAH* fatty acid amide hydrolase, *HC* hippocampus, *HyTh* hypothalamus, *NAc* nucleus accumbens, *PFC* prefrontal cortex

chronic CB exposure. In contrast to the above-discussed studies, there is also evidence that acute ethanol exposure inhibits eCB signaling in a brain region-specific manner. It was shown that acute exposure to ethanol reduces eCB levels in the hippocampus, striatum, prefrontal cortex, amygdala, and cerebellum [141, 142] without altering FAAH activity [143], suggesting that the effects of acute ethanol are not due to increased eCB metabolism.

Recent studies have indicated that the eCB system inhibits the effects of acute ethanol exposure. Acute ethanol pretreatment blocks eCB release from medium-spiny neurons in the dorsomedial striatum and prevents long-lasting disinhibition of these neurons, and this function is independent of eCB synthesis and CB1R activation [144]. Further, acutely applied ethanol was shown to block the CB1R-mediated presynaptic facilitation of GABAergic signaling in pyramidal neurons (PNs) in the central amygdala [145]. Studies of cerebellar Purkinje neurons have found that the activation of CB1R blocks ethanol-facilitated GABA release from presynaptic terminals (enhanced inhibitory postsynaptic currents, IPSC frequency) via a PKA-dependent mechanism that releases Ca^{2+} from internal stores independent of eCB synthesis [146, 147]. Our *in vivo* studies using FAAH KO mice also indicate that AEA opposes some of the acute effects of ethanol, including loss of the righting reflex and hypothermia, while exacerbating others [148]. Collectively, these findings indicate that there is cross-talk between the eCB system and some of the acute ethanol effects depending on the brain regions. It was shown that general anesthetic propofol effects were in fact mediated by the eCB system [149], suggesting that the use of anesthetics during *in vivo* studies may influence eCB signaling outcomes. However, there are no studies on the influence of urethane on the eCB system. Therefore, future studies on eCBs should consider the possible state of the organism under investigation with respect to exposure to anesthesia and alcohol and impact of such exposure on the eCB system (Table 6.2).

6.5 The eCBs System in the Reinforcing Properties of Ethanol

A large body of literature gathered over the past 20 years suggests that the eCB system plays an important but complex role in regulating the function of reward neurocircuitry for both non-drugs [150] and drugs of abuse, including alcohol [151, 152] rewarding behavior. The activity of mesolimbic DA neurons in the VTA acting on the NAc has been shown to regulate the reward and learning processes leading to compulsive drug-seeking behavior [55, 153–155]. It was shown that acute ethanol treatment increased DA release in a CB1R-dependent manner in the NAc, demonstrating that CB1R function regulates the ethanol-induced activation of VTA-DA neurons [156]. As discussed earlier, in another study, acute ethanol treatment induced an increased firing rate of VTA-DA neurons in a CB1R-dependent manner [140]. In our previous studies, acute ethanol-enhanced NAc-DA release was blocked by pharmacological blockade or genetic ablation of CB1R (CB1R KO) [157], and CB1R KO mice displayed diminished ethanol-induced conditioned place preference [158]. There is a strong association of the human CNR1 gene polymorphism with the reinforcing properties of ethanol [159]. Together, these findings suggest that alcohol reward is regulated in part by the eCB-mediated facilitation of the VTA-DA system.

6.6 The eCBs System in Ethanol Consumption/Self-Administration Behavior

Some studies have suggested that genetic variability in CB1R expression and signaling may predispose some individuals to alcohol abuse and dependence. In line with this concept, our previous studies have shown that C57BL/6 mice, which show a higher preference for ethanol, displayed lower CB1R levels [160] and signaling [161] than DBA/2 mice, which show a lower preference for ethanol. In other studies, the

Table 6.2 Influence of EC System activity on Alcohol Abuse Behaviors

Mode of modulation	Methods	Behavioral paradigm	Drug administration	Genus	Strain	Outcome	References
CB1R Activation	CP 55,940 (30 μg/kg)	Lick-based progressive ratio operant responding for beer	Systemic	Rattus	Wistar	↑	[131]
	WIN (10 mg/kg)	Deprivation-induced escalation of operant response for ethanol	Systemic	Rattus	Wistar	↑	[190]
	CP 55,940 (30 μg/kg)	Two-bottle-choice	Systemic	Mus	C57BL/6J and DBA	↑	[287]
	WIN (10 mg/kg)	Deprivation-induced escalation of operant response for ethanol	Systemic	Rattus	Wistar	↑	[189]
	WIN (0.5 mg/kg)	Drinking in dark	Systemic	Mus	C57BL/6J	↑	[288]
	WIN (0.5 μg/side)		VTA-microinjection	Mus	C57BL/6J	↑	[288]
CB1R Inactivation	WIN (3 mg/kg)	Two-bottle-choice	Systemic	Mus	CD1	↑	[289]
	SR141716 (3 mg/kg)	Two-bottle-choice	Systemic	Mus	C57BL/6J	↓	[132]
	SR141716 (5 mg/kg)	Two-bottle-choice	Systemic	Rattus	sP	↓	[133]
	SR141716 (1.5 and 3 mg/kg)	Lick-based progressive ratio operant responding for beer	Systemic	Rattus	Wistar	↓	[131, 134]
	SR141716 (3 mg/kg)	Operant ethanol self-administration in dependent animals	Systemic	Rattus	Wistar	↓	[135]
	SR141716 (3 mg/kg)	Operant ethanol self-administration	Systemic	Rattus	Long-Evans	↓	[171]
	SR141716 (3 mg/kg)	Operant ethanol self-administration	Systemic	Rattus	msP	↓	[172]
	SR141716 (3 mg/kg)	Operant ethanol self-administration	Systemic	Rattus	Wistar	↓	[170, 172]
	SR141716 (3 μg/side)	Operant ethanol self-administration	NAc microinjection	Rattus	Wistar	↓	[168]
	SR141716 (3 μg/side)	Operant ethanol self-administration	PFC microinjection	Rattus	AA	↓	[163]
	AM250 (6 mg/kg)	Two-bottle-choice	Systemic	Rattus	Fawn-hooded	↓	[290]
	AM4113 (3 mg/kg)	Two-bottle-choice	Systemic	Mus	C57BL/6J	↓	[291]
	SR141716 (3 mg/kg)	Cue-induced reinstatement to operant ethanol self-administration	Systemic	Rattus	Wistar	↓	[170, 172, 192]
	SR141716 (3 mg/kg)	Stress-induced reinstatement to operant ethanol self-administration	Systemic	Rattus	Wistar	None	[170, 192]

(continued)

Table 6.2 (continued)

Mode of modulation	Methods	Behavioral paradigm	Drug administration	Genus	Strain	Outcome	References
FAAH Inactivation	FAAH Null mice	Two-bottle-choice	–	Mus	129/SvJxC57BL/6J	↑	[148, 162]
			–	Mus	C57BL/6J	↑	[292]
	URB597 (4.0 μg or 1 mg/kg)	Operant ethanol self-administration	PFC microinjection	Rattus	Wistar	↑	[163]
			Systemic			None	[293]
	URB597 (1 mg/kg)	Two-bottle-choice	Systemic	Rattus	Wistar	None	[293]
				Mus	129/SvJxC57BL/6J	↑	[162]
	URB597 (0.1 mg/kg)	Two-bottle-choice	Systemic	Mus	C57BL/6J	↑	[292]
	URB597 (1 mg/kg)	Cue-induced reinstatement to operant ethanol self-administration	Systemic	Rattus	Wistar	None	[293]
		Yohimbine-induced reinstatement to operant ethanol self-administration	Systemic	Rattus	Wistar	None	[293]
	URB597 (0.5–1 mg/kg) after 1 day of withdrawal from 3-week chronic intermittent alcohol drinking	Two-bottle-choice	Systemic	Mus	C57BL/6J	↓	[294]
	URB597 (1 μg)	Operant ethanol self-administration	CeA and BLA microinjection	Rattus	msP	↓	[295]
FABPs inhibition	SBFI26 (20 mg/kg)	Two-bottle-choice	Systemic	Mus	C57BL/6J	↓	[296]

BLA basolateral amygdala, *CB1R* cannabinoid receptor 1, *CeA* central amygdala, *FAAH* fatty acid amide hydrolase, *FABPs* fatty acid binding proteins, *NAc* nucleus accumbens, *PFC* prefrontal cortex, *VTA* ventral tegmental area

pharmacological inhibition or genetic ablation of FAAH (FAAH KO) resulted in a higher preference for ethanol [148, 162]. Similarly, AA (alcohol-preferring) rats displayed decreased FAAH activity and reduced CB1R levels in the prefrontal cortex compared with ANA (alcohol avoiding or alcohol non-preferring) rats [163]. In the same study, pharmacological inhibition of FAAH (in PFC) enhanced alcohol preference in regular rats (AA rat phenotype) [163]. Together, these studies demonstrated that reduced FAAH activity is associated with decreased CB1R levels and increased ethanol preference. These results are consistent with the earlier discussion proposing that acute ethanol use facilitates eCB synthesis and release [138–140], and the decreased CB1R levels found in these studies are probably due to β-arrestin-mediated endocytosis of CB1R [164–167] because of heightened AEA tone (Table 6.3).

In other studies, self-administration of ethanol was shown to enhance the extracellular levels of 2-AG in the NAc, which are related to the amount of ethanol consumed but do not affect the AEA levels [168]. Further, in a mouse model of methamphetamine-induced neurotoxic lesion of nigrostriatal dopaminergic projections, enhanced ethanol consumption displayed enhanced 2-AG levels in limbic forebrain tissues comprising anterior cingulate and NAc [169]. The pharmacological inhibition or genetic ablation of MAGL (MAGL KO) also increased ethanol intake and preference [169]. However, future studies using more specific MAGL inhibitors and MAGL KO mice should be employed to discriminate between the roles of AEA and 2-AG in mediating the effects of ethanol.

Activation of CB1R likely mediates the effects of increased AEA on ethanol consumption, as several studies have established that direct activation or inhibition of CB1R modifies ethanol intake. Pretreatment with a CB1R agonist enhanced the motivation of rats to self-administer beer despite increased responses for both beer and sucrose [131]. In another study, microinjection of WIN into the posterior VTA enhanced binge-like ethanol intake in the second half of the drinking-in-the-dark model, suggesting that the activation of CB1R in VTA neurons contributes to the motivation to consume ethanol. Previously, it was shown that pharmacological blockade of CB1R reduces ethanol intake in C57BL/6 mice [132]. In a later study, SR administration decreased the self-administration of beer via lever press [134] or 10% ethanol solution [170], demonstrating that pharmacological blockade of CB1R reduces the ethanol reward. Additionally, SR administration reduced operant responses to ethanol and sucrose through sipper tube access [171]. Similarly, in Sardinian alcohol-preferring rats, SR administration decreased both ethanol and food consumption [133], and similar findings were demonstrated in AA rats trained to self-administer ethanol in an operant chamber [163]. Consistent results were also shown in a study using a self-administration model in Wistar and Marchigian Sardinian alcohol-preferring (msP) rats, in which the administration of SR reduced ethanol, saccharin, and sucrose consumption without affecting food consumption [172]. Additionally, reduced *Cnr1* mRNA levels in several brain regions in msP rats were stabilized after ethanol intake. Further, SR was also shown to reduce ethanol self-administration in ethanol-dependent rats but failed to affect control rats [135]. Although the neuroanatomical region responsible for CB1R-mediated ethanol self-administration is not well established, the available data suggest that brain regions typically associated with addiction may be involved. A study indicated that microinjection of SR into the medial PFC but not the dorsal striatum reduces ethanol self-administration [163]. Similarly, microinjection of SR in the NAc was found to reduce ethanol self-administration in another study [168]. Future studies are needed to further understand the role of other brain regions, such as the VTA and amygdala, in regulating CB1R and ethanol intake. After the initial findings, in which CB1R KO mice exhibited reduced ethanol consumption and preference [157, 173], many other studies have reproduced these findings. It was shown that CB1R KO mice exhibit reduced ethanol consumption and preference [174, 175]. Thus, preclinical studies suggest that the eCB system plays an essential role in ethanol consumption, and clinically safe tools to manipulate

Table 6.3 Repeated ethanol and withdrawal effects on the EC system

Measure	Ethanol exposure	Duration	System	Genus	Genetic background	Method of analysis	Brain region	Effect	Duration of abstinence	Withdrawal or abstinence effects	References
AEA	50–150 mM ethanol	24–72 h	In vitro	Homo	Neuroblastoma SK-N-SH cells	Cell and medium extract	–	↑	–	–	[19]
	100–150 mM ethanol	72 h	In vitro	Rattus	Wistar	Cell and medium extract	Cereb	↑	–	–	[21]
	7.2 % v/v ethanol in liquid diet, 24 h access	10–15 days	Ex vivo	Rattus	Sprague	Tissue content	AMG	–	3 h	↓	[184, 185]
							CPu	–	3 h	↓	
							HyTh	–	3 h	None	
							Cereb	None	–	–	
							HC	None	–	–	
							Limbic Forebrain	↓	–	–	
							Midbrain	↓	–	–	
							Striatum	None	–	–	
	Forced vapor inhalation	72 h	Ex vivo	Mus	Swiss-Wistar	Tissue content	Cortex	↑	24 h	None	[297]
	Oral chronic intermittent 5–6 g/kg alternate days	120 days	Ex vivo	Mus	Swiss-Wistar	Tissue content	HC	None	40 days	↑	[180]
	Forced chronic ethanol consumption and acute ethanol challenge	7 days	Ex vivo	Rattus	Wistar	Microdialysis	NAc	None	7–14 days	None	[298]
	Alcohol-dependent patients	Life-time	–	Homo	–	Plasma content	Plasma	↑	28 days	–	[299]

AEA	chronic intermittent ethanol vapor	48 days every day	Ex vivo	Rattus	Male Wistar	AEA tissue content	BLA	↓	3 days	↓	[300]
					Female Wistar	AEA tissue content	vmPFC	–	3 days	–	
					Male Wistar	Napeld mRNA	BLA	–	3 days	–	
					Female Wistar	Napeld mRNA	vmPFC	↓	3 days	↓	
					Male Wistar	Faah mRNA	BLA	–	3 days	–	
					Female Wistar	Faah mRNA	vmPFC	–	3 days	–	
2-AG	100–150 mM ethanol	48–72 h	In vitro	Rattus	Sprague	Cell and medium extract	Cereb	↑	–	–	[20]
	7.2% v/v ethanol in liquid diet	10–15 days	Ex vivo	Rattus	Wistar	Tissue content	AMG	–	3 h	None	[142]
							CPu	–	3 h	None	
							HyTh	–	3 h		
							Cereb	None	–	–	
							Cortex	None	–	–	
							HC	None	–		
							Limbic Forebrain	None	–	–	
							Midbrain	↓	–	–	
							Striatum	None	–	–	
	Oral chronic intermittent 5–6 g/kg alternate days	120 days+2 days withdrawal	Ex vivo	Mus	Swiss-Wistar	Tissue content	HC	↑	40 days	↑	[180]
2-AG	chronic intermittent ethanol vapor	48 days every day	Ex vivo	Rattus	Male Wistar	Magll mRNA	BLA	↓	3 days	↓	[300]
					Female Wistar	Magll mRNA	vmPFC	–	3 days	–	
					Male Wistar	Dagla mRNA	BLA	↓	3 days	↓	
					Female Wistar	Dagla mRNA	vmPFC	–	3 days	–	
					Male Wistar	2-AG	BLA	–	3 days	–	
					Female Wistar	2-AG	vmPFC	↓	3 days	↓	

(continued)

Table 6.3 (continued)

Measure	Ethanol exposure	Duration	System	Genus	Genetic background	Method of analysis	Brain region	Effect	Duration of abstinence	Withdrawal or abstinence effects	References
FAAH	Chronic ethanol treatment (100–150 mM)	72 h	In vitro	Rattus	Sprague	Activity and EC transport	Cereb	↓	–	–	[21]
	Forced vapor inhalation	72 h	Ex vivo	Mus	Swiss-Webster	Activity	Cortex	↓	–	–	[297]
	Alcohol dependent (patients/control)	Lifetime	Post-mortem	Homo	–	Protein and activity	NAc	↓	–	–	[186]
CB1	Continuous forced vapor inhalation	72–96 h	Ex vivo	Mus	Swiss-Webster	Binding and GTPγS binding	Whole brain	↓	24 h	None	[129, 130]
							Cortex	↓	24 h	None	[297]
							HC	↓			
							Striatum	↓			
							Whole brain	↓			
	7.2% v/v ethanol in liquid diet	10–15 days	Ex vivo	Rattus	Wistar	mRNA and binding	AMG	–	3 h	None	[184, 185]
								None	–	–	
							CPu	–	3 h	None	
								None	–	–	
							HyTh	None	–	–	
							Cereb	None	–	–	
							Cortex	–	3 h	None	
								None	–	–	
							HC	None	–	–	
							NAc	None	–	–	
	Forced consumption of ethanol (10%, v/v)	52 days	Ex vivo	Rattus	Wistar	mRNA	CPu	↓	–	–	[179]
							Cortex	None	–	–	
							HC	Region dependent	–	–	
							HyTh	↓			
	Operant ethanol self-administration	10 days	Ex vivo	Rattus	mSP	mRNA	AMG	None	–	–	[172]
							CPu	↓			
							Cortex	None			
							HC	None			

CB1	Twice daily 4 g/kg i.p.	10 days	Ex vivo	Mus	C57BL/6J	Protein	Cereb	None			[126]
							Cortex	None	–	–	
							HC	None	–	–	
							HyTh	↓	–	–	
							NAc	None	–	–	
							Striatum	None	–	–	
							VTA	↓	–	–	
	Chronic forced vapor Inhalation (intermittent)	49 days	Ex vivo	Rattus	Wistar	mRNA	Cortex	–	3 weeks	↑	[181]
	Chronic, 5–6 g/kg every other day (intermittent)	120 days	Ex vivo	Rattus	Sprague	Protein and mRNA	HC	↓	40 days	↑	[180]
CB1	Alcohol dependent (patients/control)	Lifetime	Post-mortem	Homo	–	Protein and GTPγS	NAc	↓	–	–	[186]

2-AG 2-arachidonyl glycerol, *AEA* anandamide, *AMG* amygdala, *CB1R* cannabinoid receptor 1, *Cereb* cerebellum, *CPu* caudate putamen, *FAAH* fatty acid amide hydrolase, *HC* hippocampus, *HyTh* hypothalamus, *NAc* nucleus accumbens, *PFC* prefrontal cortex

the eCB system are also unraveling. Future clinical studies using clinically safe tools to manipulate the eCB system will enable the use of the eCB system as a potential target for alcohol use disorders (AUD) treatment. S426A/S430A mutant mice, which express a desensitization-resistant form of CB1R and display an enhanced response to eCBs and Δ^9-THC, exhibit modestly increased intake and preference for low (6%) but not higher concentrations of ethanol. Although CB1Rs increase ethanol consumption, the reward, tolerance, and acute sensitivity to ethanol and other drugs (morphine) remained normal [176]. Cannabidiol (CBD), a non-psychoactive constituent of marijuana, reduced ethanol consumption, ethanol-induced hypothermia, and handling-induced convulsion without affecting the blood ethanol concentration in C57BL/6J mice. Furthermore, CBD significantly inhibited tyrosine hydroxylase (TH) gene expression in the VTA, Oprm1, Cnr1 and Gpr55 and enhanced Cnr2 gene expression in the NAc. Collectively, these results suggest that CBD reduces ethanol motivational behaviors. These findings strongly suggest that CBD may be useful for the treatment of alcohol use disorders [177].

6.7 The eCB System and Ethanol Tolerance and Dependence

The two main traits of alcohol dependence are the presence of tolerance to the acute effects of ethanol and the vulnerability to a withdrawal syndrome in the absence of ethanol. Studies from several investigators have shown that the eCB system functions in ethanol tolerance and dependence. In fact, most of the early work suggesting an interaction between ethanol and CB drugs supports the view that the eCB system is involved in mediating these two traits of addiction. However, at the time these studies were conducted, the presence of the eCB system and the mechanisms by which ethanol and CBs elicit their effects were unknown. In addition to the results of the studies discussed in the previous section, the symmetrical cross-tolerance that develops from the ataxic effects of CBs and ethanol was actually CB1R-dependent [124, 125], and this cross tolerance appears to be consistent with changes in CB1R expression [126]. It was previously shown that after chronic ethanol treatment for three days, which leads to ethanol tolerance and dependence, CB1R levels and function were reduced [129, 130]. These original findings have been reproduced by several investigators using different chronic and sub-chronic ethanol regimens. In another study, sub-chronic administration of ethanol for seven days reduced sensitivity to WIN-elicited changes in monoamine synthesis in many brain regions [178]. Another study using rats that created ethanol-dependency using 52 days of forced access to a 10% ethanol solution also showed reduced *Cnr1* gene expression in the striatum, hippocampus, and hypothalamus [179]. In another dependence study, rats were made ethanol dependent using a chronic intermittent ethanol treatment paradigm, and the study demonstrated reduced *Cnr1* gene expression and CB1R protein levels in hippocampal tissues and reduced CB1R-mediated inhibition of GABAergic synaptic transmission [180]. Interestingly, ethanol withdrawal for 40 days resulted in CB1R level recovery above control levels. Similar CB1R level recovery was observed after ethanol withdrawal for 3 weeks in chronic ethanol-exposed animals [181]. In another study, ethanol exposure for 10 days followed by 3 h withdrawal also reduced CB1R levels [143]. Further, chronic ethanol-induced changes in cortical, hippocampal, and cerebellar NMDA and GABA (A) receptor expression in wild-type (WT) mice were not found in CB1R KO mice [182]. Together, these findings demonstrate that an ethanol treatment model that produces tolerance and dependence reduces CB1R levels and function and subsequent ethanol withdrawal causes upregulation of CB1R levels as acute withdrawal symptoms diminish. Although the further effect of reduced CB1R signaling in ethanol tolerance and dependence is not well studied, CB1R KO studies suggest that diminished CB1R signaling may occur to counteract the neural adaptations to impaired NMDA and GABA (A) receptors after chronic ethanol. A plausible explanation for the reduced CB1R in the chronic

ethanol model was reported in our original experiments performed using cultured cells. Chronic incubation of cells with intoxicating concentrations of ethanol enhanced both AEA and 2-AG content [19, 20] via activation of PLA_2 followed by eCB synthesis [127, 183]. In another study, rats were forced to consume ethanol (7.2%) in a liquid diet, and AEA content increased in the limbic forebrain but was reduced in the midbrain [184], amygdala, and striatum [185]. Ethanol-dependent rats displayed increased AEA and 2-AG levels in the hippocampus that persisted 40 days into withdrawal [180]. It was shown that in cultured cerebellar neurons exposed to chronic ethanol treatment, enhanced media levels of AEA were associated with reduced FAAH and AEA transport mechanisms [21]. Data from human postmortem tissue also demonstrated increased AEA and reduced CB1R levels, FAAH expression and activity in the ventral striatum of alcoholic patients [186]. Together, these findings demonstrate that the enhanced eCB levels following chronic ethanol exposure may be due to increased synthesis and impaired inactivation mechanisms. Further availability of methodologies to selectively prevent AEA formation will help in establishing the mechanisms for the downregulation of CB1R and advance our knowledge of the molecular mechanisms responsible for the role of the eCB system in ethanol tolerance and dependence. In another study, ethanol dependence produced decreased baseline 2-AG dialysate levels and increased baseline levels of glutamate and GABA. Acute ethanol abstinence induced an enhancement of these dependence-induced effects, and the levels of 2-AG and GABA were restored upon ethanol re-exposure. Additionally, ethanol self-administration increased central nucleus (CeA) 2-AG levels in ethanol-dependent rats. Increased anxiety-like behavior and ethanol consumption were attenuated mainly by MAGL inhibitors [187]. These findings suggest a key role for eCB signaling in motivational neuroadaptations during ethanol dependence, in which a deficiency in CeA 2-AG signaling in ethanol-dependent animals is linked to stress and excessive alcohol consumption behavior. In another study, acute ethanol exposure decreased excitatory postsynaptic potential (EPSP) amplitudes in Wistar rats and in male but not female msPs. WIN decreased EPSP amplitudes in msPs and in male but not female Wistar rats. The combined application of WIN and ethanol resulted in strain-specific effects in female rats. No tonic CB1R signaling was found at glutamatergic synapses in the CeA of any groups, and no interaction with ethanol was found. Together, these findings demonstrate sex-strain-specific differences in ethanol and eCB effects on CeA glutamatergic signaling [188].

6.8 The eCB System in Alcohol Reuse Behavior (Relapse)

Because addiction is a sophisticated form of chronic disease, the goal of all addiction treatments is to entirely prevent the reuse of drugs (relapse) of abuse, including alcohol. It is possible that the eCB system, which plays an essential role in ethanol reward, consumption, and withdrawal processes, may also participate in the mechanisms associated with relapse. It was shown that noncontingent exposure to WIN during a period of ethanol withdrawal potentiated relapse-like drinking in rats [189, 190]. Additionally, sub-chronic exposure of WIN reduced DA release in the NAc shell in response to the following dose of ethanol [191]. Along the same line, many studies have reported the role of the CB1R blockade on reinstatement in ethanol self-administration. It was shown that CB1R antagonist SR141716 (SR) administration before the reinstatement paradigm reduced conditioned recovery of ethanol-seeking behavior in rats [170, 172, 192]. Further, the combined injection of subthreshold doses of the CB1R antagonist SR with either an adenosine A2A or mGluR5 antagonist also prevented relapse-like ethanol seeking [193]. This latter study is exciting and may be useful in clinical applications to minimize or avoid the adverse psychiatric side effects of higher doses of SR. Furthermore, SR administration failed to affect foot-shock-elicited relapse, indicating that CB1Rs play no role in stress-induced relapse [170, 192]. Together, these find-

ings suggest that CB1R plays a critical role in ethanol relapse behavior. However, future studies are required to explore the neuroanatomical location of CB1Rs and the role of other components of the eCB system to avert reinstatement of ethanol-seeking behavior.

6.9 The eCB System and the Susceptibility to Alcohol Abuse Disorders

Despite extensive animal data on the function of the eCB system in chronic ethanol use and withdrawal, limited studies have demonstrated that the *CNR1* gene variation contributes to inherent vulnerability to alcohol dependence. It was shown that being homozygous for the *CNR1* allele and having five or more repeats of a microsatellite polymorphism are associated with a decreased amplitude of the P300 wave of evoked related potentials (ERP) in the frontal lobes [194]. Further, reduced amplitude of the P300 wave of ERP has been identified as a physiological marker that is associated with family members with a history of alcohol dependence and attentional processes [195]. Additionally, single nucleotide polymorphisms (SNPs), such as 1359G/A (rs1049353), have been shown to facilitate the withdrawal severity experienced by chronic alcoholic patients, and those who are homozygous for the A allele show more severe symptoms than those with other genotypes [196]. In another study, it was reported that individuals with at least one copy of the C allele (rs202323) exhibited enhanced craving and salivary response to an ethanol-associated cue [197]. Further, the C allele of rs2023239 in the above study was associated with enhanced CB1R expression in postmortem tissues of the human prefrontal cortex (PFC). Additionally, C allele alcohol-dependent patients display enhanced PFC, orbitofrontal cortex, and NAc activation in response to alcohol-associated cues and reported greater personal reward following consumption of several alcoholic beverages [159]. These findings together indicate that *CNR1* C allele individuals may display more susceptibility to binge ethanol use and suggest that a genetic polymorphism in the *CNR1* gene may contribute to the development of AUDs.

6.10 The Role of Non-CB1Rs in AUDs

To date, most investigations relating to the eCB system in AUDs have focused on eCB transmitters, their related synthetic and inactivating enzymes, and CB1R. This is almost certainly due to the well-ingrained dogma in the CB field that CB1R represents the central CB receptor [8] and that CB2R is the peripheral CB receptor [198]. However, the existence and role of central CB2Rs are beginning to advance [199, 200], and a recent behavioral study has indicated that CB2R is involved in anxiogenic, pneumonic, and motoric processes [201]. Furthermore, ethanol treatment and consumption are known to alter *Cnr2* gene expression in the brain [202]. Studies have suggested that genetic ablation of the *Cnr2* gene increases the preference for and vulnerability to ethanol consumption partly through the increased ethanol-induced sensitivity of the tyrosine hydroxylase (TH) and μ-opioid receptor gene expressions in mesolimbic neurons [203, 204]. In addition to CB2R, evidence suggests that TRPV1R is also associated with components of the eCB system, such as AEA, and several studies have demonstrated that ethanol potentiates TRPV1-mediated responses [205–207]. More importantly, TRPV1 null mutant mice display higher ethanol preference and reduced sensitivity to ethanol-induced sleep and ataxia, and the reduced behavioral sensitivity to ethanol has also been observed in WT mice administered a TRPV1 antagonist [206]. Together, these findings suggest that AEA-mediated TRPV1 signaling may have a significant function in the response to ethanol and further warrant future studies on the influence of short- and long-term ethanol exposure on TRPV1 expression and function.

6.11 eCBs System During Development and Its Role in Fetal Alcohol Spectrum Disorders (FASD)

CB1Rs are widely distributed in the developing brain, and their expression pattern parallels neuronal differentiation in the embryo from the most primitive stages. Many studies have shown the CB1R mRNA expression pattern and the CB1R distribution in the fetal and neonatal rat brain [208–211]. CB1R mRNA expression and receptor binding were reported from gestational day (GD) 14 in rats, corresponding to the phenotypic expression pattern of most components of the neurotransmitter system (for review, see [212]). At this age, CB1Rs were already coupled to Gi/Go proteins, indicating that they were functional [208]. Developing human and rat brains express higher levels of CB1Rs [213, 214] compared to adult brains [209]. However, the distribution of CB1Rs is atypical in the fetal and early neonatal brain, particularly in white matter areas [211] and subventricular zones of the forebrain [208, 209], compared to the adult brain [46, 214]. This specific CB1R localization is a transient occurrence because the receptors are upregulated during late postnatal development, developing the typical distribution pattern found in the adult brain [208, 211]. The presence of CB1Rs during early brain development indicates the possible involvement of CB1Rs in cell proliferation, migration, axonal elongation and later synaptogenesis and myelinogenesis [for review, see [30, 33, 42]]. Therefore, CB1Rs contribute to creating neuronal divergence in brain regions during early brain development. CB1Rs are expressed in the presynaptic area of all brain regions that are central to the regulation of learning and memory (hippocampus), fear, anxiety (amygdala), stress (hypothalamic nuclei), depression (PFC) and addiction (striatum) activities [31, 32, 111, 215–222].

Exogenous CB exposure during the gestational period has been shown to impair the maturation of neurotransmitter systems and their activities [33, 223–226]. These negative effects were due to the activation of CB1Rs, which are expressed early in the developing brain [33, 208, 209, 226]. In the adult brain, the activity of a specific neurotransmitter is the consequence of a well-regulated sequence of events that occurs during early brain development. Exposure to CBs, at doses similar to those observed in marijuana users, was found to slow neurotransmitter maturation and cause neurobehavioral abnormalities. Therefore, adult animals exposed to CBs during the gestation period displayed several persistent behavioral abnormalities. These include male copulatory behavior [227], open-field activity [228], learning ability [229], stress response [230], pain sensitivity [231], social interaction and sexual motivation [232], drug-seeking behavior [233], neuroendocrine abnormalities [234] and others (for review, see [223–225, 229]).

Remarkably, most of these neurobehavioral defects are consequences of the maturational impairments of various neurotransmitter systems instigated by CB exposure. CB1Rs may also have a role in glial cell function, which plays a substantial role in brain development. CBs have been shown to mobilize arachidonic acid in glial cells via CB1R [235]. These findings indicate that CB1R may function in neural-glial signaling in the brain and that AEA released from the neurons may affect astrocyte function via the activation of CB1R located in these cells. Acute administration of THC markedly enhanced the proapoptotic properties of ethanol in the neonatal rat brain. However, THC did not induce neurodegeneration by itself, even though neuronal loss became disseminated and severe when THC was combined with a mildly intoxicating ethanol dose. The effects of this THC and ethanol dose combination resembled the massive neurodegeneration observed when ethanol was administered alone at much higher doses [236]. Additionally, THC and coadministration of a low dose of ethanol increased CB1R expression without affecting CB2R expression in the thalamus and dorsal subiculum brain regions. The influence of THC on neuronal cell death was mirrored by WIN (1–10 mg/kg) in a CB1R-dependent manner. Additionally, neonatal CB1R KO mice were less susceptible to the neurotoxic effects of a low dose of ethanol. Moreover, the CB1R antagonist SR prevented the apoptotic effects of ethanol [236].

The role of the CB1R signaling pathway during brain development has not been well investigated. The available evidence supports the participation of ERK1/2 via a mechanism involving the upstream inhibition of Rap1 and B-Raf (for review, see [237]). Activation of CB1Rs also prevented the recruitment of new synapses by inhibiting the formation of cAMP [238]. Although the intracellular signaling events involving MAPK coupled to the activation of CB1Rs have been determined in the embryonic developmental stage [239], they are not well defined during postnatal development. As discussed in the previous section, many studies using different cell lines have suggested that MAPK was both up- and downregulated during Δ^9-THC-mediated apoptosis [240, 241]. Furthermore, cannabis exposure during brain development also induced a variety of deficits that are similar to several specific human developmental disorders [242], which were possibly facilitated via the activation of CB1Rs. Moreover, cannabis use during brain development also induced a variety of neuronal defects that are comparable to several specific human developmental disorders [242] and may well overlap with those observed in fetal alcohol syndrome [243], which was likely mediated via the activation of CB1Rs.

Studies in postnatal day 7 (P7) mice established a specific role of CB1R-mediated ERK1/2, CREB phosphorylation, AKT and Arc protein expression in ethanol-induced neurodegeneration. P7 ethanol treatment significantly reduced the activation of ERK1/2, AKT and CREB, followed by suppression of Arc protein expression in the hippocampus and neocortex tissues [111]. Furthermore, activation of ERK1/2, CREB and Arc protein expression was prevented by SR pretreatment, but AKT activation was not affected. Likewise, CB1R KO mice, which did not show ethanol-induced neurodegeneration, were protected against P7 ethanol-induced inhibition of ERK1/2, CREB activation, and Arc protein expression, but they failed to induce the inhibition of AKT phosphorylation. Therefore, ethanol-activated CB1R-induced neurodegeneration was regulated by the CB1R/pERK1/2/pCREB/Arc pathway but not by PI3-kinase/AKT signaling in the developing brain [105, 111] (Fig. 6.4). CB1R-mediated Arc regulation via the MAPK pathway is an essential physiological mechanism by which CBs and eCBs can modulate synaptic plasticity.

According to recent studies, a higher dose of postnatal ethanol, which induces massive widespread neuronal cell death in neonatal mouse brains, enhanced the eCB system. In addition to enhanced AEA and associated biosynthetic enzymes, ethanol-induced transcriptional activation of the *Cnr1* gene results in enhanced levels of *Cnr1* mRNA and CB1R protein expression in cortical and hippocampal brain regions [111]. Remarkably, we found that postnatal ethanol exposure in mice enhances acetylation of histone (H4) on lysine 8 (H4K8ace) at *Cnr1* exon1, CB1R binding and the CB1R agonist-stimulated GTPγS binding in cortical and hippocampal brain regions [105]. Administration of SR or genetic ablation of CB1Rs (KO) before ethanol exposure prevented neuronal cell death [105, 111]. Interestingly, CB1R blockade through pharmacological or genetic deletion resulted in normal adult synaptic plasticity, learning and memory, including social memory, in mice exposed to postnatal ethanol. The enhanced AEA/CB1R signaling pathway might be directly responsible for the neurobehavioral defects accompanying FASD [105, 111].

Blockade of the NMDA receptor for a few hours during the synaptogenesis period has been suggested to trigger massive and widespread neuronal apoptosis in the rodent brain [244]. Therefore, during this developmental period, the survival of NMDA receptor-expressing neurons was dependent on the glutamatergic input being regulated within narrow time periods [244]. eCBs and CBs are well known to affect glutamatergic signaling [245, 246], and therefore, ethanol-induced eCBs [111, 138] or CB-induced alterations in glutamate levels [247–249] might contribute to neonatal apoptosis or lasting behavioral abnormalities [111, 250, 251] observed after binge-like ethanol exposure during this specific susceptible period of brain development. In addition, blockade or genetic deletion of CB1Rs

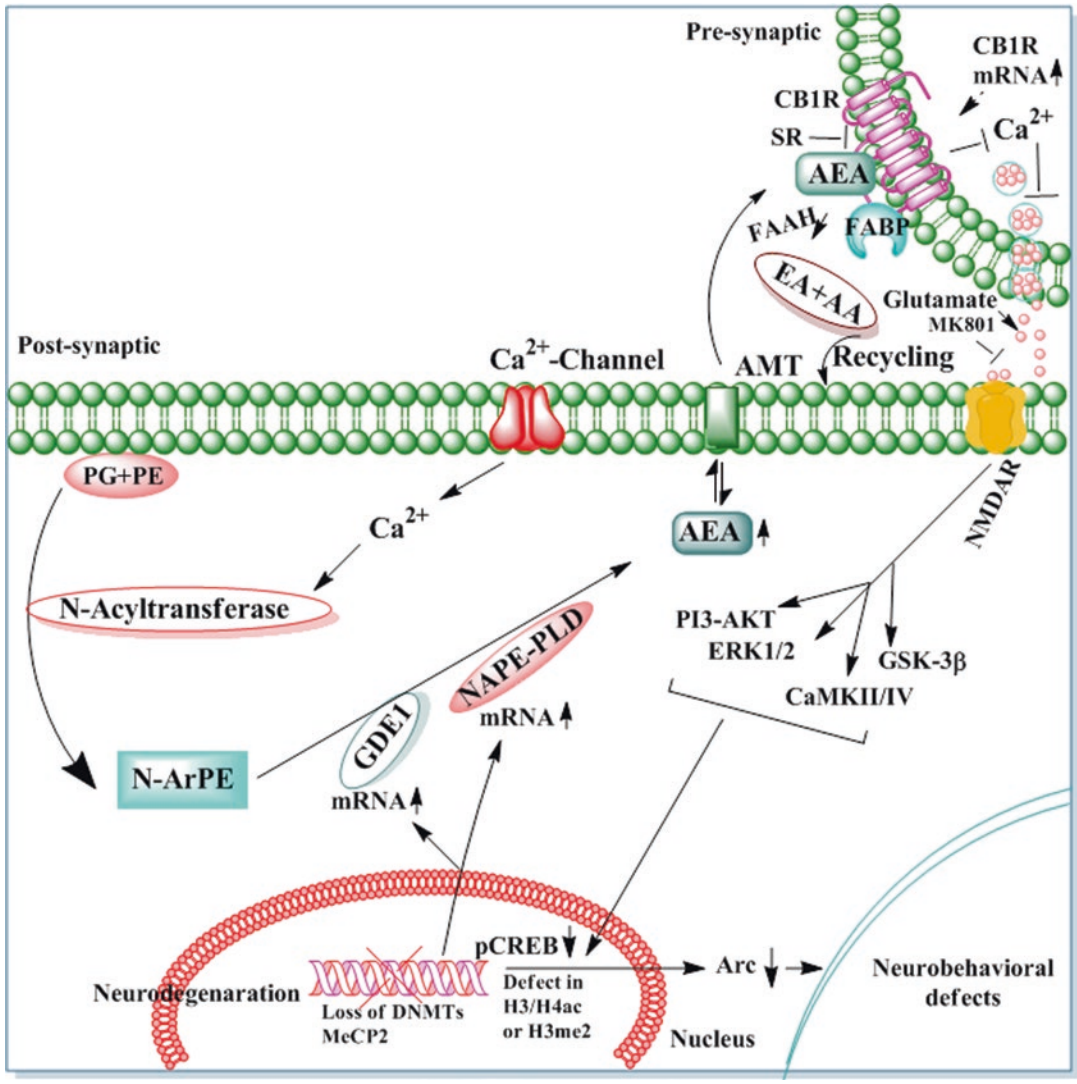

Fig. 6.4 CB1R function in the development of neurobehavioral deficits induced by developmental ethanol exposure. Postnatal ethanol exposure enhances AEA levels in the postsynaptic neuron by NAPE-PLD and GDE1 enzymes through transcriptional activation of their genes. AEA, acting through CB1Rs at the presynaptic neuron, results in decreased glutamate release, which causes NMDA receptor (NMDAR) hypofunction and CDK5, ERK1/2 and CREB hypophosphorylation deficits leading to inhibition of Arc and Rac expression followed by neonatal neurodegeneration. Earlier studies have shown that CB1R activation inhibits NMDAR function in several experimental models [253, 285], and ethanol was shown to inhibit glutamatergic neurotransmission via CB1R activation [138]. These events during postnatal development may disrupt the refinement of neuronal circuits [250, 251] and lead to long-lasting deficits in synaptic plasticity and memory in adult animals. The inhibition of CB1Rs (AEA tone) prevents CDK5 activation; pERK1/2 and CREB hypophosphorylation; loss of MeCP2, DNMT1/2 and DNA methylation; deficits in Arc and Rac expression; and neonatal neurodegeneration (Tau and caspase-3 cleavage), which results in normal neurobehavioral function in adult mice. Genetic ablation of CB1R does not affect NMDAR antagonist-induced apoptosis but does provide protection against ethanol-induced neonatal neurodegeneration and synaptic and memory deficits in adult mice. Thus, the putative AEA/CB1R/CDK5/pERK1/2/pCREB/Arc/Rac signaling mechanism may have a potential regulatory role in neuronal function in the developing brain and may be a valuable therapeutic target for FASD.

removed the eCB-mediated inhibition of glutamate release by ethanol, resulting in a reduction in ethanol-induced neuronal apoptosis (Fig. 6.4). Thus, CB1Rs serve as good candidate targets for modulating NMDA receptor function in developmental disorders. Interestingly, an NMDA receptor antagonist was able to induce neuronal apoptosis in CB1R KO mice [111], further establishing the mechanism by which postnatal ethanol exerts its adverse effects in the developing brain (Fig. 6.4). The findings obtained from neonatal rats suggested that ethanol might affect CA3 pyramidal neurons via inhibition of postsynaptic amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPARs), which results in a decrease in glutamatergic release [252]. Moreover, it was shown that exogenous CBs inhibited glutamatergic release by activating CB1R-mediated inhibition of N-type and P/Q-type calcium channels [253] and might be accountable for the enhanced vulnerability of the immature brain to ethanol neurotoxicity [236] and persistent neurobehavioral abnormalities [105, 111, 251, 254–256].

Although the molecular mechanisms are still unfolding, ethanol exposure during early postnatal development triggers synaptic dysfunction in adulthood [105, 111, 251, 254–256]. These dysfunctions were due to ethanol-increased AEA-CB1R signaling via disruption or delaying the maturation of neuronal circuits, leading to long-lasting neurobehavioral disturbances. This could explain why some cortical maps [257–260] and olfactory-hippocampal networks [250, 251] are altered in FASD models. Consistent with these findings, blockade of CB1R activity completely prevented postnatal ethanol-mediated LTP defects. Similarly, the genetic ablation of CB1Rs provided complete protection against postnatal ethanol-induced LTP defects. However, CB1R KO mice exhibited a greater LTP magnitude compared to WT or C57BL/6J saline-treated mice [105, 111, 256], as observed in other studies [107, 110]. Additionally, postnatal ethanol exposure caused object recognition and spatial and social interaction memory performance defects, which was prevented in mice by treatment with the CB1R antagonist [105, 111, 256]. Furthermore, CB1R KO mice were protected against postnatal ethanol-induced object recognition memory performance, spatial memory, and social interaction memory abnormalities as observed in LTP. It is also possible that AEA/CB1R signaling during the critical period of brain development can disturb the maturation of multiple neurotransmitter systems, including the glutamatergic, catecholaminergic, serotonergic, GABAergic and opioid systems [33, 261, 262], subsequently contributing to an impaired hippocampal network and long-term behavioral abnormalities [263]. Although more studies are warranted, heightened CB1R activity during postnatal development can cause long-term behavioral defects [264] that are regulated by NMDA receptor function [111]. Additionally, more research is required to establish the influence of heightened CB1R activity during brain development on the maturation of multiple neurotransmitter systems, which may also cause lasting morphological changes underlying the synaptic and memory defects.

Further, postnatal ethanol exposure activated caspase-3 via CB1R and led to the loss of DNA methyltransferases (DNMT1 and DNMT3A) [265], methylated DNA binding protein (methyl-CpG-binding protein 2, MeCP2) and DNA methylation in neonatal mice [265]. The CB1R KO or administration of SR prior to ethanol treatment not only inhibited caspase-3 activation but also reduced the loss of DNMT1, DNMT3A MeCP2 protein, CREB activation, and Arc expression. Together, these findings suggest that the ethanol-induced CB1R-mediated activation of caspase-3 degrades the DNMT1, DNMT3A and MeCP2 protein in the P7 mouse brain and causes long-lasting neurobehavioral deficits in adult mice. This CB1R-mediated instability of MeCP2 during active synaptic maturation may disrupt synaptic circuit maturation and lead to neurobehavioral abnormalities, as observed in this animal model of FASD. Furthermore, postnatal ethanol exposure also generates p25, a cyclin dependent kinase 5 (CDK5)-activating peptide, and suppresses Rac1 expression via an epigenetic mechanism in a CB1R-dependent manner [266]. Inhibition of CDK5 activity pre-

vents the ethanol-induced loss of Ras-related C3 botulinum toxin substrate 1 (Rac1) expression in neonatal mice. Rac1 expression is controlled by the presence of H3K9me2 and G9a, a suppressive chromatin, in the *Rac1* gene promoter region that leads to persistent loss of Rac expression in adulthood. Inhibition of CDK5 activity by roscovitine in P7 mice also prevented neurodegeneration in neonatal mice and prevented pERK1/2, pCREB, Arc signaling defects and loss of *Rac1* gene expression, synaptic plasticity and behavioral abnormalities in adult mice treated with ethanol at P7. These findings suggest that CB1R-mediated [111, 267] activation of CDK5/p25 activity followed by the persistent loss of pERK and pCREB and epigenetic suppression of Arc [268] and Rac1 expression is responsible for the persistent neurobehavioral abnormalities found in adult mice exposed to ethanol at P7. In a recent study, it was shown that treatment of P7 mice with SR before ethanol treatment prevented the activity-dependent (Y-maze behavior) signaling abnormalities such as pCaMKIV, pCREB and pCaMKII in adult mice exposed to postnatal ethanol. Administration of SR before ethanol exposure also prevents the impaired activity-dependent global epigenetic marks such as H4K8 acetylation (ac), H3K14ac and H3K9 dimethylation (me2) at the *Arc* gene promoter in adult mice exposed to postnatal ethanol [269].

6.12 Summary

The previous literature related to marijuana and ethanol interaction clearly suggests the significant role of the eCB system in the acute reinforcing properties of ethanol and the neuroadaptive changes that occur with its chronic abuse. By the end of 1990, the molecular details of the eCB system were well characterized. In the past decade, the vast majority of studies have explored the direct interaction between ethanol and the eCB system. Additionally, acute ethanol intake inhibits hippocampal neurons via an eCB-mediated inhibition of glutamate release and, if the same mechanism exists in cortical neurons, one would expect that the ethanol-induced eCB release would likely inhibit cortical output, thus producing a synergistic mechanism with that of the mesolimbic DA pathway. Many studies have shown that ethanol increases the tissue content of eCBs such as 2-AG levels in the NAc of rats during ethanol self-administration, and an infusion of CB1R agonists into the posterior VTA enhances ethanol consumption, indicating a common pathway. Further, the broad variety of treatment paradigms employed by many of these studies affords a more comprehensive viewpoint on the timescale of changes in the eCB system, particularly changes in CB1R. Studies using a 3-day ethanol exposure paradigm consistently show an increase in eCB levels that is associated with decreased FAAH and CB1R function, but these impairments reverse to basal levels after only 24 h of withdrawal. In studies where the time of ethanol exposure is somewhat longer and the blood ethanol concentration varies as subjects are not under the chronic treatment paradigm, CBR expression seems much more variable and is brain region-dependent. In the long-term exposure paradigm, followed by immediate analysis of CB1R expression, the results seem to consistently report reduced CB1R levels with elevated eCB. From these studies, it is apparent that eCB release in response to ethanol mediates the reinforcing properties of ethanol and that chronic ethanol exposure resulting in tolerance and dependence significantly changes the function of eCB signaling. However, our understanding of the mechanisms responsible for the changes in EC signaling in response to chronic ethanol is incomplete, and knowledge on the role of the eCB system in regulating specific circuits associated with addiction processes is also limited. Future work with more standardized methodologies is needed to better understand the complexity underlying the interaction between this neuromodulatory system and alcohol dependence. Additionally, strong evidence is emerging from developmental studies where AEA-CB1R/CDK5/pERK/pCREB/Arc/Rac significantly contribute to ethanol-induced developmental disorders such as FASD. In addition, novel areas of research continue to appear with fundamental discoveries surrounding the molecular constituents of the eCB system.

Acknowledgments National Institute of Alcohol and Alcoholism grant (R01 AA019443) to BSB.

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Genetic Factors in Cannabinoid Use and Dependence

7

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Abstract

Cannabinoid use and dependence are heritable traits controlled in part by genetic factors. Despite a high incidence of use worldwide, genes that contribute to the risk of problematic use and dependence remain enigmatic. Here we review human candidate gene association studies, family-based linkage studies, and genome-wide association studies completed within the last two decades. These studies have expanded the list of candidate genes and intervals. However, there is little overlap between studies and generally low reproducibility in independent samples. Reasons for this lack of coherence vary but may depend on low sample size and statistical power, and the fact that most studies leverage populations ascertained for drug dependence other than cannabis. However, recent well-powered studies on lifetime cannabis use demonstrate that the genetic architecture of cannabis use resembles that of other substance use disorders and psychiatric disease in that many small effect genes contribute in an additive fashion. This finding suggests that increasing sample size and more focused recruitment of individuals based on cannabinoid use and dependence will identify more candidate genes. Follow-up of existing high priority candidates in preclinical model systems will facilitate better understanding of the genetic architecture and genetic risk factors for cannabis use and dependence.

Keywords

Cannabis · Marijuana · Cannabinoid · Dependence · Genetics · GWAS

Abbreviations

2-AG	2-arachidonoylglycerol
AA	African American
AEA	N-arachidonylethanolamide or anandamide
CADM2	cell adhesion molecule 2
CB1	cannabinoid receptor type 1
CB2	cannabinoid receptor type 2
CD	cannabis dependence
CGAS	candidate gene association studies
Chr	chromosome
CNR1	gene encoding CB1
CUD	cannabinoid use disorder
DAG	1,2-diacylglycerol
DSM	Diagnostic and Statistical Manual of Mental Disorders
EA	European Americans
FAAH	fatty acid amide hydrolase

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A. N. Bukiya (ed.), *Recent Advances in Cannabinoid Physiology and Pathology*, Advances in Experimental Medicine and Biology 1162, https://doi.org/10.1007/978-3-030-21737-2_7

FLS	family-based linkage studies
GPCR	G-protein coupled receptor
GTEx	Genotype-Tissue Expression
GWAS	genome-wide association studies
iPSYCH	Initiative for Integrative Psychiatric Research
LD	linkage disequilibrium
LOD	logarithm of the odds
MGLL	monoacylglycerol lipase
nAChR	neuronal acetylcholine receptor
NAG	Nicotine Addiction Genetics Program
NAPE-PLD	N-acylphosphatidylethanolamine-specific phospholipase D
NCAM1	neural cell adhesion molecule 1
NRG1	neuregulin 1
PPAR	peroxisome proliferator-act ivated receptor
SCOC	short coiled-coil protein
SCOC-AS1	short coiled-coil protein antisense RNA 1
SNP	single nucleotide polymorphism
THC	Δ9-tetrahydrocannabinol
TRP	transient receptor potential (ion channel)

7.1 Introduction

Similar to other abused substances, genetic factors contribute substantially to cannabinoid use and dependence. Heritability for abuse of cannabinoids, primarily cannabis and its derivatives, ranges from 30% to 80% [1, 2]. Heritability measures the contribution of segregating gene variants to the total variation in a trait or phenotype of interest. For substance use disorders heritability has often been estimated from twin studies in which the concordance rate for cannabis-related traits is compared in monozygotic (genetically identical) and dizygotic (fraternal) twins. Higher concordance of a trait in monozygotic versus dizygotic twin pairs indicates a substantial contribution of genetic factors relative to environmental factors. Thus, high heritability is an indication that genetic factors contribute significantly to cannabinoid use disorders (CUDs). However, few of these factors have been elucidated. In this chapter we review the growing list of genes associated with CUDs based on recent candidate gene, linkage, and genome-wide association studies in humans. Finally, we discuss reasons for and possible solutions to address the paucity of known genetic factors contributing to CUDs.

7.1.1 What Is Cannabis Use Disorder?

The term "substance use disorder" is derived from the Diagnostic and Statistical Manual of Mental Disorders: DSM-5 [3]. This manual defines a set of criteria used to diagnose problematic and recurrent use of drugs or alcohol that can impact health and social well-being. Symptoms of CUD include disruption in normal function caused by cannabis use and development of tolerance, craving, and/or withdrawal symptoms associated with increased or continued use. A cluster of withdrawal symptoms, including sleep disruptions, anxiety, anger, depression have been associated with early abstinence from cannabis and may contribute to continued use. As of 2018, there are no FDA-approved treatments for CUD.

Cannabis is one of the most widely consumed psychoactive substances worldwide [4]. In the United States, policy changes in individual state legislature beginning in 1996 for medical use and continuing in 2012 for recreational use are likely to increase cannabis use in this country. As the number of users increases, so does the risk of CUDs. According to recent epidemiological studies in the United States, CUD impacts ~6% of the population [5]. Key to the identification of individuals at risk for CUD and development of pharmacological interventions for CUD is a better understanding of the genetic factors contributing to the disease.

7.1.2 Introduction to Human Genetic Association Studies

Substance use disorders, like CUDs and other psychiatric diseases, are complex traits that are driven by the actions and interactions of multiple

genetic and environmental factors. In the case of genetic factors, variation in heritable traits is caused by inheritance of different gene alleles (e.g. polymorphisms or sequence variants) that confer differential gene regulation or function. Human genes often contain multiple polymorphisms which can impact a single base (single nucleotide polymorphism or SNP) or several (insertion or deletion). These polymorphisms are typically biallelic and exist in one of two forms that represent the ancestral sequence or the altered sequence. The frequency of each allele in the population being sampled is generally a major consideration. Alleles with a minor allele frequency less than 1% will be difficult to study because few individuals with the minor allele exist in the population. In this case, detection of significant associations between inheritance of alleles and trait variation will be difficult even with moderate sample sizes. For this reason, the majority of associations identified between human disease and allelic variation involve common variants with relatively abundant allele frequencies in most human populations. Although common, these variants typically have low penetrance (probability that inheritance of the variant will cause the phenotype being measured). Genetic studies of human disease over the past decade have revealed that most human diseases are complex polygenic traits that result from the inheritance of many small effect risk alleles acting in an additive fashion [6]. These associations typically involve common variants, but rare alleles can also impact disease. Rare alleles are present at low frequency or only in specific human populations, and can only be identified using specialized study designs and populations. It is important to note that for CUDs, and most other complex diseases, the absolute number of risk alleles, their frequency in the population, and their individual contribution to disease risk (genetic architecture) in humans is unknown. Thus, the goal of human genetic association studies is to evaluate the genetic architecture of disease and identify risk genes and alleles in order to identify vulnerable individuals and design effective intervention or treatment strategies.

Three main approaches have been used towards the goal of identifying the genes and gene variants associated with CUDs in humans. These are candidate gene association studies (CGAS), family-based linkage studies (FLS), and genome-wide association studies (GWAS). In the first approach (CGAS), known allelic variants within a candidate gene are tested for an association with the disease. Often the candidate gene is selected based on *a priori* evidence regarding involvement in a disease related pathway and/or the presence of known functional variants. In a typical CGAS, inheritance of candidate gene alleles are associated with disease risk using statistical models and a case-control or family-based study design. A benefit to CGAS is that only a few associations are tested at a time, resulting in less correction for multiple testing and more significant association scores. A caveat to CGAS is the biased and limited experimental design which may lead to inflation of the contribution of the candidate gene to the disease phenotype. In contrast, FLSs and GWASs represent unbiased methods to identify gene variants contributing to phenotypic variation or disease risk.

FLSs represent a genome-wide approach to identify loci that are associated with a trait or disease risk. FLSs compare pedigrees among families to assess the likelihood that affected individuals share the same allele at a polymorphic marker more often than would be expected by chance when compared to unaffected relatives. Markers found to be significantly linked to the disease or trait by FLS are postulated to be near the causal gene variant. However, the region of linkage in FLS is often quite large. Historically this has been due to smaller sample sizes and marker panels in the size range of hundreds to thousands. For these reasons, most FLS of CUDs typically result in the identification of large (~10 Mb) linked regions that contain hundreds of potential candidate genes. Resolution to a single candidate gene in the larger linked region is not possible.

In contrast, a typical GWAS tests the association between a phenotype and allele frequency at hundreds of thousands or millions of individual polymorphisms (typically SNPs). Most mamma-

lian genomes have been sequenced and there are many high-throughput sequencing and genotyping platforms available to identify the allelic variation (genotype) at each locus on a global scale for individuals in a population. Currently, the most cost-effective and high throughput strategies include the use of genotyping microarrays that profile inheritance at millions of common variants. Due to linkage disequilibrium (LD), all variants do not need to be genotyped. LD occurs because regions of the genome are inherited as small blocks of DNA from either parent. Polymorphic genetic markers contained within each LD block will be highly correlated with one another because they are inherited as a unit. Polymorphisms in adjacent blocks will be less well correlated., Therefore, representative tag SNPs can be used as a proxy for all variants within a region of high LD (regions with high LD that are inherited together are referred to as haplotype blocks). Genotypes for adjacent polymorphisms can be imputed later for individuals based on population haplotypes. Although it is assumed that genetic polymorphisms modulate gene function or expression, the causal gene variant is generally not known following a GWAS. It is important to remember that GWAS can identify candidate gene loci, but cannot generally identify causal variants, the impact of variants on gene function, or the biological mechanism by which the gene contributes to disease.

Relative to CGAS, FLS and GWAS are unbiased approaches that can lead to the detection of multiple loci containing genes and alleles that contribute to risk. Each loci exerts a small effect on disease risk (i.e. CUDs) and the sum of all risk alleles, referred to as polygenic risk, is a better overall predictor of disease risk that captures more of the genetic variability or heritability of the disease. However, the large number of tests performed in FLS and GWAS requires correction for multiple testing and results in severe statistical penalties. To account for the many linkage or association tests performed for each marker and the phenotype of interest, empirical P-values adjusted for multiple test correction are computed. Usually the adjustment is made following the results of hundreds to thousands of permutations of genotypes for individuals in a genetic study. The adjusted *P*-value is represented as the number of times a permuted logarithm of the odds (LOD) score for association is greater or equal to the maximum observed LOD score divided by the number of permutations plus one. The empirical adjusted *P*-value is also referred to as the genome-wide corrected *P*-value. For most GWAS studies this is set very low ($P < \text{E-09}$). For these reasons, the sample sizes and association scores required to reach statistical significance are much higher compared to CGAS. Increased sample sizes in recent GWAS has led to the identification of more candidate genes and better models of polygenic risk (for a review see [6]). In addition, genotyping a larger (or infinite) number of markers using microarray or next-generation DNA sequencing has the potential to resolve linkage region or loci down to a single gene.

7.2 Candidate Genes Identified Through Human Association Studies

Relative to alcohol and other drugs of abuse the number of association studies performed for CUDs and related traits remains relatively small. In this section candidate genes and the evidence supporting them will be reviewed.

7.2.1 Candidate Gene Association Studies

For CUD most CGAS have focused on gene variants within the endocannabinoid system. Endocannabinoid signaling is critical for modulation of numerous biological processes including, response to natural rewards, learning and memory, emotional processing, motor coordination, pain, energy metabolism, fertility, development, and immune response. Major endogenous lipid ligands of the endocannabinoid system include N-arachidonylethanolamide (AEA or anandamide) and 2-arachidonoylglycerol (2-AG). Both are synthesized from membrane precursors by N-acylphosphatidylethanolamine-

Table 7.1 Results from candidate gene association studies

Phenotype	Genes	Effect	Study reference
Problematic use	CNR1 (A/G; rs806380)	Minor allele G protective	[8]
Dependence	CNR1 (A/G; rs806380)	Minor allele G protective	[7]
Withdrawal severity and craving	FAAH (C/A; rs324420)	Minor allele A protective	[13]
Withdrawal severity and craving	FAAH (C/A; rs324420)	Minor allele A protective	[14]
Dependence	FAAH (C/A; rs324420)	Minor allele A protective	[12]

specific phospholipase D (NAPE-PLD) in the case of AEA, and by 1,2-diacylglycerol (DAG) lipases DAGLα and DAGLβ in the case of 2-AG. Both endocannabinoids are catabolized by one of two enzymes—fatty acid amide hydrolase (FAAH) for AEA and monoacylglycerol lipase (MGLL) for 2-AG. Both ligands (AEA and 2-AG) act as agonists primarily at two G-protein coupled receptors (GPCRs), cannabinoid receptor type 1 (CB1) and type 2 (CB2), although they can also activate other receptors including GPCRs 55 and 119, and peroxisome proliferator-activated receptor (PPARs). AEA also acts as an agonist at transient receptor potential ion channels (TRPs). Note that each member of the endocannabinoid system is encoded by genes located in distinct genomic regions as opposed to localization within several gene clusters.

Of the known endocannabinoid signaling genes, variants in two—cannabinoid receptor type 1 (*CNR1*) and *FAAH*—have repeatedly been tested for associations with CUDs and related traits (Table 7.1). It is unknown whether variants in *CNR1* influence CB1 expression or function, however several variants have been associated with cannabis use or dependence [7, 8]. In particular the minor G allele of the rs806380 SNP is thought to exert a protective effect. A common missense variant (rs324420; C/A) that results in substitution of the amino acid threonine for proline in the FAAH enzyme has also been associated with CUDs and related traits.

In contrast to variants in *CNR1*, the missense mutation in *FAAH* has an impact on enzyme stability and function. Inheritance of both copies of the minor A allele (A/A homozygous genotype) results in lower expression and activity of the enzyme due to decreased stability and increased proteolysis [9, 10]. Of interest, the major allele associated with normal enzymatic activity is more frequently associated with risk or problematic cannabis use [11]. For example, inheritance of the major allele (C/C genotype) has been associated with CUD [12] and high cannabis withdrawal symptoms and craving [13, 14]. Although associations between traits related to CUD and variants in *CNR1* and *FAAH* have been reported, there are several studies for which these associations were not replicated [15] and overall there is no consensus regarding the involvement of these mutations in cannabis intake, withdrawal, and dependence.

Gene variants that impact the function of key enzymes involved in drug metabolism can also influence drug use and risk of developing use disorders. An example of this are functional variants in genes involved in alcohol metabolism (alcohol dehydrogenase and aldehyde dehydrogenase) which are among the strongest protective factors against development of alcohol dependence (reviewed in [16]). Functional variants in cannabinoid metabolizing genes exist but have not yet been associated with CUD. In the liver, the cytochrome P450 family of enzymes plays a role in processing cannabinoids. There are several functional variants that modulate expression or enzymatic activity of family members, in particular, polymorphisms in the P450 family member *CYP2C9* were found to influence metabolism of the synthetic cannabinoid JWH-018 that is a high affinity agonist at cannabinoid receptors [17]. Two mutations *CYP2C9*2* (cysteine substitution for arginine at amino acid residue 144) and *CYP2C9*3* (leucine substitution for isoleucine at amino acid residue 359) were found to increase or decrease metabolism of the synthetic cannabinoid, respectively. However, the impact of these variants on synthetic cannabinoid or cannabis use and dependence is not known.

7.2.2 Family-Based Linkage Studies

The first FLS was performed for adolescent cannabis use and dependence by Hopfer and colleagues [18]. The population used in this study included adolescents (ethnic distribution roughly 8% African American or AA, 37% Hispanic, 52% European American or EA, and 4% other) in a substance abuse treatment program in Denver and their genetically related siblings. Participants were part of a larger Colorado Center on Antisocial Drug Dependence study [19]. In total, 324 adolescent sibling pairs from 192 families were included. Cannabis use was also measured in an age-matched control sample drawn from the same population (community sample) consisting of 4843 individuals. The community sample was used to standardize cannabis dependence (CD) scores in the treatment samples. Repeated cannabis use was defined as using cannabis at least six times and CD was measured as the number of lifetime symptoms based on DSM-IV criteria. Cannabis use and dependence were much higher (99% and 59%, respectively) in the adolescent treatment probands relative to their siblings (55% and 14%, respectively) and compared to the community sample (5% prevalence of CD in age-matched controls). Parents and sibling pairs were genotyped for 374 markers covering the 22 autosomal chromosomes (the X and Y sex chromosomes were excluded). Two linkage regions were identified that met the criterion for suggestive linkage (P = 0.0004, LOD > 2.5) between inheritance of parental alleles at a marker and CD. Suggestive linkage regions were located on Chrs 3 (3q21 near marker D3S1267) and 9 (9q34 near marker D9S1826). No significant loci were found. The interval for linkage on Chr 3 was located roughly between markers D3S1271 and D3S1292 (101 to 132 Mb using the GRCh38/hg38 human genome assembly) and the interval for Chr 9 was located between marker D9S290 to the end of the chromosome (128.6 to 138 Mb). Because of the small number of markers used in the analysis, many genes (376 for Chr 3 and 305 for Chr 9) were located in each linkage interval. Although the Chr 3 suggestive linkage region includes *MGLL*, the gene encoding the major enzyme responsible for catabolism of the endogenous cannabinoid 2-AG, the precise genes contributing to trait variation in this first study of CD remain elusive.

Following closely behind the first linkage analysis for CD were several larger FLS. Agrawal and colleagues [20] leveraged data from the Collaborative Study on the Genetics of Alcoholism (COGA; [21]) to perform a linkage analysis based on DSM-IV criteria for CD. The COGA population was unique because it consisted of many generations of families (~90% EA and ~10 AA) at high risk for alcoholism. Genotyping was performed for 1364 individuals with genetic high-risk for alcoholism using a microarray platform consisting of 1717 SNPs. A community sample of 984 individuals was not genotyped but used to address and correct for possible confounds associated with linkage analysis (e.g. gender, race, age). A suggestive locus (adjusted $p = 0.71$, LOD = 1.9) on Chr 14 spanning ~14 Mb from markers rs759364 to rs872945 (89.3 to 103 Mb and containing 311 gene models) was associated with CD in the mostly EA COGA cohort carrying risk alleles for alcohol dependence.

Agrawal and colleagues [22] performed linkage analysis for CD based on DSM-IV requirements on 3431 individuals from 289 Australian families comprising the Nicotine Addiction Genetics Program (NAG) [23]. These families (>90% Anglo-Celtic or Northern European ethnic origin) included siblings and parents with a lifetime history of heavy smoking (40 cigarettes in a 24 h period or 20 cigarettes per day during periods of heavy smoking). A community-based control sample of 5776 individuals was used to standardize phenotypes and correct for possible confounds associated with linkage analysis in the NAG cohort. The NAG cohort was genotyped for a panel of 381 autosomal markers. Factor analysis was performed on the abuse and dependence criteria to create a single cannabis problems factor score which accounted for the majority of the variance (>60%) among measures. Suggestive linkage regions for the cannabis problems factor score were identified on Chr 1 (~10 cM interval centered on marker D1S2841 located at 78.9 Mb)

and Chr 4 (~25 cM interval centered on marker D4S419 located at 18.7 Mb). Note that the cM is a unit of genetic distance measured in map units. This unit of measure has historically been used in association studies where 1 cM corresponds to a recombination frequency of 1%. In humans, 1 cM is roughly equivalent to 1200 kb, but this varies between sexes and physical location on the chromosome. Again, linkage intervals identified in this study were too large to nominate single candidate genes.

In a separate and larger family study Agrawal and colleagues [24] performed linkage analysis for lifetime cannabis use, early-onset cannabis use, and frequency of cannabis use. The Australian cohort consisted of 5600 adult Australian twins, parents, and siblings from 2352 families genotyped at 1461 markers per individual. No markers passed the threshold for genome-wide significance. A suggestive linkage region ($P \approx 0.65$) on distal Chr 18 near marker D18S1360/GATA129F05 was identified for cannabis initiation (LOD = 1.97) and frequency of use (LOD = 2.14). A suggestive region was also located on proximal Chr 19 for early-onset cannabis use (LOD = 1.92). Marker position was not provided for all traits in the study so approximate linkage regions for this study are included in Table 7.2. Similar to previous FLS, relatively small sample sizes and marker panels provided low statistical power to detect linkage regions as well as poor resolution within suggestive linkage regions (hundreds of candidate genes located within large regions of linkage).

Ehlers and colleagues [25] analyzed a separate cohort of 1647 adults (92% Caucasian) from families with a history of alcoholism in order to identify loci associated with CD, craving, and withdrawal (feeling nervous, tense, restless, or irritable during abstinence from cannabis use). The probands were genotyped for 811 markers and a control sample of 147 individuals was used to access baseline phenotype rates. For CD, two suggestive loci were identified on Chrs 1 (LOD = 2.1, 17 cM interval near marker D1S498) and 2 (LOD = 2.6, 22 cM interval near marker D2S2361). Five loci were identified for craving on Chrs 7 (LOD = 5.7, 13 cM interval near D7S502), 3 (LOD = 4.4, 12 cM interval near D3S1279), Chr 1 (LOD = 3.6, 12 cM interval near D1S199), and 6 (LOD = 3.2, 7 cM interval near D6S281). An additional two suggestive loci for craving were identified on Chrs 9 (LOD = 2.6, 19 cM interval near D9S157) and 15 (LOD = 2.3, 9 cM interval near D15S127). For withdrawal, the strongest linkage region was identified on Chr 9 (LOF = 3.6, 10 cM interval near D9S1838). Additional suggestive loci for withdrawal were identified on Chrs 3 (LOD = 2.5, 13 cM interval near D3S1566) and 7 (LOD = 2.2, 25 cM interval near D7S506). The withdrawal loci on Chrs 9 and 3 also demonstrated evidence of linkage for a phenotype related to sleep disruptions ("sleeplessness"). Because the population under study was recruited based on a family history of alcoholism, Ehlers and colleagues examined whether linkage regions for alcohol overlapped with CD and associated traits. None of the linkage regions identified for CD or craving and withdrawal phenotypes had previously been associated with alcohol related traits measured in the same cohort.

Finally, Han and colleagues [26] used a multistage design to identify gene variants associated with CD. Linkage analysis was first performed in two different ethnic study cohorts—AA (1022 individuals from 384 families) and EA (874 individuals from 355 families). Both cohorts were ascertained for cocaine and opioid dependence and selected families included at least two affected siblings for opioid or cocaine dependence based on DSM-IV criteria. Linkage was performed in each ethnic sample separately. The strongest linkage peak was identified on Chr 8 (8p2.11, LOD = 2.9) for the AA samples and another suggestive peak for these samples was also detected on Chr 14 (LOD = 2.26). In the EA samples, a suggestive linkage peak was detected on Chr 7 (LOD = 1.85). In the next stage of the analysis the authors used an independent data set from the Study of Addiction: Genetics and Environment (SAGE) that included 4036 unrelated individuals (275 AA cases and 401 controls and 422 EA cases and 1049 controls). GWAS was performed dependent on ethnic background for the strongest suggestive linkage peak identi-

Table 7.2 Results from family-based linkage analysis

Phenotype	Individuals	Families	Study information	Criterion	Approximate linkage region	Annotated genes	Ethnicity	Replication	Study reference
Craving	1647	713	University of California San Francisco (UCSF) Family Alcoholism Study	Strong evidence	Chr1: 12 to 27 Mb	346	Caucasian	No	[25]
Dependence	3431	289	Nicotine Addiction Genetics Program	Suggestive	Chr1:73 to 85 Mb	85	Caucasian	No	[22]
Dependence	1647	713	University of California San Francisco (UCSF) Family Alcoholism Study	Suggestive	Chr1: 141 to 161 Mb	579	Caucasian	No	[25]
Dependence	1647	713	University of California San Francisco (UCSF) Family Alcoholism Study	Suggestive	Chr2: 202 to 229	304	Caucasian	No	[25]
Withdrawal	1647	713	University of California San Francisco (UCSF) Family Alcoholism Study	Suggestive	Chr3: 62 to 78 Mb	122	Caucasian	No	[25]
Dependence	648	192	Colorado Center on Antisocial Drug Dependence	Suggestive	Chr3:101 to 132 Mb	376	Mixed	No	[18]
Craving	1647	713	University of California San Francisco (UCSF) Family Alcoholism Study	Strong evidence	Chr3: 144 to 158 Mb	142	Caucasian	No	[25]
Dependence	3431	289	Nicotine Addiction Genetics Program	Suggestive	Chr4:4 to 34 Mb	374	Caucasian	No	[22]
Craving	1647	713	University of California San Francisco (UCSF) Family Alcoholism Study	Strong evidence	Chr6: 165 to 174 Mb	118	Caucasian	No	[25]
Withdrawal	1647	713	University of California San Francisco (UCSF) Family Alcoholism Study	Suggestive	Chr7: 38 to 68 Mb	312	Caucasian	No	[25]
Craving	1647	713	University of California San Francisco (UCSF) Family Alcoholism Study	Strong evidence	Chr7: 60 to 75 Mb	161	Caucasian	No	[25]
Dependence	Multiple cohorts	Multiple cohorts	Multistage Analysis	Suggestive and strong evidence	Chr8: 32.6 Mb	NRGN1 (rs17664708)	AA	Yes	[26]
Craving	1647	713	University of California San Francisco (UCSF) Family Alcoholism Study	Suggestive	Chr9: 6 to 30 Mb	151	Caucasian	No	[25]

Dependence	648	192	Colorado Center on Antisocial Drug Dependence	Suggestive	Chr9:128 to 138 Mb	305	Mixed	No	[18]
Withdrawal	1647	713	University of California San Francisco (UCSF) Family Alcoholism Study	Strong evidence	Chr9: 132 to 144 Mb	229	Caucasian	No	[25]
Dependence	1364	143	Collaborative Study on the Genetics of Alcoholism	Suggestive	Chr14:89 to 103 Mb	311	Caucasian	No	[20]
Craving	1647	713	University of California San Francisco (UCSF) Family Alcoholism Study	Suggestive	Chr15: 85 to 96 Mb	147	Caucasian	No	[25]
Initiation and frequency of use	5600	2352	Australian Twin Registry	Suggestive	Chr18: 56 to 80 Mb	219	Caucasian	No	[24]

fied on Chr 8 in the FLS. A SNP (rs17664708) located in a candidate gene for schizophrenia risk, *NRG1*, was modestly associated with CD in both AAs and EAs. The association between CD and genotype at rs17664708 was replicated in an independent sample of AAs (758 dependent cases and 280 controls) but was not able to be replicated in an independent sample of EAs (568 dependent cases and 318 controls). Of interest, the *NRG1* variant is common in EA samples but rare in AA samples and was primarily associated with CD in AA samples. The variant in *NRG1* may also be associated with drug dependence in general as it was also associated with opioid dependence in the original AA study cohort ascertained for cocaine and opioid dependence.

Some of the first genetic studies for CUD and related traits involved FLS that included less than 10,000 individuals, less than 3000 families, and less than 2000 markers. These studies were not well-powered to identify individual candidate genes. However, they were able to identify genomic regions that might harbor genes related to initial cannabis use and dependence. The one exception was the multistage analysis performed by Han and colleagues [26] which used FLS, GWAS, and independent replication cohorts to nominate variants in *NRG1* as possible genetic risk factor for cannabis and opioid dependence, particularly in AAs. Taken together, these FLS were able to demonstrate that the high heritability for CUDs ascertained from twin studies translated into the detection of large linkage regions possibly harboring gene variants mediating CUD and cannabis use traits. Few of these regions pass stringent genome-wide correction and most were not replicated in separate cohorts. Thus, we cannot exclude the possibility that some of these suggestive loci represent false positives. Of note, some of these suggestive regions overlap among studies. Genetic maps cannot be translated directly into physical maps, but in general 1 cM is roughly equivalent to 1.2 Mb. Approximate linkage regions were determined for studies that provided marker information and/or the cM interval for linkage regions by using the physical marker locations as an anchor on the physical map (human genome assembly GRCh/hg38). Genetic distances in cM were then converted into physical map distances. Approximate linkage regions are provided in Table 7.2. Although these intervals are a rough estimate, they provide support for possible overlapping linkage regions containing gene variants that may modulate CUDs. This includes a region on Chr 9 from 128 to 144 Mb that is associated with both dependence and withdrawal in two different cohorts [18, 25]. A region on Chr 7 from ~38 to 75 Mb was associated with cannabis withdrawal and craving. However, both traits were collected from the same cohort [25]. It is important to note that comparing overlapping linkage intervals is not a robust comparison method across studies. The appropriate comparison between studies would be a meta-analysis using summary scores associated with linkage between markers and traits in both studies. However, this information was not provided in some of the FLSs. Another possible reason for the general lack of replication across studies could arise from differences in how each cohort was ascertained (*e.g.* adolescent cannabis dependence versus lifetime use or genetic risk for alcohol or nicotine dependence) and these loci may confer risk of CUD or related traits only during specific developmental periods or populations (e.g. *NRG1*).

7.2.3 Genome-Wide Association Studies

Although the number of cannabis use and related GWAS is still relatively small, several large studies have identified genome-wide significant and suggestive loci containing candidate genes (Tables 7.3 and 7.4). These studies provide evidence that CUD is a polygenic disease and that increasing the GWAS sample sizes should increase the number of candidate genes and generate better genetic predictors to evaluate risk, the relationship to other diseases or behavior traits, and the role of environmental factors.

The first GWAS for CD based on DSM-IV criteria was published by Agrawal and colleagues [27]. A panel of 948,142 SNP markers was genotyped in a case-control study design with 708

Table 7.3 Results from genome-wide association studies

Phenotype	Type	Number of individuals	Number of independent genome-wide significant markers	Replication cohort/ significance	Study design	Study cohort	Ethnicity	Study reference
Dependence	GWAS	3054	0	No	Case-control: 708 cases and 2346 controls	Study of Addiction: Genes and Environment (SAGE)	EA and AA	[27]
Initiation of use	metaGWAS	10,091	0	No	Family based: 7175 Australian twins and families and 2916 UK twins and families	Australia and UK twin registries	European Ancestry	[33]
Lifetime use	metaGWAS	32,330	0	Yes/N.S.	Discovery cohort with replication (5627 individuals)	International Cannabis Consortium samples	European (discovery cohorts) and Mixed European and AA (replication cohorts)	[32]
Age at initiation	metaGWAS	24,953	5	Yes/N.S.	Discovery cohort with replication (3735 individuals)	International Cannabis Consortium (ICC)	European Ancestry	[30]
Lifetime use	metaGWAS	184,765	8 SNPS, 35 genes	No	Discovery cohort	ICC; UK Biobank; 23andMe	Primarily Caucasian	[29]
Dependence severity	metaGWAS	14,754	3	Yes/partial	Case-control discovery and replication cohorts	Yale-Penn Study, SAGE, International Consortium on the Genetics of Heroin Dependence	EA and AA	[31]
Dependence	metaGWAS	8515	1	Yes/partial	Case-control discovery and replication cohorts	COGA, SAGE and the Comorbidity and Trauma Study	European Discovery Cohort and AA and EA discovery cohort	[28]
Dependence	metaGWAS	51,372	1	Yes/yes	Case-control discovery and replication cohorts	Lundbeck Foundation Initiative for Integrative Psychiatric Research, deCODE	European discovery and repliocation cohort	[39]

Table 7.4 Results from genome-wide association studies (top marker shown for each region)

Gene	Gene new	SNP/SNP number	Chr	Mb Start	P value	Phenotype	Study reference
UCHL5	UCHL5	rs9427573	1	193,026,083	4.75E-06	Dependence	[27]
Intergenic	LINC01122	rs17552189	2	58,845,834	4.12E-06	Dependence	[27]
KYNU	KYNU	rs2381356	2	142,929,013	1.70E-05	Dependence	[27]
Intergenic	Intergenic	rs12491921	3	106,570,103	1.03E-06	Dependence	[27]
AIM1	CRYBG1	rs1462146	6	106,538,205	1.16E-05	Dependence	[27]
RPS6KA2	RPS6KA2	rs16900973	6	166,699,507	2.48E-05	Dependence	[27]
Intergenic	Intergenic	rs10858373	9	135,235,645	1.68E-05	Dependence	[27]
Intergenic	Intergenic	rs11007350	10	29,000,102	2.68E-06	Dependence	[27]
STAM	STAM	rs1007264	10	17,668,025	2.34E-05	Dependence	[27]
Intergenic	Intergenic	rs2169487	11	38,182,871	1.41E-05	Dependence	[27]
Intergenic	Intergenic	rs2068909	11	94,724,752	1.96E-05	Dependence	[27]
Intergenic	Intergenic	rs1352414	11	37,989,792	2.07E-05	Dependence	[27]
MICAL2	MICAL2	rs1609930	11	12,231,627	2.77E-05	Dependence	[27]
CHST11	CHST11	rs12811699	12	104,623,386	7.88E-06	Dependence	[27]
LGR5	LGR5	rs1280605	12	71,562,287	1.04E-05	Dependence	[27]
DKFZP779	CCDC91	rs7313862	12	28,458,596	2.61E-05	Dependence	[27]
ACADS	ACADS	rs555404	12	120,737,931	2.66E-05	Dependence	[27]
Intergenic	LINC00362	rs9507041	13	23,170,240	8.47E-06	Dependence	[27]
KATNAL1	KATNAL1	rs7317962	13	30,280,075	2.23E-05	Dependence	[27]
Intergenic	Intergenic	rs17102248	14	34,135,613	2.19E-05	Dependence	[27]
FTO	FTO	rs11865530	16	53,920,866	2.18E-05	Dependence	[27]
ANKFN1	ANKFN1	rs1019238	17	56,201,354	6.12E-07	Dependence	[27]
Intergenic	Intergenic	rs1431318	17	56,115,162	9.14E-07	Dependence	[27]
Intergenic	Intergenic	rs10521290	17	14,947,119	2.69E-05	Dependence	[27]
Intergenic	Intergenic	rs8111749	19	34,548,589	2.05E-05	Dependence	[27]
PIM3	Intergenic	rs28372448	22	49,957,323	8.00E-06	Dependence	[27]
RP11-406O16.1	LINC02549	rs12207424	6	68,275,183	6.94E-05	Initiation of use	[33]
GNG5P5	Intergenic	rs1417205	13	47,898,879	1.62E-07	Initiation of use	[33]
NELL1	Intergenic	rs1573535	11	21,599,402	8.83E-06	Initiation of use	[33]
	Intergenic	rs9900808	17	13,159,478	8.82E-05	Initiation of use	[33]
KCNT2	KCNT2	rs73067624	1	196,364,081	6.30E-05	Lifetime use	[32]
	Intergenic	rs58691539	2	52,526,521	6.40E-06	Lifetime use	[32]
	Intergenic	rs2033867	2	174,323,303	4.20E-06	Lifetime use	[32]

	SETD2	rs35053471	3	47,083,021	9.20E-05	Lifetime use	[32]
	Intergenic	rs12518098	5	61,568,390	4.70E-05	Lifetime use	[32]
	NCAM1	rs4471463	11	113,112,623	9.00E-07	Lifetime use	[32]
	Intergenic	rs7107977	11	915,514	6.40E-06	Lifetime use	[32]
	Intergenic	rs2099149	12	30,326,175	5.10E-07	Lifetime use	[32]
	Intergenic	rs4984460	15	95,880,920	2.20E-06	Lifetime use	[32]
	CADM2	NA	3	84,959,005	MTC < 0.05	Lifetime use	[32
	SCOC-AS1	NA	4	140,283,724	MTC < 0.05	Lifetime use	[32]
	SCOC	NA	4	140,257,286	MTC < 0.05	Lifetime use	[32]
	ATP2C2	rs1574587	16	84,419,200	4.00E-09	Age at initiation	[30]
	Intergenic	rs4935127	10	54,894,976	4.60E-07	Age at initiation	[30]
	Intergenic	rs2249437	6	1,594,731	5.10E-07	Age at initiation	[30]
	Intergenic	rs9266245	6	31,357,675	1.60E-06	Age at initiation	[30]
	Intergenic	rs28622199	8	5,534,331	2.70E-06	Age at initiation	[30]
	ABCC1	rs215069	16	15,997,130	3.80E-06	Age at initiation	[30]
	SPINT1-AS1	rs4924506	15	40,837,019	5.50E-06	Age at initiation	[30]
	ECT2L	rs7773177	6	138,821,701	8.50E-06	Age at initiation	[30]
	CADM2	rs2875907	3	85,469,180	1.59E-19	Lifetime use	[29]
	SDK1	rs10085617	7	3,594,829	2.93E-08	Lifetime use	[29]
	ZNF704	rs9773390	8	80,653,207	5.66E-09	Lifetime use	[29]
	NCAM1	rs9919557	11	113,006,436	9.94E-11	Lifetime use	[29]
	ATP2A1	rs10499	16	28,903,956	1.13E-09	Lifetime use	[29]
	SMG6	rs17761723	17	2,203,546	3.24E-08	Lifetime use	[29]
	KLHL21	96	1	6,590,724	7.65E-07	Lifetime use	[29]
	PHF13	84	1	6,613,696	1.99E-06	Lifetime use	[29]
	LRRTM4	3621	2	76,747,724	1.02E-07	Lifetime use	[29]
	MSANTD1	231	4	3,244,369	2.22E-06	Lifetime use	[29]
	HTRA1	64	5	122,461,525	2.41E-06	Lifetime use	[29]
	BEND6	252	6	56,955,292	2.60E-08	Lifetime use	[29]
	KIAA1586	58	6	57,046,532	1.75E-07	Lifetime use	[29]
	RAB23	86	6	57,186,992	2.32E-09	Lifetime use	[29]
	REV3L	539	6	111,299,052	1.99E-06	Lifetime use	[29]
	ARID1B	1344	6	156,777,930	1.15E-08	Lifetime use	[29]
	ADGRB1	275	8	142,464,016	1.21E-06	Lifetime use	[29]

(continued)

Table 7.4 (continued)

Gene	Gene new	SNP/SNP number	Chr	Mb Start	P value	Phenotype	Study reference
	NEURL1	17	10	103,493,979	1.83E-07	Lifetime use	[29]
	BORCS7	87	10	102,854,210	1.19E-06	Lifetime use	[29]
	AS3MT	177	10	102,869,453	1.53E-08	Lifetime use	[29]
	CNNM2	549	10	102,918,293	8.02E-07	Lifetime use	[29]
	NT5C2	389	10	103,088,018	7.64E-07	Lifetime use	[29]
	BRAP	97	12	111,642,146	5.48E-07	Lifetime use	[29]
	ACAD10	141	12	111,754,283	9.89E-08	Lifetime use	[29]
	ALDH2	112	12	111,766,933	3.61E-07	Lifetime use	[29]
	MAPKAPK5	195	12	111,842,626	5.58E-07	Lifetime use	[29]
	TMEM116	222	12	111,931,283	3.96E-07	Lifetime use	[29]
	SBK1	23	16	28,292,519	4.52E-08	Lifetime use	[29]
	NPIPB7	10	16	28,456,372	5.46E-08	Lifetime use	[29]
	CLN3	62	16	28,477,294	2.56E-09	Lifetime use	[29]
	APOBR	49	16	28,494,649	7.56E-09	Lifetime use	[29]
	IL27	57	16	28,499,362	7.48E-09	Lifetime use	[29]
	SGF29	181	16	28,553,926	4.87E-09	Lifetime use	[29]
	SULT1A2	25	16	28,591,943	6.66E-08	Lifetime use	[29]
	SULT1A1	51	16	28,605,597	1.14E-07	Lifetime use	[29]
	CDC37P1	31	16	28,700,294	1.42E-07	Lifetime use	[29]
	EIF3C	14	16	28,688,558	8.08E-08	Lifetime use	[29]
	EIF3CL	23	16	28,379,581	4.55E-08	Lifetime use	[29]
	NPIPB9	8	16	28,751,787	6.29E-08	Lifetime use	[29]
	ATXN2L	89	16	28,823,093	2.50E-09	Lifetime use	[29]
	TUFM	55	16	28,842,411	2.83E-09	Lifetime use	[29]
	SH2B1	71	16	28,863,975	5.46E-09	Lifetime use	[29]
	NFATC2IP	8	16	28,950,937	8.82E-08	Lifetime use	[29]
	RABEP2	71	16	28,904,421	2.84E-08	Lifetime use	[29]
	SRR	121	17	2,303,704	5.03E-08	Lifetime use	[29]
	TSR1	90	17	2,322,503	1.12E-08	Lifetime use	[29]
	NPC1	257	18	23,541,437	5.87E-08	Lifetime use	[29]
	LINC00526/C18orf18	132	18	5,236,724	5.65E-08	Lifetime use	[29]
	PKN2-AS1	rs74823926	1	88,263,750	1.40E-05	Dependence severity	[31]
ARHGEF33	ARHGEF33	rs114383460	2	38,938,782	1.09E-06*	Dependence severity	[31]

Gene	Gene new	SNP/SNP number	Chr	Mb Start	P value	Phenotype	Study reference
Intergenic	Intergenic	rs12621150	2	77,801,462	2.91E-04	Dependence severity	[31]
AFF3	AFF3	rs7586604	2	99,834,964	1.06E-06	Dependence severity	[31]
Intergenic	Intergenic	rs144605126	2	103,147,706	8.67E-07*	Dependence severity	[31]
Intergenic	Intergenic	rs150064803	2	117,733,075	3.41E-07*	Dependence severity	[31]
SCN9A	SCN9A	rs143020225	2	166,357,954	7.19E-07*	Dependence severity	[31]
RP11-206 M11.7	Intergenic	rs143244591	3	149,295,898	2.18E-08*	Dependence severity	[31]
PI4K2B	SEPSECS antisense RNA 1	rs73252553	4	25,199,446–25	1.66E-07	Dependence severity	[31]
SEC24D	SEC24D	rs28595532	4	118,795,545	1.13E-06	Dependence severity	[31]
CTNND2	CTNND2	rs114311699	5	11,892,022	3.78E-07*	Dependence severity	[31]
COL23A1	COL23A1	rs10066744	5	178,319,349	4.82E-07*	Dependence severity	[31]
Intergenic	Intergenic	rs17665889	6	51,356,409	2.58E-04	Dependence severity	[31]
Intergenic	Intergenic	rs12534830	7	85,323,065	4.52E-07	Dependence severity	[31]
CSMD1	CSMD1	rs77378271	8	3,215,717	4.60E-08	Dependence severity	[31]
Intergenic	Intergenic	rs10969106	9	29,364,079	7.39E-08#	Dependence severity	[31]
Intergenic	Intergenic	rs115553536	10	31,692,207	6.46E-07*	Dependence severity	[31]
RET	RET	rs74400468	10	43,097,111	6.46E-07*	Dependence severity	[31]
CCAR1	CCAR1	rs12218439	10	68,730,099	1.13E-04	Dependence severity	[31]
SLC35G1	SLC35G1	rs146091982	10	93,899,951	1.95E-07*	Dependence severity	[31]
	Intergenic	rs73443003	11	20,539,214	1.31E-06	Dependence severity	[31]
	Intergenic	rs200453611	11	81,721,913	6.81E-04	Dependence severity	[31]
	Intergenic	rs200391037	11	109,028,447	1.32E-06	Dependence severity	[31]
	Intergenic	rs193047854	12	55,880,121	7.06E-07*	Dependence severity	[31]
	Intergenic	rs199783889	20	21,725,717	3.32E-07*	Dependence severity	[31]
	Intergenic	rs78068107	21	16,646,750	1.02E-06*	Dependence severity	[31]
	Intergenic	rs186825689	21	46,585,890	1.86E-08*	Dependence severity	[31]
Intergenic	Intergenic	rs1409568	10	118,871,023	3.95E-08	Dependence	[28]
	ACYP2	rs2287641	2	54,115,568	9.00E-07	Dependence	[28]
	CHRNA2	rs56372821	8	27,459,761	9.31E-12	Dependence	[39]

dependent cases and 2346 non-dependent controls (66% EA and 34% AA). Similar to many of the FLS, the population of cases and controls for which CD was assessed were originally ascertained for alcohol dependence. A caveat of measuring cannabis or other drug-related traits in these populations is highly comorbid polydrug dependence. However, at the time no populations recruited exclusively for cannabis-related traits existed and populations ascertained for alcohol dependence were readily available. As expected based on the small sample size (<10,000 individuals), no markers met genome-wide significance. However, the large number of genotyped markers resulted in suggestive associations for markers tagging individual genes or intergenic regions. These suggestive associations were located on Chrs 1 (*UCHL5*), 2 (*LINC01122*, *KYNU*), 3 (intergenic), 6 (*CRYBG1*, *RPS6KA2*), 9 (intergenic), 10 (*STAM*), 11 (*MICAL2*), 12 (*CHST11*, *LGR5*, *CCDC91*, *ACADS*), 13 (*LINC00362*, *KATNAL1*), 14 (intergenic), 16 (*FTO*), 17 (*ANKFN1*), 19 (intergenic), and 22 (intergenic) (Table 7.4). The first GWAS for CUDs lacked statistical power and did not follow-up their suggestive loci in an independent replication cohort. However, it identified the first putative candidate genes for CD.

Later GWAS [28–33] were able to increase the number of candidate gene associations for CUDs and related traits by increasing the sample size of the discovery cohort. This was achieved primarily by combining results from smaller GWAS studies using meta-analysis (metaGWAS). Individually, each study may be underpowered to detect small effect alleles due to small sample sizes. However, when each study is combined the detection of small effect loci becomes possible due to the increased sample size. In metaGWAS summary statistics (effect size, standard error, and/or p-values) for associations between SNPs and phenotypes from multiple population studies comprised of unique individuals are combined to generate new association scores, effect estimates, and evaluate data set heterogeneity (differences in methodology between studies that could impact results). For a review of the metaGWAS approach, see [34]. As a note of caution, metaGWAS can increase sample size and power, but inclusion of samples ascertained for substance dependence other than cannabis can introduce heterogeneity and has the potential to confound results or limit reproducibility.

Several groups, starting with Verweij and colleagues [33], were able to increase the number of subjects beyond 10,000 through the use of metaGWAS and by selecting a dichotomous cannabis-related trait (yes or no to cannabis use) that could easily be assessed on a large-scale. However, few markers passed the criterion for genome-wide significance at sample sizes of 20,000 to 30,000 individuals. For example, Verweij and colleagues densely genotyped over two million SNPs from families in Australia and the United Kingdom (10,091 related-individuals) that were part of the Australia and UK twin registries (Spector & Williams 2006). Associations between SNPs and initiation of cannabis use were assessed in the Australian and UK cohort separately using family-based association tests followed by meta-analysis. Suggestive associations were observed for markers on Chrs 6, 13, 11, and 17, but no SNPs reached genome-wide significance (Table 7.4). Likewise, Stringer and colleagues [32] examined 32,330 subjects (European ancestry) for lifetime cannabis use and failed to identify any SNP associations reaching genome-wide significance. This was despite tripling the sample size used by Verweij and colleagues [33] by combining 13 discovery samples collected from around the world (International Cannabis Consortium data sets) and performing meta-analysis. Nevertheless, suggestive associations were identified on Chrs 1, 2, 3, 5, 11, 12, 15 (Table 7.4) and a less stringent gene-based analysis of 24,576 genes/genetic regions identified significant associations for the genes *NCAM1* (neural cell adhesion molecule 1, Chr11), *CADM2* (cell adhesion molecule 2, Chr3), *SCOC-AS1* (short coiled-coil protein anti-sense RNA 1, Chr4), *SCOC* (short coiled-coil protein, Chr4), and *KCNT* (Chr1) following multiple test correction (Table 7.4). The top SNP and gene associations identified in the discovery cohorts failed to replicate in an independent samples consisting of 5627 individuals (53% European and

47% AA), with the exception of suggestive associations for *SCOC-AS1* and *SCOC* in one of the 4 replication samples (AA). SNP heritability based on common SNPs in the Stringer study was estimated at 13–20% for lifetime cannabis use, which was an improvement over the 6% SNP heritability computed for Verweij and colleagues [33]. Both studies included discovery cohorts with different recruitment strategies and subsequent wide variation in the prevalence of lifetime cannabis use among cohorts may have deflated heritability estimates in both studies. Nevertheless, improvements in SNP heritability with larger samples sizes in the Verweij study confirmed that lifetime use of cannabis is a heritable trait contributed to by many loci of small effect. Thus, further increases in sample size should result in identification of more significant loci.

As proof of this concept, Pasman and colleagues [29] published the largest metaGWAS of lifetime cannabis use (184,765 individuals) and identified eight genome-wide significant independent SNPs in six regions (Chrs 3, 7, 8, 11, 16, and 17). Altogether, the identified SNPs accounted for 11% of the individual variance in lifetime use of cannabis. Using gene-based tests they identified 35 genes significantly associated with lifetime cannabis use (Table 7.4). Replication in an independent cohort was not performed, likely because the replication cohort would be much smaller and less well-powered than the discovery cohort. There was also substantial heterogeneity among cohorts used in the meta-analysis that might have limited power in some analyses and/or reproducibility or generalizability. Despite some limitations of the study, Pasman and colleagues were able to identify multiple significant loci and genes for lifetime cannabis use using a massive cohort of nearly 200,000 individuals. This study provides more evidence that the genetic architecture of lifetime cannabis use is complex and involves many small effect genes. Importantly, most of the loci identified in the study were novel and had not been identified previously.

The GWAS discussed thus far took advantage of samples recruited based on different criteria to identify loci associated with lifetime cannabis use. However, there is some debate over how lifetime use is related to development of problematic use and dependence. Early use has been associated with progression to problematic cannabis use and susceptibility for other substance use disorders [1, 35–37]. Early use may also interact with environmental and social factors. For example, the age at which individuals begin to use cannabis may depend on the overall prevalence of use within a country. Higher prevalence has been related to younger ages of initiation [38].To begin to address this issue, Minică and colleagues [30] used GWAS to identify loci associated with early cannabis use. The authors performed metaGWAS on a discovery cohort of 24,953 individuals with replication in a sample of 3735 individuals. This study also estimated heritability for age of initiation at 39% based on three cohorts consisting of 8055 twins (European descent). SNPs in the ATP2C2 gene reached genome-wide significance (Table 7.4). However, they failed to replicate in the independent cohort, and SNP-based heritability for age of initiation was not significant. Note that in both metaGWAS studies with replication examined thus far [30, 32] the replication cohort was much smaller than the discovery cohort which may have limited the power for replication in the discovery cohort.

Only three metaGWAS studies [28, 31, 39] examined CUD directly. In the first study, Sherva and colleagues [31] identified loci associated within CD severity based on DSM-IV criteria using metaGWAS and replication across three independent cohorts consisting of 14,754 individuals (AA and EA). Each cohort was ascertained separately for drug dependence as part of the Yale-Penn Study on the genetics of substance use [40], the SAGE Study on the genetics of alcohol, nicotine, and cocaine use [41], and the International Consortium on the Genetics of Heroin Dependence [42]. SNPs tagging several independent loci met the criteria for genome-wide significance ($P < E^{-7}$) in the AA samples alone or in the combined metaGWAS (Table 7.4). These SNPs were upstream of the gene for S1000 calcium binding protein (*S100B*) and within the gene for CUB and Sushi multiple domains 1

(*CSMD1*). Secondary analysis using a replication cohort found additional support for the dependence severity association score and SNPs in CSMD1 and the drug/metabolite transporter superfamily gene solute carrier family 35 member G1 (SLC35G1). Potential limitations of the study were that CD severity was significantly correlated with dependence for other drugs of abuse (alcohol, nicotine, opioids, and cocaine) and there was high heterogeneity among the sample cohorts included in metaGWAS and used for replication.

The second metaGWAS for CD relied on 8515 individuals of European descent and was drawn from five different cohorts, four of which were ascertained for substance use, including COGA, SAGE, and the Comorbidity and Trauma Study [43]. Agrawal and colleagues [28] analyzed 2080 dependent and 6435 non-dependent cannabis-using controls from this cohort using metaGWAS. The selection of non-dependent controls (based on DSM-IV criterion) with at least one reported use of cannabis was a unique aspect of the study. SNPs on Chr 10 were identified as genome-wide significant and there was modest evidence for replication of this association in AA (but not EA) individuals in a small independent replication sample (896 AA cases and 1591 controls). These SNPs were not associated with genes but the authors provided some evidence that one SNP in the Chr 10 region (rs1409568) may be located within an active enhancer. A suggestive association between dependence severity (cannabis dependence symptoms counts based on DSM-IV criteria) and SNPs on Chr 2 around marker rs2287641 was also identified but did not replicate in the independent cohort.

Finally, Demontis and colleagues [39] identified an association between SNPs located in a cluster on Chr 8 (rs56372821 index SNP) and CD using a data set consisting of 2387 dependent cases and 48,985 controls. The cohort used in this analysis differed from most of the previous studies in that it was ascertained for major mental illness (schizophrenia, bipolar disorder, attention deficit hyperactivity disorder, anorexia nervosa and autism spectrum disorder) and not drug use or dependence. All individuals were part of the Lundbeck Foundation Initiative for Integrative Psychiatric Research (iPSYCH) Danish nation-wide cohort [44]. Significant replication was observed in a replication sample of 5501 cases and 301,041 controls. Of note, expression of the neuronal acetylcholine receptor (nAChR) alpha-2 subunit gene, *CHRNA2*, in human cerebellum was found to be controlled by the variants at the Chr 8 locus using the Genotype-Tissue Expression (GTEx) dataset [45]. Taken together these results provide a possible causal mechanism by which variants at the Chr8 locus regulate *CHRNA2* brain expression and possible risk of CD.

7.3 Limitations and Future Directions

Increasing the sample size of GWAS for cannabinoid use has dramatically increased the number of markers that pass the criteria for genome-wide significance. This was clearly demonstrated by Pasman and colleagues [29] following identification of 8 independent genome-wide significant markers on Chrs 3, 7, 8, 11, 16, and 17 for lifetime cannabis use in a cohort of 184,765 individuals. Another large case-control study of 51,372 individuals identified one genome-wide significant loci on Chr 8 that replicated in an equally sized replication cohort [39]. However, these successes are modest compared with recent large GWAS studies for other diseases. For example, over 100 risk loci for Schizophrenia have been identified in GWAS combining ~50,000 individuals [6].

There are several possible reasons for the paucity of strong candidates in human association studies of cannabis use and dependence. The first reason is that the genetic architecture of cannabis use and dependence may be difference than that of other substance use disorders and psychiatric diseases. Most GWAS for substance use disorders and psychiatric diseases demonstrate a large genomic target associated with disease risk. In other words, many biological pathways and genes contribute a small amount to overall risk. Thus, many genes of small effect combine in an

additive fashion to influence disease risk. This type of genetic architecture typically results in a positive linear relationship between sample size and identification of genome-wide significant associations. However, it is possible that the genetic architecture of cannabis use and dependence is different and that rare variants and non-additive interactions or environmental interactions drive the disease more than the combined actions of many small effect loci. However, this hypothesis seems at odds with recent large GWAS for cannabis use and dependence [29, 39] and for GWAS results for other drugs of abuse, which likely share some common underlying biological and genetic mechanisms.

Perhaps a more likely explanation for the small number of high-confidence candidates is the type of data sets used in recent GWAS. For cannabis research, there is a lack of large population-based data sets for which individuals were ascertained primarily for cannabinoid or cannabis-related traits. Combining data sets ascertained for psychiatric disease or dependence for other drugs of abuse can lead to heterogeneity among samples that can actually decrease power. This may explain why so few loci were identified as genome-wide significant level for association with lifetime cannabis use in a massive data set of nearly 200,000 individuals [29].

Yet another issue with association studies over the past two decades is the lack of replication among data sets. Only a single linkage region overlapped among FLS (Table 7.2). Of all the suggestive and significant associations identified in GWAS (Table 7.4), only two genes (*CADM2* and *NCAM1*) were identified by different studies [29, 32]. However, it is important to note that both studies that identified *NCAM1* and *CADM2* included the same set of ~30,000 individuals from the International Cannabis Consortium.

Association studies in humans have the capability to identify genes and risk alleles. As the sample sizes for GWAS studies increase, so does the number of associations. If these alleles can be identified directly in humans, why the need for testing in preclinical animal models? The answer depends on biological systems and causality. Preclinical genetic animal models (specifically rodents) offer the ability to directly test the role of genes in CUDs and explore the underlying biology in ways that would be impossible in humans. Environmental factors can also be controlled and manipulated in preclinical studies in ways that are not possible in studies involving human subjects. Bi-directional translation between association studies in humans and preclinical models is essential for identifying the environmental, genetic, and molecular mechanisms contributing to disease and for design of effective therapeutics.

One of the simplest ways in which genetic preclinical models support association studies is through reverse genetic engineering. In this case, candidate genes are manipulated in the preclinical model to evaluate their role in disease. It is even possible to introduce the precise human genetic variant into a preclinical model to evaluate its impact. Such humanized mice have been used to evaluate the role of common functional variants in the catechol-O-methyltyransferase gene [46] and to model the role of alleles involved in risk of familial Alzheimer's disease [47]. Thus, genetic engineering approaches in preclinical rodent models can be used to directly evaluate the role of candidate genes evaluated in human association studies. However, relatively few genes have been evaluated for a role in cannabis or cannabinoid-related traits in rodent models. The only gene identified from human association studies that has also been independently evaluated for cannabinoid-related traits is the *NRG1* gene. Of interest, mice heterozygous for deletion of murine *Nrg1* show enhanced sensitivity to the main psychoactive cannabinoid in cannabis, Δ9-tetrahydrocannabinol or THC [48–51]. Recent advances in genetic engineering, including CRISPR/Cas9 mediated genetic engineering [52] should facilitate functional evaluation of genes and gene variants such as *CADM2* and the schizophrenia susceptibility gene, *NCAM1*, associated with cannabis use, dependence, and/or withdrawal in humans.

Preclinical genetic models can also be used for unbiased genome-wide linkage or association studies to identify genes and gene variants that contribute to disease variation. Examples of these

include rodent genetic models in which two or more inbred progenitor strains are crossed repeatedly and then inbred (recombinant inbred lines such as the BXD panel or collaborative cross panel in mice) or outcrossed repeatedly (diversity outcross and heterogenous stock mice) to create genetic panels segregating millions of variants [53–56]. For a review see [57]. As of 2018, traits related to CUD in humans (initial sensitivity, tolerance and dependence, withdrawal severity, and/or self-administration) have not been profiled in genetic rodent populations in order to identify candidate genes. A single study attempted a short-term selection in an F2 cross between C57BL/6J and DBA/2J inbred strains in order to determine if locomotor sensitivity to THC was heritable and could be selected for in order to produce progeny that carry sensitive or resistant alleles for later genetic dissection [58]. As in all systems, preclinical models have some advantages and disadvantages. The clear advantage is the ability to manipulate all aspects of preclinical studies and derive causality from these controlled manipulations. The main disadvantage is that preclinical models are not identical to humans at all levels of behavior and physiology and, as a result, there will always be some controversy regarding translatability.

7.4 Conclusions

Association studies for cannabis use and dependence over the past two decades have identified candidate linkage regions (FLS, Table 7.2) and genes (primarily through GWAS, Table 7.4). In contrast, CGAS have yielded inconsistent results. Over the next two decades, it is likely that more GWAS containing 50,000 to 1000,000 individuals will be performed for cannabis use, dependence, and withdrawal. Recruiting samples directly for these traits along with other methods to reduce heterogeneity among cohorts can be expected to increase the number of genome-wide significant associations. This should lead to a larger and more reproducible list of candidates and a better assessment of polygenic risk and genetic architecture. It is also important to remember that, despite some of the current issues with power and reproducibility, human association studies have identified candidate genes and mechanisms that should be evaluated to determine how and how much they contribute to disease risk. The stage is already set for this type of translational research in preclinical animal models.

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Pharmacology of Medical Cannabis

8

Md Ruhul Amin and Declan W. Ali

Abstract

The Cannabis plant has been used for many of years as a medicinal agent in the relief of pain and seizures. It contains approximately 540 natural compounds including more than 100 that have been identified as phytocannabinoids due to their shared chemical structure. The predominant psychotropic component is Δ^9-tetrahydrocannabinol (Δ^9-THC), while the major non-psychoactive ingredient is cannabidiol (CBD). These compounds have been shown to be partial agonists or antagonists at the prototypical cannabinoid receptors, CB1 and CB2. The therapeutic actions of Δ^9-THC and CBD include an ability to act as analgesics, anti-emetics, anti-inflammatory agents, anti-seizure compounds and as protective agents in neurodegeneration. However, there is a lack of well-controlled, double blind, randomized clinical trials to provide clarity on the efficacy of either Δ^9-THC or CBD as therapeutics. Moreover, the safety concerns regarding the unwanted side effects of Δ^9-THC as a psychoactive agent preclude its widespread use in the clinic. The legalization of cannabis for medicinal purposes and for recreational use in some regions will allow for much needed research on the pharmacokinetics and pharmacology of medical cannabis. This brief review focuses on the use of cannabis as a medicinal agent in the treatment of pain, epilepsy and neurodegenerative diseases. Despite the paucity of information, attention is paid to the mechanisms by which medical cannabis may act to relieve pain and seizures.

Keywords

Cannabinoids · CBD · THC · Medicinal

Abbreviations

Δ^9-THC	tetrahydrocannabinol
2-AG	2-arachiodonoylglycerol
AEA	anandamide
AD	Alzheimer's disease
cAMP	cyclic adenosine monophosphate
CB1	cannabinoid receptor 1
CB2	cannabinoid receptor 2
CB3	cannabinoid receptor 3
CBD	cannabidiol
CBN	cannabinol

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A. N. Bukiya (ed.), *Recent Advances in Cannabinoid Physiology and Pathology*,
Advances in Experimental Medicine and Biology 1162, https://doi.org/10.1007/978-3-030-21737-2_8

CNS	central nervous system
CHO	Chinese hamster ovary
DRG	dorsal root ganglion
EAE	experimental autoimmune encephalomyelitis
GABA	gamma-aminobutyric acid, or γ-aminobutyric acid
GPCR55	G protein-coupled receptor 55
IP3	Inositol trisphosphate
KA	kainic acid
LPI	L-α-lysophosphatidylinositol
MS	multiple sclerosis
SCBs	synthetic cannabinoids
TRPA1	transient receptor potential cation channel, subfamily A, member 1
TRPV1	transient receptor potential cation channel, subfamily V, member 1
TRPV2	transient receptor potential cation channel, subfamily V, member 2

8.1 Introduction

Extracts from the cannabis plant have been used medicinally for thousands of years. The first recorded use of cannabis as a medicinal compound appeared almost 5000 years ago in early Chinese texts by the Emperor Chen Nung [1, 2] when it was used as a treatment of malaria, constipation, rheumatic pain and analgesia in childbirth. Similar accounts of its use as a therapeutic agent occurred in ancient Egypt and India, around 3000 years ago [3, 4]. In more modern times it was listed in Canadian, US and British pharmacies for many years before concerns of its effects as a psychotropic agent led to it being criminalized and listed as an illicit drug of abuse in the 1970s. However, the last 15–20 years has seen a resurgence in interest of cannabis as a therapeutic agent for a range of illnesses and diseased conditions, and the decriminalization and legalization of cannabis will surely pave the way for much needed research on the therapeutic potential of this plant.

The origins of cannabis plant use can be traced back to central Asia [5, 6] with an appearance in the Western hemisphere in the 1500s [7]. There is general agreement among botanical taxonomists that more than one species of cannabis plant exists, with possibly up to 4 species in existence: *Cannabis sativa*, *Cannabis indica*, *Cannabis ruderalis* and *Cannabis afghanica*. The predominant form that is widely used in western society is *Cannabis sativa*, of which there are multiple chemical phenotypes (or chemotypes) which express differing chemical compositions of cannabinoids. Different chemotypes range from plants that contain Δ^9-THC as the predominant cannabinoid, to plants that contain CBD as the predominant cannabinoid, to a variety of mixtures of the two [7]. There are even chemotypes that express high titers of other less known cannabinoids such as cannabidivarin or tetrahydrocannabivarin (THCV) [7]. The wide range of chemotypes is especially pertinent for medicinal forms of cannabis where producers aim to breed specific chemical phenotypes that are high in CBD and low in THC in order to minimize unwanted psychotropic effects of Δ^9-THC.

C. sativa contains approximately 540 natural compounds of which more than 100 have been identified as phytocannabinoids due to their shared chemical structure [8]. Phytocannabinoids are neutral cannabinoids that possess a lipid backbone featuring alkylresorcinol and monoterpenes in their molecules [8, 9] (Fig. 8.1). Cannabinoids are biosynthesized as cannabinoid acids and then decarboxylated into the neutral forms [8]. Phytocannabinoids can be classified into several subclasses including the tetrahydrocannabinol type, the Δ^9-tetrahydrocannabivarin type, the cannabidiol type, the cannabinol type, and several others [8]. Of these, trans-Δ^9-tetrahydrocannabinol (Δ^9-THC) and CBD are the compounds that have been investigated to a much greater degree compared with many of the others, with CBD showing significant potential as a therapeutic agent in a several pathophysiological or diseased states.

While selective breeding of various chemotypes leads to a number of varieties that express very different titers of cannabinoids, the predominant cannabinoid in *C. sativa* which induces psychotropic effects is Δ^9-THC. It was not until the cloning of the first cannabinoid receptor

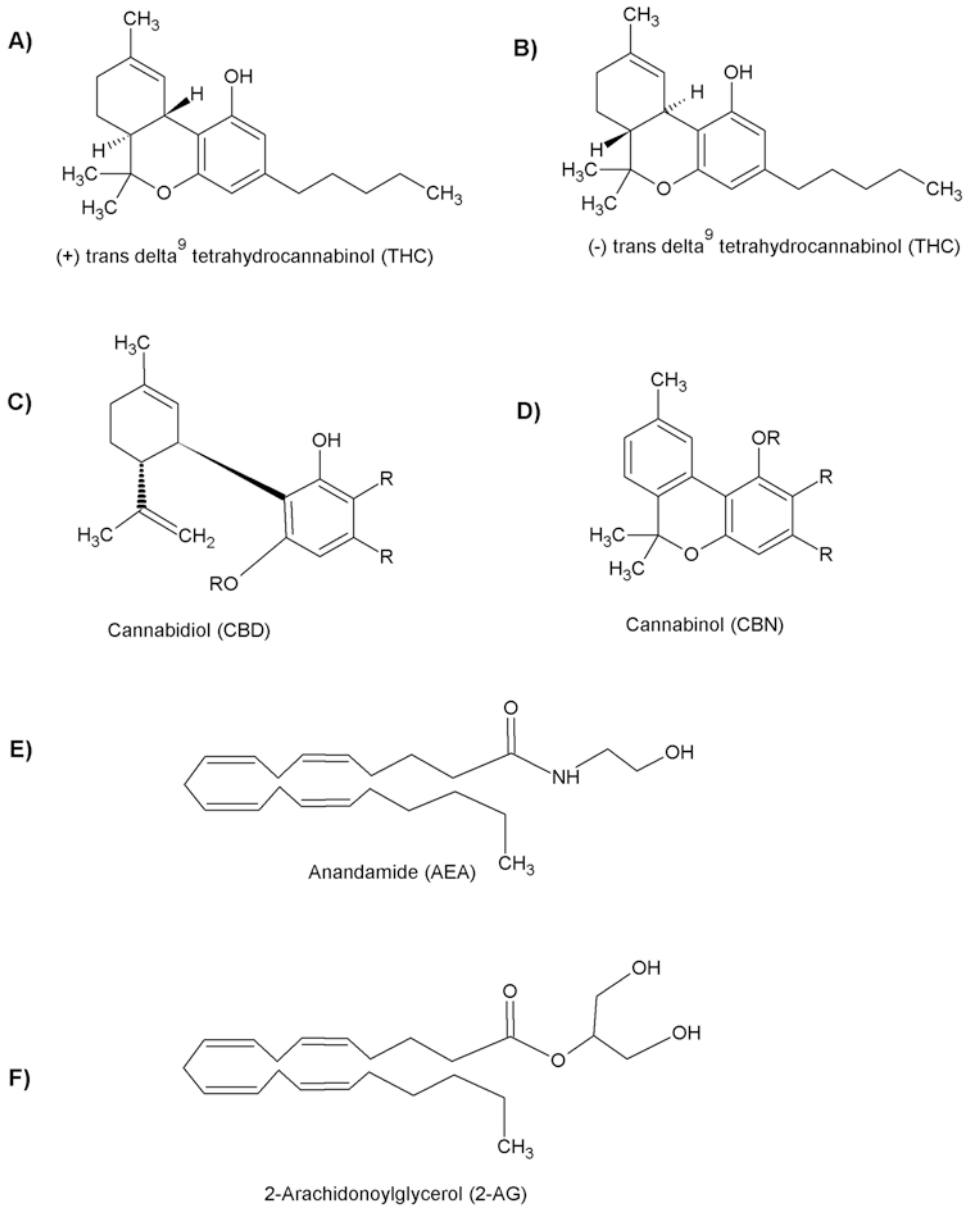

Fig. 8.1 Chemical structure of several phytocannabinoids (A-D), (+) trans-Δ^9-tetrahydrocannabidiol, (−) trans-Δ^9-tetrahydrocannabidiol, cannabidiol (CBD), cannabinol (CBN), and the endocannabinoids (E-F), anandamide (AEA) and 2-arachiodonoylglycerol (2-AG)

type (CB1) in 1990 that the pharmacodynamics of phytocannabinoids was initiated [10, 11]. Three years later, the second cannabinoid receptor type 2 (CB2) was cloned [12]. We now know that phytocannabinoids have the ability to influence many physiological states through their interactions with receptors and transmembrane proteins such as the prototypical CB receptors, transient receptor potential cation channels (e.g. TRPV1, TRPV2, TRPA1) and the serotonin receptors 5HT2 to only name a few. We first address some of the relevant receptor and protein interactions and then focus on therapeutic applications for pain relief, epilepsy and neurodegeneration.

8.2 Cannabinoid Receptors

It had long been thought that cannabinoids interact with receptors to produce their wide-ranging effects as psychotropic agents, analgesics or antiemetic compounds, but it was not until 1990 that the first cannabinoid receptor was cloned from rat cerebral cortex cDNA library [10]. The translated genetic sequence gave rise to a 473 amino acid protein of the G-protein coupled family of receptors, which contained seven putative hydrophobic or membrane-spanning domains, and several potential glycosylation sites. When expressed in Chinese hamster ovary K1 cells the protein displayed cannabinoid stereo-selectivity and cannabinoid-induced inhibition of adenylate cyclase activity [10]. Consequently, the human homologue (472 amino acid protein) and mouse homologue (473 amino acid protein) were rapidly identified [11, 13]. Three years after the initial cloning of the rat CB1 receptor, a second type of G-protein coupled cannabinoid receptor was cloned from a human promyelocytic leukaemia cell line (HL60) [12]. This receptor was highly expressed in macrophages obtained from spleen and its amino acid composition exhibited significant divergence from the CB1 receptor that was cloned from rat brain. Evidence has now accumulated to show that both CB1 and CB2 receptors are negatively coupled to adenylate cyclase and are typically expressed in very different regions of the body. CB1 receptors are mainly limited to the brain and CNS, while CB2 receptors are largely confined to the peripheral nervous system and the immune system. A detailed tissue distribution of cannabinoid receptors is reviewed elsewhere [14, 15]. Radiolabeling of CB1in the brain with the tritiated CB1 receptor agonist [^{3}H] CP55,940 showed high density expression in regions of the basal ganglia such as the *substantia nigra* pars reticulata and globus pallidus, as well as in the hippocampus and cerebellum [16]. However, expression was sparse in the thalamus and lower brainstem regions [16]. The subcellular location of receptors provided clues of their functional roles. Because CB1 receptors are highly localized to presynaptic membranes, they were thought to act as modulators of synaptic release. Indeed, physiological studies confirmed this hypothesis and showed that activation of CB1 altered synaptic transmission in a homeostatic manner. But how does this occur? What are the mechanisms that underlie these effects? To answer these questions we need to delve/examine the literature on the pharmacology of CB1 receptor activation (Table 8.1).

Table 8.1 Ki values for phytocannabinoids and endocannabinoids at CB1 and CB2 receptors

Compound name	*Ki* (at CB1 receptor)	*Ki* (at CB2 receptor)
(−) Δ^9-THC	5–80 nM[a]	3–32 nM[a]
(−) Δ^8-THC	44–48 nM[a]	39–44 nM[a]
CBD	4350 nM[a]	2860 nM[a]
CBN	120–1130 nM[a]	96–300 nM[a]
AEA	61 nM (mice)[b]	1930 nM[b]
2-AG	472 ± 55 nM[b]	1400 ± 172 nM[b]

[a]Pertwee RG (2008) The diverse CB1 and CB2 receptor pharmacology of three plant cannabinoids: Δ^9-tetrahydrocannabinol, cannabidiol and Δ^9-tetrahydrocannabivarin. *British Journal of Pharmacology* 153, 199–215; doi:https://doi.org/10.1038/sj.bjp.0707442

[b]Bow EW and Rimoldi JM (2016) The structure–function relationships of classical cannabinoids: CB1/CB2 Modulation. *Perspect Medicin Chem.* 2016; 8: 17–39; doi: 10.4137/PMC.S32171

Both CB1 and CB2 receptors are negatively linked to adenylate cyclase activity (Fig. 8.2). When the receptors are expressed in cell lines, they initiate a pertussis toxin mediated event that requires Gi/o signaling and that results in a reduction of cAMP production [17]. Ligand binding studies show that the endocannabinoid, anandamide, is capable of inhibiting adenylate cyclase activity in membranes possessing CB1 receptors [18, 19], but this same agonist shows markedly less efficacy on CHO cells expressing CB2 receptors, suggesting that anandamide has differential effects on CB1 vs CB2 receptors. In contrast, the other main endocannabinoid, 2-Arachidonoylglycerol (2-AG), acts as a full agonist at the cannabinoid receptors when inhibiting forskolin-induced cAMP accumulation [20]. A critical determinant of the downstream effects of CB receptor activation is the isoform of adenylate cyclase that associates with the receptor. For instance, ligand binding to CB receptors co-expressed with adenylate cyclase

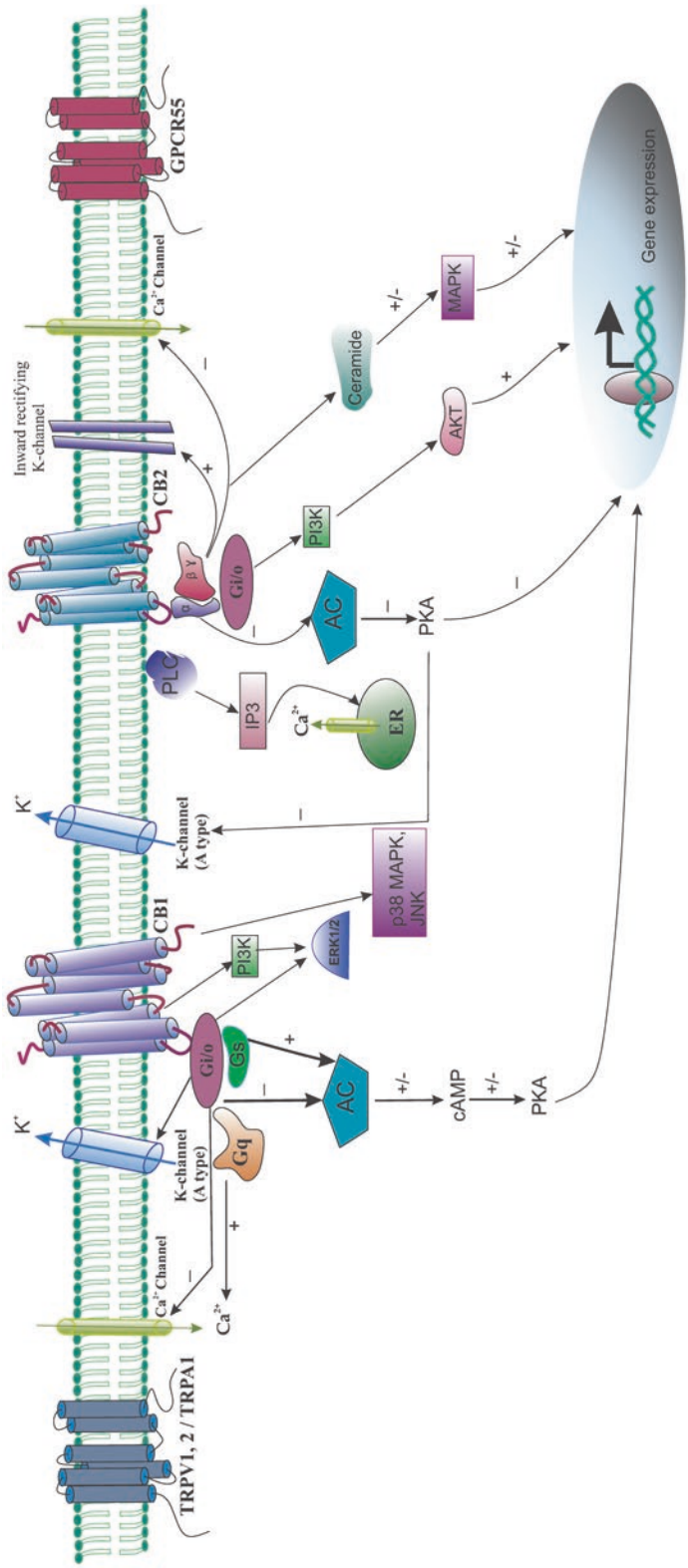

Fig. 8.2 Schematic outline for some of the possible receptors for phytocannabinoids and endocannabinoids. The prototypical G-protein coupled receptors for cannabinoids are CB1 and CB2, but GPR55 has been suggested to be a possible third cannabinoid receptor. CB1 and CB2 are negatively coupled to adenylate cyclase (AC) via Gi/o, while GPR55 is potentially linked to the IP3/DAG/Ca^{2+} system. Cannabinoids are also known to bind to transient receptor potential channels such as TRPV1, TRPV2 and TRPA1. Possible downstream effects include the regulation of genes and ion channel activity (A-type K^+ channels)

isoforms 1, 3, 5, 6 or 8 leads to inhibition of cAMP, whereas co-expression with adenylate cyclase isoforms 2, 4, or 7 leads to stimulation of cAMP production [21, 22]. Thus, CB1/CB2 are capable of activating Gq in addition to Gi/o even though much of the endogenous or physiological activity appears to lead to an inhibition of cAMP. Our understanding of the mechanisms that underlie key interactions between the cannabinoid receptors and their agonists and antagonists was further increased with the elucidation of the crystal structure of the human CB1 receptor in 2016 [23].

The search for additional cannabinoid receptors led to the presentation/publication of convincing evidence in 2007 that the orphan receptor GPR55 is a cannabinoid receptor [24]. Cloning, sequencing and expression of GPR55 showed that the CB1/CB2 receptor ligand [^{3}H]CP55940 exhibited high specificity for GPR55. Moreover, the receptor can also be activated by Δ^9-THC, anandamide, 2-AG and the CB1 selective agonist noladin ether. Interestingly, 2-AG displays almost 200-fold greater potency as an agonist at GPR55 compared with the prototypical CB1and CB2 receptors, and that Δ^9-THC has a greater efficacy at GPR55 compared with CB1 or CB2. GPR55 couples to Ga13 [24], but has also been linked to increases in intracellular Ca^{2+} via a mechanism that involves Gq, G12, RhoA, actin, phospholipase C and Ca^{2+} release from IP_3-gated stores [25]. In other words, cannabinoid receptors are linked to multiple second messenger systems that have the potential to couple enzyme activity to ion channel behavior to gene activation and more. An investigation into the role of GPR55 at presynaptic terminals of CA3-CA1 synapses show that activation of GPR55 by L-α-lysophosphatidylinositol (LPI) transiently increases calcium release probability by elevating presynaptic Ca^{2+} through activation of local Ca^{2+} stores, implying a possible role in short-term potentiation in hippocampus [26]. Based upon these findings there have been suggestions that the GPR55 receptor could be renamed a type 3 cannabinoid receptor, CB3. Nonetheless, its current classification notwithstanding, GPR55 shows significant characteristics of a true cannabinoid type receptor and fully determining its distribution within the body, subcellular localization, temporal expression patterns and downstream signaling pathways will lead to a greater understanding of the function of endocannabinoids and effects of phytocannabinoids.

There is now significant evidence for a direct interaction between cannabinoids and transient receptor potential channels such as the transient receptor potential of vanilloid type 1 and 2 (TRPV1 and TRPV2) and transient receptor potential of ankyrin type 1 (TRPA1) [27]. TRPV1 and V2 channels are cation channels that allow the passage of Na^+, K^+ and Ca^{2+} across cell membranes and are activated by capsaicin or heat above temperatures of 40 °C and above ~50 °C respectively, whereas TRPA1 are menthol and cold activated cation channels [28]. TRPV1 are activated by the endocannabinoids 2-AG and anandamide [29], while TRPV2 and TRPA1 are activated by Δ^9-THC and CBD [29–31]. TRPV1 are largely found in the cerebellum, basal ganglia, hippocampus, diencephalon and DRG neurons [32, 33]. TRPV2 tend to be localized to sensory neurons of the DRG, spinal cord, and trigeminal ganglia, but are also found in the cerebellum [34, 35]. TRPA1 is extensively colocalized with TRPV1 in sensory neurons [36–38]. Activation of these receptors typically leads to membrane depolarization and activation, but TRPV1 and TRPA1 are known to exhibit functional desensitization. In other words, activation of TRPV1 and TRPA1 by cannabinoids may lead to an immediate depolarization, but this will be followed by sensitization and subsequently inhibition because further activation by ligands, heat or cold will be muted as the channels are in a desensitized state. Some evidence exists for the direct interaction between Cannabinoids and ion channels and it has been hypothesized that some of the CB1/CB2-independent cannabinoid effects occur in this manner.

8.3 Pharmacokinetics of Cannabinoid Preparations

THC is highly lipophilic and accumulates in adipose tissue and the spleen which can act as long-term storage sites [39]. It is estimated that up to 37% of Δ^9-THC present in cigarettes can be delivered to the body during smoking while up to 30% is destroyed via pyrolysis [40]. When smoked, Δ^9-THC enters the blood stream extremely rapidly with rising levels detected in blood plasma within 1–2 min of the first inhalation [41]. In controlled experiments, puffs of a 3.5% Δ^9-THC cigarette result in peak Δ^9-THC blood plasma levels of approximately 270 ng/ml [41], and in experiments where the THC content of cigarettes was kept at either a "low" dose of 1.75% or a "high" dose of 3.55%, the blood plasma levels obtained from individuals smoking the higher dose cigarettes were variable and ranged from <90 ng/ml to >250 ng/ml [41]. These data indicate that the bioavailability varies substantially with each individual, and factors such as weight, gender, age, health and physiological background will likely impact the extent to which Δ^9-THC and other cannabinoids affect an individual. Δ^9-THC taken orally usually peaks in the circulation within 1–2 h, with blood plasma levels lower than those obtained during smoking [42]. Δ^9-THC accumulates in fatty tissue and organs such as the heart, liver and spleen [39]. It readily crosses the blood-brain barrier and can be found in high quantities in the brain [42]. THC released from fat has a half-life of several days and in some instances may take up to several weeks to fully clear from adipose tissue [41, 43].

Much of the metabolism of Δ^9-THC occurs in the liver where it is converted to 11-hydroxy-THC or 11-nor-9-carboxy-THC [41]. This conversion is rapid and occurs within minutes of THC detection in blood plasma [43–45]. Whereas 11-hydroxy-THC is psychotropically active, 11-nor-9-carboxy-THC is not [46] and is the principle component found in urine analyses as a proxy for determining cannabis consumption [43]. Numerous additional oxidative metabolites occur, but in lesser quantities.

8.4 Medicinal Cannabis

Cannabis has been used as a medicinal agent and an analgesic for many years. It is sought after as an anti-emetic (anti-nausea agent), a treatment for epilepsy, muscle spasms, multiple sclerosis, neuropathic pain, neurodegenerative diseases and cancer. Cannabis-derived pharmaceuticals such as nabilone (a compound of the same general type as Δ^9-THC), nabiximols and dronabinol (a synthetic Δ^9-THC) are prescribed to relieve chemotherapy-induced nausea and vomiting. Sativex (a combination of Δ^9-THC and CBD) has been used to alleviate neuropathic pain. We will now explore its use as a medicinal agent.

8.5 Pain

Even though the use of cannabis for the treatment of pain can be traced back to 5000 years ago, there is still only little information on its mechanisms of action. In fact, questions still arise whether or not cannabis may alleviate certain types of pain. Cannabinoids and cannabinoid-based pharmaceuticals are prescribed to alleviate neuropathic pain, which is a severe form of chronic pain arising from lesions or disease affecting the somatosensory system [47]. Evidence is mounting that THC in particular, is somewhat effective in reducing neuropathic pain [48–50], however the data is inconsistent and the potential side effects are concerning. A strong desire to find alternatives to other pain medication such as opioids has pushed cannabinoids-based pain therapies to the forefront, and while there is a general lack of well-designed studies on the effects of medical cannabis as pain medications, there is data to indicate that smoking cannabis is effective for some forms of pain.

Studies designed to compare the effects of smoked cannabis against a placebo showed that participants generally reported effective pain relief with increased efficacy linked to higher THC content [51]. Overall the pain relief was modest, and not as effective as medications prescribed specifically for pain such as, the GABA receptor agonists gabapentin and pregabalin. As a

general rule, more effective pain relief tends to occur when cannabinoids are taken together with existing pain medications as opposed to being taken on their own. For instance, oromucosal sprays such as Nabiximols (equal mixtures of Δ^9-THC and CBD), taken along with existing pain medication results in a significant reduction in pain intensity [49, 52, 53]. Similarly, Δ^9-THC/CBD spray was found to be better than placebo when comparing mean pain relief [54].

Other studies have examined the effects of medical marijuana, which contains several hundred compounds along with approximately 100 cannabinoids [7]. Systematic reviews of randomized clinical trials on the pain relief effectiveness of medical marijuana found that medical marijuana was effective in reducing neuropathic pain only in the short term, measured in days rather than weeks or months. Interestingly, medical marijuana was better than placebo in providing a minimum pain relief of 30%, but there was no statistically significant difference between medical marijuana and placebo when comparing the mean pain relief [54].

When evaluating the effectiveness of cannabinoids for relief of visceral pain such as rheumatic disease pain, the data is inconclusive. Systematic reviews of several randomized clinical trials evaluating Δ^9-THC/CBD oromucosal sprays in patients with musculoskeletal pain, fibromyalgia and rheumatoid arthritis concluded that there was insufficient evidence to recommend cannabinoids as pain relief treatment [55, 56]. However, an analysis of medical marijuana administered as a cigarette resulted in a decrease in abdominal pain and an increase in appetite of patients with Crohn's disease compared with placebo cigarettes not containing Δ^9-THC [57]. Moreover, a 3-month study on the effect of oral Δ^9-THC on chronic pancreatitis led the authors to conclude that there was no significant difference between the effects of Δ^9-THC compared with placebo [58]. Overall, the data is largely inconclusive in support of the idea that medical marijuana provides significant relief for chronic pain associated with cancer, rheumatoid arthritis, or fibromyalgia. Clearly, more research is needed to ascertain the use of cannabis or individual cannabinoids as effective analgesics. Of particular interest is the role of synthetic cannabinoids as analgesics. Synthetic cannabinoids (SCBs), also known as K2, spice, herbal incense and other names, are full agonists at CB1 and CB2 receptors, whereas Δ^9-THC is a partial agonist. Thus, SCBs have the potential to act as pain relief agents. In fact, tail immersion assays in mice, indicate that SCBs such as JWH-018 and JWH-073 do indeed act as analgesics [59]. In these studies, the tails of mice were allowed to freely hang into 55 °C water and the time taken for the mouse to remove its tail from the painfully hot stimulus was measured. Administration of JWH-018:JWH-073 in the ratios of 2:3 and 1:1 resulted in an increase in the tail immersion time, in a manner that was additive for the 1:1 ration but synergistic for the 2:3 ratio of SCBs [59], with the tails of immobilized animals hung freely and were placed in 55 °C water.

How does medical marijuana or cannabinoids (Δ^9-THC/CBD) alleviate neuropathic pain? The answer to this is unclear but several possibilities exist. First, the use of THC as a pain relief agent is problematic because of the potential side effects as a psychoactive agent, whereas CBD offers far more promise because it does not activate CB1 receptors and indeed acts as a negative allosteric modulator of CB1, meaning that it does not induce similar psychotropic effects to that of Δ^9-THC. In fact, high concentrations of CBD can be administered in vivo with relatively few complications [60]. However, care must still be taken when determining the type of patient to receive CBD based upon age, health, pregnancy status, existing illnesses etc. To act as analgesics, cannabinoids may associate with the prototypical cannabinoid receptors, CB1 and CB2Rs, but the data for CB1 is inconsistent and CBD is not an agonist of this receptor. CB1 receptors are largely limited to the CNS and not the periphery but are still associated with sensory neurons. CB1 knockouts in sensory neurons results in a reversal of cannabinoid induced anti-hyperalgesia [61], while another study found that CB1 null-mutant mice experienced significantly less anti-hyperalgesia effects, and only in the peripheral nervous system [29]. In several studies, peripheral

pain responses are studied via examining capsaicin (CAP)-induced nociception. Some of these responses were found to be independent of G-protein coupled pathways [62], implying a more direct mechanism of action such as that associated with transient receptor potential channels. Indeed, cannabinoids acting via TRP channels is a very attractive hypothesis because TRP channels are highly localized to sensory neurons and they have been shown to undergo cannabinoid-induced desensitization. Moreover, their activation does not rely on G-proteins but may rely on Ca^{2+}/calcineurin.

An area that is receiving more attention with regard to pain relief is that of cannabinoid anti-inflammatory effects. Since inflammation can contribute to acute and chronic pain, treatments that reduce inflammation may be effective pain relief agents. CBD has long been known as an anti-inflammatory compound and has been investigated for its ability to prevent osteoarthritic pain through its anti-inflammatory actions. For instance, local administration of CBD to male Wistar rats in which osteoarthritis was induced, resulted in a reduction in transient joint inflammation and blocked osteoarthritic pain [63]. Thus, the actions of cannabinoids as pain relief agents are still unclear. Anecdotally, patients who smoke marijuana espouse its analgesic effects on neuropathic pain, but there are only a few properly controlled, double blind, randomized clinical trials in existence and more are certainly needed if we are to have a clearer picture of medicinal marijuana and pain.

8.6 Epilepsy

Epilepsy is a disease in which neuronal networks in the brain become hyperexcitable and are capable of discharging synchronous activity. Epileptic seizures originate from various regions of the brain, usually cortical or sub-cortical structures, and can be classified as partial or generalized seizures. Epilepsy affects approximately 65 million people worldwide with an incidence rate of around 20–70 new cases per 10,000 people on an annual basis [64–67]. Approximately one third of individuals suffering from epilepsy are drug-resistant, meaning that their seizures cannot be controlled with the application of at least two anti-epileptic medications [68]. Thus, there is significant need for therapies capable of controlling epileptic seizures. It has long been thought that marijuana can reduce the severity and incidence of convulsions, epileptic seizures and spasticity. Animal epileptic model studies have shown that CBD has anticonvulsant abilities when tested in audiogenic seizure models [69, 70]; pilocarpine models [70, 71] and electroshock models [69]. Tests designed to evaluate the efficacy of Δ^9-THC and CBD in animal models of epilepsy clearly indicate that both Δ^9-THC and CBD have anticonvulsant effects in rodents [72]. Similarly, the endocannabinoid anandamide produces anticonvulsant effects in rodents as well [73]. Finally, synthetic agonists of CB1 receptors such as WIN55212, when used in conjunction with standard epileptic drugs, offer a greater degree of relief from seizures [74, 75]. Thus, when it comes to animal models, the evidence is overwhelmingly in support of the anticonvulsant effects of cannabinoids. But what about well-constructed, randomized clinical trials in patients? Are cannabinoids truly effective anti-seizure agents in humans?

Data from clinical trials studying the effect of CBD and CBD-enriched products on seizure frequency, safety and drug interactions is scarce and much of the information on marijuana and cannabinoid anti-seizure properties is anecdotal. One of the earliest clinical trials, reported in 1970, highlighted a randomized study of 9-patients with refractory temporal lobe epilepsy, 4 of whom received CBD for 5 weeks and 5 of whom received placebo for 5 weeks. Two of the CBD treated patients were free of seizures within 3 weeks while none of those who were administered the placebo reported relief from seizures [76]. A double-blind phase 2 study in 1980 examined 15 patients with refractory epilepsy, 8 of whom received CBD in addition to their normal anti-epileptic medication, and 7 of whom received placebo. Four of the CBD patients experienced no seizures during the study while another 3 experienced partial improvement. Only

one of the placebo group showed improvement, while the others were unaffected [77]. More recently, an observational, longitudinal study examining the effect of CBD-enriched cannabis as an antiepileptic in children and adolescents was reported. The CBD-enriched cannabis oil treatment contained a ratio of CBD:THC of 20:1 and was given to children and adolescents with refractory epilepsy in addition to their baseline standard antiepileptic treatment [78]. In total, 69 patients, with a mean age of 9.6 years, received treatment with CBD-enriched cannabis oil. Overall, there was a seizure reduction of <50% in 56% of the patients and a reduction rate of >75% in 35% of patients [78].

Antiepileptic drugs work by either reducing excitation (via blocking voltage-gated Na^+ channels or Ca^{2+} channels, usually T-type), or by increasing inhibition (often by modulating GABA related activity) in the CNS. CB1 receptors are known to regulate neuronal excitability by reducing presynaptic neurotransmitter release. In fact, CB1 receptors are considered to play homeostatic roles since increased levels of activity result in the release of endocannabinoids that feedback on presynaptic CB1 receptors. Ligand binding to these presynaptic receptors activate Gi/o or Gq which leads to a reduction in transmitter release. Activation of the CB1 receptors by endocannabinoids is involved in retrograde inhibition of transmitter release [79–81], the control of neuronal excitability [82] and even in the regulation of some forms of synaptic plasticity [80, 81, 83]. Therefore, it is plausible that increased levels of CB1 receptor activity might dampen neuronal excitation. The specific CB1 agonist WIN55212, and the cannabinoid d9-THC were both able to abolish spontaneous epileptic seizures in rats. Furthermore, levels of 2-AG and expression of CB1 protein increased in the hippocampus of pilocarpine-induced seizure animals [84]. In an elegant study by Monory and coworkers [85], the experimenters introduced conditional mutants lacking CB1 receptors in specific neuronal populations and used a kainic acid model of seizures to show that the CB1 receptors localized to hippocampal glutamatergic neurons are necessary for the CB1-dependent protection against kainic acid-induced acute excitotoxic seizures [85]. Interestingly, the CB1 receptors associated with GABAergic neurons did not appear to play a significant neuroprotective role against KA-induced seizures, only the CB1 receptors localized to glutamatergic neurons. Additionally, virus-mediated conditional overexpression of CB1 receptors in pyramidal and mossy fiber cells of the mouse hippocampus confers neuroprotection and reduces convulsions in an acute kainic acid seizure model [86]. The seizures induced the release of anandamide followed by activation of CB1 receptors. Thus, protection against epileptic-like synchronous activity and overexcitability in neural networks may be conferred by activation of CB1 receptors. In healthy individuals, the endocannabinoid system working through CB1 confers neuroprotection, and in those afflicted with refractory epilepsy, activation of CB1 might constitute an important avenue for medical intervention.

But exactly how does activation of CB1 lead to a downregulation of neural activity? This could happen via a number of mechanisms. For instance, presynaptic activation of CB1 reduces presynaptic Ca^{2+} entry through N-type Ca^{2+} channels and lowers glutamate release [87]. Activation of CB1 also leads to an enhancement of A-type voltage gated K^+ channels [88] as well as an enhancement of inward rectifying K^+ channels conductance [89]. The overall effect of activation of either of these K channel types could lead to a reduction in excitation.

8.7 Neurodegenerative Diseases

While medical marijuana and cannabinoids have been proposed to act as antiepileptics and analgesics, the evidence is mounting for use to alleviate a number of neurodegenerative diseases such as multiple sclerosis and Alzheimer's disease. Additionally, a role in schizophrenia and other psychiatric conditions has been proposed. Multiple sclerosis shares a number of pathological features with other neurodegenerative diseases such as a link with neurodegeneration, neuroinflammation and excitotoxicity. It is an

autoimmune disease that is characterized by demyelination and degeneration of motor neurons, often associated with neuropathic pain, aberrant neuronal activity and debilitating and painful muscle spasms. Cannabis plant extracts have been used with some success to relieve the symptoms of MS [90], while application of a 1:1 ratio of Δ^9-THC and CBD (sativex) via the oral-mucosal route has analgesic effects and limits neuropathic pain while also reducing muscle spasms [52]. Indeed, CBD has been shown to be capable of relieving neuropathic pain associated with MS [91]. In patients with MS, endocannabinoid levels in the circulating plasma are increased [92, 93] whereas in an experimental animal model for MS, known as experimental autoimmune encephalomyelitis (EAE), the endocannabinoid levels in the brain have actually been downregulated [94]. In fact, animals in which CB1 receptors are deficient and are then induced with EAE tend to develop neurodegeneration more rapidly than those that express CB1 receptors [95] implying a neuroprotective role for CB1.

Well-constructed, randomized, double blind clinical trials using whole plant cannabis-based medicinal extracts containing equal amounts of Δ^9-THC and CBD, on a cohort of 160 patients with MS resulted in improved scores on symptoms such as spasticity, spasms, tremor, pain and bladder control, however statistical significance was lacking [96]. A meta-analysis of three studies evaluated a total of over 660 patients with spasticity, to determine if nabiximols (Δ^9-THC:CBD extract) alleviated these symptoms [97]. The authors concluded that nabiximols reduced spasticity beyond what would occur by placebo alone.

Alzheimer's disease is an age-related neurodegenerative disease in which a pathological hallmark is the onset of neurofibrillary tangles and amyloid beta plaques in the brain. Neurodegeneration occurs and the individual presents with a progressive decline in cognition and memory. There is a concomitant activation of microglia in plaque filled regions along with neuroinflammation and oxidative stress. Cell death occurs via multiple mechanisms but in large part due to excitotoxicity. CB1 receptor expression is high in basal ganglia and hippocampus, where β-amyloid plaques tend to occur most often in AD. Neuronal CB1 expression is reduced in these two regions [98] while expression of CB1 and CB2 expressing microglia is increased [99]. These studies suggest that medications that protect from excitotoxicity and neuroinflammation have the potential to offer therapeutic benefits to individuals afflicted with AD because they relieve secondary pathologies rather than the direct cause of the disease. Links between the endocannabinoid system and Alzheimer's disease have been reported [100, 101], and evidence exists that THC may actively inhibit Aβ aggregation [102]. For instance, Δ^9-THC has been shown to be directly linked to AD [102]. In this study, Eubanks and colleagues found that Δ^9-THC competitively inhibits Acetylcholinesterase activity and reduces Aβ aggregation in vitro. Moreover, The CB1 receptor agonists anandamide and noladin ether are capable of inhibiting Aβ toxicity in a differentiated human teratocarcinoma cell line Ntera 2/cl-D1 neurons [103].

As described in a previous section this may be linked to a reduction in glutamate release through downregulation of N-type Ca channel activity, or an upregulation of K-channel activity, both of which are associated with reduced synaptic transmitter release.

8.8 Conclusions

It is clear that medicinal cannabis has the potential to play a significant role in the treatment of ailments from neuropathic pain to epilepsy, nausea, cancer and neurodegenerative diseases. Until now much of the evidence for its use as a medicinal agent has been anecdotal and limited in power. We are at the dawn of a period where legalization of cannabis for medicinal use and recreational purposes will ease the restrictions for research. In this exciting time, we stand to make significant progress in our understanding of the pharmacological basis of the actions of cannabinoids. But there are still obstacles to overcome. For instance, the unwanted psychotropic

side effects of THC limit its capacity as a therapeutic agent. Moreover, the cannabinoid receptor sites need to be fully identified and properly characterized. One can imagine that a wide array of effects such as an analgesic, anti-epileptic agent, anti-emetic or anti-inflammatory compound could occur through the action of highly selective cannabimimetics. This can only be realized following intensive research identifying the molecular targets and signaling mechanisms of cannabinoids. Indeed, there is much to learn.

Acknowledgements This research was supported by the Natural Sciences and Engineering Research Council of Canada Discovery Grant to DWA.

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A. N. Bukiya (ed.), *Recent Advances in Cannabinoid Physiology and Pathology*,
Advances in Experimental Medicine and Biology 1162, https://doi.org/10.1007/978-3-030-21737-2