

Endocannabinoid signaling at the periphery: 50 years after THC

Mauro Maccarrone^{1,2*}, Itai Bab^{3,4*}, Tamás Bíró^{5*}, Guy A. Cabral^{6*},
Sudhansu K. Dey^{7*}, Vincenzo Di Marzo^{8*}, Justin C. Konje^{9*}, George Kunos^{10*},
Raphael Mechoulam^{4*}, Pal Pacher^{10*}, Keith A. Sharkey^{11*}, and Andreas Zimmer^{12*}

¹ Center of Integrated Research, Campus Bio-Medico University, Rome, Italy

² Center for Brain Research, Santa Lucia Foundation IRCCS, Rome, Italy

³ Bone Laboratory, Hebrew University Medical Faculty, Jerusalem, Israel

⁴ Institute for Drug Research, Hebrew University Medical Faculty, Jerusalem, Israel

⁵ DE-MTA 'Lendület' Cellular Physiology Research Group, Department of Physiology, Medical Faculty, University of Debrecen, Debrecen, Hungary

⁶ Department of Microbiology and Immunology, Virginia Commonwealth University, Richmond, VA, USA

⁷ Division of Reproductive Sciences, Cincinnati Children's Research Foundation, Cincinnati, OH, USA

⁸ Endocannabinoid Research Group, Institute of Biomolecular Chemistry, National Council of Research, Pozzuoli, Italy

⁹ Department of Obstetrics and Gynaecology, Sidra Medical and Research Center, Doha, Qatar

¹⁰ National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD, USA

¹¹ Hotchkiss Brain Institute, Department of Physiology and Pharmacology, Cumming School of Medicine, University of Calgary, Alberta, Canada

¹² Institute of Molecular Psychiatry, University of Bonn, Bonn, Germany

In 1964, the psychoactive ingredient of *Cannabis sativa*, Δ^9 -tetrahydrocannabinol (THC), was isolated. Nearly 30 years later the endogenous counterparts of THC, collectively termed endocannabinoids (eCBs), were discovered: *N*-arachidonylethanolamine (anandamide) (AEA) in 1992 and 2-arachidonoylglycerol (2-AG) in 1995. Since then, considerable research has shed light on the impact of eCBs on human health and disease, identifying an ensemble of proteins that bind, synthesize, and degrade them and that together form the eCB system (ECS). eCBs control basic biological processes including cell choice between survival and death and progenitor/stem cell proliferation and differentiation. Unsurprisingly, in the past two decades eCBs have been recognized as key mediators of several aspects of human pathophysiology and thus have emerged to be among the most widespread and versatile signaling molecules ever discovered. Here some of the pioneers of this research field review the state of the art of critical eCB functions in peripheral organs. Our community effort is aimed at establishing consensus views on the relevance of the peripheral ECS for human health and disease pathogenesis, as well as highlighting emerging challenges and therapeutic hopes.

Historical introduction

Fifty years ago the major psychoactive cannabis (*C. sativa*) constituent THC (see [Glossary](#)) ([Table 1](#)) was discovered

Corresponding author: Maccarrone, M. (m.maccarrone@unicampus.it).

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*All authors contributed equally.

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[1]. At first it was believed to exhibit nonspecific activity and few studies investigated its mechanism of action. However, by the mid-1980s numerous observations suggested that specific receptors were possibly involved. Allyn Howlett found that cannabimimetic drugs inhibit cAMP accumulation in neuronal cells by a receptor-mediated cellular response [2] and THC action was found to be stereospecific, indicating a specific site of action [3]. Using a highly potent cannabinoid analog, a receptor was identified by Howlett's group in 1988 [4]. Later, Lisa Matsuda and coworkers [5] established the functional expression of the cloned cDNA. This receptor is now known as the type 1 cannabinoid receptor (CB₁). A second entity, known as CB₂, was identified by sequence homology and presumed to be mainly present in the periphery [6]. Today we know that CB₂ is also present in the central nervous system (CNS). The next step was the discovery of the endogenous ligands of CB₁ and CB₂, the so-called eCBs. As THC is a lipophilic molecule, it was assumed that any endogenous cannabinoid would probably be a lipid. Using methods for the separation of lipids, it was possible to identify the first eCB, AEA ([Table 1](#)) [7]. AEA is a derivative of arachidonic acid, a compound that serves as the precursor of a large number of endogenous molecules (e.g., prostaglandins, prostacyclin, thromboxanes, leukotrienes), an example of evolutionary conservation to save energy by the use of the same biochemical pathways for various biologically important molecules. A second eCB – again an arachidonic acid derivative, 2-AG ([Table 1](#)) – was isolated shortly thereafter [8,9].

There are many additional fatty acid amides of ethanol amines and amino acids present in the brain and (in part at least) in the periphery that do not bind to cannabinoid receptors but whose actions may be cannabinoid receptor

Glossary

Acne vulgaris: one of the commonest human skin diseases, characterized by pathologically elevated and qualitatively altered sebum production (seborrhea) and by inflammation of the sebaceous glands (and of the skin in general).

Acrosome reaction: changes in the sperm head that occur on contact with the egg, allowing it to penetrate and then fertilize the egg.

Advanced glycation end products: substances that can contribute to the development or worsening of many human degenerative diseases.

Anandamide (AEA): the amide derivative of arachidonic acid with ethanolamine (*N*-arachidonoyl-ethanolamine); named after the Sanskrit word for bliss, *ananda*.

Antigen presentation: an adaptive immune response whereby antigen-specific receptors recognize processed antigens in the form of peptides.

Arachidonic acid: common name 5,8,11,14-eicosatetraenoic acid; an essential (diet-derived) ω -6 fatty acid that serves as a precursor for eicosanoids and endocannabinoids.

Asthenozoospermia: reduced sperm motility and sperm count.

Atherosclerosis: accumulation of fats, cholesterol, and other substances (plaques) in and on artery walls that can restrict blood flow.

Autocrine: related to a substance secreted by a cell and acting on surface receptors on the same cell.

Blastocyst: an embryonic stage in mammals comprising a fluid-filled cavity (blastocoel) and two cell types – the inner cell mass and the trophectoderm.

B lymphocyte: an immune cell that makes antibodies against antigens, acts as an antigen-presenting cell, and develops into a memory cell after activation by antigen.

Cannabinoid receptors: GPCRs that bind THC as well as AEA, 2-AG, and other endocannabinoids.

Cannabinoids: natural lipophilic products found in *Cannabis sativa* extracts (e.g., hashish, marijuana). Among >60 cannabinoids present in this plant, all of which are characterized by a terpenophenol bicyclic or tricyclic structure, the best known compound is THC.

Catagen: the cessation phase of hair production that also includes also the growth (anagen) and rest (telogen) phases.

Celiac disease: a GI autoimmune disorder that results in severe damage to the mucosal lining of the small intestine when foods containing gluten are eaten, causing malabsorption, bloating, pain, and diarrhea.

Chemokine: a class of cytokines that function to attract white blood cells (leukocytes) such as lymphocytes, granulocytes, monocytes, and macrophages to sites of infection.

Chemotaxis: the movement of an organism in response to a chemical stimulus.

Cholecystokinin: a peptide released from I-type enteroendocrine cells that are predominantly found in the proximal small intestine of the GI tract. Cholecystokinin regulates food intake and coordinates the digestion of fat and protein.

Chondroclasts: multinucleated cartilage-resorbing cells.

Chondrocytes: cartilage cells derived from mesenchymal stem cells that produce extracellular matrix components of cartilage tissue.

Cumulus oophorus: the cluster of cells that surrounds the oocyte in the ovarian follicle.

Cytokine: a category of small proteins (5–20 kDa) released by immune cells that play an important role in cell signaling affecting the behavior of other cells; cytokines include chemokines, IFNs, and lymphokines.

Dendritic cells: antigen-presenting cells capable of activating T lymphocytes and stimulating the growth and differentiation of B lymphocytes.

Embryo development: development of the fertilized egg to the blastocyst stage through several rounds of mitosis.

Endocannabinoids: a family of lipid messengers that behave as endogenous agonists of CBs in animals. AEA and 2-AG are the best characterized compounds described to date and represent prototype members of the fatty acid amides and monoacylglycerols, respectively.

Endometrium: the inner lining of the uterus, comprising stromal and epithelial cells.

Endotoxemia: the presence of endotoxins in the blood, which may result in shock.

Enteric nervous system: the enteric nervous system is the third division of the autonomic nervous system. It comprises nerve cell bodies arranged in two ganglionated plexuses (the myenteric and submucosal plexuses) and an extensive network of nerve fibers that lie in the wall of the GI tract. It provides local neural control of the GI tract and is required for the coordination of the digestive and defensive functions of the gut.

Enterocytes: absorptive epithelial cells that line the lumen of the GI tract.

Enteroendocrine cells: specialized hormone-producing epithelial cells of the GI tract and pancreas. Enteroendocrine cells of the intestine are the most numerous endocrine cells in the body.

Epidermal barrier: the barrier – comprising physicochemical, immunological, and microbial aspects – formed chiefly by the outermost (i.e., epidermal) layer of the skin that protects the body against various external challenges and maintains cutaneous homeostasis.

Fatty acid amide hydrolase (FAAH): an endomembrane-bound serine hydrolyase that cleaves AEA into arachidonic acid and ethanolamine.

Fibrosis: excessive formation of connective tissue due to the overactivity of dermal fibroblasts.

Idiopathic infertility: infertility for which there is no obvious cause.

Implantation site: the site of embryo attachment in the uterus (implantation chamber).

Inflammatory bowel disease: a group of chronic inflammatory conditions of the GI tract that are characterized by abdominal pain, diarrhea, rectal bleeding, severe internal cramping, and weight loss. The two main forms of inflammatory bowel disease are Crohn's disease and ulcerative colitis.

Inotropy: alteration of the force or energy of muscular contractions.

Interleukins (ILs): a class of glycoproteins produced by leukocytes regulate immune responses.

Lipogenesis: the process by which acetyl-coenzyme A (CoA) is converted to fatty acids.

Macrophage: a type of leukocyte found in tissues that engulfs and digests cell debris, foreign substances, microbes, and cancer cells. Macrophages process foreign antigens into peptides and present them to T lymphocytes.

Microglia: resident macrophages of the brain and spinal cord.

Monocyte: a type of leukocyte that replenishes resident macrophages and moves to localized sites of infection in tissues, where they divide into macrophages and dendritic cells to elicit an immune response.

Muscular dystrophy: a pathological condition that weakens the musculoskeletal system impairing locomotion. Muscular dystrophies of genetic origin are often due to defective skeletal muscle formation and characterized by progressive skeletal muscle weakness, defects in muscle proteins, muscle inflammation, and death of muscle cells and tissues.

Muscular hypertrophy: a condition that involves an increase in the size of skeletal muscle through an increase in the size of its component cells, often as a consequence of both sarcoplasmic hypertrophy, with increased muscle glycogen storage, and myofibrillar hypertrophy, with increased myofibril size.

Natural killer (NK) cell: a lymphocyte that binds to selected tumor cells and virus-infected cells with antigen stimulation. NK cells kill target cells by inserting granules containing perforin.

Neutrophil: a type of leukocyte that constitutes one of the first responders to inflammation, particularly that due to bacterial infection. Neutrophils are a type of phagocyte normally found in the bloodstream.

(Non-)receptive uterus: a uterus that is (in)capable of supporting blastocyst growth and attachment.

Normozoospermia: normal sperm count, morphology, and motility.

Oligoasthenozoospermia: reduced sperm motility and count.

Oligozoospermia: reduced sperm count.

Osteoblasts: bone-forming cells derived from mesenchymal stem cells.

Osteoclasts: multinucleated myeloid cells resorbing bone.

Osteoporosis: a progressive degenerative bone disorder characterized by reduced bone mass and concomitantly increased fracture risk.

Ovariectomy: surgical removal of the ovaries.

Oviductal embryo transport: the journey of a fertilized egg through the oviduct and ultimately into the uterus.

Paracrine: related to a substance secreted by a cell and acting on surface receptors of nearby cells.

Parturition: the action or process of giving birth to offspring.

Perforin: a protein released by NK cells that destroys target cells by inducing pores in their membranes.

Peroxisome proliferator-activated receptors (PPARs): a superfamily of nuclear receptor transcription factors with three members: α , γ , and δ . PPAR α and γ are targets of AEA and its congeners.

Placentaion: the role of the placenta, which comprises trophoblast cells and establishes a vascular connection between the conceptus and the mother.

Polymorphism: natural variations in a DNA sequence that have been identified in a population.

Restenosis: the recurrence of a stenosis – the narrowing of a blood vessel – leading to restricted blood flow.

Sperm kinematic parameters: the parameters of movement in a forward direction and at an acceptable pace.

Steatosis: the abnormal retention of lipids within a cell.

Sympathetic and parasympathetic projections: nerve endings of the autonomic nervous system originating from cranial or sacral nerves belonging to the sympathetic or parasympathetic nervous system.

Systemic sclerosis (also known as scleroderma): a systemic autoimmune disease that can be characterized by the excessive accumulation of collagen fibers in the skin and internal organs.

T lymphocytes: immune cells that can directly kill infected cells or cancer cells; these cells assist in B lymphocyte maturation and produce cytokines.

Tight junction proteins: families of proteins, including the occludins, zonula occludens, and claudins, that together form an intercellular tight junction between adjacent gastrointestinal epithelial cells. The tight junction lies just beneath the apical surface and comprises interlinked rows of integral membrane proteins limiting the passage of water and other molecules.

Transient receptor potential vanilloid 1 (TRPV1): a 6-transmembrane receptor channel that is activated by physical and chemical stimuli as well as by AEA.

Trophectoderm: the outer layer of the blastocyst and the progenitor of the future placenta.

Δ^9 -Tetrahydrocannabinol (THC): the psychoactive ingredient of cannabis (*Cannabis sativa*) extracts like hashish and marijuana.

Zona pellucida: the glycoprotein layer surrounding the plasma membrane of mammalian oocytes.

dependent [10,11]. They should possibly also be considered *bona fide* components of the eCB family.

Although the discovery of eCBs and the recognition of their ubiquitous activities represented a major advance in biology, we should perhaps wonder why we were for so long unaware of their existence and why these molecules had not been detected through their activity in so many distinct physiological reactions. 2-AG (and possibly also AEA) is a retrograde synaptic messenger that can prevent the development of excessive neuronal activity, a regulatory action

Table 1. Major (endo)cannabinoids and the main metabolic enzymes of the ECS

Name	Chemical structure
THC	
AEA	
2-AG	
OEA	
PEA	
Biosynthetic enzymes of AEA	Intracellular localization
Ca ²⁺ -dependent NAT	Integral membrane protein
Ca ²⁺ -independent NAT (iNAT)	Mainly cytosolic
NAPE-PLD	Membrane associated
α/β -Hydrolase domain 4 (ABHD4)	Membrane associated
Protein tyrosine phosphatase, non-receptor type 22 (PTPN22)	Mainly cytosolic
Glycerophosphodiester phosphodiesterase (GDE1)	Integral membrane glycoprotein
Degrading enzymes of AEA	Intracellular localization
FAAH	Membrane associated (mainly ER)
<i>N</i> -acylethanolamine-hydrolyzing acid amidase (NAAA)	Lysosomal
Biosynthetic enzymes of 2-AG	Intracellular localization
DAGL α	Membrane associated
DAGL β	Membrane associated
Degrading enzymes of 2-AG	Intracellular localization
MAGL	Membrane associated and cytosolic
α/β -Hydrolase domain 6 (ABHD6)	Membrane associated
α/β -Hydrolase domain 12 (ABHD12)	Membrane associated
Oxidative enzymes of AEA and 2-AG	Intracellular localization
COX-2	Membrane associated
LOXs	Membrane associated and cytosolic
CYPs	Membrane associated

of major importance [12]. eCBs are involved in myriad physiological processes [13–15], yet most of the actions of these endogenous molecules were established only after the discovery of the proteins that bind and metabolize them, the so-called ECS (for recent reviews see [14,15]). Here the main ECS components are presented and the state of the art of critical eCB functions in peripheral organs is reviewed. Our community effort is aimed at establishing consensus views on the relevance of the peripheral ECS for human health and disease pathogenesis, as well as highlighting emerging challenges and therapeutic hopes.

The ECS at a glance

The two best characterized eCBs, AEA and 2-AG, bind with different affinities to CB₁ and CB₂, which are both well-characterized 7-transmembrane G protein-coupled receptors (GPCRs) [15–18]. Accumulated evidence suggests the occurrence of other targets for eCBs, like the purported 'CB₃' receptor GPR55 [19] and the transient receptor potential vanilloid 1 (TRPV1) ion channel, which has an intracellular binding site [20]. Other eCB targets such as peroxisome proliferator-activated receptor (PPAR) α and γ are localized in the nucleus, where they shuttle from/to the cytosol in a ligand-dependent manner [21]. In addition to distinct receptor targets, the ECS comprises numerous metabolic enzymes. It is widely accepted that eCBs are produced 'on demand' from membrane lipid precursors by multiple biosynthetic pathways; that is, when and where needed on (patho)physiological stimuli. AEA and 2-AG metabolism occurs through distinct routes, of which several have been described in detail [22,23]. The canonical view is that AEA is synthesized from membrane phospholipid precursors mainly by the sequential action of *N*-acyltransferase (NAT) and *N*-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD). By contrast, diacylglycerol lipase (DAGL) α/β are responsible for the synthesis of 2-AG. The eCB-mediated effects are terminated by their fast catabolism, mainly through hydrolysis by fatty acid amide hydrolase (FAAH) for AEA and by monoacylglycerol lipase (MAGL) for 2-AG. Alternatively to hydrolytic routes, AEA and 2-AG can be oxidized by cyclooxygenase-2 (COX-2), distinct lipoxygenases (LOXs), or cytochrome P450 (CYP). Interestingly, the main oxidative products of AEA and 2-AG are endowed with novel biological activities that are probably mediated by distinct receptors compared with those that bind eCBs [22,23].

Overall, it is apparent that these metabolic enzymes regulate the *in vivo* biological availability of eCBs, which together are responsible for maintaining the eCB tone [24,25]. The best characterized of these enzymes are summarized in Table 1, along with their intracellular localization (for a recent review, see [26]).

The complexity of the ECS supports its manifold activities at the periphery and may offer various targets for the development of selective drugs able to modulate eCB signaling in distinct peripheral cells. In the following sections, current knowledge of the impact of eCB signaling (mainly by activation of the THC-binding CB₁ and CB₂ receptors) in distinct peripheral organs is presented.

Cardiovascular system

Studies over the past few decades have demonstrated that CB₁ and CB₂, eCBs, and their anabolic/catabolic enzymes are present in cardiovascular tissues and may play an important role in the development and/or progression of common cardiovascular disorders [27–29]. Earlier studies focusing on the acute hemodynamic effects in various forms of shock and heart failure have demonstrated that under these pathological conditions eCBs produced by activated monocytes/macrophages contributed to hypotension and negative inotropy via activation of cardiovascular CB₁ [27]. Later studies investigated the signaling mechanisms in murine and human cardiomyocytes, endothelial and vascular smooth muscle cells, and fibroblasts/myofibroblasts using various clinically relevant cardiomyopathy/heart failure, metabolic syndrome/diabetes, and hypertension models [28–34]. These demonstrated that cardiovascular cells can also generate eCBs under pathological challenges/conditions, which in turn, through CB₁-dependent and/or -independent pathways, may promote the generation of reactive oxygen species (ROS), angiotensin II type 1 receptor signaling, accumulation of advanced glycation end products, β -myosin heavy chain isozyme switch, remodeling/fibrosis, and activation of proapoptotic mitogen-activated protein kinases, eventually resulting in decreased function of sarcoplasmic reticulum/endoplasmic reticulum (ER) Ca²⁺-ATPase and cell death, leading to cardiac dysfunction/failure [32–35]. In these experimental models, eCBs and/or CB₁ receptors were increased/upregulated in the myocardium and CB₁ antagonists ameliorated the contractile dysfunction and all of the abovementioned characteristic pathological processes. Experimental studies using CB₁ antagonists and/or CB₁ knockout mice also suggested that eCBs via CB₁ in macrophages may promote proinflammatory processes and inflammatory cell recruitment, thus contributing to the development/progression of atherosclerosis as well as to the pathological smooth muscle proliferation associated with restenosis [33]. The proinflammatory effect of CB₁ in the cardiovascular system was also confirmed using inhibitors and/or knockout of the eCB-metabolizing enzyme FAAH in models of atherosclerosis and cardiomyopathy [33]. In clinical trials in obesity/diabetes, the CB₁ antagonist/inverse agonist rimonabant decreased multiple cardiovascular risk factors, including inflammatory markers. Increased plasma levels of eCBs were strongly correlated with adverse coronary circulatory events or impaired coronary endothelial function in human obese subjects [35]. Several human reports using CB₁ antagonists clearly established that both marijuana and THC exerted CB₁-dependent cardiovascular effects and clinical trials with peripherally restricted CB₁ agonists for pain were discontinued because of development of severe adverse cardiovascular and metabolic consequences of CB₁ stimulation [35]. A recent study also raised serious concerns about the cardiovascular safety of marijuana use in humans [36], which are supported by numerous case reports in young adults consuming marijuana or mixtures of synthetic CB₁ agonists for recreational use [35].

In contrast to CB₁, CB₂ signaling appears to be protective in cardiovascular diseases [28]. In endothelium, vas-

cular smooth muscle and fibroblast CB₂ activation decreases pathological activation, proliferation, or the proinflammatory/fibrotic response and may also exert protective effects in cardiomyocytes [28]. In immune cells, CB₂ attenuates chemotaxis, adhesion of inflammatory cells, and activation [28]. These effects are responsible for the protective properties of CB₂ agonists in preclinical models of atherosclerosis, restenosis, myocardial infarction, and stroke [28]. Importantly, in the cardiovascular system eCBs via degradation to arachidonic acid metabolites or through putative novel cannabinoid or other (e.g., TRPV1) receptors, independently of CB₁ and CB₂, may also exert numerous context-dependent effects, ranging from vasodilation/vasoconstriction to anti-inflammatory/proinflammatory effects [35]. The impact of eCB signaling in the cardiovascular system is schematically depicted in Figure 1 and Table 2 summarizes the therapeutic applications of ECS-related drugs.

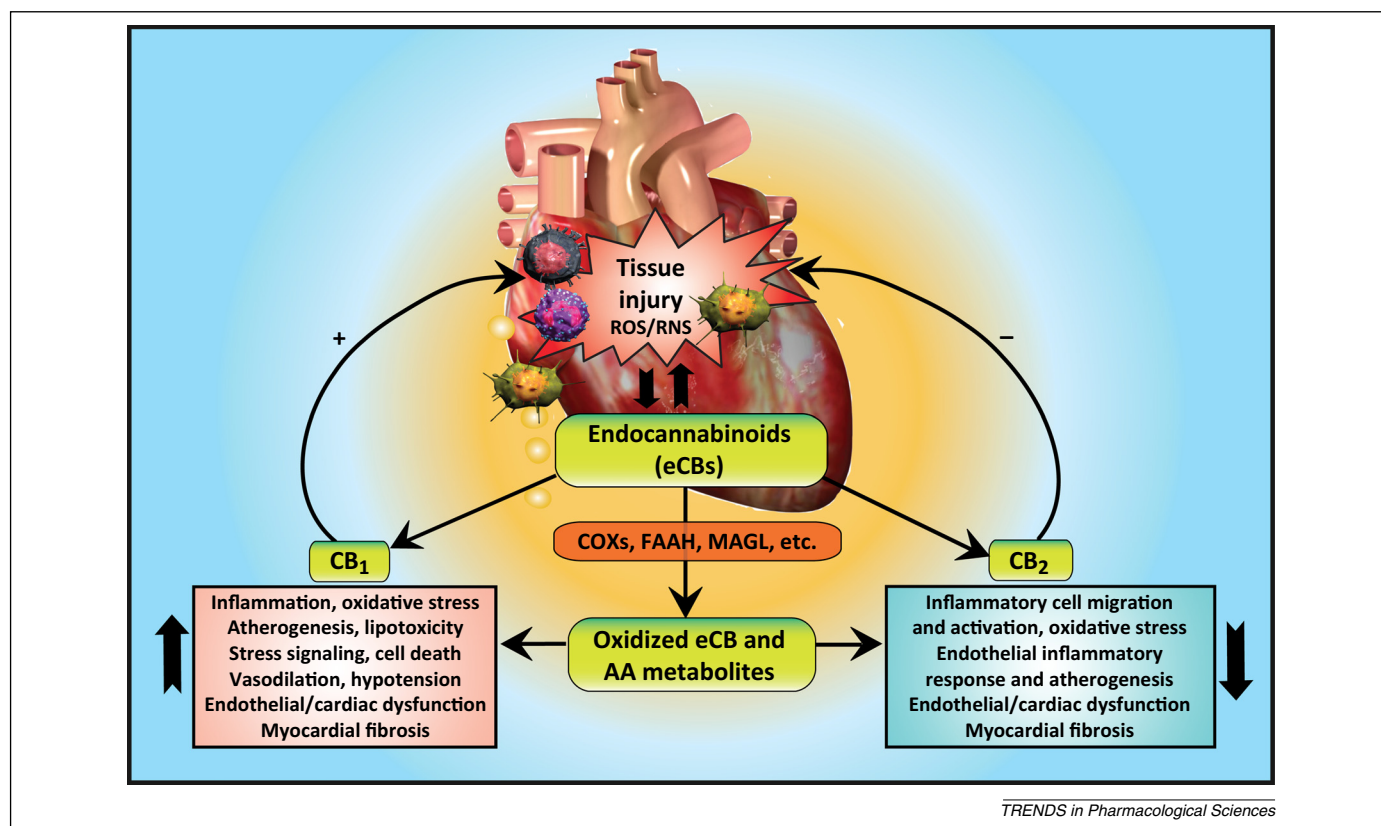
Collectively, there is strong evidence supporting the pathological function of an overactive ECS (particularly CB₁) in cardiometabolic diseases. It can be concluded that: (i) the ECS is dysregulated in cardiovascular disease; (ii) eCBs and synthetic ligands exert opposing effects on cardiovascular injury and inflammation through CB₁ and CB₂, with CB₁ promoting and CB₂ attenuating them; and (iii) peripherally restricted CB₂ agonists and CB₁ antagonists appear to be promising targets in cardiovascular disease.

Gastrointestinal (GI) tract

The mechanistic basis for the therapeutic effects of cannabis in the GI tract was apparent after the discovery of THC as the major psychoactive constituent of cannabis. Even before a specific cannabinoid receptor was cloned, it was shown that THC inhibited acetylcholine release from enteric nerves [37]. This finding paved the way to an extensive examination of the ECS in the gut and the remarkable discoveries that virtually all gut functions are regulated by eCBs and that the ECS of the gut is critical for CNS control of the metabolic and homeostatic functions of the body (Figure 2).

CB₁ and CB₂ are highly expressed on enteric nerves and throughout the intestinal mucosa on enteroendocrine cells (CB₁), immune cells (CB₁ and CB₂), and enterocytes (CB₁ and CB₂), as depicted in Figure 2. Under physiological conditions, the actions of eCBs in the GI tract are largely mediated by CB₁ [37]. Activation of the latter stimulates GI motility, suppresses acid and fluid secretion, and induces mesenteric vasodilatation [37]. The role of CB₁ in the regulation of enteric neurotransmission has recently been revealed. eCBs (and notably 2-AG) are retrograde transmitters in the brain but in the enteric nervous system they also seem to be involved in a previously unknown form of synaptic control that utilizes two retrograde messengers (eCBs themselves and a purine nucleotide) working in opposite directions to control synaptic strength. Such a phenomenon is known as metaplasticity [38].

The role of the ECS in the regulation of energy balance is multifaceted and involves the GI tract in at least three ways. First, CB₁ is localized to enteroendocrine cells and



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Figure 1. Role of the endocannabinoid (eCB) system (ECS) in cardiovascular injury/disease. Cardiovascular insult inflicted by ischemia, inflammation, or hemodynamic overload leads to increased formation of reactive oxygen and/or nitrogen species (ROS/RNS) and inflammation. These processes trigger activation of the ECS in the cardiovascular system and infiltrating immune cells. eCBs, via activation of the type 1 cannabinoid receptor (CB₁) in cardiomyocytes, endothelial cells, fibroblasts, and certain immune cells, promote processes facilitating the development of cardiovascular dysfunction, inflammation, and pathological remodeling. By contrast, via activation of CB₂ eCBs exert opposing protective effects. Moreover, eCBs through their catabolism by fatty acid amide hydrolase (FAAH) and/or monoacylglycerol lipase (MAGL) or oxidation by cyclooxygenases (COXs) or other enzymes may represent a significant source of arachidonic acid (AA) and/or other oxidized eCB metabolites with both pro- and anti-inflammatory effects. Thus, the protective or detrimental effect of eCBs in cardiovascular disease may largely be context, time, and pathology dependent.

may regulate the release of enteroendocrine peptides such as cholecystokinin, which signals hunger [39]. Second, CB₁ expression on enterocytes is regulated by the enteric microbiota [40]. Activation of this receptor enhances epithelial permeability by reducing the expression of tight junction proteins, allowing bacterial translocation and metabolic endotoxemia, leading to obesity. Blocking CB₁ expression reduces obesity, in part through the enhancement of intestinal barrier function. Third, the gut-derived eCBs AEA and 2-AG, and the related compound *N*-oleylethanolamine (OEA) (Table 1), are key signaling molecules for the control of food intake as well as for lipid sensing [41]. OEA in particular seems to be positioned to monitor dietary fat intake and does so by activating both homeostatic vagal afferent pathways, and the brain's reward circuitry [42,43]. In pathophysiological states, both CB₁ and CB₂ reduce accelerated GI motility, with CB₂ activation normalizing motility and serving as a braking mechanism to limit abnormal motility [44]. Interestingly, inhibition of FAAH, thereby elevating endogenous levels of eCBs, also normalizes accelerated GI motility in pathophysiological states but not in normal animals [45]. The ECS is also an important anti-inflammatory system and when activated reduces gastric damage and intestinal inflammation [46,47]. Recent findings have surprisingly revealed that both peripheral and central cannabinoid receptors are required to block the development of colitis [46].

Components of the ECS are altered in many GI disorders, such as celiac disease and inflammatory bowel disease [48,49]. However, the therapeutic potential to target this system in their treatment remains to be determined, while numerous other GI diseases are likely to benefit from ECS-related drugs (Table 2). Although cannabis has been used for decades, the clinical utility of the many experimental compounds that have been developed, which target distinct elements of the ECS, remains uncertain and the central effects of many of these compounds are likely to limit their usefulness. Peripherally restricted compounds could overcome these limitations and appear to have potential in some circumstances [50]. However, a note of caution is warranted. In inflammatory bowel disease, cannabis use is common and subjectively improves pain and diarrhea. In a recent short-term (8 weeks) clinical trial, inflammation was reduced with smoked cannabis in a cohort of patients with Crohn's disease [51]. However, cannabis use is associated with higher risk of surgery in patients with Crohn's disease [52]. This illustrates the problems of pharmacology in a system that is ubiquitous and involved in many diverse functions in the gut (Figure 2).

Fifty years ago, one could not have imagined the remarkable nature of the ECS in the GI tract. To date, we can conclude that: (i) eCB signaling contributes to the regulation of motility, barrier function, immune function, and the control of food intake and energy balance; (ii) CB₁ and CB₂

Table 2. Therapeutic applications of ECS-related drugs at the periphery

Compound/category	ECS target	Model	Therapeutic indication	Clinical condition	Refs
(Peripherally restricted) CB ₁ antagonists	CB ₁	Rodent/human	Cardiomyopathies, heart failure, diabetic cardiovascular complications, atherosclerosis, circulatory shock	Doxorubicin-induced and cirrhotic cardiomyopathies, diabetic cardiomyopathy and vasculopathy, circulatory shock, atherosclerosis, heart failure	[27–29,31–33,35, 63,159,160]
CB ₂ agonists	CB ₂	Rodent	Myocardial infarction, stroke, myocarditis? cardiomyopathies?	Myocardial infarction, stroke, myocarditis? cardiomyopathies?	[28,160]
(Peripherally restricted) CB ₁ agonists	CB ₁	Dog/human	Transient lower esophageal relaxation	Gastroesophageal reflux disease	[161,162]
(Peripherally restricted) CB ₁ agonists	CB ₁	Mouse/rat	Diarrhea Inflammation Visceral pain	Irritable bowel syndrome Inflammatory bowel disease Gastric ulcer	[163] [164–166] [167]
(Peripherally restricted) CB ₁ antagonists	CB ₁	Mouse	Metabolic endotoxemia Food intake Dysmotility	Obesity Paralytic ileus	[43] [40] [168,169]
CB ₂ agonists	CB ₂	Mouse/rat	Diarrhea Inflammation, visceral pain	Irritable bowel syndrome Inflammatory bowel disease	[44,170] [44,170]
FAAH inhibitors	CB ₁ , CB ₂ , PPARs	Mouse	Diarrhea Inflammation	Irritable bowel syndrome Inflammatory bowel disease Gastric ulcer	[45,171] [166,172] [173]
MAGL inhibitors	CB ₁ , CB ₂ , PPARs	Mouse/rat	Diarrhea Inflammation	Irritable bowel syndrome Inflammatory bowel disease Gastric ulcer	[174] [175] [47,176]
Peripherally restricted CB ₁ antagonists	CB ₁	DIO mice, <i>ob/ob</i> mice, <i>db/db</i> mice, ^a ZDF rats ^b	Lipogenesis, inflammation	Obesity/metabolic syndrome, fatty liver disease, type 2 diabetes	[61,177,178]
Rimonabant	CB ₁	Mouse C2C12 myoblasts	Defective myotube differentiation and muscle regeneration	Muscular dystrophy	[100]
Synthetic CB ₂ agonists	CB ₂	Mouse	Bone mineralization	Osteoporosis	[113]
PEA	CB ₁ ? CB ₂ ? TRPV1? PPARs? GPR55?	Human	Vestibulodynia, vulvodynia, proctodynia	Infertility	[179,180]
THC	CB ₁	Mouse	Parturition	Infertility	[125,128]
PEA	CB ₁ ? CB ₂ ? TRPV1? PPARs? GPR55?	Human	Inflammation, pruritus	Atopic dermatitis, prurigo, uremic itch	[140,141]
(Peripherally restricted) CB ₁ antagonists	CB ₁	Rodent	Diabetic and other nephropathies and tubulopathies	Diabetic and other nephropathies and tubulopathies	[151,152,154]
CB ₂ agonists	CB ₂	Rodent	Diabetic and other nephropathies and tubulopathies	Diabetic and other nephropathies and tubulopathies	[153,154]

^aDiet-induced obesity (DIO), *ob/ob* (defective in leptin gene), and *db/db* (defective in leptin receptor gene) mice are models of obesity.

^bZucker diabetic fatty (ZDF) rats are a model of diabetes.

located on enteric nerves, enteroendocrine cells, immune cells, and the intestinal epithelium mediate the actions of eCBs by reducing neurotransmitter release, inhibiting release of enteroendocrine hormones, suppressing immune activation, and regulating tight junctions, respectively; and (iii) the discoveries outlined above provide an exciting array of therapeutic targets and with further developments in this field the opportunities to develop new drugs for GI disorders are immense.

Liver

The ECS of the liver is normally quiescent owing to the low level of cannabinoid receptor expression. However, under

pathophysiological conditions the expression of receptors is increased and they have now been recognized as having a critical role in liver disease (Figure 2).

Although the liver was initially thought to be devoid of CB₁, it is now evident that these receptors are expressed and mediate several biological functions in various types of liver cell, including hepatocytes, stellate cells, and vascular endothelial cells, and that blockade of these receptors contributes to the beneficial metabolic effects of CB₁ blockade.

High-fat feeding promotes hepatic lipogenesis and mice deficient in CB₁ are resistant to high-fat diet-induced obesity and the associated hepatic steatosis

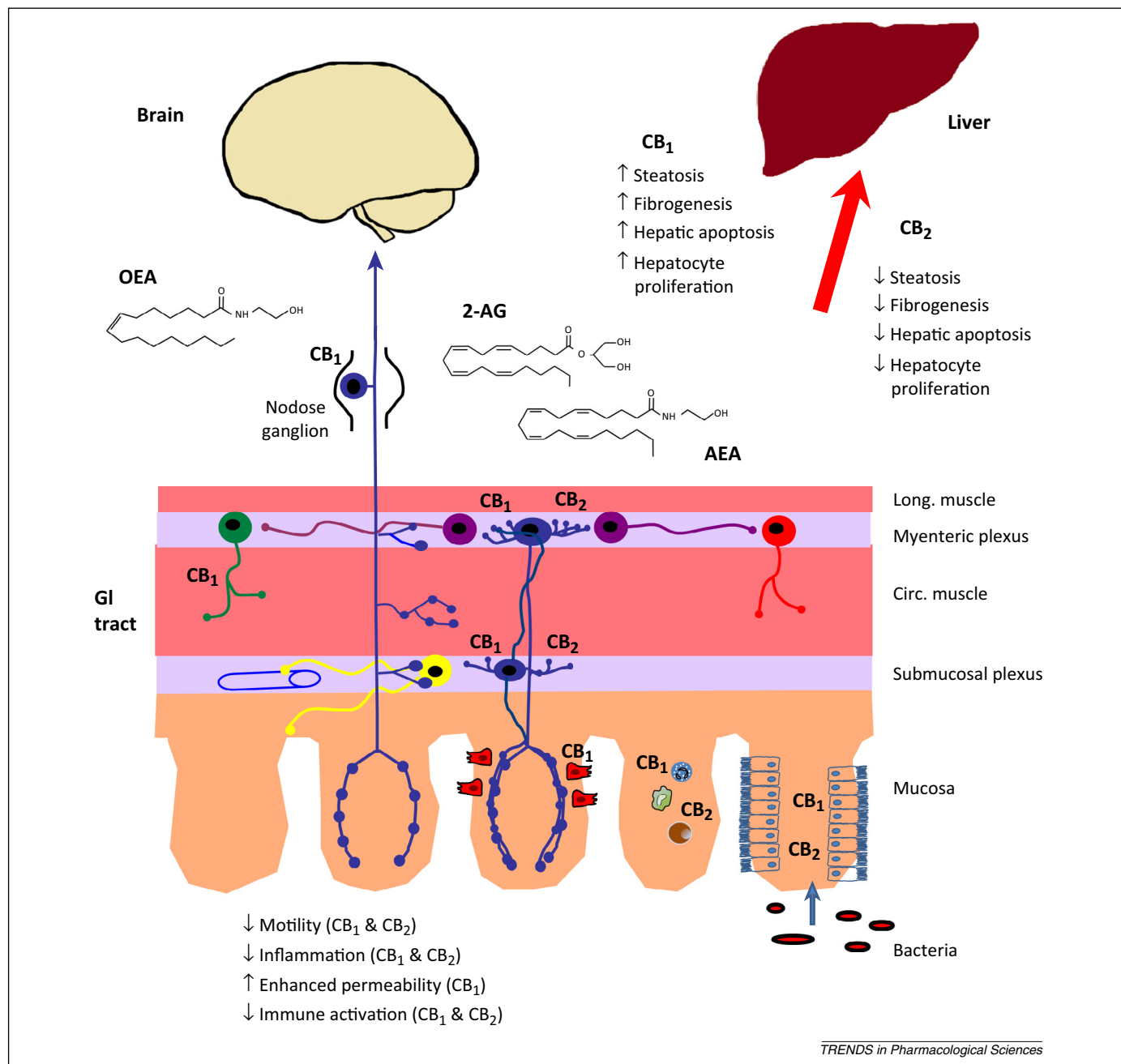


Figure 2. The endocannabinoid (eCB) system (ECS) of the gastrointestinal (GI) tract and liver. *N*-arachidonylethanolamine (anandamide) (AEA), 2-arachidonoylglycerol (2-AG), and *N*-oleoylethanolamine (OEA) are synthesized in the gut and liver, acting locally and in the brain. eCBs regulate gut motility at the level of the enteric neural plexuses, reduce intestinal inflammation through actions on the immune system, and influence intestinal barrier function at the level of the epithelium. eCBs and OEA regulate food intake by actions on enteroendocrine cells in the wall of the gut, the vagus nerve, and the brain. In the liver, the type 1 cannabinoid receptor (CB₁) and CB₂ have opposing effects, with CB₁ promoting steatosis, fibrogenesis, apoptosis, and proliferation and CB₂ inhibiting these effects.

[53], which suggests that functional CB₁ in hepatocytes promotes lipogenesis. This was documented by the ability of a CB₁ agonist to induce lipogenic gene expression and *de novo* hepatic fatty acid synthesis in mouse liver *in vivo* and in isolated hepatocytes *in vitro* and by the absence of these effects in CB₁ knockout mice and hepatocytes treated with a CB₁ antagonist [53]. CB₁-mediated hepatic lipogenesis was subsequently documented in human liver cells [54]. In contrast to increasing lipogenesis, hepatic CB₁ inhibits fatty acid oxidation, as documented in cultured liver explants [55] and in the liver of wild type mice but not in mice with hepatocyte-specific deletion of CB₁

[56]. Hepatic CB₁ also plays a dominant role in the hepatic insulin resistance that accompanies high-fat diet-induced obesity: activation of hepatocyte CB₁ inhibits insulin clearance by the insulin-degrading enzyme, contributing to hyperinsulinemia, and also triggers ER stress-dependent suppression of insulin signaling through insulin receptor substrate-1 (IRS1) and Akt-2, resulting in increased glycogenolysis [57]. Inhibition of insulin signaling by hepatic CB₁ may also involve activation of the cAMP response element-binding protein H (CREBH)/LIPIN1/diacylglycerol/protein kinase C epsilon (PKCε) signaling pathway, which results in increased

gluconeogenesis [58]. Expression of CB₁ mRNA and protein is very low in normal liver but is increased substantially in steatotic hepatocytes [53,56,59], and chronic CB₁ blockade by rimonabant reverses steatosis induced either by high-fat feeding or ethanol and the upregulation of hepatic CB₁ associated with both [59,60]. This indicates not only that increased eCB/CB₁ tone mediates the development of fatty liver of various etiologies, but also that CB₁ expression is autoinduced under these conditions. The finding that reversal of diet-induced obesity and its hepatic sequelae could be fully replicated by a non-brain-penetrant CB₁ antagonist supports the dominant role of peripheral (including hepatic) rather than central CB₁ in these effects [61]. The latter receptor is also expressed in hepatic stellate cells, where it is induced by various fibrogenic stimuli, while its genetic or pharmacological ablation protects the liver from fibrosis [62]. In cirrhosis, which represents the advanced stage of liver fibrosis, CB₁ are similarly upregulated in the vascular endothelium where they mediate hepatic vasodilatation, which predisposes cirrhotic individuals to ascites formation and variceal hemorrhage [63].

The role of CB₂ in the liver was first described by Boris Julien and colleagues, who demonstrated an antifibrogenic action of its agonists in hepatic myofibroblasts and stellate cells and that CB₂ knockout mice developed enhanced fibrosis in response to the hepatotoxin carbon tetrachloride [64]. CB₂ has since been implicated in liver fibrosis, fatty liver disease, and acute ischemic liver injury [65]. CB₂ is expressed on immune cells, Kupffer cells, and myofibroblasts but not on hepatocytes. Extending the findings of Julien and colleagues, Javier Muñoz-Luque's group [66] also used the carbon tetrachloride model to induce fibrosis of the liver and treated rats with the CB₂ agonist JWH-133. The latter substance markedly improved the extent of liver fibrosis leading to reduced portal pressure. This effect is mediated by CB₂ on myofibroblasts and is due to reduced interleukin (IL)-17 production by T helper 17 (Th17) lymphocytes [67].

Inflammation in the liver is an important feature of alcoholic fatty liver disease. Activation of CB₂ receptors in this context switches Kupffer cells from a proinflammatory (M1) phenotype to an anti-inflammatory (M2) phenotype by the induction of hemoxygenase-1. Fat accumulation in hepatocytes is also reduced, but this is an indirect effect through a reduction in steatogenic cytokines such as IL-1 β [68].

After liver transplantation, ischemia–reperfusion injury leads to marked liver damage. In mouse models of ischemia–reperfusion injury, Sandor Bátkai and colleagues revealed a remarkable role of CB₂ receptors in reducing inflammatory infiltrates, inflammatory cytokine and chemokine levels, and the expression of adhesion molecules on liver sinusoidal endothelial cells [69]. eCBs made in the liver are also capable of regulating the degree of ischemia–reperfusion injury. Wild type mice given the MAGL inhibitor JZL184 were protected from hepatic injury, as were mice lacking this enzyme [70].

It is apparent that CB₁ and CB₂ receptors are acting in opposite directions in the liver. CB₁ promotes fibrogenesis, steatosis, and the cardiovascular complications of liver

disease whereas CB₂ is protective, reducing these indices of liver dysfunction. Clinical studies of cannabis use show that it is detrimental, presumably because of its actions at CB₁ [71]. Thus, CB₁ blockade (with drugs that do not cross the blood–brain barrier) and CB₂ agonists have therapeutic potential in liver cirrhosis by targeting both the fibrotic process and its potentially lethal hemodynamic complications (Table 2).

In summary: (i) activation of hepatic CB₁ promotes vasodilatation that can lead to ascites formation and promotes fat accumulation, insulin resistance, and fibrosis; (ii) activation of CB₂ reduces proinflammatory cytokines, attenuates reperfusion injury, reduces fat accumulation, and is antifibrotic; and (iii) CB₂ agonists and peripherally restricted CB₁ antagonists have therapeutic potential in (non)alcoholic fatty liver diseases and their long-term consequences.

Immune system

The mammalian immune system can be subdivided into the innate and adaptive immune systems. The innate immune system functions nonspecifically, responds to invasive pathogens in a generic fashion, and does not confer long-lasting immunity. Elements of innate immunity include the complement system, which elicits a biochemical cascade targeting the surface of foreign cells, natural killer (NK) cells, which destroy tumor and virus-infected cells, and soluble factors such as eicosanoids and cytokines released by injured and infected cells. Typical cytokines that are elicited during this phase of the immune response include interferon-gamma (IFN- γ), interleukins, and tumor necrosis factor alpha (TNF α). These soluble factors bind to cognate receptors and other cellular targets and set in motion signaling cascades that culminate in the activation of selected genes. In contrast to the innate immune system, the adaptive immune system (Figure 3) is antigen specific, recognizes 'non-self' antigens during a process referred to as antigen presentation, and confers immunological memory. The principal players in adaptive immunity are B lymphocytes, which are critical for the humoral immune response through their capacity to produce antibodies, and T lymphocytes, which are critical to the cell-mediated immune response. In adaptive immunity, Th lymphocytes, through the facilitation of their CD4 coreceptor (CD4⁺), recognize antigens (i.e., substances that induce a specific immune response and that react with the products of that response) presented by antigen-presenting cells (APCs) in the context of their class II MHC molecules. The consequent activation of Th lymphocytes results in their release of cytokines and other stimulatory signals that activate macrophages, cytotoxic T lymphocytes, and B lymphocytes.

Bioactive lipid molecules participate in this crosstalk between different types of immune cell. Included among these are eCBs, which can bind to cannabinoid receptors and cooperate with other signaling molecules to modulate the functional activities of immune cells, particularly at the level of the adaptive immune response [72,73]. Their ligation to, and activation of, CB₁ and CB₂ sets in motion a series of signal transduction pathways that converge at the transcriptional level to regulate cell migration and the

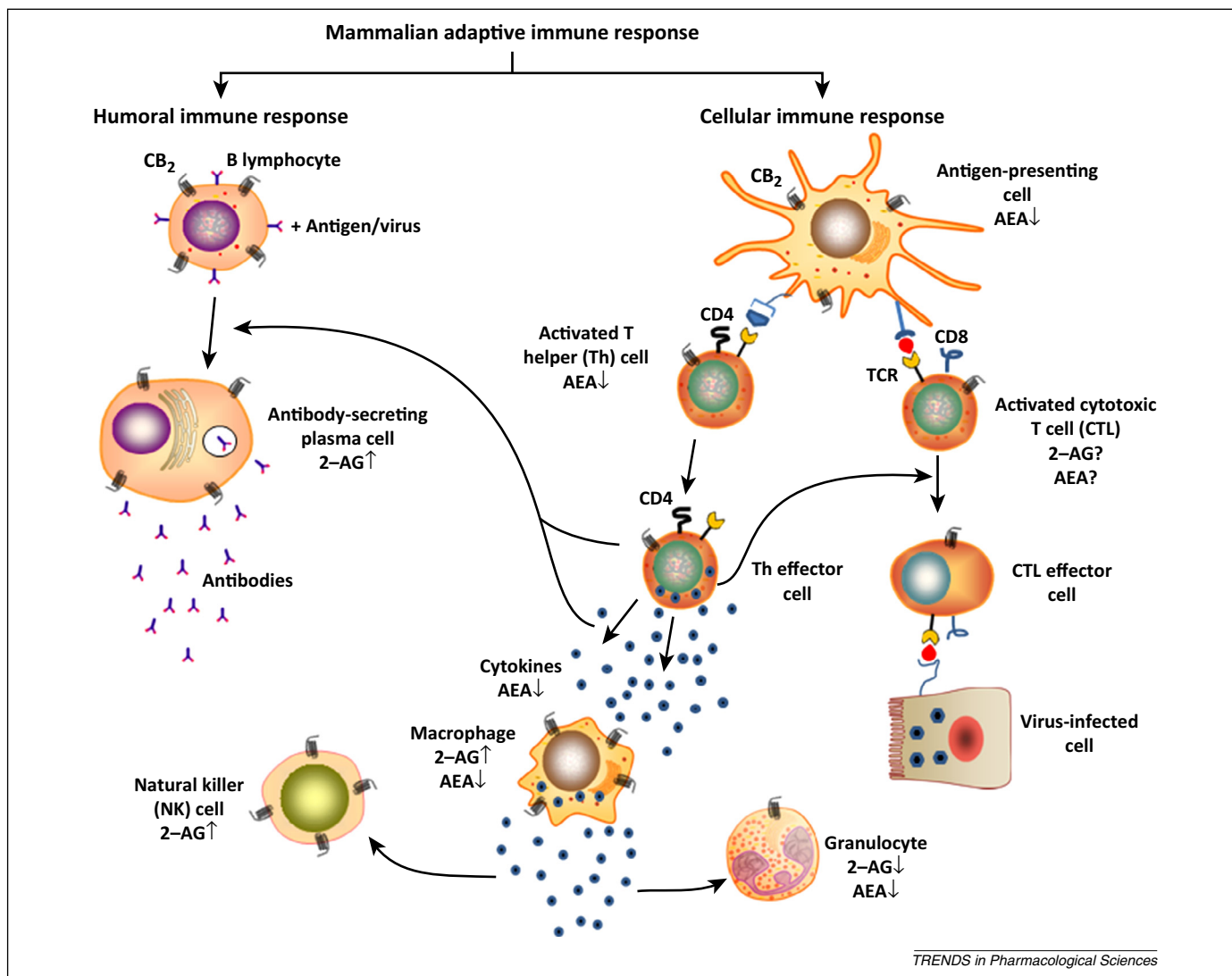


Figure 3. Cell types and the cascade of immunological events that come into play in an adaptive mammalian immune response and the respective functional activities that have been implicated to be modulated by endocannabinoids (eCBs). The adaptive immune response in mammals is highly specific to a given pathogen and can provide long-lasting protection by destroying the invading pathogen and the toxic molecules it produces. Cells that are involved in this response are white blood cells known as B lymphocytes and T lymphocytes. These cells play critical roles in the humoral and cellular immune responses, respectively, once activated by antigens (i.e., molecules or linear fragments that are recognized by receptors on the lymphocyte surface). In the humoral immune response, activated B lymphocytes secrete antibodies, or immunoglobulins. These antibodies travel through the bloodstream and bind to the cognate antigen resulting in its inactivation. Such inactivation may include prevention of its attachment to host cells. In the cellular immune response, T lymphocytes are mobilized when they encounter an antigen-presenting cell such as a dendritic cell or B lymphocyte that has digested an antigen and is displaying antigen fragments bound to its MHC molecules. During this response, cytokines (small signaling proteins) facilitate T lymphocyte maturation and the growth of more T lymphocytes. The engendered MHC-antigen complex activates T cell receptors resulting in T lymphocyte secretion of additional cytokines. Some T lymphocytes develop into T helper (Th) cells and secrete cytokines that attract other immune cells such as macrophages, granulocytes, and natural killer (NK) cells. Some T lymphocytes become cytotoxic cells (CTLs) and lyse tumor cells or host cells infected with viruses. CD4, cluster of differentiation 4 – a glycoprotein found on the cell surface and used as marker for Th cells; CD8, cluster of differentiation 8 – a glycoprotein found on the cell surface and used as marker for CTLs; TCR, T cell receptor – a molecule found on the surface of T lymphocytes that recognizes antigens bound to MHC molecules.

production of cytokines, chemokines, and other inflammatory factors. Immune cells preponderantly express CB₂. Consistent with this observation, there is a large body of data supporting a functional relevance for 2-AG acting through CB₂ to inhibit the migratory activities of a diverse array of immune cell types [74,75]. It has been suggested that 2-AG is the cognate functionally relevant eCB for CB₂. For example, Takayuki Sugiura and colleagues [76] examined the effect of 2-AG on intracellular free Ca²⁺ concentrations in human HL-60 macrophage-like cells and found that it induced a rapid transient increase in levels of intracellular free Ca²⁺. The induced Ca²⁺ transient was blocked by a CB₂ antagonist, consistent with the involvement of CB₂ in this response.

AEA also has been reported to inhibit immune function activities, particularly the production of proinflammatory cytokines. Evgeny Berdyshev and colleagues [77] reported that AEA diminished levels of the cytokines IL-6 and IL-8 from human monocytes. In keeping with these findings, Maria Teresa Cencioni and colleagues [78] showed that AEA suppresses the release of cytokines like IL-2, TNF α , and IFN- γ from activated human peripheral T lymphocytes, mainly through a CB₂-dependent mechanism. Jean-Marie Derocq and colleagues [79], using IL-3- and IL-6-dependent mouse cell lines, proposed that AEA exerted a growth promotion effect. Luigi Facci and colleagues [80] reported that mast cells, which are bone marrow-derived cells found in mucosal and connective tissues

(as well as in the nervous system) and which play a role in tissue inflammation and neuroimmune interactions, expressed a peripheral cannabinoid receptor that was differentially sensitive to AEA. These cells reportedly expressed CB₂, which exerted negative regulatory effects on mast cell activation. However, AEA did not down-modulate mast cell activation *in vitro*. Thus, unequivocal data supporting a functional linkage of AEA to a cannabinoid receptor in mediating immune cell effects remains to be obtained. Indeed, it has been proposed that the immune modulatory effects of AEA could be cannabinoid receptor independent [77,79,81]. eCBs, as typical bioactive lipids, are degraded rapidly, have a short half-life intracellularly and extracellularly, and modulate immune function in an autocrine and paracrine fashion. Thus, their immediate effective action on immune function may be at localized sites. It is speculated that, in this context, eCBs play an important role in maintaining the overall 'fine-tuning' of the immune homeostatic balance within the host. Various mammalian cell systems have been used as experimental models to validate the *in vitro* effects of eCBs on immune function. Early experiments involved the exogenous introduction of eCBs to cultures of transformed immune cells or to immune cell subpopulations obtained from mice and humans. Such studies were complemented with those using mixed cell populations that potentially replicated more closely *in vivo* conditions that integrated crosstalk between different immune cell types. Experimental animal models such as guinea pigs and mice also have been used to document the effects of eCBs on immune function [81]. Nevertheless, the conduct of *in vivo* studies using eCBs has been challenging, partially because these substances are readily degraded, necessitating that they be applied experimentally at relatively high doses. Furthermore, their intracellular fate, compartmentalization, and processing within the host may be distinct from that of exogenously introduced phytocannabinoids and synthetic cannabinoids. Given these caveats, *in vivo* studies have converged on an immune functional relevance for eCBs that parallels the one that has been obtained using various *in vitro* paradigms [82].

Recognition that immune functions can be mediated through the interaction of eCB ligands with specified receptors such as CB₂ should provide novel insights regarding potential targets in signal transduction pathways that are amenable to therapeutic manipulation. In this fashion, the manipulation and ablation of untoward immunological events, including those possibly associated with neuroinflammation linked to infection by HIV or other pathogens, may be achieved.

In summary: (i) eCBs can bind to cannabinoid receptors and cooperate with other signaling molecules to modulate the functional activities of immune cells; (ii) the latter cells preponderantly express CB₂ and there is a large body of data supporting a functional relevance for 2-AG acting through this receptor in the inhibition of the migratory activities of a diverse array of immune cell types; and (iii) the recognition that immune functions can be mediated through the interaction of eCB ligands with cannabinoid receptors should provide novel insights regarding targets for therapeutic manipulation.

Muscle

It is now well accepted that muscle cells produce eCBs and express CB₁, CB₂, and eCB metabolic enzymes, all of which seem to be somehow sensitive to the development of overweight and obesity [83–88]. While in primary skeletal muscle myotubes of lean and obese subjects, expression of *cnr1* (the gene encoding CB₁) remained unchanged [87], in rats fed a high-fat diet abdominal wall skeletal muscle expression of *cnr2* (encoding CB₂) and *magl* (encoding MAGL) was decreased and increased, respectively [85]. In genetically obese Zucker rats, CB₁ mRNA levels in soleus muscle were decreased [89], whereas AEA levels were increased [90]. However, expression of *cnr1* in the soleus from mice on a high-fat diet was increased [91] and 2-AG, but not AEA, levels were significantly elevated in parallel with the late development of obesity and hyperglycemia [92]. These reports indicate that the ECS response to the metabolic state of the organism may have muscle subtype-, species-, and diet-dependent differences and should be interpreted based on the results of pharmacological studies. The latter have shown that systemic pharmacological CB₁ antagonism in obese humans or rodents results in increased energy expenditure/oxygen consumption, which in turn is due to elevated fatty acid oxidation [93–96], possibly subsequent to increased muscle mitochondrial biogenesis [97]. In addition, chronic treatment of *ob/ob* mice and lean or obese Zucker rats with rimonabant results in increased glucose uptake in the soleus muscle [96,98], whereas administration of a single dose of the CB₁/CB₂ agonist HU210 caused CB₁-dependent reduction of whole-body glucose clearance and glucose transport into muscle but not adipose tissue and decreased insulin-dependent Akt phosphorylation in hind-leg muscle [99]. It also seems noteworthy that, in soleus muscle explant cultures from lean and insulin-resistant Zucker rats, AEA and rimonabant significantly decrease and increase, respectively, both basal and insulin-dependent glucose import [89]. Finally, AEA has been shown to inhibit insulin-dependent glucose uptake and Akt activation in skeletal muscle cells in a CB₁-dependent manner [86]. Overall, these data imply that skeletal muscle eCBs, via activation of CB₁, reduce not only mitochondrial activity but also insulin signaling and glucose uptake, possibly through inhibition of IRS1 phosphorylation and insulin-dependent extracellular signal-regulated kinase (ERK) 1/2 activation [86,88]. Thus, the overactivity of the ECS potentially occurring in skeletal muscle during obesity might contribute to defective muscle insulin sensitivity and glucose metabolism as well as to fatty acid accumulation.

More recently, the ECS was found to also play a fundamental role in skeletal muscle formation (Figure 4A). It was found that, possibly due to changes in the expression of genes involved in its metabolism, levels of 2-AG were decreased both during myotube formation *in vitro* from murine C2C12 myoblasts and during mouse muscle growth *in vivo*. 2-AG and a CB₁-specific agonist were shown to prevent myotube formation in a manner antagonized by CB₁ knock down and pharmacological antagonism, which instead stimulated differentiation *per se* [100]. *In vivo*, muscle fascicles from CB₁ knockout embryos were found to contain more muscle fibers and those from postnatal CB₁

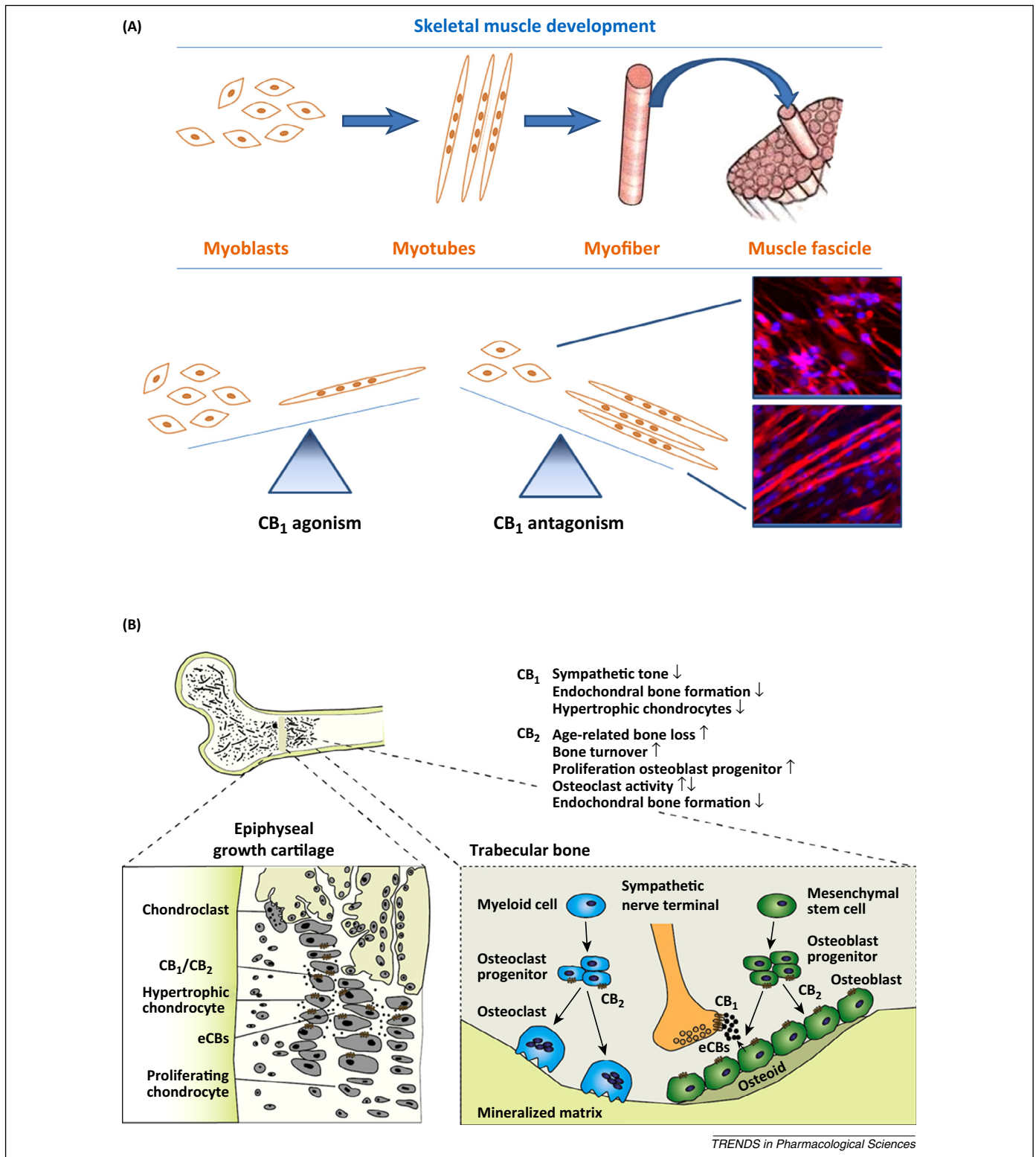


Figure 4. (A) Role of the endocannabinoid (eCB) system (ECS) in skeletal muscle formation. Simplified representation of the skeletal muscle differentiation process, from myoblasts to myotubes, myofibers, and fascicles. Activation of the type 1 cannabinoid receptor (CB₁) by eCBs inhibits myoblast-to-myotube differentiation, whereas CB₁ blockade, as in cells treated with a CB₁ antagonist or CB₁ siRNA, facilitates this process and *in vivo*, as in CB₁ null mice, leads to larger fibers. **(B)** eCB signaling in bone growth and bone remodeling. CB₁ and CB₂, as well as eCB synthetic enzymes, are expressed on hypertrophic chondroblasts in the epiphyseal growth cartilage. Mice lacking CB₂ develop longer femora and vertebral bodies, resulting in a longer stature, whereas stimulation of CB₁ restrains bone growth. Bone remodeling is stimulated in CB₂-deficient mice but with net loss of bone mass, resulting in an age-related osteoporotic phenotype. CB₂ signaling stimulates proliferation of osteoblast progenitors and affects the differentiation of osteoclasts. CB₁ is most prominently expressed on sympathetic nerve terminals and inhibits the release of norepinephrine, reducing the sympathetic tone which in turn inhibits bone formation.

null mice exhibited increased diameter relative to those of wild type littermates. The myoblast differentiation inhibitory action of 2-AG was demonstrated to occur through inhibition of the activity of the Kv7.4 channel, which plays a permissive role in myogenesis and depends on phosphatidylinositol 4,5-bisphosphate (PIP₂). CB₁ stimulation reduced both total and Kv7.4-bound PIP₂ levels in C2C12 myoblasts and inhibited Kv7.4 currents in transfected CHO cells. It was suggested that 2-AG might be an endogenous repressor of myoblast differentiation via CB₁-mediated inhibition of Kv7.4 channels [100].

Finally, it is important to remember that AEA, and to a lesser extent 2-AG, also activates TRPV1 channels, which were shown to stimulate both skeletal muscle mitochondrial biogenesis [101] and hypertrophy [102]. However, no evidence exists on the possibility that eCBs modulate these two aspects of skeletal muscle function via TRPV1 activation. Against this background, it is unsurprising that the therapeutic exploitation of ECS-related drugs in muscle diseases remains in its infancy, with as yet only one potential application (Table 2).

Taking these findings together, it can be concluded that: (i) eCBs are deeply involved in both the control of energy metabolism by skeletal muscle and the formation of new muscle fibers; (ii) both of these fundamental actions are mediated preferentially via CB₁ and involve AEA and 2-AG to varying degrees; and (iii) the ECS ultimately controls the utilization of energy in skeletal muscle by reducing either glucose oxidation by myofibers or the extent of muscle formation.

Bone

The skeleton provides a mechanical support to soft tissues and drives body growth. It is constantly adapted to changing mechanical needs by a remodeling process in which bone is removed by osteoclasts and newly formed by osteoblasts. Lack of appropriate remodeling leads to excessive mineral deposition, which reduces the flexibility of the bone and results in the accumulation of fatigue damage and stress fractures. The remodeling process is tightly coordinated by an intricate system that involves eCB signaling [103–114], as depicted in Figure 4B. Thus, AEA and 2-AG are locally produced by bone cells and reach concentrations similar to those in brain tissues [115]. Stimulation of CB₂ on osteoblast precursor cells has a mitogenic effect and results in an expansion of the preosteoblastic cell pool, while mature osteoblasts respond with increased alkaline phosphatase activity and matrix mineralization [112,113]. The role of CB₂ signaling in osteoclastogenic cultures and RAW 264.7 cells is more complex, as both inhibitory and stimulatory effects of CB₂ agonists on osteoclast formation have been reported under different experimental conditions [103,109,110,113]. Interestingly, expression of CB₂ on osteoclasts is modulated by the TRPV1 channel, which is another target for AEA and has profound effects on bone remodeling [116]. Mice lacking CB₂ displayed strikingly enhanced age-related loss of trabecular bone volume density with concomitantly increased bone turnover, a phenotype reminiscent of human postmenopausal osteoporosis [113]. Human genetic studies have also identified polymorphisms in the CB₂

coding region that are associated with low bone mass and osteoporosis [117,118]. Together, these findings implicate CB₂ signaling in pro-osteogenic processes along the osteoblast lineage and indicate a therapeutic potential for CB₂ agonists in the anabolic therapy of osteoporosis (Table 2). Studies in mice have shown that CB₂ agonists rescue ovariectomy-induced bone loss [113].

eCB signaling is also important in the CNS regulation of bone remodeling via antagonistic sympathetic and parasympathetic projections. Norepinephrine released from sympathetic fibers inhibits bone formation and stimulates bone resorption [119,120] whereas parasympathetic acetylcholine decreases bone resorption [121]. CB₁ is mostly expressed on sympathetic nerve endings [115] and its activation by 2-AG produced from closely apposed osteoblasts inhibited norepinephrine release, thus alleviating tonic inhibition of bone formation by the sympathetic nervous system. Deletion of CB₁ in congenic C57BL/6J mice resulted in a low bone mass phenotype [115]. A detailed phenotype analysis indicated that CB₁ signaling increased trabecular bone mass and radial diaphyseal growth by upregulating bone formation and downregulating bone resorption [115]. The phenotype was masked, however, in a different CB₁-deficient mouse line maintained on an outbred CD1 genetic background, which showed no changes in bone remodeling parameters [110,114].

Recently, a functional ECS was also discovered in epiphyseal growth cartilage (EGC) [122], which drives bone and consequently body growth by endochondral bone formation. In this process, chondrocyte progenitors differentiate into proliferative and then hypertrophic chondrocytes. The extracellular matrix separating the hypertrophic chondrocytes is then calcified, resorbed by osteoclasts/chondroclasts and replaced by bone. CB₁ and CB₂, as well as DAGL α and DAGL β , are expressed in EGC hypertrophic chondrocytes and 2-AG was detected at significant levels in EGC. The femora of mice lacking CB₁ or CB₂ were considerably longer at the end of the rapid growth phase compared with those of wild type animals. Importantly, THC slowed skeletal elongation in wild type and CB₂ knockout but not CB₁-deficient mice. This was probably due to a direct effect of THC on hypertrophic chondrocytes, because THC inhibited EGC chondrocyte hypertrophy in *ex vivo* cultures and reduced the hypertrophic cell zone thickness of treated animals [122]. These experimental findings are in line with human studies reporting that babies born to marijuana-using mothers have a reduced fetal growth rate resulting in reduced birth weight, shorter stature, and reduced head size at birth [123,124].

Considering the profound effects of THC on murine bone growth and the highly prevalent consumption of marijuana by adolescents during their growth phase, it will be important to examine the possible postnatal growth-retarding effects of THC in humans. Overall, it can be stated that eCB signaling regulates bone elongation and bone remodeling by modulating: (i) the proliferation and differentiation of bone cells; (ii) communication between bone cells; and (iii) the neuronal control of bone remodeling.

Reproductive system

Anecdotal and epidemiological evidence suggested adverse effects of marijuana on male and female reproduction well before direct molecular evidence that phytocannabinoids affect female reproduction was provided. CB₁ and CB₂ were shown to be expressed in preimplantation mouse embryos, where CB₁ was primarily localized in the blastocyst trophoblast (reviewed in [125]). It was also shown that THC, as well as uterine AEA, can activate CB₁ and interfere with embryo development. Notably, CB₁ but not CB₂ was primarily expressed in the mouse female reproductive tract, and cannabinoid/eCB effects on embryo development and function were differentially executed depending on embryonic stage and ligand levels. In this respect, levels of AEA and CB₁ are higher in non-receptive uteri, dormant blastocysts, and interimplantation sites but decline to lower levels in receptive uteri, activated blastocysts, and implantation sites. This suggests that optimally balanced eCB signaling is critical to the synchronization of preimplantation embryo development and the preparation of the endometrium for implantation (reviewed in [125]). Therefore, site-specific levels of AEA and/or other endogenous ligands of CB₁ in the uterus may spatially regulate blastocyst implantation in addition to effects from embryo-based eCB signaling. Subsequent studies showed that eCBs were important for several aspects of female reproduction, including oviductal embryo transport, placentation, and parturition [125] (Figure 5A).

Before embryo implantation, oviductal transport of the embryo was also impacted by cannabinoid/eCB exposure. It was shown that a critical balance between AEA synthesis and degradation in mouse embryos and oviducts created a local ‘AEA tone’ that determined normal embryo development and oviductal transport. Defects in oviductal embryo transport under aberrant eCB signaling led to deferred on-time implantation and poor pregnancy outcome. These studies uncovered novel regulation of preimplantation processes by eCB signaling, which could be clinically relevant for fertility regulation in women. For example, CB₁ is expressed at low levels within both the Fallopian tubes and the endometrium of women with ectopic pregnancy, suggesting that aberrant eCB signaling within the Fallopian tubes may lead to this pathology [126–128]. Aberrant eCB signaling was also observed to confer premature trophoblast stem cell differentiation and defective trophoblast development and invasion. These defects were reflected in retarded fetal development and compromised pregnancy outcome [129]. Thus, a tightly regulated eCB signaling threshold across multiple early pregnancy events is critical for female reproductive success [130]. Separate studies further supported these findings, suggesting a similarly important role for eCB signaling in human pregnancy [125]. Aberrant eCB signaling may be associated with increased rates of miscarriage and human ectopic pregnancy [126,131–133].

The effects of eCB signaling on the reproductive organs led to the conclusion that exposure to marijuana during pregnancy may have many adverse effects [125]. With the current increased legalization of marijuana in the USA and high usage among young adults, there is a critical need for more research into the effects of this drug and for strong

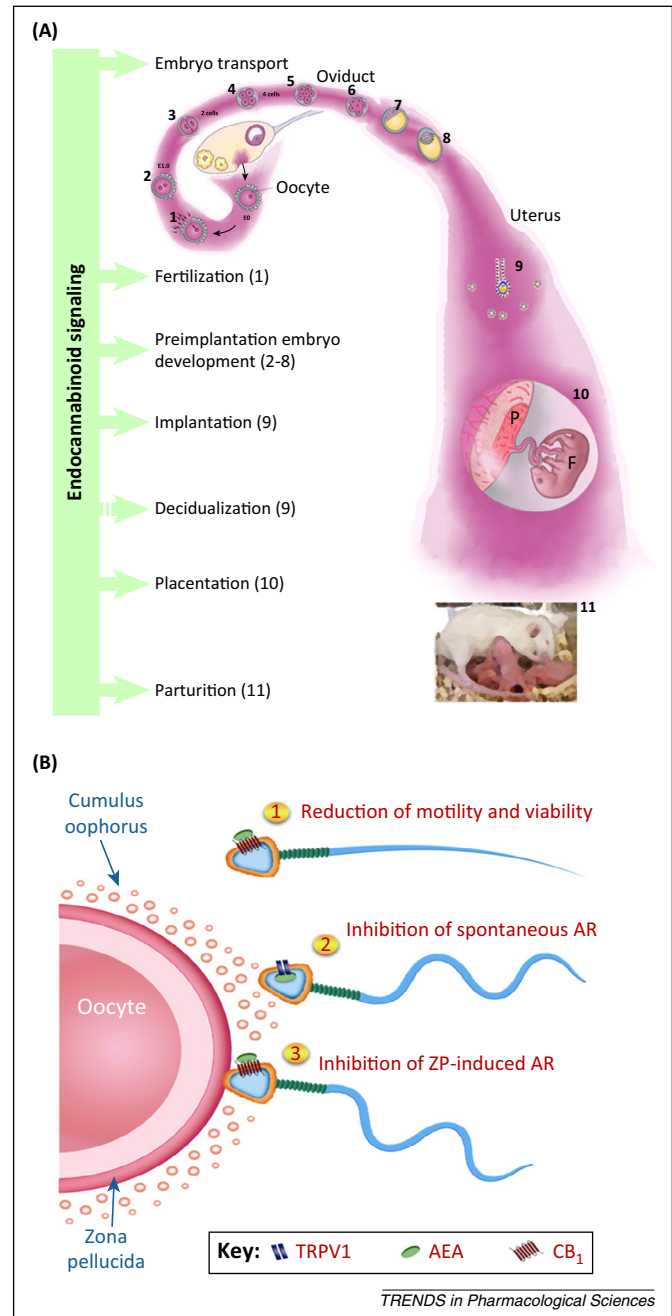


Figure 5. (A) Schematic diagram showing pregnancy events in mice that are influenced by endocannabinoid (eCB) signaling. Stage-specific eCB signaling during pregnancy is designated numerically (1–11). Implantation and decidualization are both designated (9) since they are overlapping events. P, placenta; F, fetus. (B) Functional activity of eCB system (ECS) elements in human sperm. Far from the oocyte, activation of the type 1 cannabinoid receptor (CB₁) reduces sperm motility and viability (1) and activation of transient receptor potential vanilloid 1 (TRPV1) inhibits a spontaneous acrosome reaction (AR) that would be useless for fertilizing the egg (2). Next to the oocyte, activation of CB₁ inhibits zona pellucida (ZP)-induced AR (3), thus avoiding fertilization of the egg by more than one sperm (polyspermy).

educational efforts to inform the public of the dangers associated with consuming marijuana when pregnant or trying to become pregnant. By contrast, ECS-based drugs have reached the market as treatments for female infertility, and creams containing *N*-palmitoylethanolamine (PEA) (Table 1) appear useful for the treatment of pain-related dysfunctions like chronic vestibulodynia, vulvodynia, and vaginismus (Table 2).

Taking these findings together, it can be concluded that: (i) eCB signaling operates in all critical stages of pregnancy; (ii) both silenced and amplified eCB signaling affect pregnancy events; and (iii) eCB signaling in female reproductive events is primarily mediated by CB₁.

On the male side, it should be recalled that nearly 1/6 couples suffers from infertility, with male factors accounting for 50% of cases [134]. Although anatomical factors explain a significant number of these, several remain unexplained, primarily because factors that are involved in the modulation of spermatogenesis and sperm function are poorly understood. Numerous molecules/systems have been suggested to play a pivotal role in this regard and one such system is the ECS. The fact that marijuana compromises male fertility provides support for this. AEA, OEA, and PEA have been detected at nanomolar levels in seminal plasma [135,136]. Furthermore, both the receptors (CB₁, CB₂, TRPV1) and the enzymes (NAPE-PLD, FAAH) regulating these eCBs are differentially expressed in human sperm [137]. FAAH and NAPE-PLD are localized mainly in the postacrosomal region and in the middle piece, while CB₁ is localized in the plasma membrane over the acrosomal region, the middle piece, and the tail and CB₂ is localized in the plasma membrane of the head. TRPV1 is localized in the postacrosomal region [135]. The localization of these components would suggest that both synthesis and degradation occur within human sperm, and significant evidence is emerging to confirm a functional and physiological role for the ECS in the process of fertilization, as summarized in Figure 5B (for a recent review, see [137]).

Several *in vitro* studies have shown concentration-dependent inhibition of mammalian sperm functions by AEA mediated by CB₁ activation. One is inhibition of mitochondrial activity, which preserves energy and ensures the gradual acquisition of fertilization capacity during ascent up the female reproductive tract. Sperm fertilizing ability has been shown to be enhanced by the binding of AEA to CB₁, which results in the induction of the acrosome reaction pivotal to the fertilization capacity of the sperm. More recently, a CB₂-dependent mechanism has also been implicated in this process [138].

Quantification of eCB levels in seminal plasma from an infertility clinic showed AEA, PEA, and OEA levels to be significantly lower in men with either asthenozoospermia or oligoasthenozoospermia compared with normozoospermia [134,136] and supraphysiological levels of methanandamide (a non-hydrolyzable analog of AEA) decreased sperm motility and viability through CB₁-mediated inhibition of mitochondrial activity. Furthermore, PEA and OEA were shown to improve *in vitro* sperm motility and maintain viability without affecting mitochondrial activity [134]. Taken together, these findings suggest that the maintenance of a normal eCB tone is likely to be necessary for the preservation of normal sperm function and hence of male fertility. There is evidence that PEA and OEA, which possess anti-inflammatory, antimicrobial, and antioxidant activities, may protect sperm from oxidative damage, inflammation, and microbial infections [134]. In men with idiopathic infertility, it has been shown that supplementation with PEA and OEA enhances sperm antioxidant

activity and consequently improves sperm kinematic parameters and hyperactivation *in vitro*. Ultimately, this protects sperm from oxidative damage and could be beneficial in cases of unexplained male infertility [139].

It can be concluded that several observations indicate ECS involvement in the preservation of normal sperm function, and thus male fertility, and that abnormalities in this system may have adverse reproductive consequences. This is a rapidly evolving domain and, in the future, manipulation of the ECS might improve the fertility of men with various forms of sperm dysfunction.

Skin

An increasing body of evidence suggests that the ECS is a key player in the regulation of biological processes of the skin, the largest organ of the human body. As reviewed previously [140], multiple cellular compartments of the skin (i.e., epidermal keratinocytes, hair follicles, sebaceous and sweat glands) produce prototypic eCBs. In addition, several elements of the ECS, like CB₁, CB₂, and metabolic enzymes, were identified on most cutaneous cell types; hence, the ECS was implicated in a wide array of skin functions in all layers of the skin (Figure 6).

In the epidermis, AEA was shown to inhibit proliferation and reduce differentiation of human epidermal keratinocytes via CB₁-coupled signaling (reviewed in [141]). CB₁ also controls melanogenesis in epidermal melanocytes [142]. In addition, further highlighting the role of the ECS in epidermal functions, in an animal model using gene-deficient mice, absence of CB₁ delayed whereas lack of CB₂ accelerated recovery of the epidermal permeability barrier [143], the establishment of which is strongly dependent on keratinocyte proliferation and differentiation. Of further importance, CB agonists as well as phytocannabinoids were found to inhibit the proliferation of hyperproliferative keratinocytes, while various cannabinoids exerted complex antitumor activity (antiproliferative, proapoptotic, and antiangiogenic effects together with reduced metastasis formation) in multiple models of melanoma and non-melanoma skin tumors (for a recent review, see [140]).

The ECS is also involved in the biology of skin appendages. CB₁-coupled signaling was shown to inhibit human hair shaft elongation and induce premature organ involution (catagen), whereas CB₂ was found to 'tonically' stimulate homeostatic lipogenesis in human sebaceous gland-derived sebocytes [140]. Interestingly, AEA inhibited the proliferation and induced the secretory activity of sweat gland epithelial cells via non-CB₁/CB₂-dependent mechanisms [144].

The pioneering mouse study of Meliha Karsak and colleagues [145] has clearly shown that augmentation of the cutaneous eCB tone, as well as certain phytocannabinoids, exerts remarkable anti-inflammatory activities [140]. This effect was recently also proven in human models. Tonic activation of CB₁ was shown to keep the maturation and degranulation of human skin mast cells under constitutive control and the 'pro-inflammatory' degranulation of mast cells was found to be prevented by AEA and CB₁ agonists [146]. Of further importance, the prototypic non-psychoactive phytocannabinoid cannabidiol – by modulating a complex signaling network involving TRPV4 ion

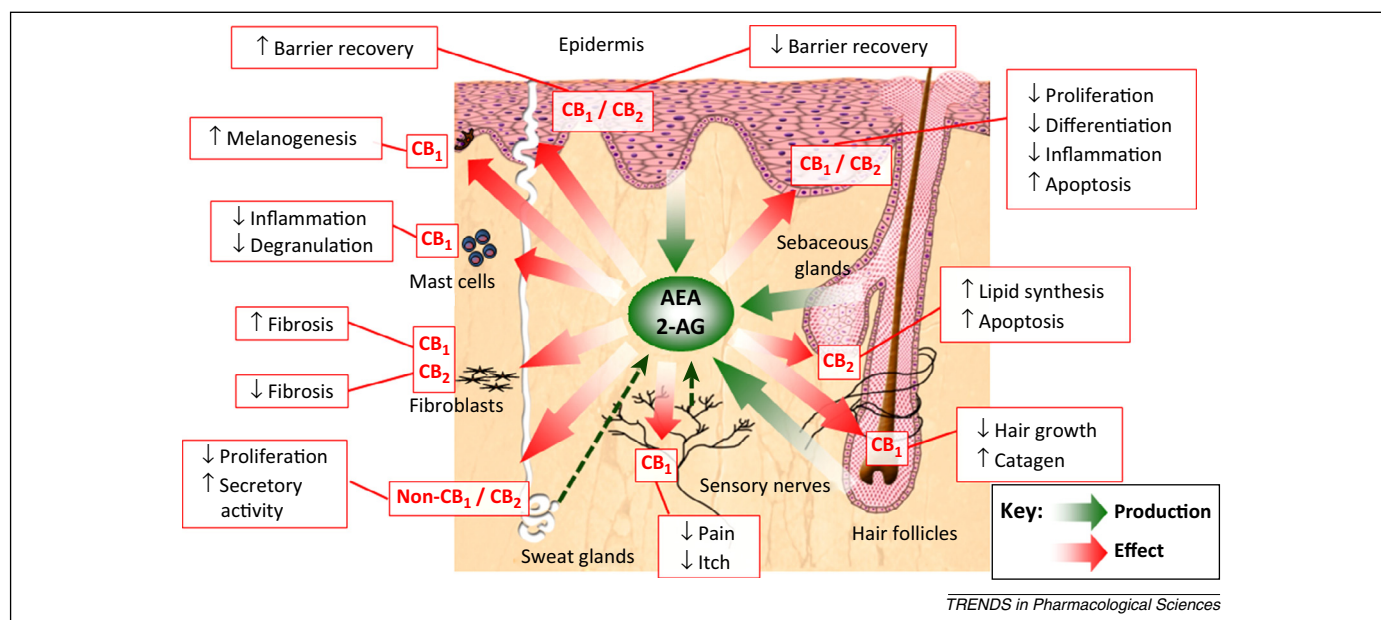


Figure 6. Summary of the effects of the cutaneous endocannabinoid system (ECS) on multiple skin functions. Adapted from [140].

channels, adenosine A2a receptors, and multiple downstream elements [e.g., the P65–nuclear factor kappa B (NF- κ B) pathway, tribbles homolog 3] – exerted dramatic sebostatic (i.e., suppression of unwanted lipogenesis) and anti-inflammatory effects in a human cellular/organ culture model of acne vulgaris, the most prevalent human skin disease [147].

Opposing roles of CB₁ and CB₂ were observed in dermal fibroblasts and various fibrosis models. FAAH was found to be downregulated in the dermal fibroblasts of systemic sclerosis (or scleroderma) patients (SScDFs), suggesting that the eCB tone might be elevated. Pharmacological or genetic abrogation of FAAH activity worsened bleomycin-induced fibrosis in mice via CB₁-coupled signaling [148]. Accordingly, CB₁ null animals were found to be protected against the profibrotic effects of bleomycin [149]. However, activation of CB₂ expressed by leukocytes was able to ameliorate fibrosis in the same animal model; likewise, CB₂ stimulation in SScDFs also resulted in significant antifibrotic effects [140,150].

Collectively, the (highly selected) data presented above highlight that appropriate, targeted manipulation of the cutaneous ECS and/or the administration of certain phytocannabinoids indicates completely new therapeutic opportunities in several cutaneous pathologies. Clinical trials are urgently required to assess the putative *in vivo* efficiency of ECS-oriented treatments in, for example, hyperproliferative skin conditions (tumors, psoriasis) as well as in allergic and inflammatory skin diseases (acne vulgaris, atopic dermatitis). As yet, only PEA has been used as an effective therapeutic agent for these skin diseases (Table 2). It can be concluded that: (i) the ECS is fundamentally involved in the regulation of a multitude of skin functions such as proliferation, differentiation, cell survival, and immune responses; (ii) possibly the most robust function of the ECS is in suppressing cutaneous inflammation; and (iii) the effects of eCBs are chiefly mediated by both CB₁ and CB₂ expressed by various skin cells.

Other organs

The impact of eCB signaling in other organs, such as the respiratory tract and urinary system, awaits better clarification through more systematic investigations. However, recent studies have proposed an important role for CB₁ and CB₂ in kidney disease. CB₁ antagonists (globally acting or peripherally restricted) and selective CB₂ agonists protected against type 1 and/or type 2 diabetic nephropathy [151–153] and tubular nephropathy induced by cisplatin [154,155], so some have therapeutic potential (Table 2). Such protection was achieved by attenuating oxidative–nitrosative stress, inflammation, and consequent cell death in glomerular podocytes and/or proximal tubular epithelial cells [151–155].

Finally, the impact of eCB signaling in the CNS appears immense and to help to navigate the *mare magnum* of related data, comprehensive reviews have been recently published [14,15].

Concluding remarks

To date, the therapeutic exploitation of ECS-based medicines appears to remain behind our scientific knowledge of eCB signaling, and also because there are outstanding questions awaiting an answer (Box 1). A summary of the potential applications of ECS-related drugs for peripheral diseases is presented in Table 2. In this context, it should be recalled that peripherally restricted CB₁ agonists failed in clinical trials for pain, owing to cardiovascular and metabolic side effects, and synthetic CB₁ agonists are damaging to the kidneys in young children, in addition to causing serious cardiovascular adverse effects. In keeping with this, over 50 case reports of the cardiovascular side effects of THC/marijuana alone have been reported so far. Additionally, global CB₁ antagonist clinical trials were successful in diabetes and obesity but failed because of unwanted side effects within the CNS. Interestingly, in rodent models of obesity and type 2 diabetes, peripherally restricted CB₁ antagonists show therapeutic promise as

Box 1. Outstanding questions

- When will we have conclusive controlled clinical trials to evaluate the overall safety/toxicity of marijuana and synthetic cannabinoids?
- Enhancement of eCB levels is seen in many disease states. Is CB₂ activation (and in some cases CB₁ activation) a general protective mechanism? What is the role of localization and intracellular trafficking in driving distinct eCB signal cascades in distinct peripheral cells? To what extent can these factors determine the efficacy of ECS-oriented therapies?
- When will we have proof-of-principle clinical trials to evaluate the therapeutic potential of peripherally restricted CB₁ antagonists and CB₂ agonists in cardiovascular disease? A clear assessment of the importance of the peripheral ECS (especially CB₁) and its therapeutic relevance in humans will have to await the results of clinical studies with novel, peripherally restricted ligands.
- Many GI disorders are triggered or exacerbated by stress. How does stress alter the ECS in the gut? What is the balance between CB₁ and CB₂ activation in GI disorders? CB₂ appears to be an important homeostatic regulator in the gut. How is it differentially regulated in GI diseases? How are the enzymes of eCB biosynthesis and degradation regulated in the gut in health and disease?
- Do AEA and 2-AG as bioactive lipids act in opposition to each other to maintain a fine-tuned balance of immune function activities? Since CB₂ is found preponderantly on immune cells, can it serve as a selective and specific molecular target for the ablation of pathological processes characterized by hyperinflammation?
- Insight regarding how activation of cannabinoid receptors alters immune function activities has been obtained through the study of their ligation to phytocannabinoids. However, the latter substances and eCBs exhibit differential intracellular fates. How can the assessment of such differential fates provide insights into phytocannabinoid superimposed dysregulation of eCB-mediated activities?
- What is the role of the ECS in genetically inherited muscle dystrophies such as Duchenne's disease? What is the role of TRPV1 channels in eCB regulation of skeletal muscle formation?
- What is the mechanism of eCB signaling in bone elongation? How is the hypertrophy of chondrocytes modulated by eCBs? In which processes are CB₂ receptors involved?
- Evidence shows that both silencing and amplification of eCB signaling can adversely impact female reproductive functions. Is the underlying mechanism the same or are different mechanisms operative? How may manipulation of the ECS influence fertility, especially in those with idiopathic male infertility?
- Is there multilateral crosstalk between cutaneous ECS and the complex neuroimmunoendocrine network (e.g., hormones, cytokines, growth factors, neuropeptides) of the skin?

they retain the full metabolic efficacy of globally acting CB₁ antagonists but are devoid of their neurobehavioral effects. Furthermore, FAAH or MAGL inhibitors appear to be effective in some preclinical models of pain, but they promote cardiovascular inflammation and cause metabolic undesirable consequences and have already failed in human trials for pain. Finally, there are other cannabis-derived phytocannabinoids with biological activity and therapeutic potential that we chose to exclude from this review due to space limitations. One of these, cannabidiol, is being increasingly approved by the FDA for orphan and other indications (e.g., refractory epilepsy, glioblastoma). Interestingly, a near 1:1 ratio of cannabidiol and THC (nabiximol; trade name Sativex) has beneficial effects in multiple sclerosis patients, who can use it to alleviate neuropathic pain, spasticity, overactive bladder, and other symptoms. However, the effects of Sativex cannot be reproduced by THC alone, suggesting that some or many could

be due to cannabidiol, via molecular mechanisms yet to be disclosed, or to the fact that coadministration of cannabidiol can widen the therapeutic window of THC, allowing its administration at higher doses with fewer side effects. Since overwhelming evidence from preclinical studies also suggests that cannabidiol may be beneficial in myocardial infarction, stroke, diabetic cardiovascular complications, and even nephropathy, investigations into its mechanisms of action appear urgent.

Against this background, one should admit that the therapeutic exploitation of ECS-oriented drugs may not be close, and also because of the apparent complexity of eCB signaling regulation. Fifty years since the discovery of THC and 20 years since that of AEA (1992) and 2-AG (1995), it only recently emerged that correct interaction between the different components of the ECS is essential for the proper functioning of eCBs as signaling molecules. For instance, distinct eCB-binding receptors can exchange signals with each other and other receptors, but to do so they must be properly located on the plasma membrane and/or within the cell [15]. Another emerging concept (already demonstrated for AEA) is that eCBs are transported within the cell by distinct carriers, collectively called eCB intracellular transporters (EITs) (see [22] and the references therein). The need for proper localization and the existence of intracellular trafficking seem to add a new dimension to the already complex regulation of eCB signaling. Thus, to understand the many-faceted actions elicited by eCBs (and hence by eCB-related drugs), it appears now crucial to understand how, after getting to the right place, eCBs can be concentrated to suitable levels for receptor activation at the right time. In this framework, after 50 years of cannabinoid/eCB research a re-examination of the widely accepted dogma that eCBs are exclusively synthesized and released on demand could be proposed, whereby intracellular trafficking, in addition to storage in specific reservoirs like adiposomes [156], could play a key role in determining signal transduction triggered by the same eCB in different cellular contexts. In line with this concept, recent evidence based on pharmacological and genetic manipulation has demonstrated that different EITs may drive AEA signaling at different receptors and/or AEA may be metabolized by different enzymes [157,158]. Thus, compounds selectively directed at one or more of these novel EITs could lead to the development of ECS-based therapeutics with limited side effects and abuse liability. The chances are that we can take advantage of these novel drugs in the near future.

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