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# Microbial Neuro-Immune Interactions and the Pathophysiology of IBD

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Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/64832>

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## Abstract

Inflammatory bowel disease (IBD), encompassing Crohn's disease (CD) and ulcerative colitis (UC), is a group of debilitating disorders affecting patient's quality of life and with unknown aetiology. The collected evidence indicates that individuals can develop IBD as a result of genetic susceptibility, a dysregulated immune response and the influence of certain environmental factors. Common symptomatology includes abdominal pain, fever and bowel diarrhoea with blood and/or mucus excretion. The location and extent of disease differ between UC and CD, affecting the mucosal layer in the colon in UC patients, whereas in CD patients, a transmural inflammation is found anywhere in the gastrointestinal tract. Factors associated with IBD pathophysiology include alterations in immune responses, characterized by an atypically T helper (Th)-2 profile in UC, and a Th1/Th17 profile in CD, modifications in epithelial barrier function and alterations in the commensal microbiota composition with blooming of specific pathobionts, for example, adherent-invasive *Escherichia coli* (AIEC), and with diet. Recent research has uncovered that inflammation, *per se*, can activate the enteric nervous system inducing neurogenic inflammation and increasing visceral sensitivity, leading to pain. Similarly, alterations in the commensal microbiota composition/ligands have also led to modifications in intestinal nociceptive markers and in visceral pain. In this chapter, we aim to review the mechanisms implicated in microbial neuro-immune axis and its potential contribution to IBD pathophysiology and symptomatology. We focus on the findings identified in animal models and in IBD patients and on the prospective translation of targeting the microbial neuro-immune axis as future therapeutic treatment for intestinal inflammatory conditions.

**Keywords:** IBD, microbiota, intestinal neuro-immune interaction, visceral pain, microbiota-gut-brain axis

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## 1. Introduction IBD

Inflammatory bowel disease (IBD) is a group of diseases comprising mainly two entities, ulcerative colitis (UC) and Crohn's disease (CD), of unknown aetiology. Ulcerative colitis was described in the late nineteenth century by Wilks and Moxon [1] and CD was described by Crohn et al. in the early 1930s as terminal ileitis [2]. Since the beginning of the twenty-first century, the incidence of IBD is increasing worldwide, especially in Westernized areas such as the United States, Europe, Australia and New Zealand as well as in South America, Asia and the Middle East and in specific populations, for example, paediatric-onset IBD. The prevalence in the Western World is currently up to 0.5% of the population [3].

IBD affects the patients' quality of life and is characterized by unpredictable flares of remission and relapses with symptoms of bloody diarrhoea, abdominal pain and rectal bleeding. The onset of IBD is at a young age ranging initially from 20 to 39 years and with a second onset in patients over 60 years of age [4]. IBD affects both males and females, with a higher prevalence of CD in females and no major differences in UC patients [5]. The inflammation in UC is localized to the colonic superficial mucosa while the inflammation in CD is transmural and can be found anywhere along the gastrointestinal (GI) tract, although the inflammation is predominantly located to the ileo-caecal area and the proximal colon [6, 7]. Ulcerative colitis is characterized by the formation of crypt abscesses, formed by extravasation of neutrophils through the intestinal epithelium while CD is characterized by the presence of skip lesions, granulomas, fibrosis and strictures. Extra-intestinal features in CD can result in major complications, for example, fibrotic strictures, and a subsequently need for surgery [8, 9]. To date, there is no cure for IBD, with most treatments primarily aiming to suppress disease severity and to keep the patient in remission by using biologics, anti-suppressants and steroids.

The cause of IBD is unknown but the collected evidence suggest that IBD can be manifested in genetically susceptible individuals who mount inappropriate local immune responses against microbial antigens after exposure to environmental factors [7, 10].

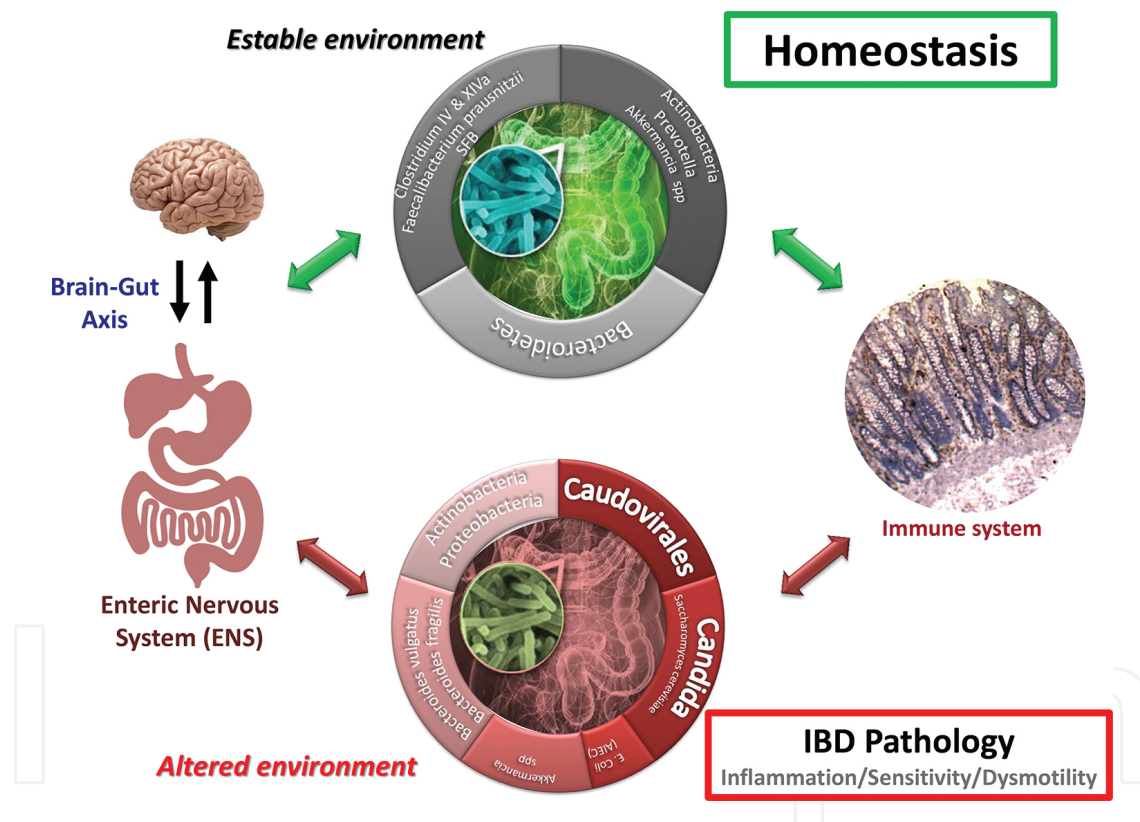
To date, genomewide association studies (GWAS) have identified at least 163 susceptible genes for IBD, with loci associated to bacterial recognition (NOD2) and autophagy (ATG16L1, IRGM) conferring a higher risk for CD. In contrast, genes involved in mucosal barrier function (e.g. HNF4a, CDH1, LAMB1, ECM1), IL-10 signalling and HLA haplotype DRB1\*0103 have been associated with UC. Interestingly, genes linked with adaptive immune responses such as IL-23R, IL-12B and STAT3 confer a higher risk for both CD and UC. Despite the large number of loci identified, only approximately 20–25% of patients are linked to at least one of these loci suggesting that there are most likely other factors potentiating its development [11–13].

Alterations in barrier function, dysregulation in tight junction proteins and increased bacterial uptake has been reported in experimental models of colitis and in patients with UC and CD supporting the GWAS identified genes on barrier function [14, 15]. Others have also suggested that Peyer's patches are the sites of initial lesions in CD with M cells playing an important role in sampling microbes from the gut lumen and presenting to immune cells to mount inflam-

matory responses [16, 17]. Furthermore, unaffected relatives of CD patients have shown increased intestinal permeability [14, 15].

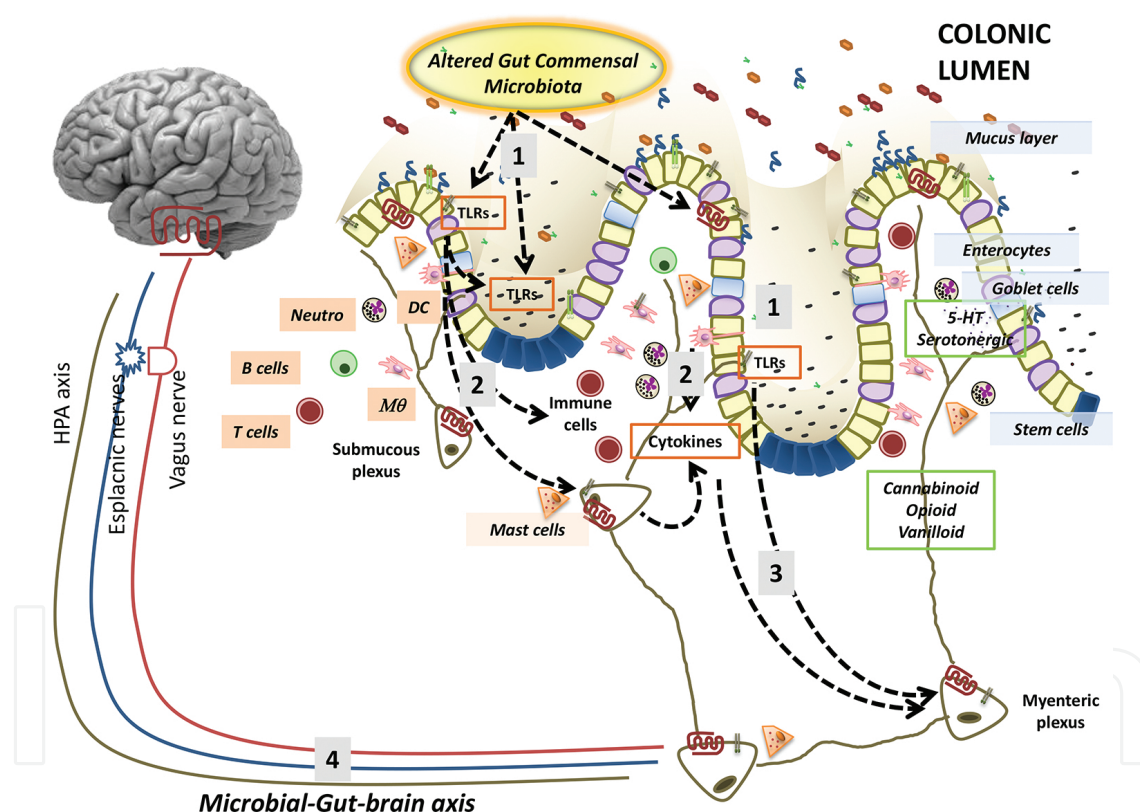
## 2. Intestinal immune mechanisms and IBD

The main function of the intestinal immune system is to protect the host from harmful signals, for example, pathogens, by mounting specific responses as well as to keep a tolerance against A myriad of food and microbial antigens. A robust immune response against invading pathogens is critical for their clearance but an excessive or uncontrolled inflammation can result in chronic inflammation and lead to the development of inflammatory conditions such as IBD (**Figure 1**). The collected evidence to date suggest that the aberrant immune response in IBD patients is attributed to the dysregulated adaptive and innate



**Figure 1.** The gastrointestinal (GI) tract harbours up to  $10^{14}$  bacteria, 10 times more than the number of cells of the human body. These bacteria include up to 1000 bacterial strains but are covered in few phyla. The most important ones in mammals are the Firmicutes (including *Clostridium* and *Lactobacilli*) and Bacteroidetes. Traditionally, it has been described that GI function is controlled by the intestinal immune system. Recent research has also highlighted that the enteric nervous system (ENS) and the gut commensal microbiota system play a crucial and an active role in influencing gut homeostasis. The ENS, mainly represented by the myenteric and the submucous plexus, Also known as the second brain due to it can work alone. The gut is connected to the CNS by the brain-gut axis, which maintains a bidirectional communication. When these three systems are balanced, there is a physiological homeostasis. An imbalance in any and/or all of these three systems can lead to the development of functional GI disorders and chronic inflammatory GI disorders such as IBD.

immune responses [7, 10]. The innate immune response is the first line of defence against harmful agents. Pattern recognition receptors (PRRs) detect microbial 'pathogen-associated molecular patterns' (PAMPs) or host-derived 'damage-associated molecular patterns' (DAMPs) inducing innate immune responses. Among PRRs, the intestinal Toll-like receptors (TLRs) are critical both in keeping intestinal homeostasis and in mounting innate immune responses. In humans, a total of 10 TLRs have been described, with the majority of them, except TLR3, signalling via the adaptor protein MyD88. Activation of TLRs via MyD88 induces several pathways including the transcription factor nuclear factor-kappa light-chain enhancer of activated B cells (NF- $\kappa$ B), mitogen-activated protein kinase (MAPK) and AP1, while the MyD88-independent pathway activates the interferon regulatory factor 3/7 (IRF-3/-7) signalling pathway [18]. In recent years, it has become evident that bacteria can penetrate/translocate through the intestinal barrier of IBD patients thereby inducing TLR-induced responses both by mucosal non-immune cells (e.g. epithelial cells) and innate immune cells (e.g. macrophages, dendritic cells). TLRs are expressed by



**Figure 2.** Representative schema of some of the putative mechanisms involved in IBD pathophysiology associated with microbial neuro-immune changes. The intestinal microbiota and microbial-derived products interact with the host bacterial recognition systems (such as TLRs) (1) generating a signalling cascade (2) that will lead to a local immune activation including mast cells, macrophages, T cells, neutrophils, dendritic cells and neuroendocrine systems (such as enterochromaffin cells) that seems to persist even when the overt inflammation is resolved. This persistent activation has the potential to influence sensory neural mechanisms within the gut depending upon the ENS (3) and the extrinsic innervation. In addition, the bidirectional communication between the gut and the CNS (4) is also altered that can offer an explanation for the altered perception of sensory signals and therefore altered manifestation of pain in patients suffering from IBD. Neutro—neutrophils; M $\phi$ —macrophages; DC—dendritic cells; TLRs—Toll-like receptors.



both epithelial and immune cells, and alterations in TLRs expression have been reported in both UC and CD tissue including increase expression of TLR4, TLR2 and TLR5 [18]. Activation of TLRs, for example, TLR4, leads to the activation of NF- $\kappa$ B pathway, which is responsible for the transcription of various pro-inflammatory cytokines and chemokines associated with IBD pathology (**Figure 2**). Other PRRs involved in CD pathology include NOD2, which is a cytosolic receptor belonging to the nucleotide-binding domain and leucine-rich repeat containing family (NLRs). NOD2 recognizes muramyl dipeptide (MDP), present in both Gram-positive and Gram-negative bacteria and activates the NF- $\kappa$ B pathway. NOD2 has also been identified as a susceptible gene for CD with 3 SNPs linked to ileal CD, suggesting that a defect in recognition and clearance of bacteria might be associated with CD development. However, the specific inflammatory mechanisms associated with NOD2 mutations are still largely unknown [18].

The intestine of IBD patients presents a chronic inflammation that differs in terms of immune cell subsets and cytokine profile. The colons of UC patients are heavily infiltrated with neutrophils, T and B cells with high levels of several pro-inflammatory cytokines including IL-1 $\beta$ , IL-6 and TNF- $\alpha$  and an atypical T helper (Th2) profile (IL-5, IL-10 and IL-13) [7, 10, 19]. Other chemokines such as IL-8 and GRO- $\alpha$  are highly increased in UC mucosa, with IL-8 levels correlating with the degree of inflammation and disease activity [20, 21]. Although neutrophils are indispensable for eliminating pathogens, their excessive presence in the tissue and their resistance to apoptosis [22] can lead to extensive tissue damage in UC, which can be caused by the persistent release of cytokines (IL-17, IL-6), reactive oxygen species (ROS) and proteases, all of which highly associated with patients with active UC [7, 19, 23]. The intestinal wall of CD patients is highly infiltrated by macrophages and T cells. It is acknowledged that CD is primarily mediated by Th17/Th1 cells as well high levels of innate pro-inflammatory cytokines including IL-1 $\beta$ , IL-6 and TNF- $\alpha$  [6, 10]. Further, the elevated levels of circulating and tissue B cells as well as their activity have also been reported in IBD patients [24, 25].

### 3. Intestinal neural pathways and visceral pain in IBD

A particular characteristic of the GI tract is the presence of an intrinsic nervous system, the enteric nervous system (ENS) also known as the second brain. Within the intestine, the ENS presents a clear distribution in two neuronal plexuses localized within the submucosa (submucosal or Meissner's plexus) and between the circular and longitudinal smooth muscle layers (myenteric or Auerbach's plexus). The ENS can maintain GI functions alone by the network around the gut wall formed by both plexi. It is composed by around  $10^8$  neurons consisting of intrinsic primary afferents, interneurons and motor neurons [26–28]. Enteric neurons are supported by glial cells (counterparts of the central nervous system (CNS) astrocytes), which can communicate with the mucosal immune system and the intestinal epithelium by producing different mediators including cytokines. The ENS controls intestinal motility, secretion and absorption, mucosal growth, local blood flow, the immune and barrier function and also carries nociceptive (painful) stimuli to the CNS [29–31] (**Figure 2**).

The gut receives also extrinsic innervation from the autonomic nervous system (ANS) and the spinal afferent nerve fibres that coordinate its activity. The ANS is composed of the sympathetic nervous system (SNS) and the parasympathetic nervous system (PSNS). The extrinsic innervation consists of vagal and spinal sensory nerves, vagal and sacral parasympathetic motor neurons, and sympathetic neurons from prevertebral ganglia, and it plays a key role in maintaining the bidirectional communication with the CNS as well as it is the anatomical basis of the gut–brain–gut axis [32–34].

The vagus nerve (cranial nerve X interfacing with the PSNS) has a motor and a sensorial division and three different endings in the gut: the intraganglionic laminar endings within the myenteric plexus, the intramuscular arrays within the smooth muscle layers and the mucosal fibres within the mucosa [32]. The SNS suppresses GI functions under vagus nerve's activation, cell bodies arise from the paravertebral sympathetic chain ganglia, adjacent to the spinal column and innervating the GI vasculature, as well as the prevertebral (celiac and superior/inferior mesenteric) ganglia, which controls motility and secretomotor neurons. Axons extend to the gut by the mesenteric nerves but also by the vagus nerve, cranially, which also contacts with the ENS [32, 35]. The spinal innervation of the gut comes directly from the dorsal root ganglia (DRG) of the spinal cord, and it is less extensive when compared to the ANS. They extend to the gut by the splanchnic (cranially) and the parasympathetic pelvic nerves (distally). The colon, which harbours large amounts of bacteria, has specific DRG in their innervation [32].

#### 4. Visceral hypersensitivity

Nociception is the neural processes of encoding and processing noxious stimuli that can be accompanied, or not, with pain [26, 36]. Visceral pain originates from the internal organs and is initiated by nociceptors, which can detect mechanical, thermal or chemical changes above a basal threshold [37]. Perception of visceral pain relies mainly in spinal C and A $\delta$  afferents fibres from DRG although vagal afferent stimulation can also mediate pain [38]. The strong compression, as well as chemical stimuli or irritation, of the colon generates afferent signals that can hypersensitize afferent nerves and become nociceptive [39–41].

Although most of the intestinal functions can be carried out by the ENS, extrinsic innervation is necessary to maintain a coordinated activity with the rest of the body and for sensory functions related to visceral pain perception within the gut. This is particularly important because visceral pain and/or altered visceral sensitivity (hypersensitivity) are frequent symptoms in several GI diseases including irritable bowel syndrome (IBS) and IBD. Visceral hypersensitivity generally originates from a local inflammation leading to an enhanced response to a painful stimulus (hyperalgesia) as a result of activation of the immune system, stressful conditions and the intestinal microbiota [42–44] (**Figures 1 and 2**). Alterations in sympathetic neural activity have specifically been implicated in IBD [25, 45]. A decrease in noradrenaline release from sympathetic varicosities in inflamed and un-inflamed regions of the GI tract has consistently been reported in animal models of colitis,

which appears to be due to the inhibition of N-type voltage-gated  $\text{Ca}^{2+}$  current in postganglionic sympathetic neurons [46]. However, specific alterations of sympathetic function and its role in IBD remain unclear [25]. In the last two decades, numerous morphological, pharmacological and molecular studies have characterized sensory-related systems within the gut, among them the serotonergic system, the endocannabinoid system, endogenous opiates and the vanilloid system have received particular attention due to their potential benefit as pharmacological targets for the treatment of visceral pain. A short description of each of these systems is outlined below.

#### 4.1. The intestinal serotonergic system

The serotonergic system involves the neurotransmitter serotonin (5-hydroxytryptamine; 5-HT), which is mainly stored in mucosal enterochromaffin (EC) cells and in a lesser extent within the enteric neurons (up to 95% of body 5-HT is present in the gut). Tryptophan hydroxylase (TPH) is the limiting enzyme mediating 5-HT synthesis. Two TPH isoforms exist, namely TPH1, mainly expressed in EC cells, and TPH2, expressed in central and enteric neurons. TPH expression/activity is regarded as a reliable indicator of 5-HT availability, whereby high expression levels are indicative of a high rate of serotonin production and release [47–49]. Within the GI tract, 5-HT participates in motor, sensory and secretory functions modifying gut motility/sensation in several ways [50]. For example, 5-HT present within the enteric nerves and acting on 5-HT<sub>3</sub> receptors of the vagal afferent nerve fibres can stimulate intestinal secretion and motor reflexes. 5-HT can also act on the receptors 5-HT<sub>3</sub>, 5-HT<sub>4</sub> and 5-HT<sub>1P</sub> present on enteric neurons, thereby contributing to peristalsis and stimulating intestinal transit [51]. Expression of 5-HT<sub>7</sub> receptor has been found on intestinal immune cells and demonstrated a key role in development of experimental colitis [52]. Intestinal inflammation is accompanied by alterations in enteroendocrine cells, among which EC is the most abundant. These cells are distributed throughout the GI tract, with many of them concentrated in the small intestine and rectum and in between epithelial cells, where they act as sensors of the intraluminal milieu. 5-HT release from EC cells is mediated by luminal or neuronal stimuli including mucosal stroking and endogenous chemical stimuli such as adenosine. Changes in the content, release and reuptake of 5-HT as well as increase numbers of EC cells have been reported in both inflamed and non-inflamed gut of IBD patients and in experimental models of IBD [53, 54]. Some studies have also shown that changes in the microbial composition or stressful conditions can induce 5-HT release from EC cells, leading to the initiation of intestinal inflammation and the generation of abnormal sensory-related responses (i.e. altered viscerosensitivity) [48, 55–57]. The sodium-dependent serotonin transporter (SERT), a member of the  $\text{Na}^+/\text{Cl}^-$  neurotransmitter transporter family, is expressed by epithelial cells and neurons in the gut [47] and is involved in the reuptake of 5-HT. SERT expression is reduced in the inflamed and in the healing colonic mucosa of UC patients, thereby increasing 5-HT levels [25, 58]. Furthermore, deletion of SERT increases the severity of 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis in mice [59] and mice treated with the SERT inhibitor paroxetine presented alterations in GI motility and sensitivity [60]. Interestingly, the regulatory cytokine transforming growth factor-beta 1 ( $\text{TGF-}\beta 1$ ) was recently shown to stimulate SERT function suggesting a novel neuro-immune therapeutic strategy to treat GI disorders [61]. These



findings implicate that 5-HT signalling and its SERT-mediated termination can contribute to the symptoms associated with IBD pathophysiology and suggest that drugs targeting this pathway may benefit patients suffering from IBD and other inflammation-related gut disorders [25, 62].

#### 4.2. The intestinal opioid system

The endogenous opioid system is composed by three G protein-coupled receptors:  $\mu$ ,  $\delta$  and  $\kappa$  opioid receptors. Within the GI tract, intestinal opioids, ligands and receptors are found in myenteric and submucosal neurons and in epithelial, endocrine and immune cells (including myeloid and CD4<sup>+</sup> T and CD8<sup>+</sup> T cells). Opioids have a well-characterized analgesic activity in visceral sensitivity [63, 64], which is mainly linked to the activation of  $\mu$  and, to a lesser extent,  $\kappa$  receptors. The expression of  $\delta$  opioid receptor together with  $\mu$  receptor is increased after administration of the inflammatory irritant mustard oil, thereby evoking allodynia and visceral hyperalgesia [25, 65].  $\mu$ -Opioid receptors (MOR) are overexpressed in active IBD mucosa, most likely as a compensatory analgesic mechanism generated in states of potentially increased sensitivity. MOR are also significantly enhanced by pro-inflammatory cytokines and repressed by NF- $\kappa$ B inhibitors in myeloid and lymphocytic cell lines [66]. Increased numbers of  $\beta$ -endorphin immunoreactive CD4<sup>+</sup> T cells and CD11b<sup>+</sup> macrophages are found in murine colonic lamina propria in chronic dextran sodium sulphate (DSS)-induced colitis, where the release of endogenous opioids decreases nociceptive signalling through the activation of  $\mu$ -opioid receptors [67]. Therefore, it is speculated that the anti-nociceptive actions of peripheral opioids in colitis may indirectly result from a reduction of the neurogenic 'pro-nociceptive' components of inflammation, by decreasing CGRP and Substance P (SP) release that could counteract the pro-nociceptive effects of inflammatory mediators such as TNF- $\alpha$  during inflammation [68]. Recent studies have suggested that probiotics and microbial-related products can also module the intestinal expression of MOR [66, 69–72].

#### 4.3. The intestinal endocannabinoid system

The endocannabinoid (CB) system comprises of two main receptors, CB1 and CB2, their endogenous ligands and their metabolizing enzymes, Mainly the fatty acid amide hydrolase, FAAH. Because of their chemical characteristics, endocannabinoid ligands are difficult to determine; therefore, the expression of CB1, CB2 and the enzyme FAAH, are used to assess endocannabinoid functionality. Within the GI tract, the endocannabinoid system controls intestinal motility, nociception and intestinal inflammation. CB1 and CB2 receptors are expressed on intestinal ganglionic neural cells within the ENS, in epithelial cells and immune cells in the gut [73–76]. The CB1 receptor is predominantly found in neural and epithelial cells, whereas the CB2 receptors are predominantly expressed in immune cells [77]. Upon activation, both receptors mediate analgesic effects and appear to have anti-inflammatory properties [75, 77–80]. Probiotics, bacterial products and stressful stimuli have been postulated to influence the endocannabinoid system [70, 81–83]. In IBD, an increased in CB1 expression has been identified in inflamed mucosa, while a reduction in the endocannabinoid agonist anandamide and no increase in CB2 expression were found.

Ex vivo cultures of IBD biopsies and immune cells with the non-hydrolysable AEA analogue methanandamide (MAEA) resulted in a reduction in IFN- $\gamma$  and TNF- $\alpha$  secretion [84]. In animal models of colitis, the CB2 agonist JWH-133 attenuates colitis in IL-10 $^{-/-}$  mice and in DSS-induced colitis by decreasing the number of mucosal immune cells (including CD4 $^{+}$  T cells, neutrophils, Mast cells and natural killer cells) [85]. Recent studies in humans and animals have identified a new strategy for the endocannabinoid system, whereby targeting of the enzyme FAAH can prove to be a better approach due to the potentially less side effects when compared to the currently available CB compounds [86–88]. Overall, the preclinical findings indicate that manipulating the endocannabinoid system can have beneficial effects in IBD patients, and therefore, the use of *Cannabis sativa* has also been studied, although further research is necessary in this context [89, 90].

#### 4.4. The intestinal vanilloid system

The vanilloid system consists of one of six subfamilies of the transient receptor potential (TRP) channel family, with six types of transient receptor potential vanilloid (TRPV1-6) [91]. These receptors are calcium permeable, non-selective cation channels involved in thermo- and chemo-sensitive transduction [92]. In the intestine, TRPV1, 3 and 4 have been linked to viscerosensitivity and are characterized as pro-algesic receptors [79, 92–94]. TRPV are mainly expressed in intestinal afferent nerves, although they can also be found in EC cells as well as epithelial and immune cells [95–97]. In agreement with their pro-algesic effects, TRPV are upregulated in states of intestinal inflammation and visceral hypersensitivity; for example, TRPV1 is highly increased in immunoreactive nerves in IBD tissue and in quiescent IBD with IBS-like symptoms such as pain [98–102]. TRPV1 deletion prevented the development of post-inflammatory visceral hypersensitivity and pain-associated behaviours, while SP can sensitize TRPV1 function leading to a pro-algesic state [101, 103]. TRPV1 has been linked to the crosstalk between the microbiota and the neuro-immune response in the gut, because TRPV1 and CGRP can modulate cytokine response to lipopolysaccharide (LPS) independently of the adaptive immune response. It has been proposed that TLR4 can activate TRPV1 via intracellular signalling thereby inducing the subsequent release of anti-inflammatory CGRP to maintain mucosal homeostasis [104]. In addition, blocking of TRPV4 has also been shown to alleviate colitis and pain associated with the intestinal inflammation induced by TNBS in mice [105]. Similarly, intrathecal injection of antisense oligonucleotides to TRPA1, another member of the transient receptor potential channel family, decrease its expression and attenuates visceral hyperalgesia in TNBS-induced colitis [25, 65].

#### 4.5. Neurotransmitters, neuropeptides and neurotrophins

More than two dozens of putative neurotransmitters have been described to date, with neurons usually expressing a combination thereof. Most of these mediators have been implicated in the neuro-immune communication associated with gut homeostasis and in the pathophysiology of intestinal inflammation but their specific functions are still to be established [106]. A short description of the most relevant mediators is outlined under this section.

Substance P (SP), an 11-amino acid peptide secreted by nerves and immune cells (including monocytes, macrophages, eosinophils and lymphocytes) belongs to the tachykinins family and acts by binding to the neurokinin-1 (NK-1) receptor. It functions in smooth muscle contraction, vasodilation and epithelial ion transport. It is a mediator of neurogenic inflammation due to stimulation of cytokine release from immune cells (e.g. macrophages, mast cells) and endothelium causing tissue damage and neurodegeneration [25, 107]. High expression of SP and NK-1 receptor was reported in the myenteric plexus and inflamed mucosa of patients with IBD. This is associated with a shift from mainly cholinergic innervation to a more extensive SP innervation, which correlates with the severity of UC and may be part of the neuronal basis for the observed altered motility disturbance seen in these patients [106, 108–110]. Antagonists of NK-1 receptors have been shown to ameliorate inflammation and protect from T-cell-induced colitis. Based on these findings, tachykinin antagonists have been proposed as potential anti-inflammatory treatment for IBD [25, 108, 111, 112].

Vasoactive intestinal polypeptide (VIP), a 28-amino acid peptide belonging to the pituitary adenylate cyclase-activating polypeptide (PACAP)/glucagon superfamily, is highly expressed in the myenteric plexus of the colon. VIP inhibits the peristaltic reflex in the circular muscle layer, controls intestinal blood flow and modulates the immune system by binding to both G protein-coupled VIP receptors 1 and 2. VIP is released from nerve terminals that contain nitric oxide synthase (NOS). These two peptides are thought to be the primary intestinal components of non-adrenergic, non-cholinergic nerve transmission. VIP expression is increased in colonic neurons of CD patients but not in UC patients [25, 113–115]. Treatment with VIP in murine TNBS-induced colitis reduces colitis severity and Th1-cell response [116, 117]. In addition, glucagon-like peptide 2 (GLP-2), a regulator of absorption with anti-inflammatory properties, decreases mucosal inflammation in TNBS-induced colitis in rats by activating VIP neurons of the submucosal plexus [118]. Neurotrophins are a family of proteins regulating neuronal activity in the CNS and PNS, belonging to a class of growth factors and playing a major role in visceral hypersensitivity in the inflamed gut. This is, partly linked to the effects of peripheral neurotrophic factors (NTFs) on local afferent neurons. Among these, nerve growth factor (NGF) is primarily involved in the regulation of growth, maintenance, proliferation and survival of certain target neurons and in innate and adaptive immune responses; brain-derived neurotrophic factor (BDNF) links the commensal microbiota and the CNS [119, 120]; and the family of glial cell line-derived NTFs (including GDNF, artemin and neurturin) are implicated in sensorial alterations observed in inflammatory and functional GI disorders [112].

## 5. Intestinal neuro-immune interactions

Intestinal inflammation, even if mild, causes significant alterations in neurally controlled gut functions including pain and altered motility. These symptoms are caused, in part, by persistent hyperexcitability of enteric neurons that can occur even after the resolution of colitis. Among cells generating inflammatory signals within the gut mucosa and affecting neural signalling in the ENS, mast cells and enterochromaffin cells seem to play a big role. Both of

them are increased in the colonic mucosa of IBD patients [25]. The ENS and the mucosal immune system have the ability to regulate each other functions. In the intestinal wall, nerve cells are localized in close proximity to immune cells and they share several chemical mediators. The collected evidence point towards a major role of inflammatory signals affecting the enteric neurons and most likely generating IBD-associated symptoms [25, 121, 122] (**Figure 2**).

Inflammation-related alterations in the ENS are divided into those that alter the structural morphology of neurons and glial cells of the ENS and those that modify enteric neurotransmitters [25, 122–124]. During intestinal inflammation, morphological and functional alterations, including remodelling of visceral afferents, are also observed outside the primary region affected by the insult [112]. ENS structural changes are more marked in CD than in UC patients and are often associated with the extent of inflammatory infiltrate. In fact, it has been suggested that severe and extensive necrosis of gut axons may be a distinct feature in CD [25]. In support of this notion is the ablation of myenteric neurons, accompanied by a high neutrophil infiltration and an excessive production of the Th1 cytokines IFN- $\gamma$ , TNF- $\alpha$  and IL-12 present in models of colitis. Interestingly, neuronal loss persisted for up to 56 days, that is when the inflammation had resolved in these models [125–128]. Others proposed mechanisms implicated in neuron loss, arisen from animal models and IBD patients, Which involve an increase in immune cell infiltrates, including eosinophils, lymphocytes, plasma cells and mast cells, in myenteric ganglia [25, 123, 126, 128, 129] as well as the activation of apoptotic pathways [130]. Furthermore, enteric glial cell ablation induces a significant decrease in the number of myenteric neurons, which appear to be associated with the loss of NOS-containing neurons in the myenteric plexus, likely underlying the alterations observed in smooth muscle relaxation and intestinal transit time [25, 131]. It is also believed that the reduced availability of neuroprotective factors due to neuronal cell loss may increase the susceptibility of enteric neurons to insults such as oxidative stress, which can have an important role in IBD pathophysiology. Overall, the collected data indicate that the loss of nerve cells is dependent on the time needed to develop inflammation, the type of inflammatory cells and the mediators profile required for nerve-immune interactions [107].

Immune cells found in the intestine, including dendritic and mast cells, lymphocytes and macrophages, express receptors for small molecule neurotransmitters and neuropeptides and produce cytokines targeting the enteric neurons [106]. Neuro-immune regulation includes degranulation of mast cells and influx of neutrophils due to neuronal activation. Neuropeptides released by enteric nerves including SP and VP can stimulate lymphocytes to induce their differentiation and alter immunoglobulin production. Signalling between immune cells and enteric neurons can also evoke alterations in gut function. Hyperexcitability of intrinsic primary afferent neurons may be secondary to activation of cyclooxygenase (COX)-2 and production of prostaglandins (PGE<sub>2</sub>) from inflamed colon [25, 132]. Intestinal kinases have also been involved in intestinal inflammation. Protein kinase A activity in nerve terminals increases in previously inflamed colon and facilitates a fast synaptic transmission and the release-ready pool of synaptic vesicles [25, 133, 134]. There is also evidences that pro-inflammatory cytokines such as IL-1 $\beta$  and TNF- $\alpha$  exhibit pro-secretory effects in the human distal colon. Both IL-1 $\beta$



and IL-6 are reported to increase excitability in submucous and myenteric neurons and to mediate effects on cholinergic and non-cholinergic transmission [135–137].

Mast cells are a major player in the innate immune response. Apart from their prominent role in immunoglobulin E (IgE)-dependent hypersensitivity, mast cells can release and modulate the release of several mediators including cytokines, growth factors, chemokines as well as histamine, proteases, and probably serotonin 2 receptors that regulate multiple important biological processes including neural actions in the human ENS [137]. Neuropeptides released from enteric and visceral afferent nerves regulate human intestinal mast cell mediator's release. In healthy individuals, mast cells are generally located in the lamina propria, in fewer amounts in the submucosa and sporadically found in the muscle layers or in the serosa. An estimated 70% of intestinal mucosal mast cells are in direct contact with nerves, and another 20% are within a 2- $\mu$ m distance. Mast cells respond to neurotransmitters and nerves and can thereby regulate their activation threshold [137, 138], submucous neurons would respond with a transient excitation mediated primarily by 5-HT<sub>3</sub> receptors [139]. Cytokines and chemokines can have different effect on mast cell functions. For example, the chemokine, macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ) is required for optimal mast cell degranulation in mice [140]. In contrast, the regulatory cytokine TGF- $\beta$ 1 can dose dependently inhibits stem cell factor-dependent growth of human intestinal mast cells by both enhancing apoptosis and decreasing proliferation [141] as well as it can influence mediator secretion by reducing histamine, cysteinyl leukotrienes and TNF- $\alpha$  release while prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) generation and COX1 and 2 expressions are upregulated. Mucosal mast cells can also respond to other mediators including adenosine triphosphate (ATP), somatostatin, calcitonin gene-related peptide (CGRP) and SP. Colorectal biopsies from patients with active CD or UC incubated with SP induce mast cell degranulation and histamine release [30, 142].

Histamine, proteases and TNF- $\alpha$  are stored as granules in mast cells and can be released within seconds. Other mediators such as lipid mediators and most cytokines are synthesized once the mast cells are activated. The most important mast cell mediator identified so far is histamine. Histamine influences fluid and ion transport, which is partly nerve mediated and directly excites submucous extrinsic sensory neurons [137, 142, 143]. There are four histamine receptors (H<sub>1</sub>, H<sub>2</sub>, H<sub>3</sub> and H<sub>4</sub>), which are found as receptor clusters on submucous neurons, with the most frequent clusters being H<sub>1</sub>/H<sub>3</sub> (29%), H<sub>2</sub> (27%) and H<sub>1</sub>/H<sub>2</sub>/H<sub>3</sub> (20%), respectively. The implication of histamine on sensory neurons comes from studies in rodents [142]. Rat dorsal root ganglion cells with projections to the viscera increased Ca<sup>2+</sup> responses to a TRPV4 agonist and enhanced TRPV4 expression, when adding histamine or serotonin [144]. The pathophysiological relevance of histamine in both allergic and non-allergic conditions including IBD and IBS is established [141, 145]. In IBD, it has been reported that histamine secretion is increased in the jejunum of active CD and in urine of UC patients [146], although in a recent study, no differences in serum levels of histamine were identified [147].

Proteases, in particular the serine protease tryptase, are prominent mediators released from mast cells. Tryptase is present in almost all human mast cells, comprising up to 25% of their total proteins [148]. Proteases signal to nerves is mediated through protease-activated recep-



tors (PARs), with four cloned PAR receptors identified in humans. PAR1, PAR3 and PAR4 are predominantly activated by thrombin, and PAR2 is activated by trypsin and mast cell tryptase [137]. In patients with UC, tryptase induces the release of inflammatory cytokines and chemokines, some of which may exert their effects through nerve pathways as outlined above [149]. Supernatants from stool of IBS and UC patients contain increased protease levels and when supernatants from UC patients were injected to mice, it promoted hypoalgesia, which was dependent on cathepsin-G-PAR4 activation [150]. PAR2 activation in mice increases intestinal permeability, which is mediated by SP and capsaicin-sensitive spinal afferent nerves while in rats PAR2 evoked visceral hypersensitivity [151]. Interestingly, PAR positive cells were increased in mucosa of UC patients and preferentially co-localized with tryptase<sup>+</sup> cells suggesting that mast cells activation via PAR2 might be involved in the pathogenesis of UC [149]. Similarly, mucosal biopsy supernatants from UC patients can activate mouse DRG neurons innervating the colon, via TNF- $\alpha$  regulation [137, 152].

## 6. Microbial alterations in IBD

The microbial community of the GI tract is composed by bacteria, virus, fungi, protozoa and yeasts. Gut colonization starts at birth and, when completed, it harbours about 100 trillion microbial commensals and symbionts belonging approximately to 5000 distinct species divided in the phyla Firmicutes, Bacteroidetes, Proteobacteria, Verrucomicrobia, Actinobacteria, Fusobacteria and Cyanobacteria [153–155]. The intestinal microbiota is not homogeneously distributed along the GI tract. For example, *Proteobacteria* spp. (mainly Enterobacteria) and Lactobacillales preferentially populate the small intestine while Bacteroidetes and Clostridia populate the large intestine. The density of bacterial cells in the gut increases caudally with the maximal counts ( $10^{11}$ – $10^{12}$  cells/g of content in both human and rodents) localized in the ceco-colonic region [156–159]. Intestinal bacteria can be transient i.e. bacteria introduced during adult life; they do not permanently colonize the gut and can have positive (probiotics) or negative (pathogens) effects on the host, or be innocuous, or permanent. The latter ones are long-term colonists of the gut, the true commensals, and they can have immunostimulatory effects (so called autochthonous), or they can confer detrimental effects under certain specific conditions (so called pathobionts) [160].

Overall, the commensal microbiota serves the host with protection against pathogens, metabolizing complex lipids and polysaccharides and neutralizing drugs and carcinogens; but it can also modulate intestinal motility, influence the maturation of the intestinal immune system and modulate visceral perception [33, 161]. Changes in the normal composition of the microbiota, termed generally in the literature as dysbiosis, have been associated with chronic inflammatory and functional GI disorders such as IBD and IBS [154, 162] (**Figure 1**). Dysbiosis can occur in parallel to intestinal pathogenesis and can be either a consequence or a cause of the disease [163]. In fact, the causal effects of the microbiota in IBD are still a matter of discussion, with some authors considering that dysbiotic state a consequence and/or a perpetuating factor, rather than a cause of the disease [164, 165].

Many pathogenic organisms have been investigated as causing agents of IBD, including *Mycobacterium avium* subsp paratuberculosis *Helicobacter* spp, non-jejuni/*coli campylobacter* and *Escherichia coli* as well as viruses including Epstein–Barr virus, cytomegalovirus, paramyxoviruses and others [166, 167]. However, to date any pathogenic organism has proven to be a causative agent or even correlate to IBD severity. Recently, the focus has shifted with the conception that the gut commensal microbiota as a whole and/or in relationship to the host can influence disease outcome. This shift has arisen from reports showing that distal ileum and colon (containing the highest microbiota loads) are most susceptible to inflammation and that germ-free animals do not develop inflammation. Similarly, antibiotics and certain probiotics have shown therapeutic efficacy in certain IBD cohorts. An altered bacterial composition (dysbiosis) is associated with IBD patients, characterized by a reduction in bacterial diversity, especially the alpha diversity, which denotes the numbers of bacterial species and their abundance [168, 169].

Pathobionts have been identified and linked to intestinal pathology. For example, *Bacteroides vulgatus* can induce colitis in HLA/B27- $\beta$ 2m rats, but not in IL-10<sup>-/-</sup> mice and it can even prevent colitis in IL-2<sup>-/-</sup> mice [170, 171]. In stool samples and mucosal specimens from IBD patients, an increased abundance of Enterobacteriaceae (belonging to Proteobacteria), especially *E. Coli*, is repeatedly observed. Among this, the adherent-invasive *E. coli* (AIEC), which selectively colonizes the ileum of up to 40% of CD patients, has been suggested to be a strain-specific microbial factor in the pathogenesis of CD (**Figure 1**). The definition of AIEC was based on the ability of the AIEC-LF82 strain to adhere and invade epithelial cells and to persist within macrophages without induction of cell death and by inducing the secretion of pro-inflammatory cytokines such as TNF- $\alpha$  [169, 172–175].

In terms of commensals, a reduction in Firmicutes and a spatial reorganization of the Bacteroidetes has been described in patients with IBD [176–178]. For example, *Bacteroides fragilis* is responsible for a greater proportion of the bacterial mass in these patients. Some specific strains of Bacteroidetes and their polysaccharide A have been linked to harbour immunomodulatory potential, as shown by their protective effect on intestinal inflammation by suppressing IL-17 production and enhancing the production of IL-10 by intestinal CD4<sup>+</sup>Foxp3<sup>+</sup> T regulatory cells [156, 179–181]. A higher abundance of *Actinobacteria* and a loss of *Prevotella* spp are identified in CD patients. A loss of the commensal *Faecalibacterium prausnitzii* (belonging to Clostridia) abundance has been described in IBD [177, 182]. *F. prausnitzii* was shown to have beneficial immune-regulatory effects on the host, with the A2-165 strain ameliorating inflammation in experimental models. *F. prausnitzii* has also been linked with a new subset of CD4<sup>+</sup>CD8 $\alpha$ <sup>+</sup> T cells with regulatory/suppressive functions, a cell type that is less abundant in IBD patients. In addition to the anti-inflammatory properties, *F. prausnitzii* is an important supplier of butyrate to the colonic epithelium and it is found adherent to the gut mucosa where oxygen diffuses from epithelial cells thereby improving barrier function [183]. The loss of *F. prausnitzii* is speculated to be an indicator for increased IBD risk [184–187]. *Clostridium* spp. constitutes one of the largest families of the commensal microbiota and, probably due to *C. difficile* infections, it has traditionally been regarded as a pathogenic bacteria. However, recent data suggest that

some members of the Clostridia group, , *Clostridium IV* and *Clostridium XIVa*, might have an anti-inflammatory potential in immune responses [180, 188] (**Figure 1**). Moreover, the Clostridia-related group of segmented filamentous bacteria (SFB) has been associated with both intestinal inflammation and immune regulation [180], but their role in human IBD pathogenesis is uncovered. Other commensal strains such as Lactobacilli and Bifidobacteria strains are typically considered to confer health benefits to the host and are frequently used as probiotics [189]. Interestingly, *L. acidophilus* seems to modulate sensory mechanisms leading to visceral analgesia [70] while Bifidobacteria can act as immunostimulants [190]. Probiotic treatment in IBD patients has, to date, not being as successful as in, for example, patients with pouchitis when compared to current treatments in UC patients. In CD patients, probiotic treatment appears to be even less beneficial [191–193]. Verrucomicrobia are a mucus-degrading group of bacteria that seems to affect intestinal barrier function through the degradation of the epithelial mucus layer [194] and some Verrucomicrobia spp such as *Akkermansia muciniphila* alleviate experimental colitis and can also mediate intestinal immune tolerance [195, 196]. A reduction in *Akkermansia* spp has been identified in IBD patients [197] (**Figure 1**).

Recent research has identified diet as a major factor influencing commensal microbiota composition. Dietary fibres are often associated with reducing the risk of IBD as well as alterations in bacterial carbohydrate metabolism [177]. Fibres are metabolized to short-chain fatty acids (SCFA) by commensal microbiota in the distal GI tract. SCFA can influence the growth of pathogens, increase intestinal barrier function, influence visceral sensitivity and serve as energy source for colonocytes, and they can facilitate the generation and differentiation of intestinal regulatory T cells [198, 199]. Patients with CD and UC are associated with impairment in SCFA production [185], which is linked to a reduction in butyrate-producing bacteria, including *Roseburia inulinivorans*, *Ruminococcus torques*, *C. lavalense*, *B. uniformis* and *F. prausnitzii* as well as a reduction in butyrate levels. Less butyrate is linked to changes in visceral hypersensitivity [169, 200]. In contrast to dietary fibres, Westernized high-fat diet, full of refined carbohydrates, is strongly associated with the development of colitis in different IBD animal models, contrary to a diet highly based on fruits, vegetables and polyunsaturated fatty acid-3, which has a protective effect against disease progression in these models. Recent data have also revealed that specific changes in dietary intake, for example, feeding of milk-fat diet, can modify the composition of the gut microbiota, resulting in the emergence of pathobionts (*Bilophila wadsworthia*). The correlations of these ‘Westernized’ diets and blooming of pathobionts in human IBD onset, development and/or relapse are Still to be further investigated [201–203].

The composition of the gut microbiota has recently been linked to the uptake and signaling effects of bile acids. Some members of the *Eubacterium* and *Clostridium XIVa* clusters possess the ability to 7 $\alpha$ -dehydroxylate which are involved in secondary bile acid production. In fact, alteration in bile acid profiles may have the potential to protect against pathogens (such as *C. difficile*) [204] or pathobionts (such as *B. wadsworthia*). The latter one exacerbates colitis in IL-10<sup>-/-</sup> mice and is known to respond to alterations in bile acid profiles [201, 205].

Apart from bacteria, there are also alterations in the commensal fungi composition as well as the virome. Fungal microbiota is skewed in IBD; for example, CD patients show reduced fungal diversity together with an increased *Candida* taxa [206] and an increased Basidiomycota/Ascomycota ratio, and a decreased proportion of *Saccharomyces cerevisiae* has also been reported. Overall, the data indicate that the IBD gut environment might favour fungi at the expenses of bacteria [207]. An increase abundance of *Caudovirales* bacteriophages has also been reported in IBD patients. Some authors are suggesting that viral dysbiosis *per se* contributes to IBD pathology and changes in the bacterial ecosystem due to their predator–prey relationship [207, 208] (**Figure 1**).

## 7. Microbiota–gut–brain axis and IBD

There is a bidirectional signalling pathway between the GI and the brain, mainly through the vagus nerve, in which the commensal microbiota have an active role, denoted as the ‘microbiota-gut-brain axis’. This axis is vital for maintaining homeostasis and it may be also involved in the aetiology of intestinal dysfunctions/disorders (**Figures 1 and 2**). There are evidences of the ability of the gut microbiota to communicate with the brain and thus modulate behaviour and pain and also transfer and eliminate micro-organisms for selecting the commensal profile. The proposal of a ‘microbiota-gut-brain’ implies that through a dynamic alignment, the microbiota inhabiting the intestinal lumen will affect the host’s superior functions by changing CNS activity and vice versa, that is the brain activity and will also impact on microbiota development and composition. Apart from cognitive and vegetative functions, the ‘microbiota-gut-brain axis’ has been studied in visceral pain [209–212]. Although it has been traditionally studied in the context of IBS pathology, some of those findings can be translated to IBD, since IBD shares some overlapping mechanisms with IBS [161, 213, 214]. This includes the dysfunction of the brain-gut axis, the implication of TNFSF gene, the abnormal microbial composition and altered host functions, the low-grade inflammation and the presence of IBS symptoms in patients with IBD in remission [215]. Overall, there is evidence that host–microbe alterations might be not only divergent regarding the abundance of microbial community members but also in their metabolic activity.

The intestinal TLRs are critical for bacterial recognition and initiation of innate immune responses. In particular, TLR2, 4 and 7 have been directly implicated in the modulation of nociceptive markers and visceral hypersensitivity and pain [72, 104, 210, 216–221]. It has also been proposed that a neurochemical ‘delivery system’ exists whereby gut bacteria can send messages to the brain. This delivery system links the commensal gut microbiota to a number of neurotransmitters including GABA, serotonin, noradrenaline, dopamine, acetylcholine and melatonin, all of which are crucial for brain-regulated functions including visceral pain, brain development, anxiety or behaviour [33, 222, 223].

Some of the mechanisms described in the microbiota-gut-brain axis imply the activation of TLRs. Among them, TLR2, expressed in enteric neurons, glia and smooth muscle cells of the intestinal wall appear to regulate intestinal inflammation by controlling ENS structure and



neurochemical coding, along with intestinal neuromuscular function. Colitis in Tlr2<sup>-/-</sup> mice is more severe compared to wild-type mice that is associated with altered ENS architecture and neurochemical profile, intestinal dysmotility, abnormal mucosal secretion, reduced levels of GDNF and impaired signalling via Ret-GFR- $\alpha$ 1. Treatment with GDNF to Tlr2<sup>-/-</sup> mice led to improved colitis [219].

TLR4, increased in IBD patients, has also been associated with severe colitis with impaired epithelial barrier, altered expression of anti-microbial peptide genes and altered epithelial cell differentiation [221]. A putative LPS–TLR4–TRPV1 axis has been described, directly implicating microbiota in changes of the nervous system by means of the innate immune system, that is the TLRs. In line with this notion, the local stimulation of TLR4 but also TLR7, both expressed in epithelial, immune and neural cells, can induce an immune activation that leads to changes in different nociceptive markers, implicating mainly the cannabinoid and the vanilloid system, without having an overt inflammatory response [216, 217]. These findings address some of the putative mechanisms associated with microbial neuro-immune responses, which can contribute to IBD pathophysiology (**Figure 2**).

## 8. Conclusions and perspectives

The intestinal immune system has as its main function to protect the host against invading pathogens as well as to tolerate the myriad of our commensal micro-organisms. If this crosstalk is altered due to genetic predisposition and/or environmental factors, the steady state will be broken and it will result in the development of chronic inflammation such as IBD. Recent research has also identified a third player, the nervous system consisting of both the ENS and the CNS, which can directly regulate the intestinal immune system (**Figure 1**). In this chapter, It is summarized the findings linking the intestinal neuronal pathways with the intestinal immune system and the microbiota in IBD patients. In several cases, the degree of inflammation appears to determine the alteration in neuronal pathways, for example, serotonin, the endocannabinoid system, the loss of neural axons, or the increase in EC and ILC cell numbers [25, 26, 84, 106, 224–226]. However, it is worthy to note that an altered neuronal signalling can persist long after inflammation is apparently resolved in patients with inactive disease and in animal models after disease is resolved [227].

In conclusion, further studies addressing the triad gut microbiota nerves will be a major challenge in the future. Fundamental understanding of neuronal pathways in inflammatory conditions such as IBD is crucial for the discovery of future target strategies. These will in particular target the regulation of functional bowel symptoms such as abdominal pain, visceral sensitivity, which are prevalent in IBD patients with quiescent disease and are regulated by several of the outlined pathways. To date, the evidence on the gut–brain–microbiota axis in human IBD is scarce but future research will aim to delineate this axis in depth, with the goal to evolve our understanding on GI function, to elucidate the complex interaction of this axis with systemic organs and to cover new potential treatments.



## Acknowledgements

This publication has emanated from the financial support of Science Foundation Ireland (SFI) under Grant Number SFI/12/RC/2273. The authors want to thank Dr Vicente Martinez for helpful discussions and valuable comments on the content of this chapter.

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