Role of the Endocannabinoid System in the Regulation of Intestinal Homeostasis

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SUMMARY

The endocannabinoid system is a lipid mediator signaling system widely distributed throughout the gastrointestinal tract. The endocannabinoid system plays a pivotal role in the maintenance of intestinal homeostasis and gut barrier integrity, responding to internal and external environmental factors while also serving as a homeostatic effector system.

The maintenance of intestinal homeostasis is fundamentally important to health. Intestinal barrier function and immune regulation are key determinants of intestinal homeostasis and are therefore tightly regulated by a variety of signaling mechanisms. The endocannabinoid system is a lipid mediator signaling system widely expressed in the gastrointestinal tract. Accumulating evidence suggests the endocannabinoid system is a critical nexus involved in the physiological processes that underlie the control of intestinal homeostasis. In this review we will illustrate how the endocannabinoid system is involved in regulation of intestinal permeability, fluid secretion, and immune regulation. We will also demonstrate a reciprocal regulation between the endocannabinoid system and the gut microbiome. The role of the endocannabinoid system is complex and multifaceted, responding to both internal and external factors while also serving as an effector system for the maintenance of intestinal homeostasis. (Cell Mol Gastroenterol Hepatol 2022;https://doi.org/10.1016/j.ccmgh.2022.05.015)

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The maintenance of intestinal homeostasis is of fundamental importance to health. Intestinal homeostasis requires the integration of digestive and defensive functions of the gut to protect against the insults that arise from digestion, harmful pathogens and toxins, and the commensal microbota that live in the gut, while simultaneously promoting the efficient utilization of food. A central determinant of intestinal homeostasis is intestinal barrier function.1-3 The intestinal barrier is a dynamic arrangement composed of a physical barrier (tight junctions) between the epithelial cells and a variety of secretory processes that together preserve the integrity of the epithelium, while being sufficiently permeable to allow for antigen sampling and the passage of nutrients, electrolytes, and water. The gut is poised to trigger effective mechanisms to rapidly rid itself of unwanted luminal contents through enteric neuroimmune mechanisms and has enormous capacity to mount immune responses to microbial pathogens and potentially harmful food antigens.4-6 Dysregulation of intestinal homeostasis has serious consequences, driving a variety of pathologic conditions, including inflammatory bowel disease (IBD), celiac disease, and diseases of gut-brain interaction such as irritable bowel syndrome.7-9

Because of the importance of preserving the integrity of the gastrointestinal (GI) tract, it is not surprising that there are numerous, highly sophisticated systems controlling various aspects of intestinal homeostasis. These include intrinsic cellular mechanisms, autocrine and paracrine factors, immune and microbial mediators, and intercellular signaling molecules, as well as intrinsic and extrinsic nervous mechanisms.10-12 Accumulating evidence indicates that the endocannabinoid system (ECS) is a vital nexus in the brain-gut-microbiota axis, serving as a critical regulator of intestinal homeostasis.13,14 The ECS is a widely distributed lipid mediator signaling system affecting a multitude of physiological and pathophysiological processes throughout the body.15,16 In this review we will highlight the complex
role the ECS plays in the maintenance of intestinal homeostasis and gut barrier integrity. We will develop the idea that it is pivotal because it responds to internal and external environmental factors, while also serving as a homeostatic effector system. We have not addressed the effects of the ECS on gut motility, because this topic has been comprehensively reviewed.14,17,18

Endocannabinoid System

The ECS or endocannabinoid system has a multitude of functions in physiological and pathophysiological processes in the brain and body.19 It is perhaps best known for playing important roles in food intake and energy metabolism, regulation of the hypothalamic-pituitary-adrenal axis, pain transmission, and a variety of emotional and behavioral conditions.14,19-25

The ECS was discovered after the isolation of Δ9-
tetrahydrocannabinol (THC) as the major psychoactive constituent of cannabis.26 This finding led to the development of ligands that were used to identify the receptors for cannabis. There are now 3 major classes of cannabinoids that are recognized: phytocannabinoids that are derived from the cannabis plant, such as THC and cannabidiol (CBD); synthetic cannabinoids, such as the chemical agonists HU210, CP55,940, and WIN55,212-2 and antagonists rimonabant, AM250, and AM630; and endocannabinoids, the lipid mediators, anandamide (AEA) and 2-arachidonoylglycerol (2-AG), that we will discuss further in this review.27

Interested readers are directed to excellent reviews of cannabinoids.15,27-30

In its original description, the ECS consists of two G
c-protein-coupled receptors (GPCRs), cannabinoid (CB) receptor 1 (CB1) and 2 (CB2), their endogenous ligands endocannabinoids, and the biosynthetic and degradative enzymes that control the availability of these ligands.15,21 Since this original description, many other receptors and ligands are now considered part of the ECS,19 as we will outline below.

Endocannabinoids are membrane-derived lipid signaling molecules. The 2 primary endocannabinoids are N-arachidonoyl ethanolamine (AEA) and 2-AG. Unlike classical neurotransmitters that are stored in vesicles, endocannabinoids are synthesized “on demand” in response to a stimulus.22 Primary stimuli for their synthesis are an elevation of intra-
cellular calcium and activation of a number of GPCRs.22 The synthesis and degradation pathways of the 2 main endo-
cannabinoids are shown in Figure 1.

Anandamide synthesis begins with the conversion of phosphatidylethanolamine into N-arachidonoyl phosphati-
dylethanolamine (NAPE) by the calcium-dependent enzyme
N-acyltransferase. Anandamide is then produced from the hydrolysis of NAPE by N-acylphosphatidylethanol-
amine-hydrolyzing phospholipase D (NAPE-PLD). Three additional pathways for AEA synthesis have also been described.23-25 (1) Phospholipase C (PLC) converts NAPE into phosphoanandamide, which is then dephosphorylated to form AEA. (2) α/β-hydrolase 4 produces glycerophosphoanandamide from NAPE, which is then converted into AEA by glycerophosphodiesterase-1, and (3) soluble phospholipase A2 acts on NAPE to form 2-lyso-NAPE, which is then converted to AEA by lyso-PLD. The primary enzyme responsible for the degradation of AEA is fatty acid amide hydrolase (FAAH). FAAH hydrolyzes AEA into arachidonic acid and ethanolamine.36,37

The synthesis of 2-AG is dependent on the calcium-
dependent enzyme PLC and the activity of diacylglycerol lipase (DAGL). PLC hydrolyzes arachidonic acid-containing membrane lipids to produce diacylglycerol, which is then converted into 2-AG by DAGL.8 In addition, 2-AG may be made from the conversion of 2-arachidonoylphosphoinositol to 2-arachidonoyl-lyso-phosphoinositol by phospholipase A1, which is subsequently converted to 2-AG by lyso-
phosphoinositol-PLC.38,39 2-AG is primarily metabolized by monoacrylglycerol lipase ( MAGL) into glycerol and arachidonic acid.39,40 However, studies in mice have revealed 2 additional degradation pathways via the enzymes α/β-hydrolase domain-containing protein (ABHD)-6 and ABHD-12.41-43

In the GI tract, the primary cellular sources of endo-
cannabinoids are identified by the expression of the genes for the relevant synthesizing enzymes and include enteric neurons and glia and the extrinsic projections from neurons in the dorsal root ganglion.14-16 Recent single cell RNA expression studies have suggested other interesting, albeit minor, cellular sources including Paneth and goblet cells.47 However, although we are gaining an understanding of the relevant ECS-related genes, the functional cellular sources of endocannabinoids in the gut are not entirely clear, and we do not have a complete understanding of how sources of endocannabinoids change during pathophysiological conditions.

The CB receptors are GPCRs coupled to Gi/o proteins leading to an inhibition of adenyl cyclase and decreased production of cyclic adenosine monophosphate (cAMP).48 The release of βγ dimers after CB receptor activation also causes inhibition of N- and P/Q-type Ca2+
channels and activation of inwardly rectifying and A-type potassium channels.32,49 The βγ dimers initiate Src-mediated signaling cascades leading to activation of mitogen-activated protein kinases and focal adhesion kinases. Activation of CB re-
ceptors is generally inhibitory, serving as a “molecular brake”, to limit excitation across the synapse, reduce secretory processes by epithelial cells, and reduce mediator release from immune cells. Interaction of ligand-bound CB receptor with β-arrestin is required for internalization and termination of extracellular signaling, but β-arrestins are themselves signal transducers for intracellular signaling.

Cannabinoid receptors may exist in multiple forms as
heterodimers or homodimers, are very widely distributed in...
essentially every organ and tissue in the body, and are subject to dysregulation in pathophysiological conditions. Of particular relevance, CB1 is expressed in the enteric nervous system (ENS) and the autonomic and central nervous systems (CNS) and is one of the most abundant GPCRs in the brain. In the GI tract, CB1 is expressed presynaptically on all classes of enteric neurons, except nitric oxide synthase-expressing inhibitory motor neurons. It is also found on the terminals of primary afferent nerves innervating the gut and on glucose-dependent insulinotropic polypeptide producing (K cells) and cholecystokinin producing (I cells) enteroendocrine cells in the upper small intestine. Activation of the CB1 receptor on enteric nerves leads to a decrease in neurotransmitter release into the synapse and therefore a depression of excitatory neurotransmission in the ENS. CB2 is predominantly expressed by immune cells including B cells, neutrophils, mast cells, and macrophages; however, it is also found in limited regions of the CNS, in the ENS, and on the terminals of primary afferent nerves. Like CB1, activation of CB2 inhibits the release of immune mediators or neurotransmitters.

To rapidly activate their receptors, endocannabinoids, as lipids, require a carrier protein to overcome the hydrophilic environment of the extracellular space or synapse. Fatty acid-binding proteins, albumin, and heat shock protein 70 have been described as endocannabinoid carrier proteins. Fatty acid-binding proteins were discovered as mediators of the intracellular transport of AEA from the plasma membrane for degradation by FAAH. Whether they are used by AEA extracellularly remains uncertain. Fatty acid-binding proteins 5 was found to regulate 2-AG signaling at excitatory glutamatergic synapses in the brain. The source of the fatty acid-binding protein 5 was astrocytes, which suggests that fatty acid-binding proteins are extracellular carrier proteins. These data suggest that glial cells serve to limit the extent of synaptic endocannabinoid signaling. Whether this mechanism exists in vivo remains to be determined.

Recent single cell RNA sequencing of neurons and glia in the ENS reveal fatty acid-binding protein 5 gene is highly expressed by enteric nerves, enteroendocrine cells, and also in enteric glia. What role it plays in the ENS remains to be determined. It also remains to be determined...
**Figure 2. Signaling pathways induced by endocannabinoid receptor activation.** Anandamide is produced from N-arachidonoyl phosphatidylethanolamine (NAPE) by the action of N-acylphosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD). It is degraded by fatty acid amidase hydrolase (FAAH). FAAH hydrolyzes AEA into arachidonic acid (AA) and ethanolamine (EA). 2-Arachidonoylglycerol (2-AG) is primarily produced through the action of diacylglycerol lipase (DAGL). It is degraded by monoacylglycerol lipase into AA and glycerol. Inhibition of these degradative enzymes increases levels of endocannabinoid to enhance signaling. Both anandamide and 2-AG signal through the CB1 and CB2 receptors where anandamide is a partial agonist (light arrows) and 2-AG is a full agonist (thick arrows). Upon ligand binding, both receptors activate \( G_{q,11} \), which then interacts with \( \beta \) and \( \gamma \) subunits to initiate downstream signaling. The primary response is inhibition of adenylyl cyclase (AC) and therefore reduction in the cytosolic levels of cyclic adenosine monophosphate (cAMP). There is evidence suggesting that these receptors can also activate MAP kinase (MAPK) pathways including ERK, JNK, and p38MAPK. Activation of the CB1 receptor also triggers \( \beta \)-arrestin, which is involved in receptor internalization, desensitization, and degradation, and which may also be involved in intracellular signaling pathways. Figure created with Biorender.com.

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The ECS is not restricted to the actions of its 2 primary ligands. Several related bioactive lipids have been identified that structurally resemble AEA and 2-AG. These include the N-acylethanlamides (NAEs), N-palmitoylethanolamide (PEA), N-oleylethanolamide (OEA), N-stearoylethanolamide, and N-linoleoylethanolamide, as well as lipid mediators from the acylglycerol family, 2-palmitoylglycerol (2-PG) and 2-oleoylglycerol (2-OG). These bioactive lipids are thought to modulate endocannabinoid signaling through interactions with synthetic and degradative enzymes and other non-canonical endocannabinoid receptors.

In addition, endocannabinoids do not interact exclusively with the canonical CB receptors, but also with several other classes of receptors. One of the best examples of endocannabinoid action at a non-cannabinoid receptor is the affinity of AEA for the transient receptor potential vanilloid type-1 (TRPV1) channel. Endocannabinoids and NAEs also have affinity for peroxisome proliferator-activated receptor (PPAR-\( \alpha \), PPAR-\( \gamma \), G protein-coupled receptor 55 (GPR55), GPR119, and GPR18).

Thus, the ECS consists of lipid mediators, their associated biosynthetic and degradative enzymes, receptors, and intracellular signaling pathways capable of exquisite local regulatory control in physiological and pathophysiological conditions. There remains much we do not know about this system in the gut, notably the exact sites and specific conditions in which endocannabinoids are released. We know little about the genetic and epigenetic regulation of CB receptor expression in the GI tract, or that of other components of the ECS in the gut. Moreover, we know relatively little about how the ECS is regulated or dysregulated in pathophysiological conditions, although we have evidence for alterations in the expression of the components of the ECS in various GI diseases.

In the following sections of this review, we will discuss how the ECS is involved in the control of intestinal homeostasis by considering the elements that make up the physical, secretory, and immunologic components of the intestinal barrier (Figure 3). Finally, we will consider the reciprocal regulation of the ECS and the gut microbiome.

**Regulation of Epithelial Tight Junctions by the Endocannabinoid System**

A single layer of epithelial cells lines the GI tract and is all that separates the contents of the gut from the rest of the body. The intestinal epithelium is a selectively permeable barrier that facilitates the transcellular and paracellular transport of nutrients, water, and electrolytes, enables antigen sampling for oral tolerance, while inhibiting the passage of possibly harmful substances such as toxins, foreign antigens, pathogens, and commensal bacteria.

Paracellular transport across the epithelial barrier is regulated by tight junctions. Tight junctions are protein complexes located between adjacent epithelial cells that form a selectively permeable barrier. There are 4 groups of transmembrane proteins: occluding, claudins, juncational adhesion molecule, and tricellulin. Scaffold proteins, such as zonula occludens (ZO) proteins, anchor the intracellular domains of the transmembrane proteins to the actin cytoskeleton. The regulation of tight junctions is dynamic and controls the paracellular permeability of ions, nutrients, and water. When the integrity of the tight junction barrier is compromised, paracellular permeability increases and allows the passage of large molecules and microbes that reside in the gut lumen. This can lead to inflammation and tissue damage from the infiltration of bacteria and their metabolites into the mucosa.

The intestinal epithelium expresses CB receptors, although this varies according to species, location along the GI tract, and pathophysiological state. The human colon expresses CB1 receptors with what appears to be primarily apical expression based on immunohistochemical studies. Colonic epithelia also express CB2 receptors in Crohn’s...
disease. The epithelium of mouse small intestine and colon do not display a substantial degree of CB1 expression under normal conditions.\textsuperscript{80,81} Caco-2 cells, a cancer cell line often used to study epithelial cell biology, expresses CB1 receptors.\textsuperscript{82}

The ECS has been shown to play a role in the regulation of tight junction proteins essential for the maintenance of intestinal barrier function.\textsuperscript{82–84} In an in vitro model of the intestinal epithelium using Caco-2 cell monolayers, Muccioli et al\textsuperscript{83} demonstrated that simultaneous application of the CB1 agonist HU210 and lipopolysaccharide reduced trans-epithelial electrical resistance (TEER), and this was associated with a reduction in the expression of tight junction proteins occludin and ZO-1. The effect on TEER and tight junction protein expression was blocked by the CB1 antagonist rimonabant.\textsuperscript{83} Similarly, Alhamoruni et al\textsuperscript{84} showed that apical application of the endocannabinoids AEA and 2-AG reduced TEER, increased mRNA expression of ZO-1, and reduced mRNA expression of claudin-1. This effect was also blocked by the CB1 antagonist AM251, suggesting that ligands of the ECS modulate intestinal permeability under baseline conditions by altering the expression of tight junction proteins.

Moreover, the ECS has also been shown to play a role in the regulation of intestinal permeability in conditions where baseline intestinal permeability is perturbed.\textsuperscript{82,84–86} In an in vitro model of ethylenediamine tetraacetic acid–induced increased permeability, basolateral application of AEA and 2-AG decreased permeability in Caco-2 cell monolayers.\textsuperscript{84} Although the effect of AEA and 2-AG is mediated by CB1, AEA relied on the additional recruitment of TRPV1.\textsuperscript{84} In a model of inflammation-induced increase in permeability where Caco-2 cells are exposed to tumor necrosis factor and interferon gamma, basolateral application of AEA and 2-AG had no effect on permeability.\textsuperscript{86} However, in both models, apical application of AEA and 2-AG increased permeability through a CB1-dependent mechanism.\textsuperscript{82,83,86} Apical application of the phytocannabinoids THC and CBD were protective and reduced permeability through a CB1-dependent mechanism.\textsuperscript{82,83,86} Interestingly, co-application of PEA to the basolateral compartment and cannabidiol to the apical compartment decreased permeability, and this was dependent on PPAR\textsubscript{a} and CB1, respectively.\textsuperscript{82} Altogether, these data demonstrate that the ECS is involved in the regulation of gut barrier function largely through a CB1-dependent mechanism but may also rely on the recruitment of TRPV1 and PPAR\textsubscript{a}.

Additional studies in vivo have further supported the role of the ECS in the regulation of intestinal permeability. Chronic activation of the CB1 receptor with the potent full agonist HU210 in wild-type mice led to an increase in
permeability to 4 kDa FITC-dextran.\textsuperscript{83} In contrast, Zoppi et al\textsuperscript{97} demonstrated that CB\textsubscript{1} knockout mice experience a greater degree of intestinal barrier dysfunction after exposure to immobilization and acoustic stress compared with wild-type mice, suggesting that the CB\textsubscript{1} receptor exerts a protective role in the colon in the regulation of paracellular permeability. Consistent with these findings, Chen et al\textsuperscript{98} showed that the beneficial effect of resveratrol on intestinal permeability in a high-fat diet-induced nonalcoholic steatohepatitis model in rats was blocked by the synthetic CB\textsubscript{2} receptor agonist arachidonyl-2-chloroethylamide. Interestingly, more recent work demonstrated that CB\textsubscript{1} receptor agonists and antagonists reduce intestinal permeability in mice exposed to a 2-week high-fat diet. Although changes in the expression of claudin-2 may underlie the effect of the CB\textsubscript{1} agonist, further studies are required to understand the mechanism by which the CB\textsubscript{1} antagonist reduces intestinal permeability.\textsuperscript{99}

Obesity is a metabolic disorder associated with an altered gut microbiota, defects in barrier function, and increased endocannabinoid tone. In genetically obese (Ob/Ob) mice and dietary-induced obese mice, treatment with the CB\textsubscript{2} antagonist rimonabant reduced plasma lipopolysaccharide levels and led to a change in the distribution and localization of tight junction proteins ZO-1 and occludin, suggesting a decrease in intestinal permeability.\textsuperscript{83,90,91}

Together, these data suggest that the ECS is indeed involved in the chronic regulation of intestinal permeability in vivo through a CB\textsubscript{1}-dependent mechanism. CB\textsubscript{2} agonists disrupt the gut barrier and, as suggested by Cani et al,\textsuperscript{13} act as a gate opener (increasing intestinal permeability), whereas CB\textsubscript{1} antagonists protect the gut barrier and are considered gatekeepers. This CB\textsubscript{2}-mediated disruption of intestinal permeability could be explained partially by changes in the distribution and localization of tight junction proteins after CB\textsubscript{1} receptor activation. CB\textsubscript{1} receptor blockade restores the tight junction barrier and the integrity of the gut barrier. However, in recent studies, we found that CB\textsubscript{1} agonists could also reduce intestinal permeability in vitro under baseline conditions,\textsuperscript{92} suggesting that the pharmacologic regulation of CB\textsubscript{1} receptors may depend on the level of endocannabinoid tone or other factors.

The literature has suggested that AEA and 2-AG, two endogenous CB\textsubscript{1} agonists, exert differential effects on gut barrier integrity, acting themselves as a gate opener and gatekeeper, respectively. Although the literature consistently establishes the role of AEA as a gate opener, the evidence to support 2-AG as a gate keeper is rather limited. The role of 2-AG as a gate keeper is somewhat challenging to understand because 2-AG is a full CB\textsubscript{1} agonist and typically produces effects associated with CB\textsubscript{1} agonism.\textsuperscript{91} If 2-AG is indeed a gate keeper, then this CB\textsubscript{1} agonist is behaving more like an antagonist, because CB\textsubscript{1} antagonists have been shown to decrease permeability. On the other hand, AEA is a partial CB\textsubscript{1} agonist with lower efficacy than 2-AG\textsuperscript{92} but is considered a gate opener and is therefore behaving like a CB\textsubscript{1} agonist. On the basis of this information, one might predict the opposite of what has been shown, such that 2-AG would be the gate opener and AEA would be the gate keeper. Whether 2-AG is indeed a gate keeper remains unclear and warrants further investigation. Further studies will need to address the concept of biased agonism in CB\textsubscript{1}-mediated effects on epithelial permeability; AEA and 2-AG, while both acting at epithelially expressed CB\textsubscript{1} receptor, could activate different signaling pathways resulting in different cellular responses. In addition, it is not known whether differences in the polarization of distribution of CB\textsubscript{1} receptor, apical vs basolateral, could be associated with coupling to different signaling pathways and, hence, different effects on epithelial cell function.

### Regulation of Secretory Function by the Endocannabinoid System

The secretion of fluid, mucus, antimicrobial peptides, and secretory immunoglobulin A are key elements of intestinal barrier function.\textsuperscript{12,93–95} The role of the ECS in the control of intestinal mucus, antimicrobial peptide, and secretory immunoglobulin A secretion is not well-understood. However, the ECS is involved in the regulation of neurogenic intestinal fluid secretion under baseline conditions\textsuperscript{96–98} and when fluid secretion is enhanced to respond to luminal toxin. Oral administration of choleragen toxin to mice results in a large increase in fluid accumulation in the small intestine.\textsuperscript{99} This is associated with increased levels of AEA and increased expression of the CB\textsubscript{2} receptor mRNA. These responses are involved in the homeostatic control because endogenous CB\textsubscript{2} receptor agonists reduce secretion to control levels, and a CB\textsubscript{2} receptor antagonist further exacerbates secretion.\textsuperscript{95} In in vitro preparations of guinea pig ileum, CB\textsubscript{1} agonism seems to reduce chloride ion transport (the main driver of fluid secretion) through prevention of neurotransmitter release from primary sensory afferents, not through direct effects on the epithelium.\textsuperscript{96} There is limited evidence supporting a role for CB\textsubscript{2} receptors in the regulation of intestinal secretion.\textsuperscript{98}

### Regulation of Local Gastrointestinal Immune Function by Endocannabinoids

The ECS is an important regulator of the immune system.\textsuperscript{100–102} Of note, cells of the innate and adaptive immune systems express CB\textsubscript{2} receptors that control their activity. Within the GI tract, neutrophils, macrophages, and T and B cells express CB\textsubscript{2} receptor as well as other receptors of the endocannabinoidome (eg, PPAR\textgreek{a} and GPR55).\textsuperscript{81,103–107} The
ECS acts as a regulator of immune homeostasis in the gut. For example, CB2 receptor regulates the numbers of CX3CR1+ macrophages in the intestinal lamina propria and their tolerogenic potential.\textsuperscript{104} Similarly, activation of CB\textsubscript{2} enhances the expansion of regulatory T cells in the gut with concomitant anti-inflammatory actions.\textsuperscript{104–107} In the section below we will expand on these homeostatic actions by focusing on the role of the ECS in regulating intestinal homeostasis in response to bacterial pathogens and intestinal inflammation.

**Role of the Endocannabinoid System in Regulating Intestinal Homeostasis in Response to Bacterial Pathogens and Intestinal Inflammation**

One of the first lines of defense against invading pathogens is the recruitment of neutrophils to the site of infection. The rapid transepithelial migration of neutrophils is a critical response to combat enteric infection. However, the products of neutrophil degranulation and the oxidative burst that are used to kill bacteria are associated with significant damage to surrounding tissues if unchecked can lead to tissue damage and dysfunction.\textsuperscript{108} There are various lipid mediators involved in the resolution of inflammation and tissue restitution including the resolvins and lipoxin A\textsubscript{4},\textsuperscript{109,110} but recently, endocannabinoids were discovered as endogenous regulators of the proinflammatory actions of the eicosanoid hepoxilin A3. Szabady et al\textsuperscript{103} investigated the role of the epithelial P-glycoprotein efflux pump in countering the hepoxilin A3 proinflammatory pathway. They discovered that N-acylethanolamines (AEA, OEA, and \(\alpha\)-linolenylethanolamide), effluxed via P-glycoprotein, suppressed neutrophil migration mediated by hepoxilin A3. This effect was sensitive to FAAH, but not MAGL, and was shown to be mediated by the CB\textsubscript{2} receptor localized on neutrophils. Interestingly, Szabady et al\textsuperscript{103} did not find evidence for a role for 2-AG or its congeners in inhibiting neutrophil migration, despite the fact it is a full CB2 receptor agonist.\textsuperscript{103}

Thus, although AEA is a (relatively weak) CB\textsubscript{2} agonist, exactly how other NAEs regulate CB\textsubscript{2} receptor function and the specific receptor mechanisms responsible for these effects remain to be explained.

Consistent with these data, CB\textsubscript{2} receptor knockout mice treated with either dextran sodium sulfate (DSS) or trinitrobenzene sulfonic acid (TNBS) to induce colitis have more severe disease and dramatic increases in neutrophil accumulation in the intestinal lumen.\textsuperscript{103,111} Similarly, activation of CB\textsubscript{2} receptors directly with specific agonists or indirectly by inhibiting FAAH and elevating endogenous ligands protects against experimental colitis in mice.\textsuperscript{105,112–115} In accordance with these findings, the CB\textsubscript{2}-Q63R variant contributes to the risk for pediatric IBD, particularly Crohn’s disease,\textsuperscript{116} illustrating the clinical significance of endocannabinoid receptor mechanisms to intestinal homeostasis. In fact, the endocannabinoide is markedly dysregulated in IBD,\textsuperscript{117} as are the expression and distribution of the ECS receptors, biosynthetic and degradative enzymes.\textsuperscript{118} Although it is tempting to speculate that these changes contribute to the breakdown in homeostasis in these diseases, this remains to be directly demonstrated.

Szabady et al\textsuperscript{103} did not find a role for the NAE, PEA, in inhibiting neutrophil migration; however, PEA has been shown by others to be a potent anti-inflammatory mediator acting via CB\textsubscript{2} receptors and, in addition, GPR55 and PPAR\textalpha, as well as through the modulation of TRPV1.\textsuperscript{115} In elegant work, Esposito et al\textsuperscript{119,120} demonstrated that PEA dose-dependently reduced colonic damage, inflammatory mediator expression and release, and immune cell infiltration in DSS colitis and inflammatory mediator expression and release in biopsy samples from patients with ulcerative colitis via PPAR\textalpha activation. They demonstrated that the effects of PEA were mediated through an action on enteric glia by reducing the expression of toll-like receptor 4 and S100B.\textsuperscript{120} In an interesting extension of this work, this group recently developed a probiotic-based delivery system for PEA. Using genetic engineering, they developed a strain of Lactobacillus paracasei with the human NAPE-PLD gene inserted into it to produce an in situ delivery system for the release of PEA in the GI tract. They demonstrated that this approach was effective in a mouse model of Clostridium difficile colitis where colonic damage, inflammatory mediator release, and tight junction protein expression were all improved.\textsuperscript{121} It was also similarly effective in DSS colitis.\textsuperscript{121} The effects of the probiotic bacterium were abolished in PPAR\textalpha knockout mice, suggesting they were mediated by PEA. However, because NAPE-PLD can synthesize a variety of NAEs and these were not assessed, it remains to be determined whether the effects observed are solely mediated by PEA. These studies placed enteric glia as critically important cellular intermediaries in the regulation of intestinal inflammation and homeostasis, a role that is gaining increasing recognition and clinical relevance.\textsuperscript{122–124} For example, it was recently shown that toll-like receptor 4 on enteric glia is critical for the development of necrotizing enterocolitis.\textsuperscript{125} Little is known about how the ECS regulates enteric glial function. Sharkey and colleagues showed that CB\textsubscript{2} receptors could attenuate activation of enteric glia, which is consistent with other actions of cannabinoids, but beyond that this remains an area for further investigation.\textsuperscript{127}

The anti-inflammatory actions of PEA in the gut have been extended to intestinal inflammation in mouse models of Alzheimer’s disease, radiation injury, and ischemia-reperfusion injury.\textsuperscript{126–128} Although the actions of PEA are terminated by hydrolysis by FAAH, they are also regulated by N-acylethanolamine-hydrorylizing acid amidase (NAA).\textsuperscript{129} Inhibiting NAA elevates levels of PEA, while not altering those of AEA. In TNBS colitis in mice, a NAA inhibitor significantly reduced the degree of colitis and the release of inflammatory mediators,\textsuperscript{129} indicating that endogenously produced PEA can regulate inflammation and mucosal integrity. Interestingly, the related NAE, OEA, is also anti-inflammatory in DSS...
Reciprocal Regulation of the Endocannabinoid System and the Gut Microbiome

The gut microbiota is a critical environmental determinant of host physiology. Studies using germ-free mice, or mice treated with antibiotics to deplete the gut microbiota, have revealed that the microbiota shapes gut function and enteric neural control mechanisms. Microbial dysbiosis is associated with a breakdown in epithelial barrier function and is associated with both local GI diseases, eg, IBD, and various systemic conditions, eg, obesity and diabetes. Investigations into the regulation of the ECS by the gut microbiome and vice versa are at an early stage, but some intriguing results have emerged that support the general hypothesis that the ECS regulates intestinal homeostasis through interactions with the microbiota.

In the absence of a gut microbiota, significant changes in the expression of the genes for receptors, biosynthetic and degradative enzymes of the ECS were observed throughout the gut. For example, cnr1 (CB1 receptor) is markedly elevated in the colon of germ-free mice. Overall, more striking changes were seen in the small intestine compared with the colon, and when mice were younger (4 weeks compared with 13 weeks of age). In concert with these changes, there were also alterations in the levels of endocannabinoids, NAEs, and other related lipid mediators, to a variable degree along the length of the gut. Most of the changes observed were reversible when a normal gut flora was introduced using fecal microbial transplant. However, it should be noted that the germ-free animals were only re-colonized in these studies for 1 week, which may not have been sufficient for stable microbial recolonization. Nevertheless, these data demonstrate that the gut microbiota is directly impacting all aspects of the ECS.

The ECS has also been examined after antibiotic administration was used to deplete the gut microbiota. Modest changes to AEA levels were noted along the length of small intestine with no changes to 2-AG; however, there were marked changes to NAEs and other endocannabinoid ligands. Interestingly, increased antibiotic treatment resulted in increased CB2 receptor expression. CB2 receptor expression was further increased in animals exposed to antibiotics who were also exposed to a period of water-avoidance stress, which is illustrative of how the ECS responds to perturbations that lead to elevated visceral sensitivity.

Taken together, these studies reveal how the ECS is relatively altered by marked changes in the microbial environment, although the mechanism underlying these alterations remains to be determined. Importantly, microbial changes to endocannabinoid signaling result in biologically relevant functional effects at the level of the gut and the brain; it should be noted that commensal bacteria make endocannabinoid ligands, although their role in GI physiology remains obscure.

Administration of prebiotics or probiotics alters the composition of the gut microbiota (transiently) and can be used therapeutically. The benefits of probiotics may be...
mediated, at least in part, by the ECS. For example, treatment with the probiotic *Lactobacillus acidophilus* upregulated CB2 receptors on intestinal epithelial cells. When animals with visceral hypersensitivity were treated with this probiotic, it gave rise to a pronounced visceral analgesia to colorectal distention, sensitive to CB2 receptor antagonism, assessed in vivo. The exact mechanisms behind these interesting effects remain to be determined because the source and nature of the ligand activating the CB2 receptors were not determined. However, the inhibitory actions of CB2 receptor activation are consistent with recent studies that show that optogenetic inhibition of the colonic epithelium reduces visceral hypersensitivity in mice with DSS colitis. In another example, treatment with the probiotic *Lactobacillus plantarum* reduced despair behavior and increased hippocampal neurogenesis associated with a chronic stress paradigm by altering endocannabinoid levels in the hippocampus. The gut-brain pathways involved in these effects remain to be determined.

Just as probiotics can alter the ECS in ways that are beneficial, altering the microbiota can also give rise to effects that are detrimental. Recently, Markey et al. colonized healthy mice with the commensal fungus *Candida albicans* for 48 hours. *Candida* colonization caused no changes to the cecal bacterial populations in the gut and no intestinal inflammation. However, there were marked increases in anxiety-like behavior, accompanied by elevations in plasma corticosterone that were inversely correlated with forebrain AEA levels. Colonization with *Candida* disrupted the metabolism of endocannabinoids in the gut, notably the NAEs. Consistent with these observations, when mice were treated with the FAAH inhibitor URBS97, corticosterone levels were reduced to control levels, as was anxiety-like behavior. This finding is illustrative of the importance of the microbiota-gut-brain axis in regulating behavior and how the ECS is an important component of this signaling system.

In an interesting study in human subjects, Vijay et al. studied the relationships between the gut microbiome, ECS, and inflammatory cytokines after 6-week exercise intervention. They demonstrated that under baseline conditions, the NAEs were positively associated with bacterial alpha diversity and with short-chain fatty acid producing bacterial species including *Bifidobacterium* and *Faecalibacterium* and negatively associated with the pathogenic bacterium *Escherichia Shigella*. The NAEs increased significantly after the exercise intervention. Changes in AEA correlated with elevated butyrate levels, increases in AEA and FAAH correlated with reductions in the inflammatory cytokines, tumor necrosis factor and interleukin 6, whereas 2-AG and OEA levels were correlated with anti-inflammatory cytokine interleukin 10. This study again highlights the interactions between the ECS and the gut microbiota and reveals that the ECS is involved in mediating homeostatic anti-inflammatory actions in humans.

The connection between gut microbiota and the ECS has been studied in the context of the regulation of energy homeostasis. Diet-induced obesity in mice is associated with an altered gut microbiota. Chronic administration of the CB1/CB2 partial agonist, THC, prevented the change in gut microbiota in the DIO mice. Chronic THC administration in DIO mice increases the abundance of *Akkermansia muciniphila*. Interestingly, THC had no effect on gut microbiota in lean mice. Administration of *A. muciniphila* in DIO mice also increases levels of 2-AG, 2-OH, and 2-PG in the gut. Furthermore, blockade of the CB1 receptor with the CB1 antagonist rimonabant increased the abundance of *Lachnospiraceae* and *Erysipelotrichaceae*. Insights into how the ECS might regulate the gut microbiota in the context of a high-fat diet were obtained by Everard et al. using molecular genetics. They selectively knocked out nape-pld (the gene for NAPE-PLD) in intestinal epithelial cells in mice fed a high-fat diet. This resulted in marked changes to the gut microbiota composition that were shown not to be due to the diet per se. These data suggest that epitheliad derived NAEs are the mediators of signaling that ultimately alter microbial composition. It remains to be shown how this occurs, but one possibility is through the regulation of local innate immune mechanisms.

Specific changes in the gut microbiota through several models (eg, prebiotic treatment, high-fat diet, antibiotic treatment, and germ-free mice) selectively alter CB1, FAAH, and MAGL mRNA expression in the colon. The gut microbiota appears to modulate intestinal endocannabinoid tone in the colon but has no effect in the small intestine, which is likely due to the greater bacterial load in the colon. Although evidence suggests that changes in the composition of the gut microbiota affect colonic endocannabinoid tone, the exact mechanism involved in this regulation remains unknown.

**Conclusions and Perspective**

A summary of the material we have presented in this review is presented in Figure 4. Although there are numerous unanswered questions, the wealth of evidence accumulated to date suggests that the ECS is intimately involved in the physiological processes that underlie the control of intestinal homeostasis. The pivotal role the ECS plays in the maintenance of gut barrier integrity is complex and multifaceted because it responds to internal (microbial) and external (diet, stress, etc) environmental factors, while also serving as a homeostatic effector system. A lot of the evidence for the role of the ECS is based on the use of pharmacologic tools or a global knockout of the CB receptors or degradative enzymes. The application of cell-specific gene deletion technologies is required to causally determine the (patho)physiological role of the ECS. Where this has been used, for example to understand the role of epithelial NAPE-PLD in DIO, very novel findings have emerged that greatly advanced our understanding of the physiology of the ECS.

An important consideration of the ECS that needs to be borne in mind is the context dependent nature of CB receptor activation by endocannabinoids. By this we refer to the fact that the 2 major endocannabinoids AEA and 2-AG are partial and full agonists of the CB1 receptor,
respectively, and as we have discussed, they can have opposite effects in the GI tract or individual effects rather than identical actions. Because of the complexity of lipid signaling in inflammatory states in particular, where there may be a diversity of precursors according to the expression of the rate limiting enzymes for the synthesis of the lipid moieties, ECS signaling may be altered in ways that are not easy to predict. Furthermore, many experimental, synthetic cannabinoids have biased signaling effects at CB receptors that do not precisely reflect signaling effects observed with endocannabinoids. Hence, future research that takes an integrative lipidomic approach to studies of the GI tract in health and disease are much needed to reconcile some of the disparate observations in the literature.

The ECS has been proposed as a good therapeutic target for the treatment of GI inflammatory diseases and conditions that involve a breakdown in intestinal homeostasis. However, because of the ubiquitous distribution of the ECS in the GI tract and its complex physiology, careful consideration needs to be given to the best molecular target. This is currently challenging because the precise sites of endocannabinoid production are not well-understood, and a detailed description of the distribution of the biosynthetic and degradative enzymes for the expanded ECS is also lacking. Future studies addressing these limitations will greatly advance our understanding of the potential of the ECS to be selectively targeted for the treatment of disease as well as shedding new light on the physiology of the ECS in the GI tract.

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Figure 4. Overview of the endocannabinoid system in the gut. Endocannabinoids in the intestine are produced primarily by enteric neurons and by subpopulations of extrinsic neurons. Some epithelial subtypes, including goblet and Paneth cells, may also produce endocannabinoids, based on the presence of synthetic enzymes identified in single cell expression studies. The main endocannabinoids are anandamide (AEA) and 2-arachidonoylglycerol (2-AG). Other N-acylenolamides and acylglycerols are endocannabinoids that act to regulate gut functions. Endocannabinoids act at CB1 and CB2 receptors to regulate various functions in the gut. Various immune cells express CB2 receptors, which function primarily to dampen immune responses. Enteric neurons express CB1 receptors, which modulate enteric neural control of various gastrointestinal functions. Various epithelial subtypes express CB1 receptor under physiological conditions, which acts to suppress secretory responses. CB1 activation also decreases epithelial permeability by altering tight junction protein expression and localization. Endocannabinoids have a reciprocal relationship with the gut microbiota; endocannabinoids can affect the composition of the microbiota, and different commensals (bacteria, fungi) can alter the endocannabinoid system. 2-OG, 2-oleoylglycerol; 2-PG, 2-palmitoylglycerol; LEA, N-linoleoylethanolamide; OEA, N-oleoylethanolamide; PEA, N-palmitoylenthanolamide; SEA, N-stearoylethanolamide. Figure created with BioRender.com.


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