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## Endocannabinoid signaling at the periphery: 50 years after THC

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### Abstract

Fifty years ago (in 1964) the psychoactive ingredient of *Cannabis sativa*, <sup>9</sup>-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 altogether form the eCB system. eCBs control basic biological processes, including cell-choice between survival and death, and progenitor/stem

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cell proliferation and differentiation. Not surprisingly, in the past two decades, eCBs have been recognized as key mediators of several aspects of human pathophysiology, and thus have emerged 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 eCB system for human health and disease pathogenesis, as well as to highlight emerging challenges and therapeutic hopes.

### Keywords

bones; cardiovascular system; gastrointestinal tract; immune system; liver; localization; muscles; (female and male) reproductive system; signaling pathways; skin

### Historical introduction

Fifty years ago the major psychoactive cannabis (*Cannabis sativa*) constituent, <sup>9</sup>-tetrahydrocannabinol (THC; Table 1) was discovered [1]. At first it was believed to exhibit non-specific activity, and few studies investigated its mechanism of action. However, by the mid-1980's, numerous observations suggested that specific receptors were possibly involved. Allyn Howlett found that cannabimimetic drugs inhibit cyclic AMP 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]. Indeed, by using a highly potent cannabinoid analogue, a receptor was identified by Howlett's group in 1988 [4]. Later on, Lisa Matsuda and coworkers [5] established the functional expression of the cloned cDNA. This receptor is now known as type 1 cannabinoid receptor (CB<sub>1</sub>). A second entity, known as CB<sub>2</sub>, was identified by sequence homology and presumed to be mainly present in the periphery [6]. Today we know that CB<sub>2</sub> is also present in the central nervous system (CNS). The next step was the discovery of the endogenous ligands of CB<sub>1</sub> and CB<sub>2</sub>, the so-called endocannabinoids (eCBs). As THC is a lipophilic molecule, it was assumed that any endogenous cannabinoid would probably be a lipid. Indeed, by the use of methods for the separation of lipids it was possible to identify the first eCB, *N*-arachidonoyl ethanolamine, named anandamide (AEA; Table 1) [7]. AEA is a derivative of arachidonic acid, a compound which serves as precursor of a large number of endogenous molecules – prostaglandins, prostacyclin, thromboxanes, leukotrienes and others – an example of evolutionary conservation to save energy by the use of the same biochemical pathways for a variety of biologically important molecules. A second eCB – again an arachidonic acid derivative, 2-arachidonoylglycerol (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, which do not bind to cannabinoid receptors, but whose actions may be cannabinoid receptor-dependent [10, 11]. They should possibly also be considered to be *bona fide* components of the eCB family.

Although the discovery of eCBs and the recognition of their ubiquitous activities represent 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 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 eCB system (ECS) (for recent reviews see refs 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 to highlight emerging challenges and therapeutic hopes.

## The ECS at a glance

The two best characterized eCBs, AEA and 2-AG, bind with different affinities to CB<sub>1</sub> and CB<sub>2</sub>, which are two well-characterized 7-transmembrane G protein-coupled receptors (GPCRs) [15-18]. Accumulated evidence suggests the occurrence of other targets for eCBs, like the purported “CB<sub>3</sub>” receptor GPR55 [19], and the transient receptor potential vanilloid 1 (TRPV1) ion channel that has an intracellular binding site [20]. Other eCB targets such as the peroxisome proliferator-activated receptors (PPAR)  $\alpha$  and  $\gamma$  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, *i.e.* when and where needed upon (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*-acyl-phosphatidylethanolamines-specific phospholipase D (NAPE-PLD). Instead, two diacylglycerol lipases (DAGL $\alpha/\beta$ ) 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 cytochromes P450 (CYPs). Interestingly, the main oxidative products of AEA and 2-AG are endowed with novel biological activities, that are probably mediated by distinct receptors compared to those that bind eCBs [22, 23].

Overall, it is apparent that these metabolic enzymes regulate the *in vivo* biological availability of eCBs, which altogether are responsible for keeping 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 ref 26).

The complexity of the ECS supports its manifold activities at the periphery, and may offer different 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 CB<sub>1</sub> and CB<sub>2</sub> receptors) in distinct peripheral organs is presented.

## Cardiovascular system

Studies over the past few decades demonstrated that CB<sub>1</sub> and CB<sub>2</sub>, 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 the hypotension and negative inotropy via activation of cardiovascular CB<sub>1</sub> [27]. Later studies investigated the signaling mechanisms in murine and human cardiomyocytes, endothelial, 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 CB<sub>1</sub>-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,  $\beta$ -myosin heavy chain isozyme switch, remodelling/fibrosis, and activation of pro-apoptotic mitogen-activated protein kinases, eventually resulting in decreased function of sarcoplasmic/endoplasmic reticulum Ca<sup>2+</sup>-ATPase and cell death, leading to cardiac dysfunction/failure [32-35]. In these experimental models, eCBs and/or CB<sub>1</sub> receptors were increased/upregulated in the myocardium, and CB<sub>1</sub> antagonists ameliorated the contractile dysfunction and all above mentioned characteristic pathological processes. Experimental studies using CB<sub>1</sub> antagonists and/or CB<sub>1</sub> knockout mice also suggested that eCBs via CB<sub>1</sub> in macrophages may promote pro-inflammatory processes and inflammatory cell recruitment, thus contributing to the development/progression of atherosclerosis, as well as to pathological smooth muscle proliferation associated with restenosis [33]. The pro-inflammatory effect of CB<sub>1</sub> in the cardiovascular system was also confirmed by using inhibitors and/or knockouts of eCB-metabolizing enzyme FAAH in models of atherosclerosis and cardiomyopathy [33]. In clinical trials of obesity/diabetes the CB<sub>1</sub> 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 CB<sub>1</sub> antagonists clearly established that both marijuana and THC exerted CB<sub>1</sub>-dependent cardiovascular effects, and clinical trials with peripherally restricted CB<sub>1</sub> agonists in pain were discontinued because of development of severe adverse cardiovascular and metabolic consequences of CB<sub>1</sub> stimulation [35]. A recent study also raised serious concerns about the cardiovascular safety of marijuana use in humans [36], which is also supported by numerous case reports in young adults consuming marijuana or mixture of synthetic CB<sub>1</sub> agonists for recreational use [35].

In contrast to CB<sub>1</sub>, CB<sub>2</sub> signaling appears to be protective in cardiovascular diseases [28]. In endothelium, vascular smooth muscle and fibroblast CB<sub>2</sub> activation decreases pathological activation, proliferation or pro-inflammatory/fibrotic response, and it may also exert

protective effects in cardiomyocytes [28]. In immune cells, CB<sub>2</sub> attenuates chemotaxis, adhesion of inflammatory cells and activation [28]. These effects are responsible for the protective properties of CB<sub>2</sub> 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 CB<sub>1</sub> and CB<sub>2</sub>, may also exert numerous context-dependent effects, ranging from vasodilation/vasoconstriction to anti-inflammatory/pro-inflammatory effects [35]. The impact of eCB signaling in the cardiovascular system is schematically depicted in Figure 1, whereas Table 2 summarizes therapeutic applications of ECS-related drugs.

Collectively, there is strong evidence supporting the pathological function of an overactive ECS (in particular CB<sub>1</sub>) in cardiometabolic diseases. Indeed, it can be concluded that: *i*) the ECS is dysregulated in cardiovascular disease; *ii*) eCBs and synthetic ligands through CB<sub>1</sub> and CB<sub>2</sub> exert opposing effects on cardiovascular injury and inflammation, with CB<sub>1</sub> promoting and CB<sub>2</sub> attenuating them; and *iii*) peripherally restricted CB<sub>2</sub> agonists and CB<sub>1</sub> antagonists appear as promising targets in cardiovascular disease.

## Gastrointestinal tract

The mechanistic basis for the therapeutic effects of cannabis in the gastrointestinal (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 metabolic and homeostatic functions of the body (Figure 2).

CB<sub>1</sub> and CB<sub>2</sub> are highly expressed on enteric nerves and throughout the intestinal mucosa on enteroendocrine cells (CB<sub>1</sub>), immune cells (CB<sub>1</sub> and CB<sub>2</sub>) and enterocytes (CB<sub>1</sub> and CB<sub>2</sub>), as depicted in Figure 2. Under physiological conditions, the actions of eCBs in the GI tract are largely mediated by CB<sub>1</sub> [37]. Activation of the latter stimulates GI motility, suppresses acid and fluid secretion and induces mesenteric vasodilatation [37]. The role of CB<sub>1</sub> 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, CB<sub>1</sub> is localized at enteroendocrine cells, and it may regulate the release of enteroendocrine peptides such as cholecystokinin, which signals hunger [39]. Second, CB<sub>1</sub> expression on enterocytes is regulated by enteric microbiota [40]. Activation of this receptor enhances epithelial permeability by reducing the expression of tight junctional proteins, allowing bacterial translocation and metabolic endotoxemia, leading to obesity. Blocking CB<sub>1</sub> expression reduces obesity, in part through the

enhancement of intestinal barrier function. Third, gut-derived eCBs AEA and 2-AG, and the related compound *N*-oleoylethanolamine (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 CB<sub>1</sub> and CB<sub>2</sub> reduce accelerated GI motility, with CB<sub>2</sub> 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, *e.g.* celiac disease and inflammatory bowel disease [48, 49]. Yet, the therapeutic potential to target this system in their treatment remains to be determined, whereas quite a number of other GI diseases are likely to benefit from ECS-related drugs (Table 2). Whilst 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 will likely limit their usefulness. Peripherally-restricted compounds could overcome these limitations, and appear to have potential in some circumstances [50]. But 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 week) 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*) CB<sub>1</sub> and CB<sub>2</sub> 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 medicines 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 receptor expression 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 CB<sub>1</sub>, it is now evident that these receptors are expressed and mediate a number of biological functions in different types of liver cells, including hepatocytes, stellate cells and vascular endothelial cells, and that blockade of these receptors contributes to the beneficial metabolic effects of CB<sub>1</sub> blockade.

High-fat feeding promotes hepatic lipogenesis and mice deficient in CB<sub>1</sub> are resistant to high-fat diet-induced obesity and the associated hepatic steatosis [53], which suggests that functional CB<sub>1</sub> in hepatocytes promote lipogenesis. This was indeed documented by the ability of a CB<sub>1</sub> agonist to induce lipogenic gene expression and *de novo* hepatic fatty acid synthesis in mouse liver *in vivo* or in isolated hepatocytes *in vitro*, and by the absence of these effects in CB<sub>1</sub> knockout mice or hepatocytes treated with a CB<sub>1</sub> antagonist [53]. CB<sub>1</sub>-mediated hepatic lipogenesis was subsequently documented in human liver cells [54]. In contrast to increasing lipogenesis, hepatic CB<sub>1</sub> inhibit 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 CB<sub>1</sub> [56]. Hepatic CB<sub>1</sub> also plays a dominant role in hepatic insulin resistance that accompanies high-fat diet-induced obesity: activation of hepatocyte CB<sub>1</sub> inhibits insulin clearance by the insulin degrading enzyme, thus contributing to hyperinsulinemia, and also triggers endoplasmic reticulum (ER) stress-dependent suppression of insulin signaling through insulin receptor substrate-1 (IRS1) and Akt-2, resulting in increased glycogenesis [57]. Inhibition of insulin signaling by hepatic CB<sub>1</sub> may also involve activation of the CREBH/LIPIN1/diacylglycerol/PKC $\epsilon$  signaling pathway that results in increased gluconeogenesis [58]. The expression of CB<sub>1</sub> mRNA and protein is very low in normal liver, but is increased substantially in steatotic hepatocytes [53, 56, 59], and chronic CB<sub>1</sub> blockade by rimonabant reverses steatosis induced either by high-fat feeding or ethanol and the upregulation of hepatic CB<sub>1</sub> associated with both [59, 60]. This indicates not only that increased eCB/CB<sub>1</sub> tone mediates the development of fatty liver of various etiologies, but also that CB<sub>1</sub> expression is auto-induced under these conditions. The finding that reversal of diet-induced obesity and its hepatic sequelae could be fully replicated by a non-brain-penetrant CB<sub>1</sub> antagonist supports the dominant role of peripheral (including hepatic) rather than central CB<sub>1</sub> in these effects [61]. The latter receptor is also expressed in hepatic stellate cells, where it is induced by various fibrogenic stimuli, whereas its genetic or pharmacologic ablation protects the liver from fibrosis [62]. In cirrhosis, which represents the advanced stage of liver fibrosis, CB<sub>1</sub> are similarly up-regulated in the vascular endothelium where they mediate hepatic vasodilation, which predisposes cirrhotic individuals to ascites formation and variceal hemorrhage [63].

The role of CB<sub>2</sub> in the liver was first described by Boris Julien and colleagues who demonstrated an anti-fibrogenic action of its agonists in hepatic myofibroblasts and stellate cells, and that CB<sub>2</sub> knockout mice developed enhanced fibrosis in response to the hepatotoxin carbon tetrachloride [64]. Since then, CB<sub>2</sub> has been implicated in liver fibrosis, fatty liver disease, and acute ischemic liver injury [65]. CB<sub>2</sub> 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 CB<sub>2</sub> agonist JWH-133. The latter substance markedly improved the extent of liver fibrosis leading to reduced portal pressures.

This effect is mediated by CB<sub>2</sub> on myofibroblasts and is due to reduced interleukin 17 (IL-17) production by Th17 lymphocytes [67].

Inflammation in the liver is an important feature of alcoholic fatty liver disease. Activation of CB<sub>2</sub> receptors in this context switches Kupffer cells from a pro-inflammatory (M1) phenotype to an anti-inflammatory (M2) phenotype by the induction of hemeoxygenase-1. Fat accumulation in hepatocytes is also reduced, but this is an indirect effect through reduction in steatogenic cytokines such as IL-1 $\beta$  [68].

After liver transplantation, ischemia-reperfusion injury leads to marked liver damage. In mouse models of ischemia-reperfusion injury, Sandor Batkai and colleagues revealed a remarkable role of CB<sub>2</sub> 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 CB<sub>1</sub> and CB<sub>2</sub> receptors are acting in opposite directions in the liver. CB<sub>1</sub> promotes fibrogenesis, steatosis and the cardiovascular complications of liver disease, whereas CB<sub>2</sub> is protective, reducing these indices of liver dysfunction. Clinical studies of cannabis use show that it is detrimental, presumably because of its actions at CB<sub>1</sub> [71]. Thus, CB<sub>1</sub> blockade (with drugs that do not cross the blood-brain barrier) and CB<sub>2</sub> 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 CB<sub>1</sub> promotes vasodilation that can lead to ascites formation, promotes fat accumulation, insulin resistance, and fibrosis; *ii*) activation of CB<sub>2</sub> reduces proinflammatory cytokines, attenuates re-perfusion injury, reduces fat accumulation and is antifibrotic; and *iii*) CB<sub>2</sub> agonists and peripherally restricted CB<sub>1</sub> 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 non-specifically, responds to invasive pathogens in a generic fashion, and does not confer long-lasting immunity. Elements of innate immunity include the complement system that elicits a biochemical cascade targeting the surfaces of foreign cells, natural killer (NK) cells that destroy tumor and virus-infected cells, and soluble factors such as eicosanoids and cytokines that are released by injured and infected cells. Typical cytokines that are elicited during this phase of the immune response include interferon- $\gamma$ , interleukins, and tumor necrosis factor- $\alpha$ . These soluble factors bind to cognate receptors and other cellular targets and set in motion signaling cascades that culminate in the activation of select 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. Principal players in adaptive immunity are B lymphocytes, that are critical players in the humoral



immune response through their capacity to produce antibodies, and T lymphocytes that are critical to the cell-mediated immune response. In adaptive immunity, helper T lymphocytes through the facilitation of their CD4 co-receptor (CD4+), recognize antigens (*i.e.*, substances that induce a specific immune response and that react with the products of that response) that are presented by antigen-presenting cells (APCs) in the context of their class II major histocompatibility (MHC) molecules. The consequent activation of helper T 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 cross-talk between different types of immune cells. Included among these are eCBs, that 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, CB<sub>1</sub> and CB<sub>2</sub> sets in motion a series of signal transduction pathways that converge at the transcriptional level to regulate cell migration and the production of cytokines, chemokines, and other inflammatory factors. Immune cells preponderantly express CB<sub>2</sub>. Consistent with this observation, there is a large body of data that supports a functional relevance for 2-AG as acting through CB<sub>2</sub> to inhibit migratory activities for a diverse array of immune cell types [74, 75]. Indeed, it has been suggested that 2-AG is the cognate functionally relevant eCB for CB<sub>2</sub>. For example, Takayuki Sugiura and colleagues [76] examined the effect of 2-AG on intracellular free Ca<sup>2+</sup> concentrations in human HL-60 macrophage-like cells, and found that it induced a rapid transient increase in levels of intracellular free Ca<sup>2+</sup>. The induced Ca<sup>2+</sup> transient was blocked by a CB<sub>2</sub> antagonist, consistent with the involvement of CB<sub>2</sub> in this response.

AEA also has been reported to inhibit immune functional activities, particularly the production of pro-inflammatory 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 release of cytokines like IL-2, tumor necrosis- $\alpha$  and interferon- $\gamma$  from activated human peripheral T-lymphocytes, mainly through a CB<sub>2</sub>-dependent mechanism. Jean-Marie Derocq and colleagues [79], using IL-3-dependent and IL-6-dependent mouse cell lines, proposed that AEA exerted a growth-promotion effect. Luigi Facci and colleagues [80] reported that mast cells, that are bone marrow-derived cells found in mucosal and connective tissues (as well as in the nervous system) and that play a role in tissue inflammation and neuroimmune interactions, expressed a peripheral cannabinoid receptor that was differentially sensitive to AEA. These cells reportedly expressed CB<sub>2</sub> that exerted negative regulatory effects on mast cell activation. However, AEA did not down-modulate mast cell activation *in vitro*. Thus, unequivocal data that supports 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 functions 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. A variety of 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 sub-populations obtained from mice and humans. Such studies were complemented with those using mixed cell populations that potentially replicated more closely an *in vivo* condition that integrated crosstalks between different immune cell types. Experimental animal models, such as guinea pigs and mice, also have been used to document effects of eCBs on immune functions [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 distinctive 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 CB<sub>2</sub>, should provide novel insights regarding potential target points in signal transductional pathways that are amenable to therapeutic manipulation. In this fashion, the manipulation and ablation of untoward immunological events, including those possibly associated with neuroinflammatory events linked to infection by the human immunodeficiency virus (HIV) and 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 CB<sub>2</sub>, and there is a large body of data that supports a functional relevance for 2-AG as acting through this receptor to inhibit migratory activities for a diverse array of immune cell types; and *iii*) recognition that immune functions can be mediated through the interaction of eCB ligands with cannabinoid receptors should provide novel insights regarding target points for therapeutic manipulation.

## Muscles

It is now well-accepted that muscle cells produce eCBs and express CB<sub>1</sub>, CB<sub>2</sub> and eCB metabolic enzymes, all of which seem to be somehow sensitive to the development of overweight and obesity [83-88]. Whilst in primary skeletal muscle myotubes of lean and obese subjects, expression of *cnr1* (the gene encoding for CB<sub>1</sub>) remained unchanged [87], in rats fed a high-fat diet, abdominal wall skeletal muscle expression of *cnr2* (encoding for CB<sub>2</sub>) and *magl* (encoding for MAGL) genes was decreased and increased, respectively [85]. In genetically obese Zucker rats, CB<sub>1</sub> mRNA levels in soleus were decreased [89], whereas AEA levels were increased [90]. Yet, 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 to the late development of obesity and hyperglycaemia [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 CB<sub>1</sub> 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 CB<sub>1</sub>/CB<sub>2</sub> agonist HU210 caused CB<sub>1</sub>-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 seems also 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 CB<sub>1</sub>-dependent manner [86]. Overall, these data imply that skeletal muscle eCBs, via activation of CB<sub>1</sub>, reduce not only mitochondrial activity but also insulin signaling and glucose uptake, possibly through inhibition of IRS1 phosphorylation and insulin-dependent ERK1/2 activation [86, 88]. Thus, the over-activity 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 play a fundamental role also 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 CB<sub>1</sub>-specific agonist were shown to prevent myotube formation in a manner antagonized by CB<sub>1</sub> knockdown and pharmacological antagonism, which instead stimulated differentiation *per se* [100]. *In vivo*, muscle fascicles from CB<sub>1</sub> knockout embryos were found to contain more muscle fibers, and those from postnatal CB<sub>1</sub> 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 (PIP<sub>2</sub>). Indeed, CB<sub>1</sub> stimulation reduced both total and Kv7.4-bound PIP<sub>2</sub> 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 CB<sub>1</sub>-mediated inhibition of Kv7.4 channels [100].

Finally, it is important to remember that AEA, and to a lesser extent 2-AG, also activate 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 not surprising that the therapeutic exploitation of ECS-related drugs in muscle diseases is still in its infancy, showing as yet only one potential application (Table 2).

Taken together, it can be concluded that: *i*) eCBs are deeply involved in both the control of energy metabolism by the skeletal muscle and the formation of new muscular fibers; *ii*) both these fundamental actions are mediated preferentially via CB<sub>1</sub> and involve AEA and 2-AG

to varying degrees; and *iii*) the ECS ultimately controls the utilization of energy in the skeletal muscle, by either reducing glucose oxidation by myofibers or the extent of muscle formation.

## Bones

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. The lack of appropriate remodeling leads to excessive mineral deposition, which reduces the flexibility of the bone and results in an 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 brain tissues [115]. Stimulation of CB<sub>2</sub> 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 CB<sub>2</sub> signaling in osteoclastogenic cultures and RAW 264.7 cells is more complex, as both inhibitory and stimulatory effects of CB<sub>2</sub> agonists on osteoclast formation have been reported under different experimental conditions [103, 109, 110, 113]. Interestingly, expression of CB<sub>2</sub> on osteoclasts is modulated by TRPV1 channel, which is another target for AEA and has profound effects on bone remodeling [116]. Mice lacking CB<sub>2</sub> displayed a strikingly enhanced age-related loss of trabecular bone volume density with a concomitantly increased bone turnover, a phenotype reminiscent to human postmenopausal osteoporosis [113]. Human genetic studies have also identified polymorphisms in the CB<sub>2</sub> coding region that are associated with low bone mass and osteoporosis [117, 118]. Together, these findings implicate CB<sub>2</sub> signaling in pro-ostogenic processes along the osteoblast lineage and indicate a therapeutic potential of CB<sub>2</sub> agonists in the anabolic therapy of osteoporosis (Table 2). Indeed, studies in mice have shown that CB<sub>2</sub> agonists rescue ovariectomy-induced bone loss [113].

eCB signaling is also important in the central nervous system 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]. CB<sub>1</sub> 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 CB<sub>1</sub> in congenic C57BL/6J mice resulted in a low bone mass phenotype [115]. A detailed phenotype analysis indicated that CB<sub>1</sub> signaling increased trabecular bone mass and radial diaphyseal growth by up-regulating bone formation and down-regulating bone resorption [115]. The phenotype was masked, however, in a different CB<sub>1</sub> 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 the 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. CB<sub>1</sub> and CB<sub>2</sub>, as well as DAGL $\alpha$  and DAGL $\beta$  are expressed in EGC hypertrophic chondrocytes, and 2-AG was detected at significant levels in the EGC. Femora of mice lacking CB<sub>1</sub> or CB<sub>2</sub> were considerably longer at the end of the rapid growth phase compared to wild-type animals. Importantly, THC slowed skeletal elongation of wild-type and CB<sub>2</sub> knockouts, but not that of CB<sub>1</sub> 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 a reduced birth weight, a shorter stature and a 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*) proliferation and differentiation of bone cells; *ii*) communication between bone cells; and *iii*) neuronal control of bone remodelling.

## 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. CB<sub>1</sub> and CB<sub>2</sub> were shown to be expressed in the preimplantation mouse embryos, where CB<sub>1</sub> was primarily localized in the blastocyst trophectoderm (reviewed in ref. 125). It was also shown that THC, as well as uterine AEA, can activate CB<sub>1</sub> and interfere with embryo development. Notably CB<sub>1</sub>, but not CB<sub>2</sub>, 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 CB<sub>1</sub> 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 synchronizing pre-implantation embryo development and preparing the endometrium for implantation (reviewed in ref. 125). Therefore, site-specific levels of AEA and/or other endogenous ligands of CB<sub>1</sub> 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], as depicted in Figure 5a.

Prior to 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, CB<sub>1</sub> is expressed in low levels within both the Fallopian tube and 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]. Independent studies further supported these findings, suggesting a similarly important role for eCB signaling in human pregnancy [125]. Indeed, aberrant eCB signaling may be associated with increased rate of miscarriages and human ectopic pregnancies [126, 131-133].

The effects of eCB signaling on reproductive organs led to the conclusion that also exposure to marijuana during pregnancy may have many adverse effects [125]. With the current legalization of marijuana in the United States and high usage among young adults, there is a critical need for more research into the effects of this drug, and for strong 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 treatment for female infertility, and creams containing PEA (*N*-palmitoylethanolamine; Table 1) appear useful for the treatment of pain-related dysfunctions like chronic vestibulodynia, vulvodynia and vaginismus (Table 2).

Taken together, it can be concluded that: *i*) eCB signaling is operative in all critical stages of pregnancy; *ii*) both silenced or amplified eCB signaling affects pregnancy events; and *iii*) eCB signaling in female reproductive events is primarily mediated by CB<sub>1</sub>.

On the male side, it should be recalled that nearly 1 in 6 couples suffers from infertility, with male factors accounting for 50% of cases [134]. Although anatomical factors explain a significant number of these, several of them remain unexplained, primarily because factors that are involved in the modulation of spermatogenesis and sperm function are poorly understood. A number of molecules/systems have been suggested as playing a pivotal role in this regard, and one such system is the ECS. The fact that marijuana compromises male fertility indeed provides support for this. Concentrations of AEA, OEA and PEA have been detected at nanomolar levels in seminal plasma [135, 136]. Furthermore, both the receptors (CB<sub>1</sub>, CB<sub>2</sub>, TRPV1) and the enzymes (NAPE-PLD, FAAH) regulating these eCB are differentially expressed in human sperm [137]. Indeed, FAAH and NAPE-PLD are localized mainly in the post-acrosomal region and in the middle piece, while CB<sub>1</sub> is localised in the plasma membrane over the acrosomal region, the middle piece and tail, and CB<sub>2</sub> is localised in the plasma membrane of the head. Finally, TRPV1 is localized in the post-acrosomal region [135]. The localization of these components would suggest that both synthesis and degradation occur within human sperm, and indeed significant evidence is emerging to confirm a functional and physiological role of ECS in the process of fertilization, as summarized in Figure 5b (for a recent review see ref. 137).



Several *in vitro* studies have shown a concentration-dependent inhibition of mammalian sperm functions by AEA, mediated by CB<sub>1</sub> activation. One of these is inhibition of mitochondrial activity that preserves energy and ensures gradual acquisition of the fertilisation capacity during ascent up the female reproductive tract. Sperm fertilizing ability has been shown to be enhanced by the binding of AEA to CB<sub>1</sub> that results in the induction of the acrosome reaction pivotal to the fertilization capacity of the sperm. More recently, also a CB<sub>2</sub>-dependent mechanism has been implicated in this process [138].

Quantification of eCB levels in seminal plasma from an infertility clinics showed AEA, PEA and OEA levels to be significantly lower in men with either asthenozoospermia or oligoasthenozoospermia, compared to normozoospermia [134, 136], and furthermore that supraphysiological levels of methanandamide (a non-hydrolyzable analogue of AEA) decreased sperm motility and viability through CB<sub>1</sub>-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 the maintenance of a normal eCB tone is most likely to be necessary for the preservation of normal sperm function, and hence of male fertility. There is indeed some 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 kinematics parameters and hyperactivation *in vitro*. Ultimately this protects sperm from oxidative damage, and could be beneficial in cases of unexplained male infertility [139].

Taken together, it can be concluded that several observations point to ECS involvement in the preservation of normal sperm function, and thus male fertility, and that abnormalities in this system may have implications for adverse reproductive consequences. This is a rapidly evolving domain, and in the future it could be that manipulation of ECS turns out to improve the fertility capacity of men with various forms of sperm dysfunction.

## Skin

An increasing body of evidence suggests that ECS is a key player in the regulation of biological processes of the skin, the largest organ of the human body. As was 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 CB<sub>1</sub>, CB<sub>2</sub> and metabolic enzymes were identified on most cutaneous cell types; hence, ECS was implicated in a wide array of skin functions in all layers of the skin (summarized in Figure 6).

In the epidermis, AEA was shown to inhibit proliferation and reduce differentiation of human epidermal keratinocytes via CB<sub>1</sub>-coupled signaling (reviewed in ref. 141). Moreover, CB<sub>1</sub> also controls melanogenesis in epidermal melanocytes [142]. In addition, further highlighting the role of ECS in epidermal functions, in an animal model using gene-deficient mice, absence of CB<sub>1</sub> delayed whereas lack of CB<sub>2</sub> accelerated recovery of the epidermal

permeability barrier [143], whose establishment is strongly dependent on keratinocyte proliferation and differentiation. Of further importance, CB agonists as well as phytocannabinoids were found to inhibit proliferation of hyperproliferative keratinocytes, whereas various cannabinoids exerted complex anti-tumor activity (anti-proliferative, pro-apoptotic and anti-angiogenic effects together with reduced metastasis formation) in multiple models of melanoma and non-melanoma skin tumors (for a recent review see ref 140).

ECS is also involved in the biology of skin appendages. CB<sub>1</sub>-coupled signaling was shown to inhibit human hair shaft elongation and induce premature organ involution (catagen), whereas CB<sub>2</sub> was found to “tonically” stimulate the homeostatic lipogenesis in human sebaceous gland-derived sebocytes [140]. Interestingly, AEA inhibited proliferation and induced secretory activity of sweat gland epithelial cells, via non-CB<sub>1</sub>/CB<sub>2</sub>-dependent mechanisms [144].

The pioneering mouse study of Meliha Karsak and colleagues [145] has clearly presented that augmentation of the cutaneous eCB tone, as well as certain phytocannabinoids, exerts remarkable anti-inflammatory activities [140]. This effect was recently proven also in human models. “Tonic” activation of CB<sub>1</sub> was shown to keep 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 or CB<sub>1</sub> agonists [146]. Of further importance, the prototypic non-psychoactive phytocannabinoid cannabidiol – by modulating a complex signaling network involving TRPV4 ion channels, adenosine A2a receptors, and multiple downstream elements (*e.g.*, P65-NFκB 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 CB<sub>1</sub> and CB<sub>2</sub> were observed in dermal fibroblasts and various fibrosis models. FAAH was found to be down-regulated in dermal fibroblasts (SScDF) of systemic sclerosis (or scleroderma) patients, suggesting that the “eCB tone” might be elevated. Indeed, pharmacological or genetic abrogation of FAAH activity worsened bleomycin-induced fibrosis in mice via a CB<sub>1</sub>-coupled signaling [148]. Accordingly, CB<sub>1</sub> null animals were found to be protected against the pro-fibrotic effects of bleomycin [149]. However, activation of CB<sub>2</sub> expressed by leukocytes was able to ameliorate fibrosis in the same animal model; likewise, CB<sub>2</sub> stimulation in SScDFs also resulted in significant anti-fibrotic effects [140, 150].

Collectively, the (highly selected) data presented above highlights that appropriate, targeted manipulation of cutaneous ECS and/or administration of certain phytocannabinoids point to completely new therapeutic opportunities in several cutaneous pathologies. Clinical trials are therefore urgently required to assess the putative *in vivo* efficiency of ECS-oriented treatments in *e.g.* 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 for these skin diseases (Table 2). Taken together, it can be concluded that: *i*) 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 to suppress cutaneous inflammation; and *iii*) effects of eCBs are chiefly mediated by both CB<sub>1</sub> and CB<sub>2</sub> expressed by various skin cells.

## Other organs

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

On a final note, 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 still behind our scientific knowledge of eCB signaling, also because there are outstanding questions that still await for an answer (Box 1). A summary that points out the potential applications of ECS-related drugs for peripheral diseases is shown in Table 2. In this context, it should be recalled that peripherally restricted CB<sub>1</sub> agonists failed in clinical trials of pain, owing to cardiovascular and metabolic side effects, and synthetic CB<sub>1</sub> agonists are damaging to 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 CB<sub>1</sub> antagonist clinical trials were successful in diabetes and obesity, but failed because of unwanted side effects within the central nervous system. Interestingly, in rodent models of obesity and type-2 diabetes, peripherally restricted CB<sub>1</sub> antagonists show therapeutic promise, as they retain the full metabolic efficacy of globally acting CB<sub>1</sub> antagonists, but are devoid of their neurobehavioral effects. Furthermore, FAAH or MAGL inhibitors appear to be good in some preclinical models of pain, but they promote cardiovascular inflammation and cause metabolic undesirable consequences, and indeed already failed human trials in 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 them, cannabidiol, is getting more and more approvals by Food and Drug Administration for orphan and other indications (*e.g.*, refractory epilepsy and glioblastoma). Interestingly, a near 1:1 ratio of cannabidiol and THC (nabiximols, trade name Sativex) has beneficial effects for multiple sclerosis patients, who can use it to alleviate neuropathic pain, spasticity, overactive bladder, and other symptoms. Yet, the effects of Sativex cannot be reproduced by THC alone, suggesting that some or many of them could be due to cannabidiol, via molecular mechanisms yet to be disclosed, or to the fact that co-administration of

cannabidiol can widen the therapeutic window of THC, thus allowing its administration at higher doses with less side effects. Since overwhelming evidence from preclinical studies also suggests that cannabidiol may be beneficial in myocardial infarction, stroke, diabetic cardiovascular complications, and even in nephropathy, investigations into its mechanism(s) of action appear urgent.

Against this background, one should admit that the way towards a therapeutic exploitation of ECS-oriented drugs may not be quite close, also because of the apparent complexity of eCB signaling regulation. After 50 years since the discovery of THC, and nearly 20 years since that of AEA (1992) and 2-AG (1995), only recently it is emerging that the correct interaction between the different components of ECS is essential for a proper function of eCB as signaling molecules. For instance, distinct eCB-binding receptors can exchange signals to each other and with other receptors, and 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) [22, and references therein]. The need of a proper localization and the existence of an intracellular trafficking seem to add a new dimension to the already complex regulation of eCB signaling. Thus, in order 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 amounts 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” issuing 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 to 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 intensely searched ECS-based therapeutics with limited side effects and abuse liability. Chances are that we can take advantage of these novel drugs in the near future.

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**Dedication:** This paper is dedicated to our dear colleague and friend Itai Bab, sorrowfully missed last October 2014.

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## Glossary

<b>Acne vulgaris</b>	one of the commonest human skin diseases, characterized by a pathologically elevated and qualitatively altered sebum production (seborrhoea) and by inflammation of the sebaceous glands (and of the skin in general).
<b>Acrosome reaction</b>	changes in the sperm head that occur with contact with the egg, allowing it to penetrate and then fertilize the egg.
<b>Advanced glycation end products</b>	substances that can contribute to development or worsening of many human degenerative diseases.
<b>Anandamide (AEA)</b>	the amide derivative of arachidonic acid with ethanolamine ( <i>N</i> -arachidonoyl-ethanolamine), named after the Sanskrit word for bliss, “ananda”.
<b>Antigen presentation</b>	an adaptive immune response whereby antigen-specific receptors recognize processed antigens in the form of peptides.
<b>Arachidonic acid</b>	common name of 5,8,11,14-eicosatetraenoic acid, an essential (diet-derived) $\omega$ -6 fatty acid that serves as precursor for eicosanoids and endocannabinoids.
<b>Asthenozoospermia</b>	reduced sperm motility and sperm count.
<b>Atherosclerosis</b>	building up of fats, cholesterol and other substances (plaques) in and on artery wall, which can restrict blood flow.
<b>Autocrine</b>	related to a substance secreted by a cell and acting on surface receptors of the same cell.
<b>B lymphocyte</b>	an immune cell that makes antibodies against antigens, acts as an antigen-presenting cell, and develops into a memory cell after activation by antigen.
<b>Blastocyst</b>	an embryonic stage in mammals and is comprised of a fluid-filled cavity (blastocoel) and two cell types – inner cell mass and trophoctoderm.
<b>Cannabinoid receptors (CBs)</b>	G protein-coupled receptors that bind THC, as well as AEA, 2-AG and other endocannabinoids.
<b>Cannabinoids</b>	natural lipophilic products found in <i>Cannabis sativa</i> extracts ( <i>e.g.</i> , hashish or marijuana). Among >60

	<p>cannabinoids present in this plant, which are all characterized by a terpeno-phenol bicyclic or tricyclic structure, the best known compound is <sup>9</sup>-tetrahydrocannabinol (THC).</p>
<b>Catagen</b>	<p>the cessation phase of hair production, that includes also growth (anagen) and rest (telogen) phases.</p>
<b>Celiac disease</b>	<p>a gastrointestinal autoimmune disorder that results in severe damage to the mucosal lining of the small intestine when foods with gluten are eaten, causing malabsorption, bloating, pain and diarrhea.</p>
<b>Chemokine</b>	<p>a class of cytokines that functions to attract white blood cells (leukocytes) such as lymphocytes, granulocytes, monocytes, and macrophages to sites of infection.</p>
<b>Chemotaxis</b>	<p>is the movement of an organism in response to a chemical stimulus.</p>
<b>Cholecystokinin</b>	<p>a peptide released from “I”-type enteroendocrine cells that are predominantly found in the proximal small intestine of the gastrointestinal tract. Cholecystokinin regulates food intake, and coordinates the digestion of fat and protein.</p>
<b>Chondrocyte</b>	<p>cartilage cells derived from mesenchymal stem cells that produce extracellular matrix components of cartilage tissue.</p>
<b>Chondroclast</b>	<p>multinucleated cartilage resorbing cells.</p>
<b>Cytokine</b>	<p>a category of small proteins (5-20 kDa) that are released by immune cells that play an important role in cell signaling affecting the behavior of other cells; cytokines include chemokines, interferons, and lymphokines.</p>
<b>Cumulus oophorus</b>	<p>cluster of cells that surround the oocyte in the ovarian follicle.</p>
<b>Dendritic cell</b>	<p>antigen-presenting cells capable of activating T lymphocytes and stimulating the growth and differentiation of B lymphocytes.</p>
<b>Embryo development</b>	<p>development of the fertilized egg to the blastocyst stage through several rounds of mitosis.</p>
<b>Endocannabinoids</b>	<p>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 fatty acid amides and monoacylglycerols, respectively.</p>

<b>Endometrium</b>	the inner lining of the uterus and consists of stromal and epithelial cells.
<b>Endotoxemia</b>	the presence of endotoxins in the blood, which may result in shock.
<b>Enteric nervous system</b>	The enteric nervous system is the third division of the autonomic nervous system. It consists of 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 gastrointestinal tract. It provides local neural control of the gastrointestinal tract and is required for the coordination of digestive and defensive functions of the gut.
<b>Enterocyte</b>	absorptive epithelial cells that line the lumen of the gastrointestinal tract.
<b>Enteroendocrine cells</b>	specialized hormone producing epithelial cells of the gastrointestinal tract and pancreas. Enteroendocrine cells of the intestine are the most numerous endocrine cells of the body.
<b>Epidermal barrier</b>	the barrier – consisting of physico-chemical, immunological and microbial aspects – formed chiefly by the outermost ( <i>i.e.</i> , epidermal) layer of the skin, that aims at protecting the body against various external challenges and at maintaining cutaneous homeostasis.
<b>Fatty acid amide hydrolase (FAAH)</b>	endomembrane-bound serine hydrolase that cleaves AEA into arachidonic acid and ethanolamine.
<b>Fibrosis</b>	excessive formation of connective tissue, due to the over-activity of dermal fibroblasts.
<b>Idiopathic infertility</b>	infertility for which there is no obvious cause.
<b>Implantation site</b>	the site of embryo attachment in the uterus (implantation chamber).
<b>Inflammatory bowel disease</b>	a group of chronic inflammatory conditions of the gastrointestinal 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.
<b>Inotropy</b>	alteration of the force or energy of muscular contractions.
<b>Interleukin</b>	a class of glycoproteins that is produced by leukocytes for regulating immune responses.

<b>Lipogenesis</b>	the process by which acetyl-CoA is converted to fatty acids.
<b>Macrophage</b>	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.
<b>Microglia</b>	resident macrophages of the brain and spinal cord.
<b>Monocyte</b>	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.
<b>Muscular dystrophy</b>	a pathological condition that weakens the musculoskeletal system thereby 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.
<b>Muscular hypertrophy</b>	a condition that involves an increase in 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.
<b>Natural killer (NK) cell</b>	a lymphocyte that binds to select tumor cells and virus-infected cells with antigen stimulation; NK cells kill target cells by inserting granules containing perforin.
<b>Neutrophil</b>	a type of leukocyte that constitutes one of the first responders to inflammation, particularly due to bacterial infection; neutrophils are a type of phagocyte normally found in the bloodstream.
<b>(Non) receptive uterus</b>	the uterus which is (in) capable for supporting blastocyst growth and attachment.
<b>Normozoospermia</b>	normal sperm count, morphology and motility.
<b>Oligoasthenozoospermia</b>	reduced sperm motility and count.
<b>Oligozoospermia</b>	reduced sperm count.
<b>Osteoblast</b>	bone-forming cells derived from mesenchymal stem cells.
<b>Osteoclast</b>	multinucleated myeloid cells resorbing bone.

<b>Osteoporosis</b>	a progressive degenerative bone disorder, characterized by reduced bone mass and a concomitantly increased fracture risk.
<b>Ovariectomy</b>	surgical removal of the ovaries.
<b>Oviductal embryo transport</b>	the journey of fertilized eggs through the oviduct and ultimately into the uterus.
<b>Paracrine</b>	related to a substance secreted by a cell and acting on surface receptors of nearby cells.
<b>Parturition</b>	the action or process of giving birth to offspring.
<b>Perforin</b>	a protein released by NK cells that destroys target cells by inducing pores in their membranes.
<b>Peroxisome proliferator-activated receptors (PPARs)</b>	superfamily of nuclear receptor transcription factors, that include three members: $\alpha$ , $\gamma$ and $\delta$ . PPAR- $\alpha$ and - $\gamma$ are targets of AEA and its congeners.
<b>Placentation</b>	role of the placenta which is comprised of trophoblast cells and establish vascular connection between the conceptus and the mother.
<b>Polymorphism</b>	natural variations in a DNA sequence that have been identified in a population.
<b>Restenosis</b>	literally means the recurrence of stenosis, a narrowing of a blood vessel, leading to restricted blood flow.
<b>Sperm kinematics parameters</b>	these refer to the parameters of movement in a forward direction and at an acceptable pace.
<b>Steatosis</b>	the process that describes the abnormal retention of lipids within a cell.
<b>Sympathetic and parasympathetic projections</b>	nerve endings of the autonomic nervous system originating from cranial or sacral nerves belonging to the sympathetic nervous system or the parasympathetic nervous system.
<b>Systemic sclerosis (also known as “scleroderma”)</b>	a systemic autoimmune disease, which can be characterized by the excessive accumulation of collagen fibers in the skin and in the internal organs.
<b>T lymphocyte</b>	immune cells that can directly kill infected cells or cancer cells; these cells assist in B lymphocyte maturation and produce cytokines.
<b>Tight junction proteins</b>	families of proteins, including occludins, zonula occludens and claudins, which together form an intercellular tight junction between adjacent

gastrointestinal epithelial cells. The tight junction lies just beneath the apical surface and consists of interlinked rows of integral membrane proteins, limiting the passage of water and other molecules.

**Transient receptor potential vanilloid-1 (TRPV1)**

a six-transmembrane receptor-channel that is activated by physical and chemical stimuli, as well as by AEA.

**Trophectoderm**

the outer layer of the blastocyst and is the progenitor of future placenta.

**<sup>9</sup>-Tetrahydrocannabinol (THC)**

psychoactive ingredient of cannabis (*Cannabis sativa*) extracts like hashish and marijuana.

**Zona pellucida**

glycoprotein layer surrounding the plasma membrane of mammalian oocytes.



**Box 1****Outstanding questions**

1. When will we have conclusive controlled clinical trials to evaluate the overall safety/toxicity of marijuana and synthetic cannabinoids?
2. Enhancement of eCB levels is seen in many disease states. Is indeed CB<sub>2</sub> activation (and in some cases CB<sub>1</sub> 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 efficacy of ECS-oriented therapies?
3. When will we have proof of the principle clinical trials to evaluate the therapeutic potential of peripherally restricted CB<sub>1</sub> antagonists and CB<sub>2</sub> agonists in cardiovascular disease? A clear assessment of the importance of the peripheral ECS (especially CB<sub>1</sub>) and of its therapeutic relevance in humans will have to await the results of clinical studies with novel, peripherally restricted ligands.
4. Many GI disorders are triggered or exacerbated by stress. How does stress alter the ECS in the gut? What is the balance between CB<sub>1</sub> and CB<sub>2</sub> activation in GI disorders? CB<sub>2</sub> 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? This important question remains to be fully explored.
5. Do AEA and 2-AG as bioactive lipids act in opposition to each other, in order to maintain a fine-tuned balance in immune functional activities? Since CB<sub>2</sub> is found preponderantly on immune cells, can it serve as a selective and specific molecular targets for ablating untoward pathological processes characterized by hyperinflammation?
6. Insight as to how activation of cannabinoid receptors alters immune functional activities has been obtained through the study of their ligation to phytocannabinoids. Yet, 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?
7. 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?
8. What is the mechanism of eCB signaling in bone elongation? How is the hypertrophy of chondrocytes modulated by eCBs? In which processes are CB<sub>2</sub> receptors involved?
9. 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?

- 10.** Is there a multilateral crosstalk between cutaneous ECS and the complex neuro-immuno-endocrine network (*e.g.*, hormones, cytokines, growth factors, neuropeptides) of the skin?

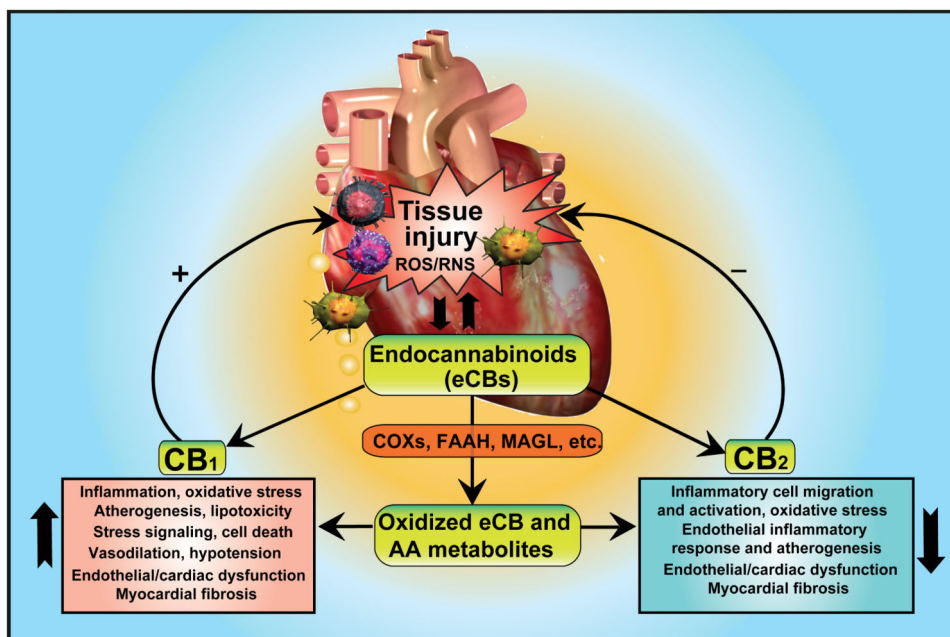
### Highlights

50 years ago the psychoactive ingredient of *Cannabis sativa*,  $\Delta^9$ -tetrahydrocannabinol (THC), was isolated.

We critically review current status of endocannabinoids (eCB) functions in peripheral organs.

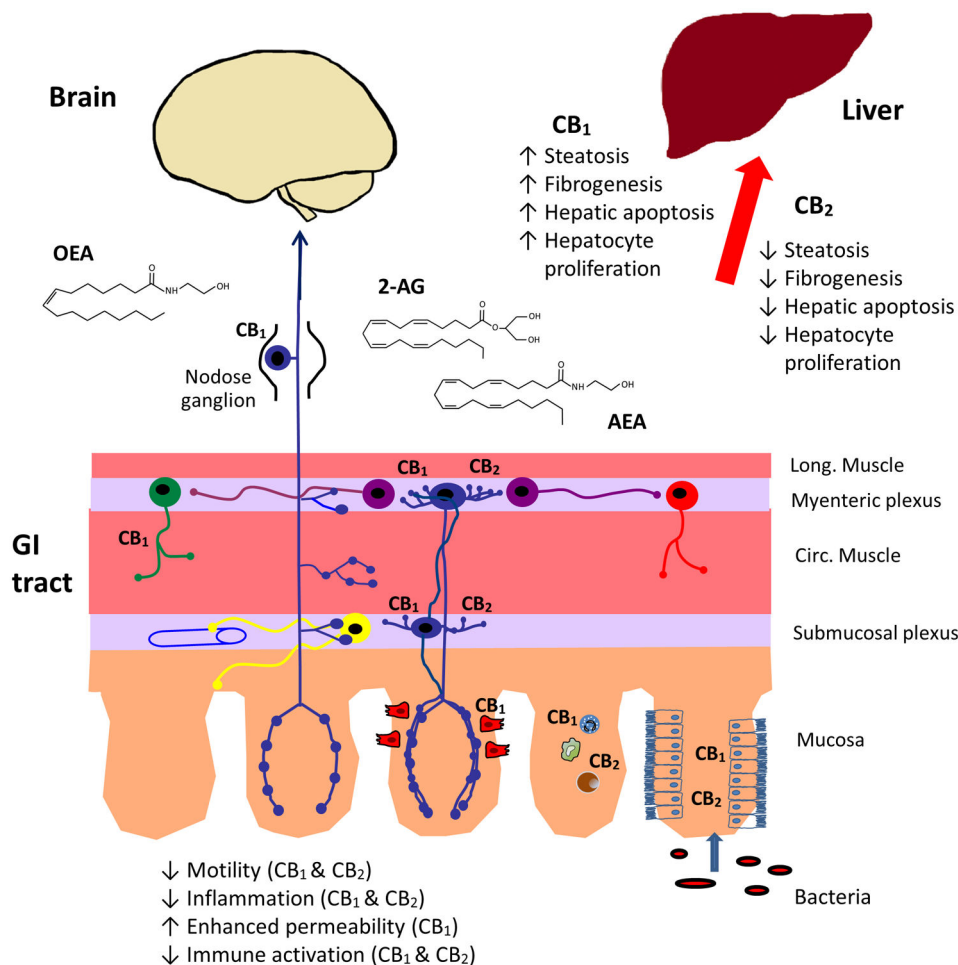
We discuss the relevance of the peripheral eCB system for human health and disease pathogenesis

We highlight emerging challenges and therapeutic hopes.



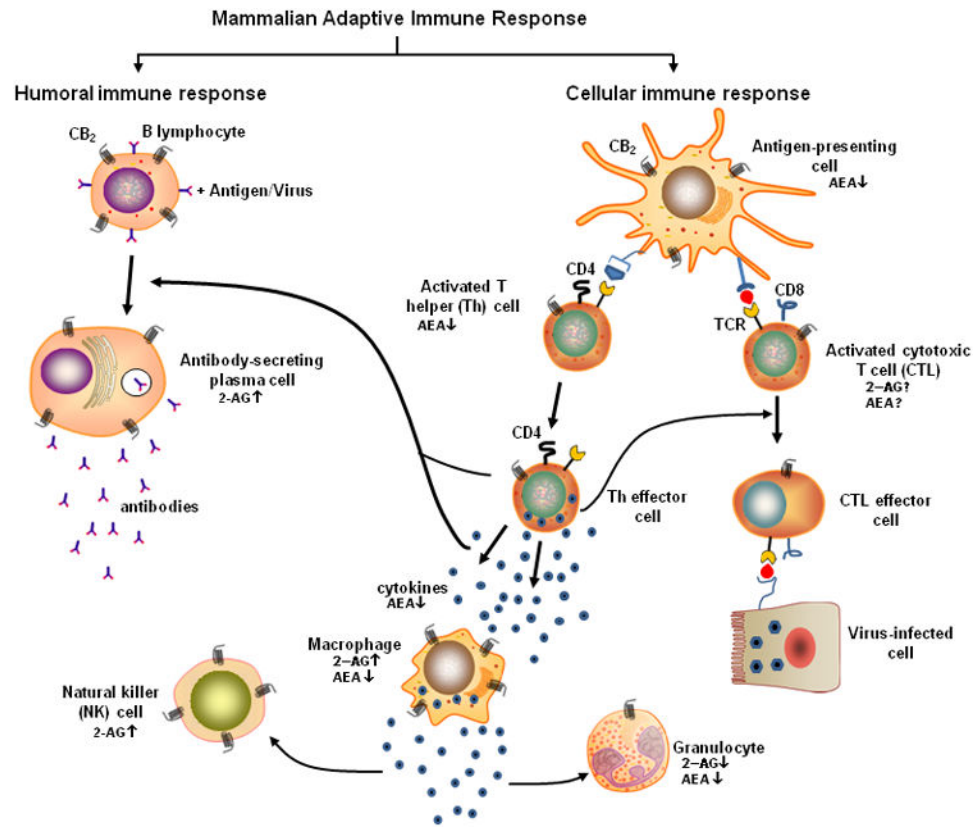
**Figure 1.**

Role of 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 ECS in cardiovascular system and infiltrating immune cells. eCBs via activation of CB<sub>1</sub> in cardiomyocytes, endothelial cells, fibroblasts and certain immune cells promote processes facilitating development of cardiovascular dysfunction, inflammation and pathological remodeling. In contrast, eCBs via activation of CB<sub>2</sub> exert opposing protective effects. Moreover, eCBs through their catabolism by FAAH and/or 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 diseases may largely be context-, time- and pathology-dependent.



**Figure 2.**

The ECS of the gastrointestinal (GI) tract and liver. AEA, 2-AG, and OEA are synthesized in the gut and liver, where they act locally and in the brain. eCBs regulate gut motility at the level of the enteric neural plexuses, they reduce intestinal inflammation through actions on the immune system and they influence intestinal barrier function at the level of epithelium. eCBs and OEA regulate food intake by actions on enteroendocrine cells in the wall of the gut, the vagus nerve and in the brain. In the liver, CB<sub>1</sub> and CB<sub>2</sub> have opposing effects, with CB<sub>1</sub> promoting steatosis, fibrogenesis, apoptosis and proliferation and CB<sub>2</sub> inhibiting these effects.



**Figure 3.**

Cell types and cascade of immunological events that come into play in an adaptive mammalian immune response, and respective functional activities that have been implicated as modulated by 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, respectively, play a critical role in humoral and cellular immune responses 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 an 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 major histocompatibility (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 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.

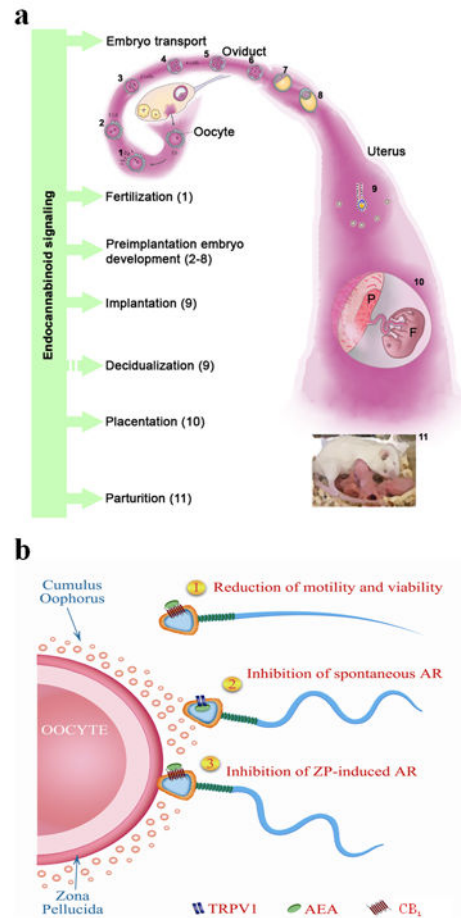
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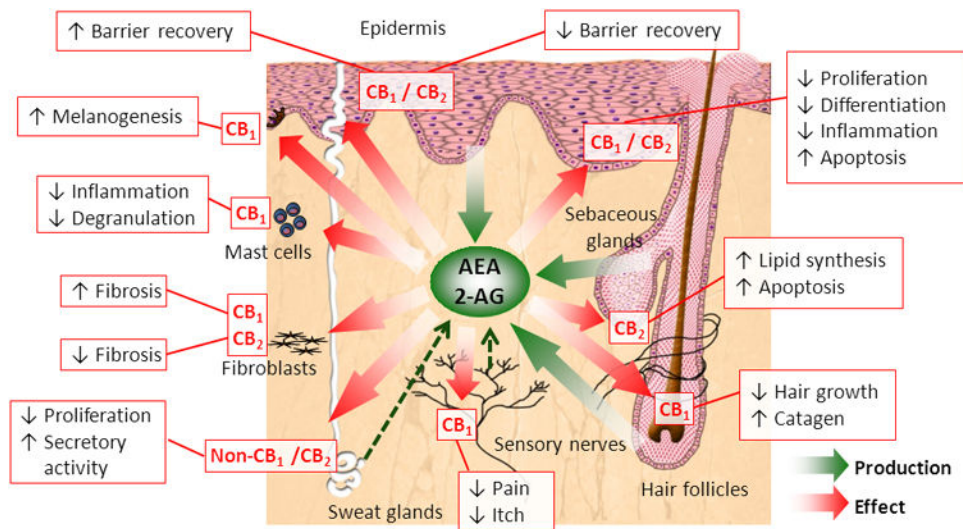
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**Figure 5.**

**a)** Schematic diagram showing pregnancy events in mice that are influenced by eCB signaling. Stage-specific eCB signaling during pregnancy is numerically designated (1-11). Implantation and decidualization both are designated by (9), since they are overlapping events. P, placenta; F, fetus. **b)** Functional activity of ECS elements in human sperm. Far from the oocyte activation of CB<sub>1</sub> reduces sperm motility and viability (1), and activation of TRPV1 inhibits a spontaneous acrosome reaction (AR), that would be useless to fertilize the egg (2). Next to the oocyte activation of CB<sub>1</sub> inhibits zona pellucida (ZP)-induced AR (3), thus avoiding fertilization of the egg by more than one sperm (polyspermy).



**Figure 6.** Summary of the effects of cutaneous ECS on multiple skin functions. Adapted from ref. 140.

Table 1

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

Name (abbreviation)	Chemical structure
<sup>9</sup> -Tetrahydrocannabinol (THC)	
<i>N</i> -Arachidonylethanolamine (Anandamide, AEA)	
2-Arachidonoylglycerol (2-AG)	
<i>N</i> -Oleylethanolamine (OEA)	
<i>N</i> -Palmitoylethanolamine (PEA)	
<b>Biosynthetic enzymes of AEA</b>	<b>Intracellular localization</b>
Ca <sup>2+</sup> -dependent <i>N</i> -acyltransferase (NAT)	Integral membrane protein
Ca <sup>2+</sup> -independent <i>N</i> -acyltransferase (iNAT)	Mainly cytosolic
<i>N</i> -acyl-phosphatidyl ethanolamines (NAPE)-specific phospholipase D (NAPE-PLD)	Membrane-associated
$\alpha/\beta$ -hydrolase domain 4 (ABHD4)	Membrane-associated
Protein tyrosine phosphatase, non-receptor type 22 (PTPN22)	Mainly cytosolic
Glycerophosphodiester phosphodiesterase (GDE1)	Integral membrane glycoprotein
<b>Degrading enzymes of AEA</b>	<b>Intracellular localization</b>
Fatty acid amide hydrolase (FAAH)	Membrane-associated (mainly ER)
<i>N</i> -acylethanolamine-hydrolyzing acid amidase (NAAA)	Lysosomal
<b>Biosynthetic enzymes of 2-AG</b>	<b>Intracellular localization</b>
Diacylglycerol lipase $\alpha$ (DAGL $\alpha$ )	Membrane-associated
Diacylglycerol lipase $\beta$ (DAGL $\beta$ )	Membrane-associated
<b>Degrading enzymes of 2-AG</b>	<b>Intracellular localization</b>
Monoacylglycerol lipase (MAGL)	Membrane-associated and cytosolic
$\alpha/\beta$ -hydrolase domain 6 (ABHD6)	Membrane-associated
$\alpha/\beta$ -hydrolase domain 12 (ABHD12)	Membrane-associated
<b>Oxidative enzymes of AEA and 2-AG</b>	<b>Intracellular localization</b>
Cyclooxygenase-2 (COX-2)	Membrane-associated
Lipoxygenases (LOXs)	Membrane-associated and cytosolic
Cytochromes P450 (CYPs)	Membrane-associated

Table 2

Therapeutic applications of ECS-related drugs at the periphery.

Compound/Category	ECS target	Model (animal/human)	Therapeutic indication(s)	Clinical conditions	References
(Peripherally-restricted) CB <sub>1</sub> antagonists	CB <sub>1</sub>	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 <sub>2</sub> agonists	CB <sub>2</sub>	Rodent	Myocardial infarction, stroke, myocarditis? cardiomyopathies?	Myocardial infarction, stroke, myocarditis? cardiomyopathies?	[28, 160]
(Peripherally-restricted) CB <sub>1</sub> agonists	CB <sub>1</sub>	Dog / human	Transient lower esophageal relaxations	Gastroesophageal reflux disease	[161, 162]
(Peripherally-restricted) CB <sub>1</sub> agonists	CB <sub>1</sub>	Mouse / rat	Diarrhea Inflammation Visceral pain	Irritable bowel syndrome Inflammatory bowel disease Gastric ulcer	[163] [164-166] [167]
(Peripherally-restricted) CB <sub>1</sub> antagonists	CB <sub>1</sub>	Mouse	Metabolic endotoxemia Food intake Dysmotility	Obesity Paralytic ileus	[43] [40] [168, 169]
CB <sub>2</sub> agonists	CB <sub>2</sub>	Mouse / rat	Diarrhea Inflammation, visceral pain	Irritable bowel syndrome Inflammatory bowel disease	[44, 170] [44, 170]
FAAH inhibitors	CB <sub>1</sub> CB <sub>2</sub> PPARs	Mouse	Diarrhea Inflammation	Irritable bowel syndrome Inflammatory bowel disease Gastric ulcer	[45, 171] [166, 172] [173]
MAGL inhibitors	CB <sub>1</sub> CB <sub>2</sub> PPARs	Mouse / rat	Diarrhea Inflammation	Irritable bowel syndrome Inflammatory bowel disease Gastric ulcer	[174] [175] [47, 176]
Peripherally restricted CB <sub>1</sub> antagonists	CB <sub>1</sub>	DIO mice, <i>ob/ob</i> mice, <i>dlb/dlb</i> mice, ZDF rats	Lipogenesis, inflammation	Obesity/metabolic syndrome, fatty liver disease, type-2 diabetes	[61, 177, 178]
Rimonabant	CB <sub>1</sub>	Mouse C2C12 myoblasts	Defective myotube differentiation and muscle regeneration	Muscular dystrophy	[100]



Compound/Category	ECS target	Model (animal/human)	Therapeutic indication(s)	Clinical conditions	References
Synthetic CB <sub>2</sub> agonists	CB <sub>2</sub>	Mouse	Bone mineralization	Osteoporosis	[113]
N-Palmitoyl/ethanolamine	CB <sub>1</sub> ? CB <sub>2</sub> ? TRPV1? PPARs? GPR55?	Human	Vestibulodynia, vulvodynia, proctodynia	Infertility	[179, 180]
THC	CB <sub>1</sub>	Mouse	Parturition	Infertility	[125, 128]
N-Palmitoyl/ethanolamine	CB <sub>1</sub> ? CB <sub>2</sub> ? TRPV1? PPARs? GPR55?	Human	Inflammation, pruritus	Atopic dermatitis, prurigo, uremic itch	[140, 141]
(Peripherally-restricted) CB <sub>1</sub> antagonists	CB <sub>1</sub>	Rodent	Diabetic and other nephropathies and tubulopathies	Diabetic and other nephropathies and tubulopathies	[151, 152, 154]
CB <sub>2</sub> agonists	CB <sub>2</sub>	Rodent	Diabetic and other nephropathies and tubulopathies	Diabetic and other Nephropathies and tubulopathies	[153, 154]

DIO (diet-induced obesity), *ob/ob* (defective in leptin gene), and *db/db* (defective in leptin receptor gene) mice are models of obesity. Zucker diabetic fatty (ZDF) rats are a model of diabetes.