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Functional and molecular alterations of the enteric nervous system in murine models of gut neuropathies

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RIASSUNTO

L'interazione fra costituenti della parete intestinale e microflora commensale costituisce il principale artefice del mantenimento della barriera mucosale, della promozione dello sviluppo del tratto gastrointestinale (GI) e della modulazione di funzioni GI, quali motilità, secrezione, immunità mucosale e sensibilità viscerale. Un'alterata microflora è stata associata a disordini GI (malattie infiammatorie croniche intestinali, MICI o sindrome dell'intestino irritabile, IBS) mentre cambiamenti del microbiota intestinale durante le fasi dell'infanzia e dell'adolescenza, causati da infezioni o antibiotici, predispongono all'insorgenza di queste malattie. Inoltre, disfunzioni del SNE quali anomalie strutturali e/o variazioni nel contenuto di neurotrasmettitori, sono state associate all'insorgenza sia di MICI che di IBS. In questo contesto, giocano un ruolo chiave i recettori Toll-like (Toll-like receptors, TLRs), un sofisticato sistema di proteine che mediano la risposta infiammatoria contro agenti patogeni e attivano segnali benefici assicurando l'integrità tissutale in condizioni fisiologiche e patologiche. Polimorfismi nei geni che codificano i TLRs, inclusi il TLR2 ed il TLR4, sono stati associati a fenotipi diversi di malattia nei pazienti affetti da disordini GI. In questo studio sono state caratterizzate le alterazioni strutturali e funzionali del SNE murino indotte da i) anomalie nella composizione del microbiota, ii) cambiamenti nel segnale dell'immunità innata, mediato dai recettori TLR2 e 4 ed implicato nel riconoscimento di profili molecolari derivanti dalla microflora intestinale e iii) variazioni di espressione della proteina catecol-O-metiltransferasi (COMT), implicata nel turnover di neurotrasmettitori del SNE e del sistema nervoso centrale, come la dopamina e altre catecolamine. Tramite studi funzionali e strutturali in vitro utilizzando topi maschi C57BL/6J WT e TLR2$^{-/-}$ di pari età (21±5 giorni di età) è stata evidenziata la presenza di alterazioni significative della contrattilità intestinale e dell'architettura dell'ENS nei preparati di ileo provenienti dai topi geneticamente modificati. Una volta dimostrato che la delezione del gene codificante per il recettore TLR2 determinava anomalie strutturali e funzionali nel SNE, è stato valutato se il segnale mediato dal TLR2 derivasse da cellule immunocompetenti ematopoietiche oppure dalle diverse popolazioni cellulari esprimenti questo recettore e residenti nell'intestino, come i neuroni, la glia, le cellule muscolari lisce e le cellule endoteliali. Sono stati così generati topi chimera cioè portatori di cellule geneticamente distinte originate da topi 'donatori' WT e TLR2$^{-/-}$ di pari sesso ed età, quali: i) topi WT che hanno ricevuto il midollo osseo da topi WT donatori (WT→WT), ii) topi WT che hanno ricevuto il midollo osseo da topi TLR2$^{-/-}$ donatori (TLR2$^{-/-}$→WT), iii) topi TLR2$^{-/-}$ che hanno ricevuto il midollo osseo da topi TLR2$^{-/-}$ donatori (TLR2$^{-/-}$→TLR2$^{-/-}$), iv) topi TLR2$^{-/-}$ che hanno ricevuto il

midollo osseo da topi WT donatori (WT→TLR2$^{-/-}$). Esperimenti di contrattilità condotti nei topi chimera hanno dimostrato che le alterazioni della funzione neuromuscolare non sono dipendenti dal segnale TLR2 mediato dalle cellule ematopoietiche. Pertanto la valutazione dei topi deficienti per il TLR2 ha evidenziato il ruolo primario di questo recettore nel conservazione dell'integrità strutturale e funzionale del SNE. Al fine di approfondire il ruolo dell'asse TLR2-microbiota nell'omeostasi del SNE e della muscolatura liscia enterica è stato messo a punto un modello animale di deplezione di microbiota intestinale attraverso la somministrazione intragastrica di 4 antibiotici, ampicillina (100 mg/kg), metronidazolo (100 mg/kg), neomicina (100 mg/kg) e vancomicina (50 mg/kg) due volte al giorno per 14 giorni a topi WT adolescenti (21±5 giorni di età; topi ABX). Da una prima valutazione il trattamento antibiotico ha determinato l'aumento delle dimensioni e del peso del cieco, caratteristica già osservata in topi adulti *germ-free*, ossia privi di microrganismi dalla nascita. Questa condizione, già evidenziata in soggetti affetti da IBS, sembra dovuta ad un ritardato svuotamento delle feci dal cieco ingrossato, ad indicare una compromissione della motilità dimostrata da un ritardato transito a livello del colon. Successivamente gli studi funzionali in vivo hanno rivelato una diminuzione significativa del transito gastrointestinale, accompagnata da alterazioni nella frequenza del pellet fecale e nel contenuto di acqua nelle feci. Tali risultati hanno evidenziato che il trattamento antibiotico determina una riduzione della motilità intestinale con possibili effetti a livello della barriera mucosale. In seguito, analisi immunoistochimiche su preparati di ileo provenienti da topi ABX hanno evidenziato anomalie nella distribuzione ed espressione della proteina marcatore pan-neuronale HuC/D, della proteina gliale strutturale GFAP (glial fibrillary acidic protein) e della subunità β della proteina S-100, marcatore gliale nucleare e citoplasmatico in grado di legare il calcio. Tali osservazioni sembrano confermare come la deplezione del microbiota intestinale possa influenzare l'integrità della rete neuronale e gliale enterica. Data l'importanza di una corretta composizione del microbiota commensale nel mantenimento della rete nervosa e del codice neurochimico del SNE è stata valutata la funzione intestinale in vitro mediante esperimenti di contrattilità utilizzando la tecnica dell'organo isolato su segmenti di ileo provenienti da topi CNTR e ABX. Queste analisi hanno evidenziato anomalie nell'attività contrattile neuromuscolare associate dopo trattamento antibiotico sottolineando l'importanza di una corretta composizione del microbiota nel mantenimento dell'attività motoria enterica.

In parallelo, è stato ulteriormente dimostrato il ruolo primario del segnale mediato dal TLR2 nel controllo dell'attività neuromuscolare, saggiando l'effetto del Pam3CSK4, ligando del TLR2, sulla disfunzione contrattile indotta dalla terapia antibiotica. La somministrazione di Pam3CSK4 per via intraperitoneale, durante la seconda settimana di trattamento antibatterico ha

prevenuto l'entità del danno motorio intestinale, a sostegno della presenza di un dialogo tra il microbiota ed TLR2, fondamentale per la modulazione della funzione neuromuscolare. Per evidenziare l'importanza dell'asse microbiota-TLR2-SNE nel mantenimento della funzione intestinale e nello sviluppo del SNE è stato somministrato l'OxPAPC, inibitore dei segnali mediati sia da TLR2 che da TLR4, a topi WT adolescenti per via sottocutanea per 7 giorni consecutivi. Il trattamento con OXPAPC ha causato un'alterazione significativa della risposta neuromuscolare sia recettore-mediata che non nei topi trattati rispetto al controllo, già evidenziata in topi deficienti per il recettore TLR2, confermando l'importanza del segnale mediato da tali recettori nell'assicurare l'integrità funzionale e strutturale del SNE durante l'adolescenza. In parallelo sono state studiate le vie di neurotrasmissione, coinvolte nella sensibilità viscerale, nel modello animale di deplezione della microflora, analizzando l'espressione genica di GluN1, subunità funzionale del recettore NMDA del glutammato. E' stato osservato un incremento dei livelli di mRNA di GluN1 nel plesso mienterico dei preparati di ileo provenienti dai topi ABX, evidenziando gli effetti di alterazioni della composizione del microbiota intestinale sulla sensibilità viscerale.

Infine è stato approfondito il ruolo dell'asse 'cervello-intestino' attraverso la valutazione strutturale e funzionale del SNE in un modello animale di malattie psichiatriche, caratterizzato dalla riduzione genica di COMT, enzima responsabile della degradazione delle catecolamine. In animali di sesso femminile è stato dimostrato che gli aumentati livelli di catecolamine, dovuti alla riduzione genica di COMT, determinano alti livelli di NO e sofferenza neuronale con conseguente accelerato transito GI e sensibilità viscerale, a confermare il ruolo primario di polimorfismi funzionali di COMT in donne affette da IBS.

ABSTRACT

The interaction between cellular constituents of gastrointestinal (GI) tract and commensal microflora is essential for the maintenance of mucosal barrier, promotion of the development of the GI system and modulation of enteric functions such as motility, secretion, mucosal immunity and visceral sensitivity. Alterations in the composition of the gut microflora have been associated to several GI disorders (e.g. inflammatory bowel disease, IBD, and irritable bowel syndrome, IBS) while changes in intestinal microbiota during infancy and adolescence, caused by infection or antibiotic therapy, appear to predispose to the onset of these diseases. Furthermore, dysfunctions of the enteric nervous system (ENS) such structural abnormalities and/or changes in the content of neurotransmitters, have been associated with the onset of IBD and IBS. In this context, a sophisticated system of proteins, so-called Toll-like receptors (TLRs), plays a key role in mediating the inflammatory response against pathogens and triggers beneficial signals to ensure tissue integrity under physiological and pathological conditions. Polymorphisms in genes encoding TLRs, including TLR2 or TLR4, have been associated with different phenotypes of disease extent and severity in patients with GI disorders. In this study we characterized structural and functional alterations of murine ENS induced by: i) anomalies in the composition of the microbiota, ii) changes in innate immunity response, mediated by TLR2 and/or TLR4 following recognition of gut commensal microflora, iii) alterations in the expression of the protein catechol-O-methyltransferase (COMT), involved in the turnover of several neurotransmitters present in both the ENS and the central nervous system, such as dopamine and other catecholamines. Functional and structural studies in male mice C57BL/6J WT and $TLR2^{-/-}$ (21 ± 5 days old) highlighted the presence of significant alterations of intestinal contractility and ENS architecture in ileal preparations of genetically modified mice. Once demonstrated that the deletion of the gene encoding for TLR2 determines ENS structural and functional abnormalities, it was examined whether TLR2-mediated functional anomalies were hematopoietic cell-independent. Therefore, bone marrow chimeric mice were generated and experimental transfers were as follows: WT donors into WT recipients (WT→WT), WT donors into $TLR2^{-/-}$ recipients (WT→ $TLR2^{-/-}$), $TLR2^{-/-}$ donors into $TLR2^{-/-}$ recipients ($TLR2^{-/-}$→ $TLR2^{-/-}$), and $TLR2^{-/-}$ donors into WT recipients ($TLR2^{-/-}$→ WT). Contractility experiments conducted in bone marrow chimeric mice evidenced that the structure and function of ENS were similar in WT mice given either WT or $TLR2^{-/-}$ bone marrow, indicating that TLR2 signaling in nonhematopoietic cells is a main contributor to ENS health. To investigate the role of the TLR2-microbiota axis in the homeostasis of ENS and enteric smooth muscle we depleted gut

microbiota by intragastric administration of a cocktail of broad spectrum antibiotics (50 mg/kg vancomycin, 100 mg/kg neomycin, 100 mg/kg metronidazol and 100 mg/kg ampicillin) twice a day for 14 days in adolescent mice (aged 21 ± 5, ABX). Mice resulted to be successfully depleted after antibiotic treatment and displayed significantly smaller spleens and enlarged ceca, macroscopically phenocopying *germ-free* mice. This condition, already highlighted in IBS subjects, appears to be due to a delayed emptying of feces from enlarged cecum, due to impaired motility. Functional studies in ABX mice revealed a significant decrease in gastrointestinal transit, accompanied by alterations in the rate of fecal pellet expulsion and stool water content, to suggest that continuous presence of microbial stimuli is required to control intestinal motility and potentially mucosal barrier permeability. Immunohistochemical analysis of ileal preparations from ABX mice showed abnormalities in the distribution and expression of the the pan-neuronal marker HuC/D, the glial structural protein GFAP (glial fibrillary acidic protein) and the cytoplasmatic and nuclear glial calcium-binding protein S100β. Overall these observations highlight the primary role of commensal microbiota in the preservation of the structural integrity of the enteric neuronal and glial network. Given the importance of proper composition of commensal microbiota in the maintenance of neuronal network and neurochemical coding of the ENS intestinal contractility was evaluated in isolated ileal segments from control and ABX mice. These analyses evidenced impaired neuromuscular function associated to antibiotic treatment to further underline that proper neuromuscular function relies on a correct composition of gut microbiota.

The primary role of TLR2 signaling in controlling gut motor function was further confirmed by testing the effect of TLR2 engagement by Pam3-CSK4 (a TLR2/TLR1 agonist) in ABX mice. Intraperitoneal supplementation with Pam3CSK4, during the second week of antibiotic treatment, partially corrected these anomalies in ENS structure and intestinal contraction, supporting the presence of a dialogue between commensal microbiota and TLR2, essential for the modulation of neuromuscular function. To highlight the key role of gut microbiota−TLR2-ENS axis in maintaining intestinal function and development of the ENS, male C57Bl/6 mice (2 weeks old) were daily treated subcutaneously with OxPAPC (a TLR2 and TLR4 inhibitor) for 7 days. In vivo inhibition of both TLR2 and TLR4 determined a significant alteration of receptor and non-receptor-mediated neuromuscular responses, in a manner similar to that found in TLR2-deficient mice, providing evidence that TLR2 and TLR4 signaling is essential in ensuring the structural and functional integrity of the SNE during adolescence. Then, we investigate changes in gene expression of GluN1 subunit of N-Methyl-D-Aspartate (NMDA) receptor of the neurotransmitter glutamate in the myenteric plexus of ileal preparations from control and ABX mice. Antibiotic-mediated depletion of commensal microflora determined increased mRNA

levels of GluN1, suggesting that commensal microbiota is involved in modulating visceral sensitivity.

Finally, the role of brain-gut axis in ENS homeostasis was assessed in an animal model of psychiatric disease, characterized by the genetic reduction of catechol-o-methyltransferase (COMT), an enzyme responsible for the degradation of catecholamines. In female animals genetic-driven COMT defective activity determined increased levels of NO associated to altered ENS architecture, neurochemical coding and visceral sensitivity. We cannot exclude that such changes may be involved in the pathogenesis of IBS in female patients, underlining a potential link with psychiatric disorders.

1. INTRODUCTION

1.1 The Enteric Nervous System

The digestive tract is unique among internal organs because it is exposed to a large variety of physical and chemical stimuli from the external environment in the form of ingested food. Therefore, the intestine has developed a rich repertoire of coordinated movements of its muscular apparatus to ensure the appropriate mixing and propulsion of contents during digestion, absorption, and excretion (Costa et al., 2000). An important arbiter of these processes is the enteric nervous system (ENS), a network of neurons and glial cells within the wall of the bowel that controls most aspects of intestinal function (Lake and Heuckeroth, 2013). The ENS is located in the walls of the entire gastrointestinal tract from the esophagus to the anus and associated glands (salivary glands and the pancreas) and the gallbladder. Originally, the ENS was thought to be part of the autonomic component of the peripheral nervous system, and its neurons located in the gut wall were considered to be parasympathetic postganglionic neurons (Goyal and Hirano, 1996). The hypothesis of 'little brain' in the gut arised in the 1899, when the physiologists W.M Bayliss and E.H. Starling proposed the existence of enteric reflex pathways by means of the classic approach, i.e. inducing specific and localized stimuli and recording the reflex-like response. They demonstrated that intestinal contractions were coordinated by short ascending excitatory pathways and longer descending inhibitory pathways where majority of enteric neurons did not interact directly with the parasympathetic axons of the central nervous system (CNS) (Bayliss and Starling, 1899; Goyal and Hirano, 1996). These findings rised the hypothesis, so called "the law of the intestine", which is now known as the peristaltic reflex. Further evaluations of the functional and chemical diversity of enteric neurons revealed that the ENS is closely similar to the CNS (Furness, 2000). The ENS contains more than 100 million neurons; approximately the number of neurons residing in the spinal cord (Furness and Costa, 1987). Therefore, in these late years the ENS has been assumed as a separate portion of the CNS with which it is in continuous communication through sympathetic and parasympathetic afferent and efferent neurons. On the other hand, the CNS is connected with the enteric neurons throughout the central autonomic neural network. Through these bidirectional connections the ENS provides neural control of all functions of the gastrointestinal tract (Goyal and Hirano, 1996).

The ENS is endowed with a wide array of restorative, maintenance and adaptive functions. Enteric reflex circuits detect the physiological condition of the gastrointestinal tract, integrate

information about its state, and provide outputs to control gut movements, fluid exchange between the gut and its lumen, epithelial permeability and local blood flow (Gershon, 2005; Furness, 2006). Enteric neurons also interact with the immune and endocrine systems of the gut and all of these functions contribute to the maintenance of the integrity of the intestinal epithelial barrier (Mach, 2004; Furness, 2006; Savidge et al., 2007).

The importance of the ENS is highlighted by the varied range of enteric neuropathies that are triggered as a result of a failure in one or more of its components. These neuropathies have been grouped as congenital or developmental neuropathies (e.g. Hirschsprung disease), sporadic and acquired neuropathies (e.g. Chagas disease or irritable bowel syndrome, IBS), neuropathies associated with other disease states (which can be secondary to the other disorder, such as diabetic gastroparesis and other diabetes-related motility disorders), and iatrogenic or drug-induced neuropathies (e.g. postoperative ileus) and those associated with intestinal transplantation, such as neuropathy following ischemia and reperfusion injury (Furness, 2012).

Anatomy of ENS

The ENS is composed of small aggregations of nerve cells, enteric ganglia, the neural connections between these ganglia, and nerve fibers that supply effector tissues, including the muscle of the gut wall, the epithelial lining, intrinsic blood vessels, GI and pancreatic endocrine cells (Furness, 2012). The nerve-cell bodies are grouped into small ganglia which are connected by bundles of nerve processes to form the two major plexuses, so-called the myenteric (or Auerbach's) plexus and the submucous (or Meissner's) plexus (Figure 1.1). A few small ganglia occur in the mucosa, close to the *muscularis mucosae* (mucous plexus) (Hansen, 2003a).

The myenteric plexus is positioned between the outer longitudinal and circular muscle layers, where forms a continuous network of ganglia and connecting axon bundles that extends from the upper esophagus to the internal anal sphincter (Furness, 2012). It primarily provides motor innervation to the two muscle layers and secreto-motor innervation to the mucosa. There are numerous projections from the myenteric plexus to the submucosal ganglia and to enteric ganglia of the gallbladder and pancreas (Kirchgessner and Gershon; 1990). Moreover, a substantial number of projections from the myenteric neurons are connected to the sympathetic ganglia (Goyal and Hirano, 1996; Hansen, 2003a) (Figure 1.1). The myenteric plexus shows a high density of neurons compared to the submucous plexus with an average ratio of the sensory, interneurons and motor neurons of 2:1:1, respectively (Costa et al., 2000; Hansen, 2003a).

The submucous plexus is located in the submucosa and in large mammals; it consists of more than one layer: an inner network located at the serosal side of the *muscularis mucosae* (Meissner's plexus) and an outer (Schabadasch's) plexus adjacent to the luminal side of the circular muscle layer. Moreover, in the human intestine, a third intermediate plexus lies between Meissner's and Schabadasch's plexus. Non-ganglionated plexuses also supply all the layers of the gut (Costa et al. 2000; Furness 2000; Hansen, 2003a). Submucosal ganglia and connecting fiber bundles form plexuses in the small and large intestines, but these ganglia are extremely rare in the stomach and esophagus (Furness, 2012) (Figure 1.1).

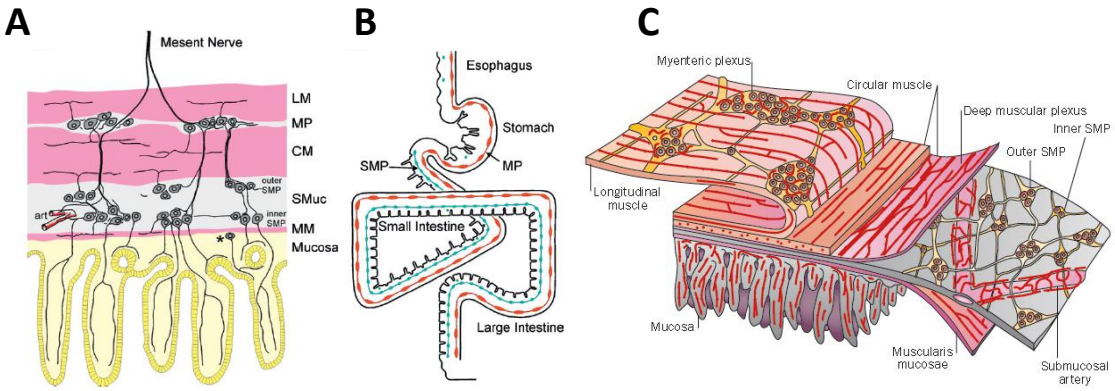

Figure 1.1. Anatomy of ENS. Panel A: In the small and large intestines, the nerve cells are contained in ganglia of the myenteric plexus (MP) between the longitudinal (LM) and curcular muscle (CM) layers and in ganglia within the submucosa (SMuc), depicted in tranverse section through the gut wall. The ganglia and fibers in the submucosa form inner and outer submucosal plexus (SMP). Panel B: The distribution of ganglia along the gastrointestinal tract. Panel C. Neuromuscular layers along the small and large intestines (modified from Furness, 2012).

Classification of enteric neurons

The ENS is composed of a large number of enteric neurons that can be identified according to their location, neurochemistry, shape, projections, proportions, connections, and function (Costa et al., 2000; Furness, 2012).

Sensory neurons

There are many types of sensory neurons in the gut. The sensory neurons are represented by an extrinsic dense network of vagal and spinal afferents neurons with their cell bodies outside the gut wall and by intrinsic primary afferent neurons [IPANs, also termed enteric primary afferent neurons (EPANs)] with their cell bodies within the gut wall, in both myenteric and submucosal ganglia (Furness, 2000; Hansen 2003b). They respond to luminal chemical stimuli, to mechanical deformation of the mucosa, and to radial stretch and muscle tension. Together

with endocrine and immune cells, IPANs form a functional network of surveillance. A bidirectional communications exists between intrinsic neurons and extrinsic afferent neurons (Holzer 2002). Whereas IPANs are essential for the neuronal control of the digestion, extrinsic afferent neurons provide information to the brain about processes that are relevant to energy and fluid homoeostasis together with the sensation of discomfort and pain (Holzer 2002; Hansen 2003b). IPANs represent about 30% of myenteric neurons and 14% of submucosal neurons, and they display a distinct Dogiel type II shape and have a long after-hyperpolarization following action potentials (Costa et al., 2000).

The class of sensory neurons also includes the mechano-, chemo- and thermoreceptors. Mechanoreceptors are activated by distension and generate tonic muscle contractions, and if distension is maintained, they respond by generating peristaltic activity. These mechanoreceptors are low-threshold nociceptors. Conversely, high-threshold afferents are thought to be the sensory analogues of pain (Furness et al. 2000; Kirkup et al. 2001). Acute visceral abdominal pain seems to emerge from activation, whereas chronic forms of visceral pain appears to result from peripheral sensitization of the high-threshold nociceptive fibers. The high-threshold mechanoreceptors normally remain sleeping and silent, but they can be awakened under such conditions as injury or inflammation. The silent nociceptors become sensitized by second order neurons (interneurons and motor neurons) via both slow and fast excitatory postsynaptic potentials (Furness et al., 2000; Kirkup et al. 2001).

Sensory nerves express a variety of membrane receptors, responsible of modulating their activity. Some receptors (e.g. for substance P, calcitonin gene-related peptide, and vasoactive intestinal polypeptide, VIP) are involved in the normal sensory neurotransmission, whereas molecules derived from multiple cellular sources during ischemia, injury or inflammation activate others. Key agents in visceral abdominal hypersensitivity after tissue damage and inflammation are bradykinin, prostaglandins, leukotrienes, interleukin-1β, tumor necrosis factor α, 5-HT, adenosine triphosphate (ATP), glutamate, pituitary adenylate cyclase-activating polypeptide (PACAP), and adenosine. The mechanisms involved in gastrointestinal sensation include direct activation (e.g. opening of ion channels), sensitization, and alteration of the mediators of the afferent nerve. As such, receptor antagonists for these substances will be useful therapeutically to treat gastrointestinal hypersensitivity in acute and chronic inflammatory conditions. For example, opioid agonists selective for the mesenteric μ-receptors on vagal afferents are promising as visceral antinociceptive drugs (Eastwood and Grundy 2000; Kirkup et al. 2001; Yiangou et al. 2001).

Interneurons

Interneurons form multisynaptic pathways in the length of the gut tube controlling the distances along the intestine where peristaltic waves are propagated. They are interposed between the primary afferent neurons and the motor or secretomotor neurons. Interneurons are both after-hyperpolarization and usually Dogiel type II neurons (Costa et al., 2000). Interneurons involved in motor reflexes are directed orally or anally and are designated as ascending or descending, respectively (Goyal and Hirano, 1996). The ascending interneurons are mainly cholinergic, whereas the descending ones, which are the majority, have a complex chemical coding including acetylcholine (Ach), nitric oxide (NO), VIP, 5-HT, and somatostatin. Interneurons which contain and release ACh/NO/VIP/somatostatin are involved in local motility reflexes, while ACh/5-HT neuros are involved in the local secretomotor reflexes. Two non-cholinergic, fast excitatory postsynaptic potentials, one mediated by ATP and the other by 5-HT, mediate interneuronal transmission (Furness 2000; Hansen, 2003b). The dual projection of these interneurons to both myenteric and submucous ganglia represents the functional link between motor, secretory, and vasomotor pathways (Costa et al., 2000).

Motor Neurons

There are three broad types: muscle motor neurons, secretomotor or vasodilator neurons and neurons innervating enteroendocrine cells. Motor neurons are Dogiel type I and synaptic-type neurons.

Muscle motor neurons

Muscle motor neurons innervate the longitudinal and circular muscles and the *muscularis mucosae* throughout the digestive tract. The muscle motor neurons are either excitatory or inhibitory and, thus, release neurotransmitters provoking muscle contractions or relaxation). For the excitatory neurons, transmission is predominantly sustained by muscarinic cholinergic (ACh) and tachykinergic (substance P and neurokinin A) signaling. They act directly on smooth muscle and possibly indirectly via the network of interstitial cells in the deep muscular plexus (Costa et al., 2000). Regarding the inhibitory neurons, responsible of most of the descending accommodating inhibitory reflexes, the neuromediators released are NO, VIP, ATP, and possibly PACAP, gamma-aminobutyric acid (GABA), neuropeptide Y and carbon monoxide. The inhibitory neurons are normally switched off in the aboral direction, resulting in contractile activity that propagates from the esophagus to the distal part of intestine. In a situation of vomit, the opposite takes place. Loss or malfunction of inhibitory motor neurons underlies

several forms of chronic idiopathic constipation and esophageal sphincteral achalasia (Wood et al. 1999; Hansen 2003b).

Secretomotor and vasomotor neurons

Secretomotor and vasomotor (vasodilatator) neurons control secretions and blood flow, respectively. There are two main types of intestinal secretomotor neurons, cholinergic and non-cholinergic. ACh released from the cholinergic neurons acts on muscarinic receptors on the mucosal epithelium. The non-cholinergic neurons appear to mediate most of the local reflex responses and utilize VIP as a neurotransmitter. The delicate balance of epithelial transport processes (mainly secretions) and blood flow is accomplished by the intrinsic secretomotor neurons through local reflex circuits. These reflexes are under extrinsic modulation, primarily via the sympathetic transmission (Furness 2000; Hansen, 2003b). Neurons with a similar function and neurochemistry are also present in the submucous ganglia. Some of the VIP submucous neurons also project to the myenteric ganglia and may represent the basis for a functional connection between secretion and motility (Costa et al., 2000).

Enteric glial cells

The ENS is composed mainly by neurons and enteric glial cells (ECGs), which are in number substantially larger than neurons. ECGs are usually small cells with highly irregular, stellate-shaped body, associated to neuronal cell bodies in enteric ganglia in an intimate physical connection, highly reminiscent of the relationship between astrocytes and neurons in the CNS (Figure 1.2) (Gulbransen and Sharkey, 2012). ECGs also show connections with enteric nerve fiber bundles, which are similar to peripheral Schwann cells, but differ from these by the function (Lomax et al., 2005; Bassotti et al., 2007).

All populations of enteric glia originate from a common pool of neural-crest-derived progenitors (Laranjeira and Pachnis, 2009) however, each population of ECGs residing in microenvironmental compartments of the gut wall represents a morphologically and functionally distinct subtype of enteric glia (Gulbransen and Sharkey, 2012).

The ECGs ‘type I’ or ‘protoplasmic’ display star-shaped cells with short, irregularly branched processes resembling protoplasmic astrocytes of the CNS and closely embrace neuronal cell bodies and fibres within myenteric and submucosal ganglia (intraganglionic ECGs). Enteric glia ‘type II’ represents the elongated glial cells within interganglionic fibre tracts, which are similar to fibrous astrocytes of the white matter in the CNS. The subepithelial glia consists of several

long branches that reach the mucosal epithelial cells, and thus could be grouped as 'mucosal' or 'type III' ECGs. The fourth type of enteric gliocytes are distributed between smooth muscle cells, running with neuronal fibres in the musculature, thus these cells are 'intramuscular' or 'type IV' ECGs (Hanani and Reichenbach, 1994)(Figure 1.2).

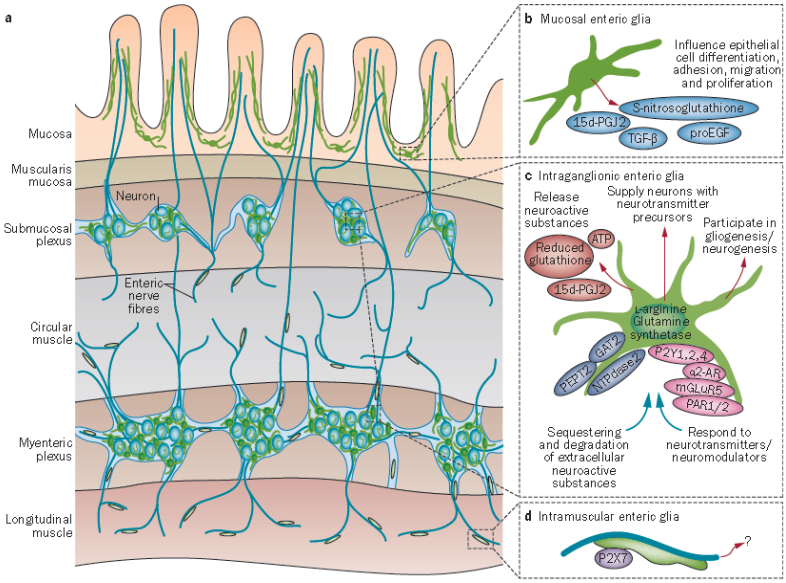

Figure 1.2. Subpopulations of enteric glia. Panel A: Several subpopulations of enteric glia located within the gut wall with different proposed physiological functions and signaling mechanisms. Panel B: Mucosal enteric glia lie in the mucosa directly beneath the epithelial cells. Panel C: Intraganglionic glia surround neurons (blue) within the enteric nerve plexuses (submucosal and myenteric plexus). Panel D: Intramuscular glia are associated with enteric nerve fibers innervating the smooth muscle coats (circular muscle and longitudinal muscle) (modified from Gulbransen and Sharkey, 2012).

ECGs resemble astrocytes in several ways, including the expression of the intermediate filament glial fibrillary acidic protein (GFAP; Jessen and Mirsky, 1980) and the Ca^{2+} binding protein, S100β (Ferri et al., 1982). Moreover, the expression of the transcription factors SOX8, SOX9, and SOX10 has been demonstrated in early ECGs of developing mouse ENS (Hoff et al., 2008).

Since enteric glia share many phenotypical features with astrocytes, ECGs were thought for a long time to provide sustenance and mechanical support for neurons, acting as a 'glue' (Boesmans et al., 2013). However, in the last two decades, it is becoming increasingly clear that ECGs play a role in the regulation of synaptic transmission, in the maintenance of intestinal epithelial barrier function and in the communication between the nervous and immune systems (Savidge et al., 2007; Van Landeghem et al., 2009; Gulbransen and Sharkey, 2012). ECGs can modulate enteric neural circuits in several ways, including terminating the actions of neurotransmitters from synapses (Fletcher et al., 2002; Braun et al., 2004; Lavoie et al., 2011), supplying neurons with neurotransmitter precursors (Nagahama et al., 2001) and by

producing neuroactive substances (Gulbransen and Sharkey, 2012). For instance, ECGs, but not neurons, appear to contain L-arginine immunoreactivity, suggesting that this NO precursor is provided to enteric neurons by glial cells (Nagahama et al., 2001). In the ENS, EGCs express the γ-amino butyric acid (GABA) reuptake transporter GAT-2, which suggests its participation in the clearance of the neurotransmitter from the synaptic cleft (Fletcher et al., 2002). Furthermore, the glutamine synthetase expression has been detected in ECGs, suggesting that these cells are involved in the maintenance of glutamatergic signalling (Jessen and Mirsky, 1983). Recent evidences demonstrate that purines are the most ubiquitous mediators of enteric neuron-to-glia crosstalk. EGCs were shown to express an extracellular surface-bound ectonucleotidase triphosphate diphosphohydrolase 2 (NTPDase2) (Braun et al., 2004), which can be important in the regulation of extracellular ATP levels after release from activating neurons. Furthermore, neuronal released ATP can activate the glial P2 receptors in multiple experimental models (Gomes et al., 2009; Gulbransen and Sharkey, 2009). Besides the well documented 'protective role', EGCs are activated by means of inflammatory insults and may directly contribute to an inflammatory condition working as an antigen presenting cell-type promoting a variegate release of cytokines, such as interleukin-6 (IL-6) and IL-1β (Rühl et al., 2001; Neunlist et al., 2008; Murakami et al., 2009). Furthermore, ECGs may act as "receptors" for cytokines, produced by themselves (Cirillo et al., 2011b). In the CNS of higher vertebrates, astroglia can react to inflammatory insults with proliferation, increased cytokines secretion and overexpression of both GFAP and S100β proteins, a phenomenon known as reactive gliosis (Sofroniew and Vinters, 2010; Cirillo et al., 2011a). As a member of the cytoskeletal protein family, GFAP is thought to be important in modulating astrocyte motility and shape by providing structural stability to astrocytic processes (Eng et al., 2000). During astrogliosis, a rapid synthesis of GFAP occurs, which is demonstrated by the increase in protein content or by immunostaining with GFAP antibody (Eng et al., 2000).

The S100β protein belongs to the S100 protein family that includes more than 20 EF-hand Ca^{2+}-Zn^{2+} binding proteins (Zimmer et al., 1995; Gonzalez-Martinez et al., 2003). In the brain, S100β exerts either trophic or toxic effects depending on its concentration in extracellular milieu (Cirillo et al., 2011a). Nanomolar concentrations of S100β promote neuronal survival, neurite outgrowth (Bramanti et al., 2010) and astrocytic proliferation (Zimmer et al., 2005). On the other hand, micromolar amounts of S100β protein have been observed in several neuropathologies such as Alzheimer's disease and Down's syndrome (Griffin et al., 1989; Van Eldik and Griffin, 1994).

As the GI tract is continuously exposed to a variety of stimuli such as bacterial and food antigens, it is considered to be in a state of ongoing physiological inflammation (Lomax et al.,

2005). Recent findings have demonstrated that S100β overexpression is associated with the onset and maintenance of inflammation in human gut (Cirillo et al., 2009). S100β upregulation, observed in celiac disease, is accompanied by enhanced iNOS protein expression and consequent release of NO, a crucial pro-inflammatory mediator in inflammatory bowel disease (IBD; Esposito et al., 2007; Cirillo et al., 2009). In humans, GFAP expression is altered in the mucosa of patients with IBD such as ulcerative colitis (UC) and Crohn's disease (Cornet et al., 2001).

Role of ECGs in neurogenesis and neuron survival

Embryonic development of the ENS involves rostro-caudal migration of cells derived from the neural crest- through the developing GI tract, with subsequent colonization and differentiation of these cells into neurons or glia (Le Douarin and Teillet, 1974). These multipotent precursor cells constitute a heterogeneous population that changes progressively as a function of developmental age and enviromental stimuli during migration or when are embedded in the gut wall (Gershon and Ratcliffe, 2006). The development of the ENS does not stop at birth. Final differentiation of enteric neurons still occurs after birth, when GFAP and SOX10-positive progenitor cells apparently downregulate their neurogenerative capacity (Joseph et al., 2011; Laranjeira et al., 2011). The maturation of enteric neurons into ganglia also persists in early postnatal life with ongoing adaptation of the three-dimensional architecture of enteric nerve fibers within the first 2 weeks of life (Schäfer et al., 1999; Faure et al., 2007; Collins et al., 2014). Once developmental neurogenesis is complete, GFAP and SOX10-positive progenitor cells retain the ability to generate glia in the adult and a low level of gliogenesis occurs in the ENS during adulthood (Schäfer et al., 1999; Faure et al., 2007).

An additional analogy between glia of the CNS and the ECGs of the ENS is the potential to exert neuroprotective effect through the production of neurotrophic factors such as the glial cell line derived neurotrophic factor (GDNF) and neurotrophin 3 (NT-3) (Anitha, et al., 2006). GDNF, an important neurotrophic factor for the ENS (Heuckeroth et al., 1998), acts with the co-receptor GDNF-receptor α (GFRα1) to activate the Ret tyrosine kinase receptor, stimulating both the MAPK and PI3K pathways (Takahashi, 2001). During development, GDNF is produced in the gut mesenchyme and its signalling through the proteins RET and GFRa1expressed on neural crest cells regulates the development of the ENS (Rodrigues et al., 2011). Several studies have previously shown that the proliferation, maturation, migration, and survival of the enteric neurons are critically dependent on the activity of the Ret tyrosine kinase receptor that is expressed not only in the developing but also in mature enteric neurons (Anitha et al., 2006; Gershon, 2010; Wang et al., 2010). Mutations in the human RET gene are responsible for

approximately 50% of familial cases of Hirschprung's disease, a pathology characterized by an aganglionic terminal colon in children (Hickey et al., 2009). Moreover a reduction of GDNF availability contributes to changes in ENS architecture and function during diabetic neuropathy (Anitha et al., 2006). GDNF holds also the ability to promote neuronal survival under inflammatory conditions in the CNS (Mucke and Eddleston, 1993), that potentially could also exert on enteric neurons. There is evidence that GDNF production is up-regulated during colitis, plausibly for its potent anti-apoptotic effect on colonic epithelial cells which limits mucosal damage in the inflammed tissue (Steinkamp et al., 2003).

1.2 The human intestinal microbiota

Through the course of evolution, a complex microbial ecosystem has colonized the skin, oral cavity, respiratory, urogenital and GI tract of all mammals, including humans. The community of commensal microorganisms residing in or passing through the GI tract is referred to as the intestinal microbiota (Dethlefsen et al., 2006). The term microbiota is to be preferred to the older term bacterial flora, as the latter fails to account for the many nonbacterial elements (e.g. archea, viruses, and fungi) that now are known to be normal inhabitants of the gut (Willing et al., 2011). The human intestinal microbiota is composed of an astounding number and species of microorganisms that colonize symbiotically the human gut, comprising approximately 300 to 500 bacterial species, containing nearly 2 million genes (the so called microbiome). The human intestinal tract is colonized by about ten times more microbial cells than human body cells and thus it contain about 150 times more microbial genes than the human genome (Qin et al., 2010; Wopereis et al., 2014).

Recent clinical studies demonstrated the great complexity of the human gut microbiota with hundreds of phylotypes, of which 80% still remain to be categorized (Zoetendal et al., 2008). Of the 10 bacterial phyla detected in the gut the *Firmicutes*, *Bacteroidetes* and *Actinobacteria* predominate, of which the *Firmicutes* is the most dominant and assorted phylum (Simren et al., 2013).

The gut microbiota is differently distributed along the GI tract. Per gram of intestinal content, the microbial density increases from 10^1–10^4 microbial cells in the stomach and duodenum, 10^4–10^8 cells in the jejunum and ileum, to 10^{10}–10^{12} cells in the colon and faeces (Booijink et al., 2007; Dethlefsen et al., 2006; Gerritsen et al., 2011), indicating that the greater microbial amount of the human microbiota is located in the large intestine. The composition also differs, with predominantly Gram-positive bacteria in the upper GI tract and mainly Gram-negative microorganisms and anaerobes in the colon, where the microbiota composition is totally

dominated by three phyla, *Firmicutes, Bacteroidetes* and *Actinobacteria* (Jones et al., 2009; Martinez et al., 2010). The composition of luminal intestinal microorganisms that populate the colon is quite uniform and faecal material seems to best represent the colonic microbiota composition (Eckburg et al., 2005). In contrast, there is only limited insight in the composition of the microbiota that resides in the small intestine. Especially the lower part of the small intestine, the ileum, has received until now minimal attention, mainly due to sampling difficulties caused by the inaccessibility of this region (Booijink et al., 2007). The composition of the small intestinal microbiota is largely influenced by a combination of gastric acid, bile and pancreatic juice that is secreted in the duodenum and generates a harsh environment for most microorganisms (Booijink et al., 2007; Gerritsen et al., 2011). Moreover, major differences exist between the microbiota present in the gut lumen and the microbiota embedded in the mucus layer of the GI tract (Schwiertz et al., 2010).

A major challenge in the analysis of gut microbiota is the inability to culture most of the different residing species (Simren et al., 2013). Nevertheless, more than a decade ago, thanks to the advances in whole genome sequencing (WGS) and 16S rRNA pyrosequencing techniques, all culture-independent sequencing technologies, gut ecosystems have being started to be thoroughly studied in the native state. 16S rRNA gene sequencing is extensively used for phylogenetic reconstruction, nucleic acid-based detection, and quantification of microbial diversity whereas WGS additionally explores the functions of the metagenome. Interestingly, gut microbiota retains a constant relative abundance at operational taxonomic unit (OTU) levels and altered microbial abundance has been associated with complex diseases such as symptomatic atherosclerosis, type 2 diabetes, obesity, inflammatory bowel disease and colorectal cancer (Andersson et al., 2008; Zoetendal et al., 2008; Mandal et al., 2015).

The intestinal microbiota is coexisting in a homoeostatic relationship with the host, playing a role in metabolic, nutritional, physiological and immunological processes in the human body. It exerts important metabolic activities by extracting energy from otherwise indigestible dietary polysaccharides such as resistant starch and dietary fibers (Wopereis et al., 2014). These metabolic activities also lead to the production of important nutrients, such as short-chain fatty acids (SCFA), vitamins (e.g. vitamin K, vitamin B12 and folic acid) and amino acids, all essential nutrients for human beings (Albert et al., 1980; Conly et al., 1994). In addition, the intestinal microbiota participates in the defense against pathogens by the production of antimicrobial molecules. The gut microbiota has a primary role in the development and functionality of the innate and adaptive immune responses, but also in regulating GI sensory and motor functions and intestinal barrier homeostasis (Parkes et al., 2008; Gerritsen et al., 2011).

Influence of gut microbiota on neurodevelopment

Commensal microbes widely contribute to host phenotype and for this reason, mammals have been described as 'superorganisms', and terms such as 'ecological development' have been coined to indicate that development is process merging both host genetics and microbiota-derived signals (Willing et al., 2011). Numerous studies in both invertebrates and vertebrates have established a clear connection between commensal microbes and gut physiology. The fruit fly *Drosophila melanogaster* has a relatively simple microbiome. However, comparisons between axenic and conventionally reared flies have shown multiple contributions of bacteria to host nutrition and physiology, including effects on the larval development rate (Storelli et al., 2011), adult lipid storage (Ridley et al., 2012), gut stem cell activity (Buchon et al., 2009), and mating preference (Broderick et al., 2014). The influence of the microbiota extends beyond the GI tract, playing a major role in the bidirectional communication between the gut and the CNS (Cryan and Dinan, 2012). Given the importance of gut microbiota in modulating health and neurodevelopment, the brain–gut axis has been extended to the microbiota–gut–brain axis (Rhee et al., 2009; Collins et al., 2012), which represents a complex network of communication between the gut, the intestinal microbiota, and the brain modulating immune, ENS and CNS functions (Mayer, 2011; Collins et al., 2012; Borre et al., 2014).

Prenatal period

More than a century ago, Theodor Escherich postulated that the fetus is sterile into intrauterine environment (Escherich, 1886). It has only recently been revealed by molecular investigation that microbial exposure start before birth and the fetus appears to receive microorganisms from the mother during gestation (Jimenez et al., 2008; Satokari et al., 2009). The presence of bacterial species in the fetus (such as *Escherichia coli*, *Enterococcus faecium*, and *Staphylococcus epidermidis*) could result from the translocation of the mother's gut bacteria via the bloodstream and placenta (Jimenez et al., 2008). The ENS development take place during pregnancy, when neural-crest derived cells migrate to the developing GI tract, as described in the previous section. In parallel, the brain formation in humans starts at 3–4 weeks of gestation with a process event known as neurulation. Cortical neurogenesis occurs predominantly during in utero gestation, but can continue up to 2-5 years of age (Herschkowitz et al., 1997; Workman et al., 2013). Gliogenesis also begins during the in utero period where mature astrocytes are present in the brain by 15 weeks of gestation.

Furthermore, hippocampal neurogenesis peaks around 8–9 weeks and this can persist well into the postnatal period (Rakic and Nowakowski, 1981).

Birth and weaning

Postnatally, the microbial gut colonization is dependent on the birth delivery mode. Whereas vaginally born infants are colonized by fecal and vaginal bacteria from the mother, infants born by cesarean delivery are exposed to a different bacterial milieu closely related to that of the human skin and hospital environment (Biasucci et al., 2010; Dominguez-bello et al., 2010) (Figure 1.3).

Early colonizers of the neonatal gut are mainly aerobes (such as staphylococci, streptococci and enterobacteria), while late colonizers are strict anaerobes (such as eubacteria and clostridia) as the total microbiota become more complex, more stable and converge to a common pattern (Stark and Lee, 1982; Palmer et al., 2007).

During the first days of life, gut microbiota of the infant shows a low diversity and it is unstable, then the precise composition of the developing microbiota population is dependent on whether the infant is breast or formula fed (Thum et al., 2012) (Figure 1.3). The microbiota of the formula-fed infants appears to be more diverse than breastfed infants, whose microbiota has a more stable colonized pattern (Schwartz et al., 2012; Fan et al., 2014).

Infants delivered by cesarean delivery are more likely to suffer from allergies, asthma, GI dysfunction, obesity, and diabetes later in life (Jakobsson et al., 2014). Moreover, breastfed infants demonstrate better neurodevelopmental outcomes and higher scores on intelligence tests (Kramer et al., 2008), but it is unclear whether these neurodevelopmental outcomes are a reflection of the microbiota composition. Thus, establishment of pioneer gut microbiota is a crucial stage in neonatal development and this is a critical period not only for ENS, but also for CNS development (Dominguez-Bello et al., 2010). In the CNS a considerable amount of morphological development, cell differentiation, and acquisition of function takes place during postnatal development. Synaptogenesis begins in earnest in the human brain after approximately post-birth (after the appearance of astrocytes) and synaptic density increases rapidly after birth to reach maximum levels by approximately 2 years of age (Huttenlocher, 1979; Petanjek et al., 2011).

Childhood and adolescence

The gut microbiota colonization continues to develop throughout childhood and adolescence. The microbiota continue to evolve until adulthood with a gradual increase in *Bacteroides* spp., a decline in *Lactobacillus* spp. after the age of five and a decline in *Bifidobacterium* spp. in late

teenage (Hopkins et al., 2002; Balamurugan et al., 2008). The next significant changes in the composition of the intestinal microbiota come with the introduction of solid food and weaning, since diet plays a crucial role in modulating microbiota composition (Borre et al., 2014; Wopereis et al., 2014) (Figure 1.3). Several recent studies demonstrate a less diverse microbiota with sufficiently different microbial gut communities in adolescent children in comparison to adults (Agans et al., 2011; Ringel-Kulka et al., 2013). It appears that instability and immaturity of gut microbiota during childhood and adolescence could be susceptible to several factors, such as the use of antibiotics, stress, harmful environment, diet, and infections, which could result in dysbiosis and potentially have a negative impact on intestinal and mental health, leading to development of gut and brain disorders later in life (Hviid et al., 2011; Kronman et al., 2012; Borre et al., 2014; Desbonnet et al., 2015) (Figure 1.3). The effect of microbiota on the formation of neural circuits in the mammalian gut is highlighted by studies of *germ-free* mice that demonstrated that these animals have altered spontaneous circular muscle contractions and decreased nerve density in the jejunum and ileum (Collins et al., 2014). In addition, *germ-free* mice showed reduced sensory neuron excitability, which was restored following colonization with normal microbiota (McVey Neufeld et al., 2013). In agreement with these observations, diet modifications leading to changes of microbiota composition resulted in significant alterations in gastrointestinal transit time (Kashyap et al., 2013).

Similar to ENS and gut microbiota development, brain maturation undergoes a crucial developmental phase during childhood and adolescence with various structural, neurochemical, and molecular changes occurring in response to genetic and environmental signals (Paus et al., 2008). A consequence of this major neuronal rewiring during adolescence is a high level of vulnerability to pathological insults ranging from stress to drugs, to abuse, and to dietary deficiencies, which leads to onset of numerous psychiatric disorders including schizophrenia, substance abuse, and mood disorders (Paus et al., 2008; Borre et al., 2014)

Furthermore, adolescence is associated with hormonal changes that may result in differential susceptibility of men and women to various disorders. For example, autism and schizophrenia have a higher occurrence in males (Jacquemont et al., 2014), whereas mood disorders and IBS are more prevalent in females (Loftus et al., 2002; McHenry et al., 2014).

Adulthood and Aging

The gut microbiome evolves throughout the lifespan, but microbiota diversity and stability decline with aging (Claesson et al., 2011). Changes also occur in extreme old age when *Bacteroides* spp. decrease while *Enterococcus* spp. and *Escherichia coli* increase (Heuckeroth et

al., 1998; Mulligan, 2014). It has recently been shown that the microbial composition of aged individuals is influenced by their residential community, dietary regimen, and their health status (Claesson et al., 2012).

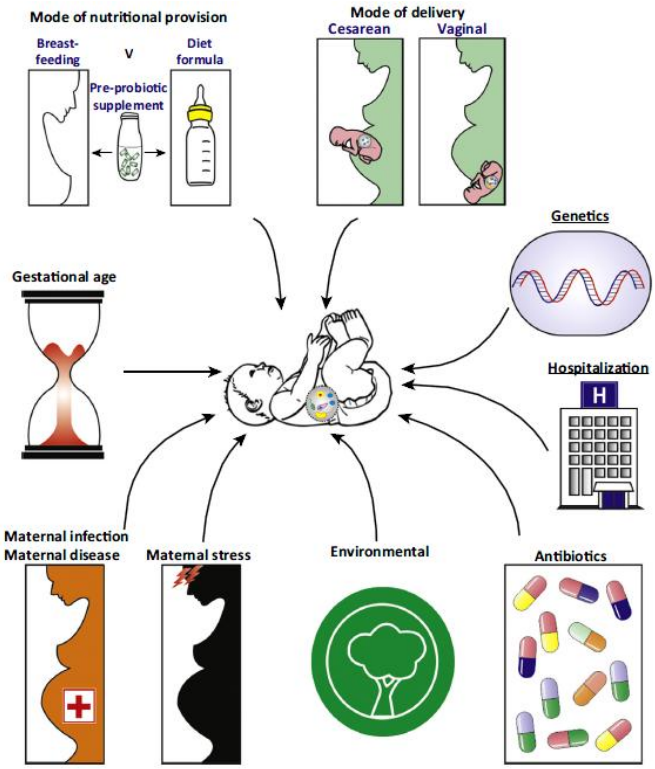

Figure 1.3. Factors influencing the development of the infant microbiota. In addition to mode of birth, mode of early nutrition, environment, other factors such as gestational age, genetics, and hospitalization, also influence the microbial composition of the infant. Infections and antibiotic usage influence the development of microbial landscape as does the selective transient enrichment by probiotics and prebiotics (modified from Borre et al., 2014).

In addition to a range of medications used by the elderly, impaired digestive and motility functions, and thus malabsorption of nutrients, and a weakened immune system contribute to compromised diversity and stability of the gut microbiota composition (Biagi et al., 2013). The ENS is plastic and continues to undergo changes throughout life, as the gut grows and responds to dietary and other environmental factors. Thus the effects of the microbiota and the complex interactions between enteric ganglion cells, mucosal immune system and intestinal epithelium indicate that other factors may well influence aging of enteric neurons (Saffrey, 2013). The CNS development continues during adulthood. The brain reaches its maximal weight by approximately 20 years of age (Dekaban, 1978), while white matter volume continues to increase and the peak of myelination is observed at approximately 50 years of age (Sowell et al., 2003). Although adulthood does not appear to be a critical or vulnerable phase, it remains a period during which alterations in the microbiota can influence brain and

behavior. Therefore, maintaining a healthy gut microbiota and mental health is an important aspect in possible prevention or attenuation brain disorders associated with aging.

1.3 Toll-like receptors

All living organisms are constantly exposed to environmental microorganisms, thus they need to cope with their potential invasion into the body. The vertebrate immune response can be divided into innate and acquired immunity. The innate immune system is the first line of host defence against pathogens and is mediated by phagocytes including macrophages and dendritic cells (DCs; Akira et al., 2006). In fact, to control the infection during the first days, the organism, through innate immune system, modulates some important functions including opsonization, activation of complement, coagulation cascades, phagocytosis, activation of proinflammatory signaling cascades and apoptosis (Janssens and Beyaert, 2003). By contrast, acquired immune responses are slower processes, in the late phase of infection, which are mediated by T and B cells, both of which express highly diverse antigen receptors that are generated through DNA rearrangement and are thereby able to respond to a wide range of potential antigens and to generation of immunological memory (Akira et al., 2006). This highly sophisticated system of antigen detection is found only in vertebrates and has been the subject of considerable research. Far less attention has been directed towards innate immunity, as it has been regarded as a relatively nonspecific system, however is able to discriminate between self and a variety of pathogens and to present antigen to the cells involved in acquired immunity (Akira et al., 2006). Also the innate immune system has an important function in activation and shaping of the adaptive immune response through the induction and release of co-stimulatory molecules and cytokines (Medzhitov, 2007) (Figure 1.4). In contrast to the clonotypic receptors, expressed by B and T lymphocytes, the innate immune system uses nonclonal sets of recognition molecules, called pattern recognition receptors (PRRs; Janssens and Beyaert, 2003) (Figure 1.4).

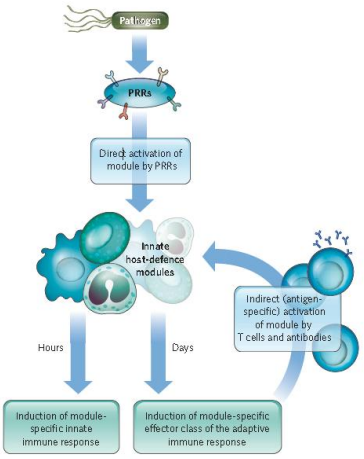

Figure 1.4. Activation of host-defence mechanisms (modified from Medzhitov, 2007).

The Toll like receptors (TLRs) are one of the most important PRRs families. The discovery of the TLRs started with the identification of the receptor 'Toll', a protein expressed in *Drosophila melanogaster* and involved in controlling embryonic development (Akira and Takeda, 2004; Okun et al., 2011). Subsequent genetic studies have led to the discovery of genes important in the dorsal-ventral patterning of the embryo (i.e., the dorsal group of genes, including Toll, tube, pelle, cactus, the NF-κB homolog dorsal, and seven genes upstream of Toll; Belvin and Anderson, 1996). Since NF-κB is involved in mammalian immunity, gradually became evident the contribution of TLRs in the signaling pathways in regulating *Drosophila* embryonic development and activating the immune system (Wasserman, 1993). In 1995 Hultmark and colleagues first identified Toll-1 as an activator of the immune response in a *Drosophila* cell line. Around the same time, a human homolog of Toll was identified and mapped to chromosome 4p14 (Taguchi et al., 1996). Later on, an in vivo study in *Drosophila* demonstrated that the Toll signaling is involved also in the antifungal response (Lemaitre et al., 1996). In 1997 the first mammalian TLR was described by the group of Medzhitov. Subsequently, five human TLRs have been characterized (Rock et al., 1998) that are involved only in controlling immune responses with no role in the development whereas the *Drosophila* Toll pathway is implicated both in immunity and developmental processes (Valanne et al., 2011). TLRs are type I transmembrane proteins responsible in the recognition of foreign pathogens referred to as pathogen-associated molecular patterns (PAMPs). PAMPs are well suited to innate immune recognition for three main reasons: i) they are invariant among microorganisms of a given class; ii) they are products of pathways that are unique to microorganisms, allowing discrimination between self and non-self molecules; iii) they have essential roles in microbial physiology, limiting the ability of the microorganisms to evade innate immune recognition through adaptive evolution of these molecules (Medzhitov, 2007). Bacterial PAMPs are often

components of the cell wall, such as lipopolysaccharide (LPS), peptidoglycan (PG), lipoteichoic acids (LTA) and cell-wall lipoproteins. An important fungal PAMP is beta-glucan, which is a component of fungal cell walls but also viral nucleic acids structures are recognized by TLRs. An important aspect of pattern recognition is that PRRs themselves do not distinguish between pathogenic microorganisms and symbiotic (non-pathogenic) microorganisms, because the receptor ligands are not unique to pathogens (Medzhitov, 2007). So far, 10 and 12 functional TLRs have been identified in humans and mice, respectively, with TLR1–TLR9 being conserved in both species. Mouse TLR10 is not functional for a retrovirus insertion, and TLR11, TLR12 and TLR13 have been lost from the human genome (Kawai and Akira, 2010) (Table 1.1). Studies in mice deficient in each single TLR type have demonstrated that every TLR has a distinct function in terms of PAMP recognition and activation of immune responses (Akira et al., 2006).

TLR1, 2, 4 and 6 recognize lipid-based structures. TLR4 recognizes LPS from Gram-negative bacteria, which causes septic shock (Akira et al., 2006). TLR2 forms heterodimers with TLR1 and TLR6 and in concert with TLR1 or TLR6 discriminates between the molecular patterns of triacyl and diacyl lipopeptide, respectively, which derived from Gram-positive bacteria, mycoplasma and mycobacteria (Kumar et al., 2009). TLR5 and 11 recognize protein ligands. TLR5 is expressed abundantly in intestinal CD11c-positive *lamina propria* cells where it senses bacterial flagellin (Uematsu and Akira, 2006). TLR3, 7, 8 and 9, being localized intracellularly, detect nucleic acids derived from viruses and bacteria. TLR3 was shown to recognize double stranded RNA (dsRNA) generally produced by many viruses during replication. TLR7 recognizes synthetic imidazoquinoline-like molecules, guanosine analogs such as loxoribine, single stranded RNA (ssRNA) derived from viruses and small interfering RNA (Akira et al., 2006) (Table 1.1). TLRs are expressed on a variety of cells, including immune cells, such as macrophages, DCs, B cells, specific types of T cells, and also fibroblasts, epithelial cells and neurons. Expression of TLRs is not static but rather is modulated rapidly in response to pathogens, an array of cytokines and environmental stressors (Akira et al., 2006). Furthermore, TLRs may be expressed extracellularly or intracellularly. While certain TLRs (TLRs 1, 2, 4, 5, and 6) are expressed on the cell surface, others (TLRs 3, 7, 8, and 9) are found almost exclusively in intracellular compartments such as endosomes, and their ligands, mainly nucleic acids, require internalization to the endosome before receptor signaling is possible (Akira et al., 2006).

The engagement of TLRs by microbial components triggers the activation of signaling cascades, leading to the induction of genes involved in antimicrobial host defense. TLRs are characterized by an ectodomain composed of leucine rich repeats (LRR) that are responsible for recognition of PAMPs and a cytoplasmic domain homologous to the cytoplasmic region of the IL-1 receptor, known as the TIR domain, which is required for downstream signalling

(Kawai and Akira, 2007).

After ligand binding, TLRs dimerize and undergo conformational changes required for the recruitment of TIR-domain-containing adaptor molecules of the TLR (Akira et al., 2006). The adaptor molecules include myeloid differentiation factor 88 (MyD88), TIR-associated protein (TIRAP)/MyD88-adaptor-like (MAL), TIR-domain-containing adaptor protein-inducing IFN-b (TRIF)/TIR-domain-containing molecule 1 (TICAM1) and TRIF-related adaptor molecule (TRAM; Oshiumi et al., 2003; Yamamoto et al., 2002) (Figure 1.5).

TLR	Location of TLR	PAMPs recognized by TLR	Co-receptor (s)	Signaling adaptor		Effector cytokines induced
TLR1/2	Plasma membrane (cell surface)	Triacyl lipopeptides (Bacteria and Mycobacteria)	Hetrodimer of TLR1/2 forms a functional receptor	TIRAP, MyD88	NFκB	Inflammatory cytokines (TNF-α, IL-6 etc.)
TLR2	Plasma membrane (cell surface)	Peptidoglycan (Gram-positive bacteria), LAM (Mycobacteria), Hemagglutinin (Measles virus), Phospholipomannan (*Candida*), Glycosylphosphophatidyl inositol mucin (*Trypanosoma*)	CD36, RP105	TIRAP, MyD88	NFκB	Inflammatory cytokines (TNF-α, IL-6 etc.)
TLR3	Endosome	ssRNA virus (WNV), dsRNA virus(Reovirus), RSV, MCMV		TRIF	NFκB, IRF3,7	Inflammatory cytokines (TNF-α, IL-6 etc.), type I IFNs
TLR4	Plasma membrane (cell surface)	LPS (Gram-negative bacteria), Mannan (*Candida*), Glycoinositolphospholipids (*Trypanosoma*), Envelope proteins (RSV and MMTV)	MD2, CD14, LBP, RP105	TIRAP, MyD88, TRAM and TRIF	NFκB, IRF3,7	Inflammatory cytokines (TNF-α, IL-6 etc.), type I IFNs
TLR5	Plasma membrane (cell surface)	Flagellin (Flagellated bacteria)		MyD88	NFκB	Inflammatory cytokines (TNF-α, IL-6 etc.)
TLR6/2	Plasma membrane (cell surface)	Diacyl lipopeptides (Mycoplasma), LTA (Streptococcus), Zymosan (*Saccharomyces*)	Hetrodimer of TLR6/2 or dectin-1 forms a functional receptor	TIRAP, MyD88	NFκB	Inflammatory cytokines (TNF-α, IL-6 etc.)
TLR7	Endosome	ssRNA viruses (VSV, Influenza virus)		MyD88	NFκB, IRF7	Inflammatory cytokines (TNF-α, IL-6 etc.), type I IFNs
TLR8[#]	Endosome	ssRNA from RNA virus		MyD88	NFκB, IRF7	Inflammatory cytokines (TNF-α, IL-6 etc.), type I IFNs
TLR9	Endosome	dsDNA viruses (HSV, MCMV), CpG motifs from bacteria and viruses, Hemozoin (Plasmodium)		MyD88	NFκB, IRF7	Inflammatory cytokines (TNF-α, IL-6 etc.), type I IFNs
TLR11[□]	Plasma membrane (cell surface)	Uropathogenic bacteria, profillin-like molecule (*Toxoplasma gondii*)		MyD88	NFκB	Inflammatory cytokines (TNF-α, IL-6 etc.)

Table 1.1 Descriptions of TLR location and characteristics (modified from Kumar et al., 2009).

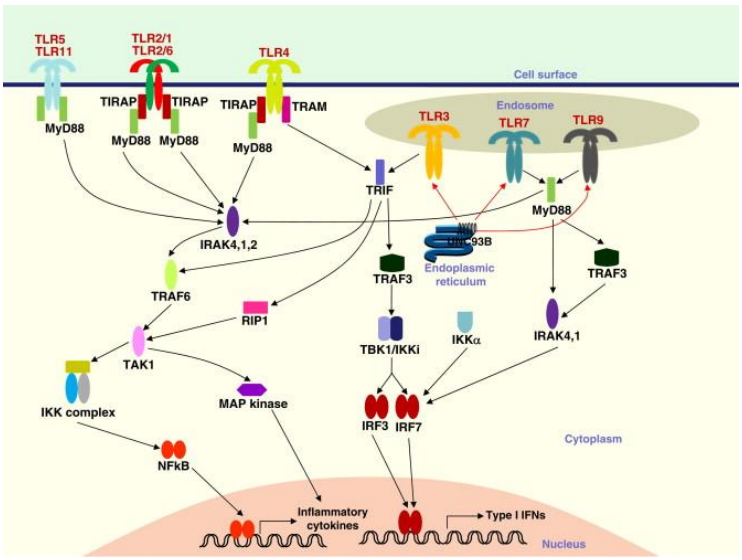

Figure 1.5. TLR signaling in conventional dendritic cells, macrophages and plasmatoid dendritic cells (modified from Kumar et al., 2009).

The differential responses mediated by distinct TLR ligands can be explained in part by the selective usage of these adaptor molecules. MyD88 and TRIF are responsible for the activation of distinct signaling pathways, leading to the production of pro-inflammatory cytokines and type I IFNs, respectively (Kumar et al., 2009).

MyD88 is a universal adapter that activates inflammatory pathways; it is shared by all TLRs with the exception of TLR3. For the complexity of the pathway, the TLR signaling pathway is categorized into MyD88-dependent and TRIF-dependent pathways (Akira et al., 2006). Upon stimulation, MyD88 associated with the portion of TLRs recruits IL-1R associated kinase (IRAK), which leads to the activation of TNF receptor associated factor 6 (TRAF6) to promote stimulation of TAK1 which results in the activation of MAP kinase or NF-κB through IKK complex, resulting in the induction of genes involved in inflammatory response (Akira et al., 2006). Also TIRAP mediates the activation of MyD88-dependent pathway. While TRIF activates an alternative pathway to induce a production of inflammatory cytokines and type I interferons (INFs). TRIF interacts with receptor-interacting protein 1 (RIP1), through a MyD88-independent way, determining the production of several cytokines (Kawai and Akira, 2007). The TRIF-dependent pathway induces INFs through IRF3 that is phosphorylated and activated by IKK-related kinase (TBK1 and IKKi) via TRAF3, a linker between TRIF and TBK1 (Kumar et al., 2009). TLR9 and TLR7 mediated INFs secretion in a MyD88-dependent manner, in contrast to TLR3 and TLR4 that produce TRIF-dependent IFN response (Kumar et al., 2009) (Figure 1.5).

TLRs play also a role in the cross-talk between the intestinal microbiota and the host, as they specifically recognize conserved microbial molecular structures, called microbe-associated

molecular patterns (MAMPs) (Martin et al., 2010). Any alterations of these interactions can lead to the development of several bowel disorders including inflammatory bowel disease (Gribar et al., 2008).

TLR4 has been found to detect LPS, a major component of Gram-negative bacteria cell walls (Okun et al., 2011). The stimulation of TLR4 by LPS determines in the activation of MyD88-dependent and MyD88-independent pathways, leading to the production of several inflammatory cytokines and IFN-beta, associated with the expression of IFN-inducible genes, (Akira and Takeda, 2004). Study performed by Anitha and colleagues (Anitha et al., 2012) focused on the role of TLR4 signaling in the colon, found that the TLR4 pathway is important for the survival of enteric neurons and for the maintenance of GI functions.

TLR2 is expressed in a wide variety of cells and plays a major role in detecting a large variety of PAMPs, in particular derived by Gram-positive bacteria. TLR2 recognizes PG and LTA, which are present in the cell membrane of Gram-positive bacteria (Schwadner et al., 1999; Takeuchi et al., 1999). TLR2 interacts physically and functionally with TLR1 and TLR6, which appear to be involved in the discrimination of subtle changes in the lipid portion of lipoproteins (Ozinsky et al., 2000). The importance of TLR2 in the host defense against Gram-positive bacteria has been demonstrated using TLR2-deficient ($TLR2^{-/-}$) mice, which have been found to be highly susceptible to challenge with *Staphylococcus aureus* or *Streptococcus pneumoniae* (Takeuchi et al., 2000; Echchannaoui et al., 2002). TLR2 appears also to have a crucial role in host defense against extracellular growing of Gram-positive bacteria (Takeda and Akira, 2004) and it seems to be a major player in gut homeostasis by exerting cytoprotective effects in intestinal epithelial cells. Indeed, the absence of TLR2 increases susceptibility to intestinal injury and inflammation (Cario et al., 2007). Polymorphisms in TLRs genes or in general a defective immune response, are involved in the initiation and perpetuation of chronic inflammation in inflammatory bowel diseases (IBD; Pierik et al., 2006).

Apart from PAMPs/MAMPs-derived ligands, TLRs also sense endogenous molecules released from stressed or dying cells—termed damage- or danger-associated molecular patterns (DAMPs), mainly derived from tissue damaged by oxidative stress. For example, TLR4 recognizes heat shock protein (Hsp) 60, Hsp 70 and fibrinogen and TLR2 recognizes Hsp 70, hyaluronan, and versican, as most recently shown (Kim et al., 2009). After recognition of DAMPs, TLRs activate and orchestrate several innate immune machineries, promoting apoptosis and shaping adaptive immune responses, but the deregulation of this response can lead to inflammatory collateral tissue damage and some forms of autoimmunity and autoinflammatory diseases (Land, 2015).

TLRs are also expressed in several residing cells of the CNS such as astrocytes, microglia, oligodendrocytes and neurons, and the regulation of their expression seems to be dynamic and associated with profoundly changes during aging (Letiembre et al., 2007). In the brain, TLRs can be activated not only after the invasion of pathogens but also in the absence of microbial infection (Zhang and Schluesener, 2006) and regulate neurogenesis through the release of growth factors (Rolls et al., 2007). Okun and colleagues suggested the existence of a paradigm in which exists an auto-regulation of the innate immune system in the CNS, which helps to prevent excessive inflammation during pathogen infections (Okun et al., 2009). Microglial cells are the “pivotal” players in the innate regulation of inflammatory responses in the CNS and, once activated bzy inflammatory stimuli, operate to maintain CNS integrity, however in case of massive and uncontrolled release of proinflammatory mediators, microglia may cause severe neuronal damage (Aravalli et al., 2007). Also it should be noted that changes in the permeability of blood-brain barrier (BBB) are crucial for the infiltration of antibodies and lymphocytes from the peripheral site, leading to the degeneration of the neuronal structure (Nguyen et al., 2002). In this case the condition is critical, because alterations of BBB are probably linked to increased vulnerability of CNS cells and excessive innate immune responses together with the production of cytokine, a condition known as excitotoxicity (Nguyen et al., 2002). Alternatively, a peripheral challenge can generate a systemic inflammation with the secretion of molecules of innate immune system that are able to cross the BBB and damage the CNS (Yang et al., 2000). In fact some authors demonstrated that perturbed stability of the BBB is pesent in neurodegenerative disease such as Alzheimer’s disease, stroke and amyotrophic lateral sclerosis (Huber et al., 2001). This instability of the barrier is associated with a severe inflammation and overexpression of TLRs, and the dynamic expression of these receptors seem to be involved in the progression of neurodegenerative pathologies in both normal aging and age-related disease (Okun et al., 2009).

1.4. Irritable Bowel Syndrome: a chronic functional gastrointestinal disorder

Functional gastrointestinal disorders (FGIDs) are the most common GI motility disorders in the general population. The term "functional" is generally applied to disorders where the body's normal activities in terms of the movement of the intestines, the sensitivity of the nerves of the intestines, or the way in which the brain controls some of these functions are impaired (Drossman, 2006). Irritable Bowel Syndrome (IBS) is one of the main chronic FGID and is reported to occur in 10-20% of the general population in developed countries (Simsek, 2011).

Widespread symptoms associated with the IBS are abnormal defecation and abdominal pain, both of which may be exacerbate by emotional stress. Abnormal defecation can be diarrhea (IBS-D) or constipation (IBS-C), and a subgroup of IBS patients may alternate from one to the other over time (mixed IBS; IBS-M). Urgency to stool often accompanies the diarrheal-state, and patients with the constipation-predominant form of IBS report abdominal tension and the feeling of incomplete evacuation (Wood, 2002). IBS is the most common disorder seen by gastroenterologists and can be associated with significant emotional distress, impaired health-related quality of life, disability, and high health care costs (Quigley, 2003). The prevalence of IBS within an industrialized community is between 10% and 25% (Jones and Lydeard, 1992; Agreus et al., 1995; Thompson et al., 2002; Husain et al., 2008). A recent meta-analysis study showed a pooled estimate of international IBS prevalence of 11.2% with variation by geographic region; the lowest occurring in South Asia (7.0%) and the highest in South America (21.0%) (Lovell and Ford, 2012). IBS is reported more frequently by women than men in Western countries, female–male odd ratio being 2:1 and seems to be more common in the ages between 20 and 40. Traditionally, IBS can affect people at any age, but it is not diagnosed in people after the age of 60, when organic diseases of the gut become more frequent (Garnett, 1999; Camilleri, 2001; Bennett and Thalley, 2002). The disorder cannot be explained by specific pathophysiologic mechanisms, since it is not associated with any structural finding or biological marker (Mach, 2004). However, the symptoms of IBS are related to combinations of several known physiological determinants such as abnormal motor reactivity, enhanced visceral hypersensitivity, altered mucosal immune and inflammatory functions (which includes changes in bacterial flora), and altered brain-ENS regulation, which is influenced by psychosocial and socio-cultural factors (Drossman, 2006; Ohman and Simren, 2010; Simren et al., 2013).

Diagnosis of IBS

The definition and classification of symptomatic manifestations of IBS is the result of careful evaluation by the clinical and scientific community for several decades. In order to standardize and define IBS and reduce unnecessary surgery, Manning and colleagues proposed six diagnostic criteria for discriminating patients with IBS from those with other organic diseases (Manning et al., 1978; Talley et al., 1990) (Table 1.2). Four symptoms have been found to be significantly more common in patients with IBS, which are visible abdominal distension, relief of abdominal pain with a bowel movement, more frequent bowel movements with the onset of abdominal pain and looser bowel movements with the onset of abdominal pain. Manning criteria were then developed through expert consensus to create the Rome criteria, of which

are known three versions: Rome I, II, and III (Thompson et al., 1999; Longstreth et al., 2006; Table 1.2).

Manning (1978)	Rome I (1989)	Rome II (1999)	Rome III (2006)
2 or more of the following symptoms: Abdominal distension Pain relief with defecation Frequent stools with pain Looser stools with pain Passage of mucus Sensation of incomplete evacuation	At least 3 months of continuous or recurrent abdominal pain: Relieved with defecation or Associated with change in stool consistency With at least 2 of the following on at least 25% of days: Altered stool frequency Altered stool form Altered stool passage Passage of mucus Bloating or abdominal distension	At least 12 weeks in past 12 months of continuous or recurrent abdominal pain or discomfort With at least 2 of the following: Relief with defecation Altered stool frequency Altered stool form Onset of symptoms more than 12 months before diagnosis	At least 3 days per month in past 12 weeks of continuous or recurrent abdominal pain or discomfort With at least 2 of the following: Relief with defecation Altered stool frequency Altered stool form Onset of symptoms more than 6 months before diagnosis

Table 1.2. Comparison of the Manning and Rome diagnostic criteria frequently used for diagnosis of IBS (modified from Canavan et al., 2014).

According to the most recent Rome III criteria, IBS, which belongs to the category C1 (bowel) of FGIDs, is more specifically defined as pain associated with change in bowel habit, and this is distinct from functional diarrhea (C4) characterized by loose stools and no pain, or functional bloating (C2) when there is no change in bowel habit. Each condition also has different diagnostic and treatment approaches (Drossman, 2006). The Rome scoring system is primarily a descriptive instrument of key value in the clinical management of IBS patients, however it may be not informative and poorly applicable to predict the molecular mechanisms and associated gene products that contribute to the different phenotypes (D'Amato, 2013).

Pathophysiology of IBS

IBS is defined as disorder with multifactorial etiology (Figure 1.6). Currently, the model for IBS incorporates several mechanisms, including visceral hypersensitivity, abnormal GI motility and secretion together with mucosal inflammation due to enteric infection, stress, food allergy and dysbiotic changes in gut microbiota (Daulatzai, 2014). The pathophysiology of IBS, still inadequately clear, is attributed to multilevel dysregulation of the nervous and intestinal system interactions (Ohman and Simrén, 2007). The brain-gut axis constitutes a bidirectional signaling pathway between the CNS and the GI tract that preserves the health of the body as a whole. This signaling pathway is regulated at multiple neural, hormonal and immunological

levels (Karling et al., 2007). Alterations presented in any of these levels (e.g. autonomic nervous system dysregulation, altered serotonin metabolism, mast cell activation) contribute to the presentation of the disorder (Gasbarrini et al., 2008; Kennedy et al., 2012) (Figure 1.6).

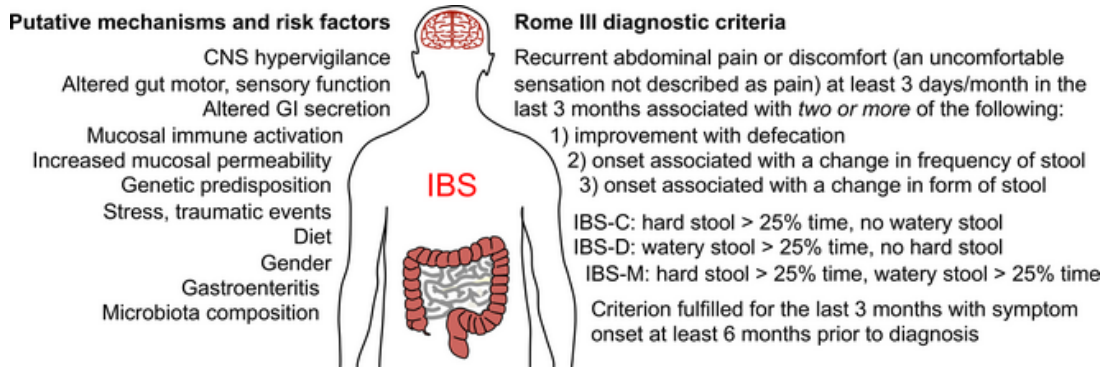

Figure 1.6. Rome III criteria for the classification of IBS, and underlying predicted risk factors and pathogenetic mechanisms (modified from D'Amato, 2013)

Abnormal GI Motility

Disturbance of GI motility occurs in about 25–75% of patients with IBS (Drossman et al., 2002). Many studies identified abnormal patterns of contractile and electrical activity in the colon of patients with IBS. In D-IBS, patients show a greater number of fast colonic contractions and propagate contractions with subsequent accelerated transit. Conversely, patients with constipation predominant IBS have a decreased number of fast colonic and propagated contractions, and fewer high amplitude propagated contractions with slowed whole gut transit (Garnett, 1999). In patients with abdominal pain, a group of brief propagated intestinal contractions were identified, which are significantly increased compared with healthy controls (Muller-Lissner et al., 1999; Mach, 2004). Several factors such as strong emotion or environmental stress can lead to increased motility in the small intestine and colon of healthy subjects. IBS, however, is characterized by having an even greater motility response to stressors (psychological or physiological) when compared to normal subjects (Locke et al., 2000; Drossman et al., 2002).

Visceral Hypersensitivity

Visceral hypersensitivity, modulated by several external and internal factors, is considered the most important factor in the pathophysiology of IBS and it helps to explain the association of pain with GI motility disorders, which leads to alterations in defecation patterns (diarrhea or constipation) (Katsanos et al., 2012). In laboratory practice, GI hypersensitivity is typically measured in response to the distension of a particular area of the GI tract. In the case of clinical studies, the subject has a balloon catheter inserted into the colorectal region and

graded isobaric or isovolumetric distensions are performed with the individual reporting their perception of the stimulus. It has been shown that many patients with IBS report lowered thresholds for all stimuli compared to healthy volunteers, and visceral hypersensitivity may be generally amplified in a subset of patients with IBS (Mertz et al., 1995; Lowen et al., 2015; Moloney et al., 2016). Repetitive balloon inflations in the colon lead to a progressive increase in pain intensity that occurs longer and to a greater degree in patients with IBS than in controls (Munakata et al., 1997; Drossman, 2006). In the gut, extrinsic nociceptors can respond to different kind of stimuli, depending on receptor expression, including stretch, pH, bacterial products, substances released from immune cells, and neurotransmitters released from the ENS or enterochromaffin cells (Sengupta, 2009). Hypersensitivity and sensitization may occur through altered receptor sensitivity at the gut mucosa, at the submucous and myenteric plexa, which may be enabled by mucosal inflammation, degranulation of mast cells close to enteric nerves, or increased serotonin activity, possibly enhanced by alteration of the bacterial environment or infection (Spiller, 2003; Barbara et al., 2004; Dunlop et al., 2005).

The nociceptors have nerve endings throughout the layers of the GI tract (mucosal, submucosal, muscular layers), and their cell bodies are located in the dorsal root ganglion (DRG) of spinal cord (Figure 1.7). Then, the nociceptive signal is transmitted to the brain throughout the controlateral side of the spinal cord, and reaches cortical areas for localization and limbic areas for the emotional component of the pain response (Figure 1.7). In IBS an increased excitability with consequent central sensitization has been hypothesized (Moloney et al., 2016). A chronic or repetitive visceral stimulation may result in the growth of the dorsal horn of spinal cord, amplifying the throughput to the CNS (Moloney et al., 2016).

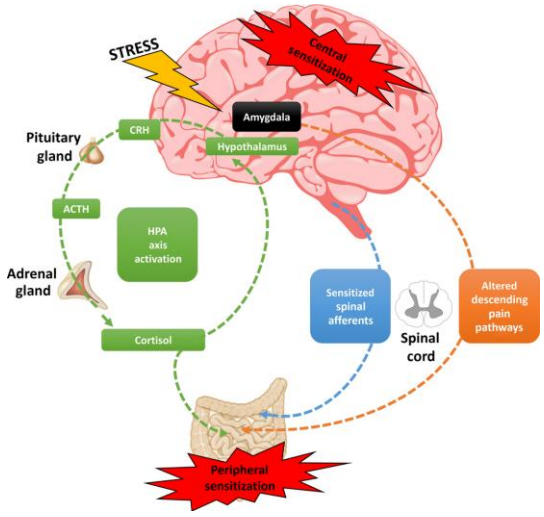

Figure 1.7. Central and peripheral pain sensitization. Increased pain perception can occur due to a combination of both central and peripheral sensitization (modified from Moloney et al., 2016).

Gut microbiota and inflammation in IBS

The composition of the human gut microbiota as well as gut host immune homoeostasis may be crucial in the healthy status (Hébuterne, 2003; Guigoz et al., 2008). In physiological conditions, the gut microbiota has a balanced composition that ensures the host health, and disruption of this equilibrium (dysbiosis) confers disease susceptibility (Gibson et al., 2014). Moreover, several animal models (Bercík et al., 2004; Akiho et al., 2005; Verdù et al., 2006; Kanazawa et al., 2014) as well as clinical observations (Rao et al., 1987) have demonstrated that altered immune function and inflammation in the GI tract (as well as GI infections and dysbiosis) affect motility and sensitivity of the gut, two of the key pathophysiological factors in IBS (Akiho et al., 2005; Verma-Ghandu et al., 2007).

Epidemiological and clinical studies revealed that a group of patients with IBS develops their chronic GI symptoms with the onset of gastroenteritis (postinfectious IBS) (Spiller and Garsed, 2009). In fact, meta-analyses demonstrated a six fold to sevenfold increased risk of developing IBS after a gastroenteritis episode, which makes GI infections the best-characterized and probably strongest known risk factor for the development of this disease (Halvorson et al., 2006; Thabane et al., 2007). Infective gastroenteritis produces a profound depletion of the commensal microbiota (Lupp et al., 2007) whose production of metabolites such as SCFAs together with the exposure to antibiotics normally determine the inhibition pathogen colonization (Barthel et al., 2003). Abnormalities in gut microbiota following enteric infections, associated with an increase in the permeability of epithelial gut barrier, might activate intestinal mast cells and monocytes triggering thus an immune response (Ohman and Simren, 2010). Mast cells are important elements in the pathogenesis of inflammatory and non-inflammatory bowel disorders, as they can cause stimulation of nerve endings, modulate inflammation and affect intestinal motility (Farhadi et al., 2007). Increased numbers of mast cells in close proximity to nerves in the colonic mucosa is one of the most frequently reported features of immune activity in IBS (Öhman et al., 2015). On the other hand, degranulation of mast cells after enteric infection or allergy might be the prime event that leads to the cascade of gut hypersensitivity through various mediators (Gui, 1998). Moreover, there is a recent demonstration that IBS patients have altered colonic mucosal expression of receptors recognizing specific microbiota-related substances (such as Toll-like receptor, TLRs). Increased mRNA levels of TLR2, TLR4 and TLR5 associated to mRNA levels of TLR7 and TLR8 have been found, in the colonic mucosa of patients with IBS, and these findings have also been confirmed at the protein level by immunohistochemical studies (Brint et al., 2011; Belmonte et al., 2012). Increased expression of TLR2 in blood monocytes from patients with IBS has also been

reported (Ohman et al., 2015). The expression of TLRs is upregulated by exposure to molecular structures of bacteria and viruses, which derived from altered microbiota. The crosstalk between luminal microbiota and the intestinal immune system influences GI motility (as demonstrated in animal models). For instance, the absence of TLR4, a receptor commonly expressed on innate immune cells that recognizes lipopolysaccharide from Gram-negative bacteria, results in substantial reduction in GI motility in mouse models (Anitha et al., 2012). Moreover, intestinal macrophages regulate peristaltic activity of the colon by changing the pattern of smooth muscle cell contractions in the presence of luminal microbiota (Muller et al., 2014; Robinette and Colonna, 2014).

Another reported immune-related feature in patients with IBS is increased levels of circulating proinflammatory cytokines such as IL‑6, IL‑8, TNF and IL‑1β (Scully et al., 2010; Chang et al., 2012), which may have an effect on epithelial barrier function. Thus, even a quite small increase in levels of proinflammatory cytokines at the epithelial barrier can lead to increased intestinal permeability, and thereby altered homeostasis at the mucosal border (Ohman et al., 2015).

Psychosocial Factors

It is well known that IBS reduces quality of life, which may have psychological consequences (Mach, 2004). In healthy subjects psychological stress affects GI function and produces symptoms that are emphasized in patients with IBS (Kroenke and Mangelsdorff, 1989). Psychiatric problems such as somatization, anxiety, hypochondriasis, depression, and phobia are common in about 50% of patients with IBS at the time of diagnosis (Quigley, 2003). These factors in patients with IBS influence not only the illness experience, but also the treatment outcome. Psychosocial and sociocultural factors include: a history of emotional, sexual, or physical abuse, stressful life events, chronic social stress, or anxiety disorder, and maladaptive coping style and often some of these may occur early in life (Drossman et al., 2002). These factors can be reduced or “buffered” by adaptive coping skills and social support, and the psychosocial response of family, society, and culture can also have a palliative effect on the illness experience.

Gut-brain axis

From the evidence discussed above, it is clear that IBS is a multifaceted disorder where both central and peripheral factors are involved; thus, it is most commonly described as a biopsychosocial disorder of the gut-brain axis. The gut–brain axis encompasses a number of fundamental elements, including the CNS, the autonomic nervous system (ANS) (sympathetic

and parasympathetic), the ENS, the neuroendocrine hypothalamic–pituitary–adrenal axis (HPA) axis, and neuroimmune systems, and more recently has expanded to include the gut microbiota, which fulfill key roles in bidirectional communication (Bercik et al., 2011; Cryan and Dinan, 2012; Burokas et al., 2015). The microbiota–gut–brain axis is pivotal in maintaining homeostasis and is involved in the control of diverse physiological functions including motor, sensory, autonomic, and secretory functions of the GI tract to regulate an array of processes from energy metabolism to mood regulation (Burokas et al., 2015; Dinan et al., 2015).

Communication between CNS and ENS implies a bidirectional connection system: the brain influences the function of the ENS, and the gut influences the brain via vagal and sympathetic afferents. The ENS independently controls gut function, the migrating motor complex, and peristalsis, but it is constantly monitored and modified by CNS via both vagal and sympathetic extrinsic nerves. Thus, the IBS symptoms may be caused by dysfunctions either primarily in the CNS, or in the gut, or by a combination of both (Moloney et al., 2016).

Genetic Predispositions

Epidemiological studies of familial aggregation suggest a genetic contribution to IBS (Camilleri and Katzka, 2012). Genetic factors may predispose some individuals to develop typical symptoms observed in IBS whereas in others, environmental factors contribute to the phenotypic expression of these conditions (Drossman, 2006).

Serotonin is predominantly known for its role in the brain, where it functions as a neurotransmitter; however, approximately 95% of serotonin in the body is contained within the gut, specifically, in the enterochromaffin cells of the mucosa and in the nerve terminals of the ENS neurons. In peripheral tissues, serotonin is involved in regulation of GI motility, secretion, and sensory perception (Costedio et al., 2007; Mc Lean et al., 2007). Its central functions include the regulation of mood, cognitive function, and central processing of sensory signals involved in pain perception (Wrase et al., 2006; Yano et al., 2015). Serotonin signaling may be a key linker in communication along gut-brain axis (O'Mahony et al., 2015), thus dysfunction of these pathways may underlie the pathological symptoms present in both GI and mood disorders, and may also explain the high comorbidity of these disorders (Camilleri and Katzka, 2012; Moloney et al., 2016). For this reason, polymorphisms related to neurotransmitter levels, such as serotonin reuptake transporter, and signaling, such as serotonin receptors, are being explored for understanding IBS pathophisiology.

Several studies also have emphasized the role of CNS-related genetic abnormalities in IBS. The ANS is involved in the regulation of GI motility and, consequently, alteration of this part of the nervous system is linked to IBS (Drossman, 2006; Katsanos et al., 2012). In general, adrenergic

transmission, as part of the autonomic nervous system, directly modulates gut function and sensation (Bharucha et al., 1997; Viramontes et al., 2001). The α2-adrenergic receptor has also been proposed as a mechanism whose genetic variation may modify motor and sensory functions in IBS (Kim et al., 2004). A relationship between IBS and commonly observed co-morbidities such as post-traumatic stress disorder, depression, and anxiety disorders is represented by the Val158Met polymorphism of catechol-O-methyltransferase enzyme. This genetic variation influences the human experience of pain and may underlie inter-individual differences in the adaptation and responses to pain and other stressful stimuli (Zubieta et al., 2003).

1.5 Role of cathecol-O-metyltrasferase in the pathophysiology of IBS

Catechol-O-methyltransferase (COMT) is one of the enzymes that catalyzes the methylation of catechol structures, including dopamine (DA), norepinephrine (NE), epinephrine, caffeine, and catechol estrogens (Axelrod and Tomchick, 1958). Expression of the COMT enzyme is under the control of two separate promoters. One gives rise to a short mRNA (1.3 kb in human and 1.6 kb in rat), which produces the soluble isoform (S-COMT) in the cytoplasm. The other produces the long mRNA (1.5 kb in human and 1.9 kb in rat), yielding both soluble and membrane-bound isoforms (MB-COMT) (Mannisto and Kaakkola, 1999). Humans *Comt* gene is located on chromosome 22q11.2 and it consists of 6 exons, the first two non-coding, while in the third are located the two promoters that control COMT isoforms expression (Salminen et al., 1990; Lundstrom et al., 1991). In peripheral tissues, the S-COMT is the most prevalent isoform of COMT enzyme and MB-COMT activity represents generally less than 5% of the total COMT activity (Guldberg and Marsden, 1975; Jeffery and Roth, 1984; Grossman et al., 1985). However, in some human tissues, like the brain, the amount of MB-COMT activity has been reported to be higher (Rivett et al., 1983; Tenhunen et al., 1994). The two isoforms of COMT are proposed to have at least partially distinct roles. It seems that at the concentrations of catecholamines (DA and NE) naturally present, MB-COMT may be more important in their metabolism (Roth, 1992). Therefore MB-COMT is the predominant enzyme at DA concentration of <10 μM and at NE concentration of <300 μM. In addition, MB-COMT also has a higher affinity for catechol substrates and a lower Km value for DA than S-COMT (Myöhänen et al., 2010). On the other hand, S-COMT is a high capacity enzyme isoform indicated by higher Vmax values than those of MB-COMT (Lotta et al., 1995).

In both mouse and human specimens, COMT expression has been detected in the liver, kidneys and GI tract (Nissinen et al., 1988). COMT has a protective role in the elimination of

biologically active or toxic catechols, particularly xenobiotics, acting as an enzymatic detoxifying barrier between the blood and other tissues (Mannisto and Kaakkola, 1999). COMT also inactivates L-DOPA, a catechol-containing drug used as a dopamine precursor in the treatment of Parkinson's disease (Ball et al., 1972; Guldberg and Marsden, 1975). Due to its harmful effect on the medication of Parkinson's disease, a great deal of interest has been directed towards the development of specific COMT inhibitors (Guldberg and Marsden, 1975; Mannisto and Kaakkola, 1999). Next to the earlier primary role of COMT in peripheral detoxification, it has emerged in the last years the importance of this enzyme in the central neurotransmission.

COMT protein is widely distributed in mammalian brain (Lundstrom et al., 1995; Mannisto and Kaakkola, 1999) and its enzyme activity regulates DA levels (Yavich et al., 2007). In the striatum, the synaptic action of DA is thought to be largely terminated by neuronal uptake by the abundant presence of DA transporters (Giros et al., 1996). However, in the prefrontal cortex DA transporters are expressed at low levels within synapses, thus alternative mechanisms, such as degradation by COMT, might be a key process in the regulation of DA availability in this brain area (Garris et al., 1993; Lewis et al., 2001; Wayment et al., 2001). In humans as well as in monkeys, rats, and mice, cortical DA signaling affects cognitive performance through a U-shaped relationship, showing that too little or too much DA having deleterious effects (Mattay et al., 2003; Vijayraghavan et al., 2007). For this reason, COMT has an important role in regulating higher cognitive processes in mammals via the modulation of central neurotransmission, especially DA (Chen et al., 2004; Papaleo et al., 2008). In CNS, genetic variations in human COMT have been associated with physiological functions (Egan et al., 2001; Winterer et al., 2006) and behavioral phenotypes related to prefrontal cortex and hippocampal information processing, including cognition (Blasi et al., 2005; Bertolino et al., 2006), anxiety (Drabant et al., 2006), obsessive-compulsive disorder (OCD) (Pooley et al., 2007), and pain sensitivity (Nackley et al., 2006). Thus, functional COMT genetic variations modulate multiple spheres of mammalian behavior, with remarkable analogy between human and mouse (Papaleo et al., 2008; Mier et al., 2010; Scheggia et al., 2012; Papaleo et al., 2012). A common polymorphism of this gene in human populations is Val158Met (rs4680). This polymorphism is a functional genetic variant determined by a valine to methionine substitution at codon 108 (S-COMT)/158 (MB-COMT) of the COMT gene and dramatically impacts COMT enzymatic activity (Lotta et al., 1995; Palmatier et al., 1999). At normal body temperature, the COMT-Val form leads to higher COMT protein levels and enzymatic activity compared with COMT-Met (Chen et al., 2004). Due to the potential importance of COMT in prefrontal DA neurotransmission, this common functional polymorphism has been studied in

association with neuropsychiatric disorders that may involve altered DA levels (Papolos et al., 1998; Eisenberg et al., 1999; Jones et al., 2001). Because there is only one COMT gene without any known tissue-specific splice variants (Tenhunen et al., 1994; Lundstrom et al., 1995), it is likely that the codon 108/158 polymorphism, causing changes in protein thermostability, also leads to functional alterations of COMT in all tissues (Lachman et al., 1996, Mannisto and Kaakkola, 1999).

Recently, it has been hypothesized an association between gut functional disorders such as IBS and COMT Val158Met polymorphism (Camilleri and Katzka, 2012; Karling et al., 2011). While IBS is multifactorial, with no single etiology to completely explain the disorder, many patients also experience comorbid behavioral disorders, such as anxiety or depression, thus IBS can be described as a disorder of the gut–brain axis (Moloney et al., 2016). In the brain, COMT-met variant, associated with low COMT activity, determines higher levels of DA and chronic activation of dopaminergic neurons which results in lower neuronal content of enkephalin and a decreased activity level of the endogenous pain inhibitory system (Zubieta et al., 2003). Experimental studies in humans (hypertonic saline infusion into the masseter muscle) showed that individuals with the met/met genotype exhibited diminished regional μ-opioid receptors activity, higher sensory and affective ratings of pain in response to painful stimuli compared to the val/val individuals (Zubieta et al., 2003; Karling et al., 2011). Low COMT activity has also been associated to chronic pain conditions such as facial pain (Marbach and Levitt, 1976; Diatchenko et al., 2005), fibromyalgia (Gürsoy et al., 2003) and with non-migraine headache (Hagen et al., 2006), whereas the val/val genotype has been associated to anxiety/ depression (Domschke et al., 2007).

On the background that both chronic pain syndromes (Whitehead et al., 2002) and anxiety/depression (Mayer et al., 2001; Garakani et al., 2003) are associated to low and high COMT activity, respectively, and both are related with IBS and IBS-like symptoms, these two genotypes may be a risk factor for the onset of IBS possibly via separate mechanistic routes, whereas the val/met (intermediate COMT activity) genotype can be considered the low risk, protective genotype (Karling et al., 2011).

2. AIM

The importance of mutual interactions among different cell populations, that constitute the enteric microenvironment (i.e. neurons, glial cells, smooth muscle cells, interstitial cells of Cajal, macrophages and microbes), in remodelling the neuronal network in response to a neuromuscular damage, is widely acknowledged, however, the molecular mechanisms are still largely unexplored. Gut microbiota seems to be directly involved in modulating the development and function of enteric nervous system (ENS), supporting the concept that changes in commensal microbiome composition, induced by infections or antibiotics, can perturb ENS integrity and activity. Several studies focused on the microbiome of animal models have underlined the "bottom-up" impact of microbes and their importance in governing ENS and CNS homeostasis. Neuronal circuitries in the ENS communicate with the CNS and vice versa via vagal and sympathetic extrinsic pathways, the so-called gut-brain axis, that in recent years has emerged as an important microbiota-to-brain communication pathway. The relationship between indigenous gut microbes and their host can shift from commensalism towards pathogenicity in diseases, such as inflammatory bowel disease (IBD), ischemia/reperfusion (I/R) injury and psychiatric disorders.

The mammalian gut must be sufficiently permeable to support absorption of nutrients, however, it must also avoid potentially damaging immune responses. Both nonpathogenic bacteria and activation of innate defenses help prevent pathogenic bacteria from crossing the mucosal barrier. When this homeostasis is perturbed, the immune system may react to both exogenous and endogenous 'danger signals' via activation of Toll-like receptors (TLRs). Polymorphisms in genes encoding TLRs, including TLR2 or TLR4, have been associated with different phenotypes of disease extent and severity in patients with GI disorders.

In this study we aimed to determine the impact of i) commensal microbiota depletion, ii) altered TLR2 and/or TLR4-mediated innate immunity response, iii) genetic-driven defective COMT activity, in the structural and functional integrity of murine ENS and consequently in gastrointestinal homeostasis.

3. MATERIALS AND METHODS

Mice

The first group of transgenic animals consists of male TLR2$^{-/-}$ (B6.129-Tlr2tm1Kir/J; postnatal days 21, P21) and age-matched wild-type (WT) C57BL/6J mice (Charles River Laboratories, Italy), that were used in order to examine the role of TLR2 signaling in maintaining ENS homeostasis and gut function. A second group consists of female heterozygous for COMT gene (C57BL/6J COMT$^{+/-}$, Charles River Laboratories, Italy) and wild-type (COMT$^{+/+}$, WT) mice (12±2 weeks) that were used to evaluate the influence of altered catecholaminergic transmission, due to COMT genetic reduction, in modulating gastrointestinal motility. These animals were provided by the research group of Dr. Francesco Papaleo from Italian Institute of Technology, Genova, Italy. Their genotypes were confirmed by PCR analysis of mouse tail DNA. All groups of mice were housed in the pathogen-free animal facility of the Department of Pharmaceutical and Pharmacological Sciences of the University of Padova under controlled environmental conditions (temperature 22° ± 2°C; relative humidity 60–70%) with free access to a standard diet and water, and maintained at a regular 12/12-h light/dark cycle. To normalize gut microbiota, mice colonies from both groups were housed in the same room and generally in the same cages and maintained by the same personnel. All experimental protocols were approved by the Animal Care and Use Ethics Committee of the University of Padova and by the Italian Ministry of Health and were in compliance with national and European guidelines for the handling and use of experimental animals.

Mice treatments

For depletion of intestinal microbiota male mice C57BL/6J (3±1 weeks old; Charles River Laboratories, Italy) were subjected to a previously published protocol that produces a germ free-like phenotype (Reikvam et al., 2011; Brun et al., 2013) which consisted in a cocktail of broad spectrum antibiotics (50 mg/kg vancomycin, 100 mg/kg neomycin, 100 mg/kg metronidazol and 100 mg/kg ampicillin, ABX group) (Figure 3.1), administered to mice by oral gavage (100 µl volume/mouse) every 12 hours for 14 days, using a stainless steel feeding tube without prior sedation of the animal. Control mice (CNTR group) were treated with vehicle (tap water). Microbiota-depletion efficacy was confirmed by performing 16S ribosomal RNA gene quantification in mouse feces, as described previously (Brun et al., 2013).

Antibiotic	Effect on the microbiota	Effect on immunity	References
Amoxicillin	*Lactobacillus* spp. depletion in SI ↓aerobic and anaerobic bacterial numbers in the colon	↓ MHC I and MHC II expression in SI and LI ↓AMPs expression in SI ↑mast cell proteases expression in SI	[54]
Metronidazole, neomycin and vancomycin	↓ bacterial numbers in SI and LI Multiple effects on composition, including: ↓ Bacteroidetes ↑ Enterobacteriaceae	↓ Reg3γ expression in SI	[4,11]
Metronidazole	Bacteroidales and *Clostridium coccoides* depletion ↑ Lactobacilli	↑ Reg3γ and IL-25 expression in colon ↑ numbers of macrophages and NK cells in colon ↓ mucus	[55]
Colistin	ND (Gram-negative spectrum)[a]	↓ numbers of ILFs	[20]
Ampicillin, neomycin, metronidazole, vancomycin	Microbiota depletion ↓ peptidoglycan levels in serum	↓neutrophil-mediated killing of pathogenic bacteria ↓Reg3γ expression by γδ T cells ↓pro-IL1β, pro-IL18, NLRP3	[21] [36] [65]
Amoxicillin/clavulanate	ND	↓ IgG serum levels	[56]
Ampicillin, gentamicin, metronidazole, neomycin, vancomycin	↓ bacterial numbers in LI Multiple effects on composition, including: ↓ luminal Firmicutes in LI ↓ mucosal associate *Lactobacillus* in LI	↓ IFNγ and IL-17 production by $CD4^+$ T cells in SI ↑ IgE serum levels ↑ basophils in blood	[5] [12]
Vancomycin	↓ Gram-positive bacteria ↑ Enterobacteriaceae	↓ Treg cells in colon ↓ Th17 in SI ↓ ILFs to a lesser extent than colistin	[20,42,45]

Figure 3.1. Antibiotic-induced changes in the microbiota composition and relative effect on immunity (modified from Ubeda and Pamer, 2012).

In order to verify the relationship between an adequate GDNF availability and a correct development of ENS, $TLR2^{-/-}$ mice were treated in vivo with rGDNF (Brun et al., 2013). Recombinant histidine-tagged (six His–tag) GDNF (rGDNF) was expressed in *Escherichia coli* and purified as described previously (Creedon et al., 1997).After elimination of endotoxin by gel chromatography (Pierce, Rockford, IL), endotoxin contamination was lower than 0.1 pg/dose (Lymulus Amoebocyte Assay; BioWhittaker, Walkersville, MD). $TLR2^{-/-}$and WT mice P14 were injected with either rGDNF (2 µg /g in 0.9% saline, subcutaneously) or endotoxin-free saline once a day for 7 days and analyzed at P21 (Wang et al., 2010).

To evaluate the influence of the activation of TLR2-mediated signaling in microbiota-depleted micePam3CysSerLys4 (Pam3-CSK4; TLR1/TLR2 heterodimer agonist; Invivogen, 2 mg/kg in 0.9 % saline, intraperitoneally) was administered daily to ABX mice during the second week of antibiotic treatment (from P28 to P35) for 7 days.

To investigate the effect of inhibition of both TLR2 and 4-mediated signaling on intestinal function, male WT mice P14 were treated with 1-palmitoyl-2-arachidonyl-snglycero-3-phosphorylcholine (OXPAPC, Invivogen; 1.5 µg/g in 0.9% saline) (Figure 3.2), a mixture of oxidized phospholipids, which was administered intraperitoneally once a day for 7 days. No signs of illness were evident during different treatments.

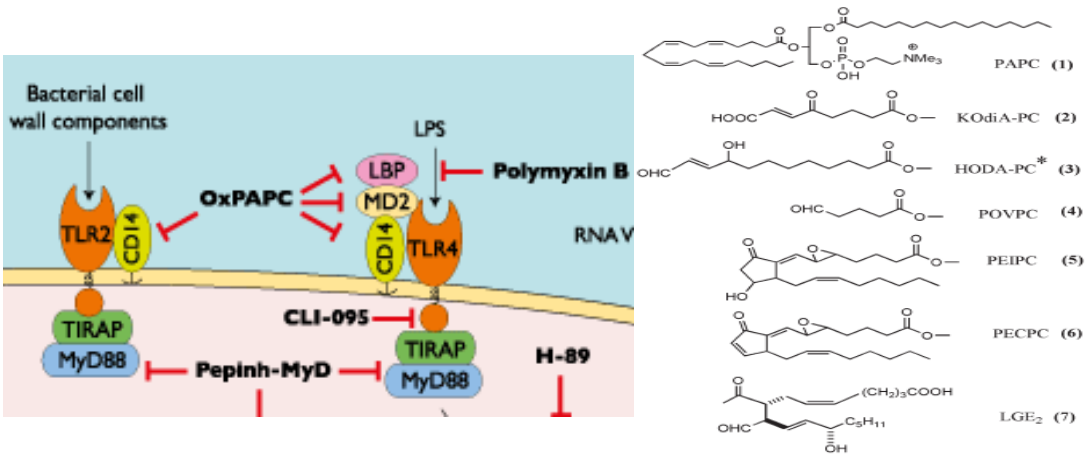

Figure 3.2. Mechanism of blocking the signaling of TLR2 and TLR4 by OXPAPC and composition of mixture of oxidized phospholipids (OXPAPC; Erridge et al., 2008).

Histopathology

Ileal specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. From each paraffin block at least three sections 4-5 µm-thick were sliced and stained with hematoxylin-eosin (H&E). Intestinal slides (n=6) were assessed in a blinded manner. A minimum of 10 independent fields per animal was examined at low (x10) and high (x40) magnification.

Immunohistochemistry

Immunohistochemical analysis was performed with the DAKO Autostainer plus (DAKO Cytomation). Paraffin-embedded ileal sections were deparaffinized in xylene, rehydrated, and pretreated in EnVisionTM FLEX Peroxidase-Blocking Reagent (DAKO) to block endogenous peroxidase activity. Sections were then rinsed in phosphate buffered saline (PBS) and incubated with anti-GDNF diluted in EnVisionTM FLEX Antibody Diluent for 30 min at room temperature. Primary antibody binding was revealed by incubation with EnVisionTM FLEX + rabbit linker (DAKO) for 15 min at room temperature and EnVisionTM FLEX/horseradish peroxidase for 20 min at room temperature. Developing solution was composed of EnVisionTM FLEX substrate buffer and EnVisionTM FLEX DAB + chromogen for 10 min at room temperature. Lastly, sections were counterstained with haematoxylin and then dehydrated, cleared with xylene and mounted. Negative controls were performed by omission of primary antibody and by pre-adsorption of antibody with recombinant GDNF for 1 hour at room temperature. Mouse brain sections were used as positive controls. Examination of the sections was performed under a Leica DM4500B microscope (Leica Microsystems) connected with a Leica DFC320 high-resolution digital camera (Leica Microsystems) and a computer equipped

with Leica Application Suite Version 2.8.1 software for image acquisition (Leica Microsystems; Brun et al., 2013).

Immunofluorescence

Immunofluorescence analysis was performed on ileal tissues embedded in optimal cutting temperature mounting medium (Tissue Tek Sakura) and frozen in dry ice (-80°C). Tissues were sectioned (7 µm-thick) with a cryostat-microtome (Leica CM 1850 UV), and then slices were fixed in 4% paraformaldehyde for 20 minutes at room temperature (RT). The aldehydes form covalent bonds between adjacent amine-containing groups through a Schiff acid-base reaction, which leads to formation of methylene bridges and other types of links (Collins and Goldsmith, 1981; Fox et al., 1985). However, unreacted aldehyde groups can emit fluorescence efficiently at the same wavelengths similar to the fluorescent probes employed for immunofluorescence assays. Fixative-induced autofluorescence was circumvented by treating fixed section for 20 minutes with 50 mM NH_4Cl, an exogenous amine-containing reagent that is able to quench fluorescence emission of unreacted aldehydes (Diez-Fraile et al., 2012). After repeated washings with Tris-buffered saline (TBS), samples were permeabilized with 0.3% Triton X-100. Non-specific binding sites were blocked incubating slides for 1 h with 2% bovine serum albumin (BSA) added together with the Triton X-100-containing solution. After TBS wash, ileal samples were incubate with primary antibodies listed in Table 3.1, diluted in TBS-0.5% BSA for 1 hour at RT. Immunocomplexes were visualized by incubating samples with the appropriate secondary antibodies listed in Table 3.2, for 1 h at RT. Nuclei were stained with TOTO-3 iodide (1:500; Life Technologies, Milan, Italy) added together with the secondary antibodies. Sections were mounted on glass slides using Mounting Medium (Sigma-Aldrich, Italy). Negative controls were obtained by incubating sections with isotype-matched control antibodies at the same concentration as primary antibody (data not shown). Images of a number of ileal areas, corresponding to the longitudinal smooth muscle layer (LM), circular smooth muscle layer (CM), myenteric ganglia (MG), submucosal ganglia (SMG) and mucosal layer (ML) were acquired with a Nikon System Spinning Disk confocal microscope. All microscope settings were set to collect images below saturation and were kept constant for all images.

Whole mount staining

Preparation of ileal whole mount samples

Animals were killed by cervical dislocation and the ileum was quickly isolated and washed with Krebs solution, to remove any contents. Then, distal ileal 10 cm-segments were filled with fixative solution consisting of 4% paraformaldehyde in PBS. Two hours were allowed for the fixation which occurred at 22°C and later tissues were cleared of fixative with2 x 30 minutes washes in PBS. Tissues were stored at 4°C in PBS containing 5% sodium merthiolate (Thimerosal, Sigma-Aldrich, Italy). Segments were cut in 0.5 cm-pieces and an incision was made along the midline of the gut. Using a dissecting microscope, tissues were pinned as a flat sheet onto wax support with the mucosa face-down and were separated into two layers: the outer musculature with adhering serosa and the submucosa/mucosa. The circular muscle was removed to yield whole-mounts of longitudinal muscle with the myenteric plexus attached (LMMP; Ruan and Burnstock, 2005). The LMMP whole-mount preparations were processed for acetylcholinesterase and NADPH-diaphorase biochemical staining or for immunohistochemical analysis.

Acetylcholinesterase and NADPH-diaphorase biochemical staining in ileal whole mount preparations

For acetylcholinesterase staining, the fixed tissue was washed in PBS and incubated in fresh copper buffer solution (100 ml dH_2O, 7.2 mg ethopropazine, 115.6 mg acetylthiocholine iodide, 75.0 mg glycine, 50.0 mg copper sulfate pentahydrate, 885.0 mg sodium acetate trihydrate; pH to 5.6 with glacial acetic acid) for 2 hours. This was followed by a wash in dH_2O, 1 minute in 1.25% sodium sulfide nonahydrate solution, a second wash in dH_2O, then mounting of the tissue on a glass slide. For NADPH diaphorase staining, the fixed tissue was washed in PBS and incubated in diaphorase solution (β-NADPH, 1 mg/ml; nitroblue tetrazolium (NBT), 0.1 mg/ml; and 0.3% Triton-X 100 in PBS) for 1 hour at 37°C. This was followed by washing in PBS and mounting of the tissue on a glass slide (Anitha et al., 2006). Samples from biochemical staining were observed using a Leica DM4500B microscope. Myenteric fiber count was performed on both acetylcholinesterase and NADPH diaphorase–stained intestines by counting fibers crossing a 0.1 mm^2 grid with 10 horizontal and 10 vertical lines. Twenty randomly selected fields per mouse were evaluated from 5 animals of each group. Total number of fibers was expressed as positive fibers per grid.

Immunohistochemistry on whole mount preparations

LMMP preparations were also used for immunohistochemistry. Ileal tissues, gently stretched and pinned down on a wax support as described before, were washed with PBS 0.3% Triton X-100 (PBS-T) for 45 minutes with slow shaking. Preparations were incubated in 2% BSA in PBS-T for 1 h at RT prior to the primary antibody incubation. Whole mount preparations were then incubated with the antibodies listed in Table 3.1 diluted in PBS-T/BSA 2%, overnight at RT. The following day, preparations were washed for 45 minutes in PBS-T prior to the appropriate secondary antibodies incubation, listed in Table 3.2, for 2 h at RT. TOTO-3 iodide (1:500; Life Technologies, Milan, Italy), a fluorescent probe for staining cell nuclei, was added to secondary antibodies solution. Three subsequent 15 minutes washes in PBS-T were given and LMMP preparations were then mounted on glass slides using a Mounting Medium (Sigma-Aldrich, Italy) and imaged with a Nikon System Spinning Disk confocal microscope.

Imaging and myenteric ganglia analysis

Preparations were imaged using a Nikon System Spinning Disk confocal microscope equipped with Nikon Plan APO 60.0X/1.40 and Plan APO 40X/0.95 oil immersion objectives. Fluorophores were visualized using a 488 nm excitation filter and 515/535 nm emission filter for Alexa Fluor 488, 543 nm excitation filter and 565/590 nm emission filter for Alexa Fluor 555, and 637 nm excitation and 650/660 nm emission filter for TOTO-3 iodide. Z-series and single 1024 x 1024 pixel images were captured. Images were composed of 10 to 15 plane forming Z-stack of 4-6 animals per group. Z-stacks of 8-µm or 10-µm depth were obtained for LMMP whole-mount preparations or for ileal frozen sections and processed as maximum intensity projections. All microscope settings were set to collect images below saturation and were kept constant for all images. In LMMP whole mount preparations we analyzed images of myenetric ganglia for HuC/D$^+$, neuronal nitric oxide synthase (nNOS)$^+$ neurons and S100β$^+$ glial cell bodies for counts and calculation of myenteric ganglia area. Number of cells was recorded within a minimum of 5 randomly selected fields covering 1.254 mm^2 from at least three LMMP preparations *per* mouse for a total of 6 animals of each experimental group. Ganglionic area was estimated using NIH Image J software. HuC/D, S100β, nNOS and GFAP immunoreactivities were determined in ileal frozen sections by measuring the area (number of pixels) and fluorescent intensity (average intensity of pixels) of staining from 24 images captured randomly in the ileal neuromuscular compartment from each CNTR and ABX tissue samples (n=4). Simultaneously, the mean intensities of HuC/D, S100β, nNOS and GFAP signals were normalized to mean TOTO-3 intensities for each neuron. Fluorescence values were expressed

as mean values in arbitrary fluorescence units (A.U.). Image analysis and quantification of the fluorescence intensity were performed using ImageJ software (version 1.48a).

ANTIGEN (HOST)	CLONE	SOURCE	DILUTION
nNOS (rabbit)	polyclonal	Life Technologies	1:100
βIII-tubulin (rabbit)	polyclonal	COVANCE	1:400
HuC/D (mouse)	Biotin conjugated 16A11	Life Technologies	1:50
GFAP (rabbit)	polyclonal	Merk Millipore	1:200
S100β (rabbit)	EP1576Y	Merk Millipore	1:100
TLR2 (mouse)	T2.5	eBioscience	1:100
Peripherin (rabbit)	polyclonal	Merk Millipore	1:100
GDNF (rabbit)	D20	Santa Cruz Biotechnology	1:100

Table 3.1. Characteristics of the primary antibodies used in immunofluorescence analysis.

ANTIGEN (HOST)	CLONE	SOURCE	DILUTION
goat anti-rabbit IgG	Rhodamine-conjugate	Chemicon	1:1000
goat anti-mouse IgG	Alexa Fuor 488-conjugate	Life Technologies	1:1000
goat anti-rabbit IgG	Alexa Fuor 555-conjugate	Life Technologies	1:1000
goat anti-rabbit IgG	Alexa Fuor 488-conjugate	Life Technologies	1:1000
Streptavidin	Alexa Fuor 555-conjugate	Life Technologies	1:1000
Streptavidin	Alexa Fuor 488-conjugate	Life Technologies	1:1000

Table 3.2. Characteristics of the secondary antibodies used in immunofluorescence analysis.

In vitro contractility studies

Intestinal contractility was examined *in vitro* by measuring tension changes on ileal samples with the isolated organ bath technique. Experiments were performed on full-thickness distal ileum segments isolated from $TLR2^{-/-}$, ABX, $COMT^{+/-}$ and respective control mice (CNTR). After the mice were sacrificed, the small intestine was placed into oxygenated (95% O_2 and 5% CO_2) Krebs solution with the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2 \cdot 2H_2O$ 2.5, $MgSO_4 \cdot 7H_2O$ 1.2, KH2PO_4 1.2, $NaHCO_3$ 25, glucose 11; pH=7.4) at 37° C. The last section of the intestine was removed and 1cm ileal segments were taken for use in this experiment. Contents

were washed with Krebs solution using a syringe. At each end of the ileal segment, thread was used to create a loop; one side was placed through the hook of an organ bath and the other side was connected to an isometric transducer. These segments were vertically mounted in 10 mL volume organ baths filled with 10 mL Krebs solution; tension was set to 1g. Isometric transducers (WPI, USA) connected to a quad bridge amplifier and PowerLab 4/30 data acquisition system using LabChart6 software (ADInstruments, UK) were used to record the changes in muscle tension. Initially each segment was allowed to equilibrate for 30 minutes. Thereafter the segment was activated with 1 µM carbachol stimulation. At first, the ileal segments were exposed to carbachol stimulations of the following concentrations (0.01-100 µM) to obtain a concentration-response curve. To evaluate the smooth muscle response, we subjected the segments to 60 mM KCl. Electrical field stimulation (EFS) was used after to evoke neuronal-mediated contractions (at 2-50 Hz; 1-ms pulse duration; 10-s pulse-trains, 40 V) by the ileal segments. For this, segments were placed between parallel electrode pairs that were connected to a Grass S88 stimulator (Grass Instrument Co. Quincy, MA.). To evaluate the response in relaxation in non-adrenergic non-cholinergic (NANC) conditions, the same EFS parameters were used for inhibition following an incubation period of 20 minutes with 1 µM atropine and 1 µM guanethidine. To block nitrergic-mediated neurotransmission, 100 µM N_{ω}-nitro-l-arginine methyl ester (L-NAME), an inhibitor of NOS, was incubated within the same NANC conditions for an additional 20 minutes and EFS occurred after this period. Concentration-response curves were subjected to a nonlinear regression analysis (fitted to a sigmoidal equation) to calculate maximal tension (Emax) values (Brun et al., 2013). The relaxation response was quantified by calculating the area under the curve defined as the integrated area under the waves (AUC).

Gastrointestinal Transit Analysis

The gastrointestinal (GI) transit was measured by evaluating the intestinal distribution of orally gavaged fluorescein isothiocyanate (FITC)-labeled dextran (70 kDa) from the stomach to the colon. With regard ABX treatment or $COMT^{+/-}$ transgenic model, treated and control mice were administered FITC-dextran dissolved in 0.9% saline (100 µl of 25mg/ml FITC-dextran solution for each mouse). Then mice were sacrificed after 30 minutes and the complete GI tract from stomach to distal colon was collected. The stomach and caecum were analyzed separately while the small intestine was divided into 10 segments of equal length and the colon was divided into 3 segments of equal length. However, for $TLR2^{-/-}$and respective controls, mice were sacrificed 60 minutes after administration of FITC-dextran and the bowel was removed.

The stomach, caecum and colon were examined separately, whereas the small intestine was divided in 8 equal segments. Luminal contents from each part (both tissue and faecal content) were collected and clarified by centrifugation (12,000 rpm for 10 minutes at 4°C). Supernatant from each sample were placed into separate wells of a 96 well plate and assayed in duplicate along with a Krebs solution control and a FITC-dextran control (1:10 part dilution of FITC-dextran and Krebs solution respectively). FITC-dextran fluorescence intensity was measured at 492/521 nm using a fluorimeter (Victor, PerkinElmer). The data collected were expressed as percentage (%) of fluorescence for each segment and GI transit was calculated as the geometric center (GC) of distribution of the fluorescent marker using the following formula (Wehner et al., 2007):

$$GC = \sum (\% \text{ of total fluorescence signal} \times \text{segment} * \text{segment number})/100$$

Pellet frequency and fecal water content

Fecal pellet output and water content was assessed in mice following antibiotic treatment. Fecal water content provides an indication of constipation, diarrhea or malabsorption. ABX and CNTR mice were placed into individual clean cages and were examined throughout a 60-minute-period. All animals were given standard chow diet and tap water ad libitum during the observation time. Fecal pellets were collected at 15 minute intervals into a 1.5-mL-microcentrifuge tube weighed beforehand. The numbers of pellets collected were tabulated; tubes were weighed to acquire the wet weight of the pellets initially. Then the pellets were dried overnight at 65°C and reweighed to obtain the dry weight. The difference in wet and dry weight was expressed over the dry stool weight to calculate fecal water content (Li et al., 2006; Anitha et al., 2012).

Bone marrow chimera mice

Two bone marrow chimera groups were produced and experimental transfers were as follows: WT donors into WT recipients (WT→WT), WT donors into $TLR2^{-/-}$ recipients (WT→$TLR2^{-/-}$), $TLR2^{-/-}$ donors into $TLR2^{-/-}$ recipients ($TLR2^{-/-}$→$TLR2^{-/-}$), and $TLR2^{-/-}$ donors into WT recipients ($TLR2^{-/-}$→WT). All recipients and donors were male between 7 and 8 weeks of age. Bone marrow cells (BMCs) were obtained as previously described (Sanders et al., 2008; Brun et al., 2013). Briefly, donor mice (C57BL/6J or $TLR2^{-/-}$) were killed and BMCs from were flushed out from femurs and tibiae with Dulbecco's Modified Eagle's Medium (DMEM), by means of a 27 gauge needle, filtered through BD Falcon cell strainers to mesh clumps, and wash twice with

DMEM. Finally, the isolated cells were sediment by centrifugation at 1500 rpm for 5 minutes at 4°C, then pellet was resuspended in DMEM. The BMCs suspension was divided into 100-µl aliquots containing at least $1x10^7$ cells, which were stored at 4°C until use. Recipient mice were exposed to a single dose of 10-Gy whole-body γ-radiation using a linear accelerator (γ-source), injected intravenously with $1×10^7$BMCs from donors via the tail vein using a 27-gauge, and allowed to reconstitute for two months. All chimeras were kept on drinking water containing 40 mg/L neomycin sulfate (Sigma Aldrich, Italy) for four to six weeks before returning to regular water. Four and six weeks after bone marrow reconstitution, peripheral blood chimerism in leukocytes expressing TLR2was assessed by fluorescence-activated cell sorter (FACS) analysis. Only recipients exhibiting peripheral blood chimerism greater than 85% were used for further analysis (Brun et al., 2013).

RNA isolation and quantitative RT-PCR

GluN1 expression in ABX or COMT experimental groups was assessed by RT-PCR in collaboration with the research group directed by Prof. Cristina Giaroni, University of Insubria, Varese. Briefly, total RNA was extracted from longitudinal muscle/myenteric plexus (LMMP) preparations with TRIzol (Invitrogen, Italy) and treated with DNase I (DNase Free, Ambion, Italy) to remove any traces of contaminating DNA. cDNA was obtained retrotranscribing 2 ug of total RNA using the High Capacity cDNA Synthesis Kit (Applied Biosystems, Life Technologies, Italy). Quantitative RT-PCR was performed on the Abi Prism 7000 real-time thermocyclator (Applied Biosystems, Italy) with Power Sybr Green Universal PCR Master Mix (Applied Biosystems, Italy) according to the manufacturer's instructions. Primer sequence was: NR1, 5-CAGGAGCGGGTAAACAACAGCAAC-3; 5-GCAGCCCCACCAGCAGCCACAGT-3; β-actin 5-ACCAGAGGCATACAGGGACA-3, 5-CTAAGGCCAACCGTGAAAAG-3. β-actin was used as housekeeping gene. For quantitative RT-PCR a final concentration of 500 nmol/L for each primer was used. Primers were designed to have a similar amplicon size and similar amplification efficiency as required for the utilization of the ΔΔCt method to compare gene expression. Experiments were performed at least five times for each different preparation. mRNA levels of LMMP preparations from ABX or COMT mice were expressed as the percentage variation *versus* values obtained in non-antibiotic-treated (CNTR) and WT preparations, respectively. GDNF mRNA levels were analyzed in collaboration with the research group directed by Prof. Ignazio Castagliuolo, Department of Molecular Medicine, University of Padova. Total RNA was obtained from homogenized LMMP of $TLR2^{-/-}$ mice, ABX or ABX/Pam3-CSK4-treated mice (and respective controls) as previously described (Brun et al.,

2013). Oligonucleotides used for qRT-PCR were: GDNF, 5'-TCCAACTGGGGGTCTACG-3'; 5'-GACATCCCATAACTTCATCTTAGA-3'; Gadph: 5'-GAGGATCCTTCTGGGAGTTTTT-3'; 5'-TAGCGAATGGGTGGATTTTC-3'. Expression of each mRNA was normalized to the level of the housekeeping gene Gapdh.

Flow cytometry

Flow cytometry experiments were performed in collaboration with the research group directed by Prof. Ignazio Castagliuolo, Department of Molecular Medicine, University of Padova. LMMP preparations, isolated from WT mice, were finely cut and incubated in collagenase type II (14 mg/ml, Gibco, Italy) and protease type I (10 mg/ml, Sigma, Italy) for 5 min at 37°C in Hanks Balanced Salt Solution (HBSS, Euroclone, Italy) and then at 37°C for 15 min in Krebs solution. Following enzymatic inactivation, cells were further dissociated by passing through a Pasteur pipette, filtered through a cell strainer and centrifuged at 900 g for 5 min. LMMP-derived cell suspension was then analyzed with flow cytometry, as previously described (Brun et al., 2013). Briefly, cells (10^6) were incubated with the permeabilization buffer (PBS containing 0,2 % Triton-X100 (eBioscience, Italy) for 20 min at 22°C. After rapid washes in PBS-BSA 0.5 %, freshly isolated cells were treated with appropriate combination of antibodies diluted in permeabilization buffer added with 0,5 % BSA, for 30 minutes. For the detection of TLR2, cells were incubated with the primary antibody listed in Table 3.3. After washes, cells double-labeled for TLR2 and F4/80, CD31, GFAP and HuC/D were incubated with the appropriate secondary antibodies listed in Table 3.4, for 30 minutes in the dark. In parallel, controls were stained using only corresponding secondary or isotypic antibodies, listed in Table 3.4. Therefore, single-cell suspensions were subjected to flow cytometry analysis which was performed using a FACSCalibur based on CellQuest software (Becton Dickinson), as previously described (Brun et al., 2013).

ANTIGEN (HOST)	CLONE	SOURCE	DILUTION
HuC/D (mouse)	polyclonal	Merck Millipore	1:100
GFAP (mouse)	GA5	Merck Millipore	1:400
CD31 (rat)	MEC 13.3	BD Pharmingen	1:200
F4/80 (rat)	CI:A3-1	Abcam	1:100
A-smooth muscle actin,SMA (mouse)	1A4 Cy3 conjugate	Sigma Aldrich	1:50
CD3 (mouse)	DaA3 FITC-conjugate	Immuno Tools	1:100
TLR2 (rabbit)	H-175	Santa Cruz Biotechnology	1:100

Table 3.3. Characteristics of the primary antibodies used in the flow cytometry.

ANTIGEN (HOST)	CLONE	SOURCE	DILUTION
goat anti-rabbit IgG	Rhodamine-conjugate	Chemicon	1:100
goat anti-mouse IgG	FITC-conjugate	Chemicon	1:400
rabbit anti-rat	Alexa Fluor 488-conjugate	Invitrogen	1:200
rat IgG isotype	Alexa Fluor 488-conjugate	Biolegend	1:100
goat IgG isotype	Alexa Fluor 488-cojugate	Biolegend	1:200
rat IgG isotype	PE/Cy7-cojugate	Biolegend	1:200

Table 3.4. Characteristics of the secondary and isotypic antibodies used in the flow cytometry.

Statistical Analysis

All results are reported as mean ± standard error of the mean (SEM), except for the geometric center, which is presented as median and range (minimum-maximum). Statistical significance was determined Statistical significance was calculated with the unpaired Student's *t* test or the 1-way analysis of variance with Newman-Keuls post-hoc test for multiple variables, or the non-parametric Mann–Whitney's U-test for independent variables using GraphPad Prism software (GraphPad Software Inc, La Jolla, USA). The differences between groups were considered significant at P-value <0.05 (Brun et al., 2013).

MATERIALS

SUBSTANCE	SOURCE
Acetylthiocholine iodide	Sigma-Aldrich, Italy
Ampicillin	Sigma-Aldrich, Italy
Atropine sulfate	Sigma-Aldrich, Italy
β-NADPH	Sigma-Aldrich, Italy
Bovine serum albumin	Sigma-Aldrich, Italy
$CaCl_2 \cdot 2H_2O$	Merck, Italy
Carbamoylcholine chloride	Sigma-Aldrich, Italy
Collagenase type II	Gibco, Italy
Copper sulfate pentahydrate	Sigma-Aldrich, Italy
D-glucose anhydrous	Sigma-Aldrich, Italy
DNase Free	Ambion, Italy
Dulbecco's Modified Eagle's Medium	
Ethopropazine hydrochloride	Sigma-Aldrich, Italy
Fluorescein isothiocyanate–dextran 70.000 Da	Sigma-Aldrich, Italy
Glycine	Sigma-Aldrich, Italy
Guanethidine	Sigma-Aldrich, Italy
KCl	Carlo Erba, Italy
KH_2PO_4	Carlo Erba, Itay
$MgSO_4 \cdot 7H_2O$	Sigma-Aldrich, Italy
Metronidazol	Sigma-Aldrich, Italy
Mounting Medium	Sigma-Aldrich, Italy
Na_2HPO_4	Merck, Italy
Na_2S	Sigma-Aldrich, Italy
NaCl	Carlo Erba, Italy
$NaHCO_3$	Carlo Erba, Italy
Neomycin	Sigma-Aldrich, Italy
NH_4Cl	Sigma-Aldrich, Italy
Nitroblue tetrazolium	Sigma-Aldrich, Italy
Nω-nitro-L-arginina metil estere cloridrato	Sigma-Aldrich, Italy
OXPAPC	InvivoGen, Italy
Pam3CysSerLys4	InvivoGen, Italy
Paraformaldehyde 8% Aqueous solution	Electron Microscopy Sciences, Italy
Power Sybr Green Universal PCR Master Mix	Applied Biosystems, Italy
Protease type I	Sigma-Aldrich, Italy

Sodium Acetate trihydrate	Sigma-Aldrich, Italy
Sodium merthiolate	Sigma-Aldrich, Italy
Sodium sulfide nonahydrate	Sigma-Aldrich, Italy
Triton X-100	Sigma-Aldrich, Italy
TRIzol	Invitrogen, Italy
Vancomycin	Pharmatex Italia S.r.l.

4. RESULTS

4.1 Toll-Like Receptor 2 in murine small intestine

Toll-like receptors (TLRs), a subgroup of pattern-recognition receptors (PRRs), play a key role in the maintenance of symbiosis between gut microbiota and the mammalian host (Kabouridis and Pachnis, 2015), protecting against microbial infection as well as controlling tissue integrity under physiological and pathological conditions (Rakoff-Nahoum et al., 2004). TLR-derived signaling is required for proper development of CNS (Rolls et al., 2007) and, recently, TLR4 was discovered to be expressed in the ENS where has been shown to regulate murine gastrointestinal motility and promote the survival of enteric neurons (Anitha et al., 2012).

4.1.1 Expression of TLR2 in ileal neuromuscular layer

Since the presence of TLRs is reported in tissues not directly exposed to microbial products (i.e. CNS), we firstly evaluated the expression of TLR2 in the ileum by confocal microscopy. Immunohistochemical analysis performed on ileal frozen sections revealed that TLR2 was expressed in smooth muscle layers of WT mice (Figure 4.1A, upper panel), but there was no staining for the protein in TLR2$^{-/-}$ mice. We also observed the distribution of TLR2 in cells expressing the neurofilament protein peripherin, indicating the presence of this receptor in myenteric neuronal cells (Figure 4.1B).

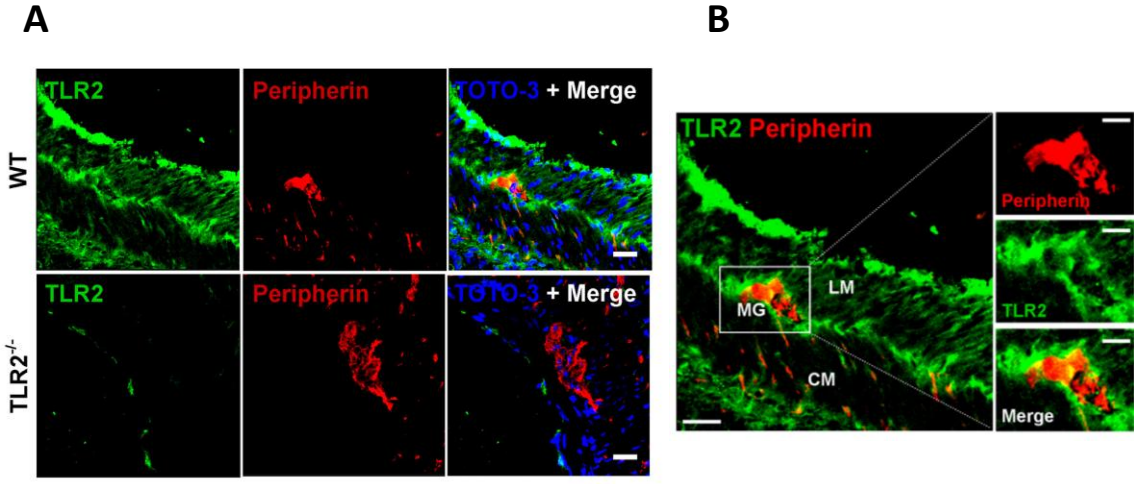

Figure 4.1. Expression of TLR2 protein in the ileal neuromuscular layers of WT mice. *Panel A*: Dual-label immunohistochemistry showing expression of TLR2 (green) and peripherin (red, cytoskeletal neuronal marker) proteins in ileal frozen cryosections from WT (upper panel) and TLR2$^{-/-}$mice (lower panel). Cell nuclei were stained with TOTO-3. Scale bar = 22 µm. For each antigen, a single optical confocal section is shown. *Panel B:* Enlarged view of peripherin^{+} and TLR2^{+} cells in LMMP from WT mice from boxed area in merge panel. Scale bar = 12.5 µm.

By performing multiparameter flow cytometry staining of cells dissociated from longitudinal muscle-myenteric plexus (LMMP) of WT mice, TLR2 expression was detected on cells co-expressing the pan neuronal marker HuC/D and glial fibrillary acid protein (GFAP, glial cells; Figures 4.2A and B). TLR2 expression was also observed in smooth muscle cells labeled for α-smooth muscle actin (α-SMA) protein, in endothelial cells and macrophages stained for CD31 and F4/80 antigens, respectively, whereas was not revealed in $CD3^+$ T-cells (Figures 4.2A and B). These findings suggest that ENS cell populations have the potential to respond directly to stimuli derived from the microbial flora throughout TLR2-mediated signaling (Barajon et al., 2009).

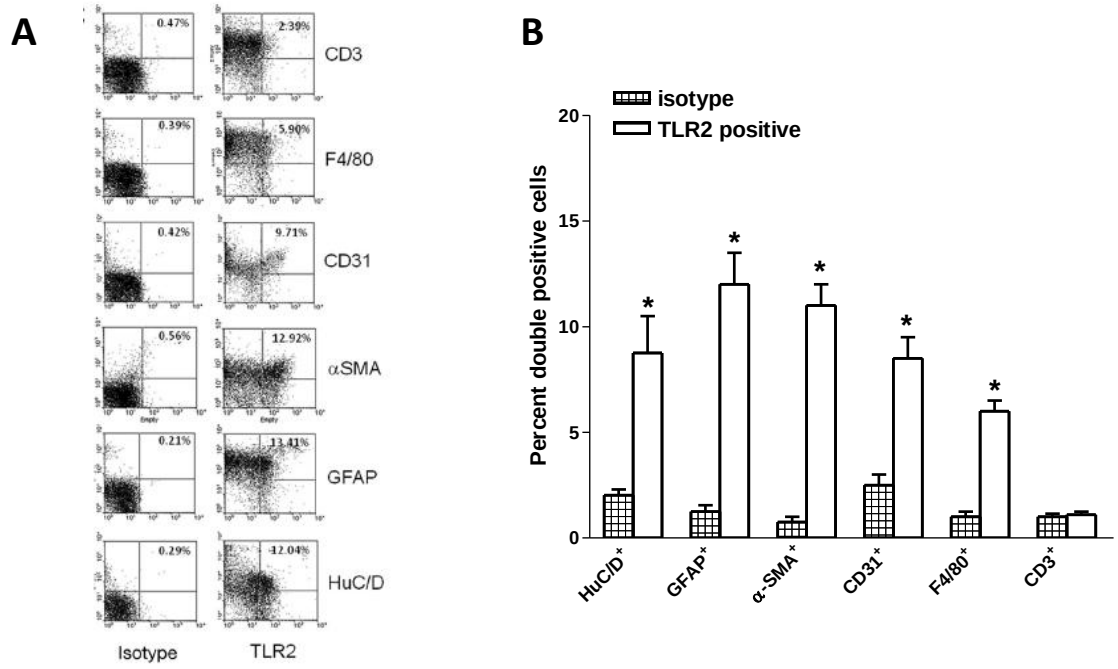

Figure 4.2. Distinct cell populations of the small intestine express TLR2. Expression of TLR2 in HuC/D^+, glial fibrillary acidic $protein^+$ (GFAP), α-smooth muscle $actin^+$ (α-SMA), $CD31^+$, $F4/80^+$ and $CD3^+$ cells harvested from LMMP of WT mice and analyzed by flow cytometry. For each LMMP, 10^6 cells were collected. *Panel A:* Representative dot plots for each experiment are reported. *Panel B:* The histogram shows the percentage of double-positive cells for each population subset (n=6 per group). *p<0.05 vs respective negative control (isotype).

4.1.2 Lack of TLR2 results in altered architecture of myenteric plexus

Since TLRs signaling play a well recognized role in regulation of postnatal neuronal plasticity and TLR2 has been implicated in differentiation of neural stem/progenitor cells into neurons in the brain (Rolls et al., 2007), this analysis aimed to assess the impact of TLR2 absence on ENS development in mice. Histological assessment showed a conserved ileal architecture in $TLR2^{-/-}$ mice together with comparable levels of inflammatory mediators between $TLR2^{-/-}$ and WT mice (Brun et al., 2013, data not shown).

Whole mount immunohistochemistry, performed on LMMP preparations, revealed an altered distribution of peripherin in myenteric neurons of TLR2$^{-/-}$ mice (Figure 4.3), associated with a significant reduction in the number of HuC/D^{+} neurons and S100β^{+} EGCs (-25.6±15% and -38.5±8% respectively, $p<0.05$; Figures 4.4A, B and C). The abnormal distribution of peripherin, a neuronal intermediate filament involved in the developing nervous system (Rauch et al., 2006), together with the reduced expression of glial regulatory protein S100β as well as the reduced number of HuC/D^{+} cells, and S100β^{+} EGC, suggests that TLR2 signaling influences enteric neurogenesis and neuroplasticity by controlling both enteric neuronal and glial cells homeostasis.

Considering the strong impact of the main inhibitory neurotransmitter nitric oxide (NO) on intestinal motility, the distribution and expression of neuronal nitric oxide synthase (nNOS) were analyzed in myenteric ganglia of mice lacking TLR2 protein. Whole mount immunohistochemistry showed an altered distribution of nNOS immunoreactivity in myenteric plexus of TLR2$^{-/-}$ mice, evidenced by the significant reduction of nNOS^{+} neurons in transgenic mice compared with WT (-29.5±4%, $p<0.05$; Figures 4.4D and E).

Acetylcholinesterase (AChE) is the enzyme involved in catalyzing the hydrolysis of the excitatory neurotransmitter acetylcholine in choline and acetic acid, thus playing an essential role in cholinergic neurotransmission (Moore and Johnson, 2005; Anitha et al., 2006; Anitha et al., 2012). AChE stained fibers were significantly decreased in TLR2$^{-/-}$ mice as compared with WT (Figure 4.4F), reflecting a dysfunction of the cholinergic system. Overall, these data support the view that intestinal TLR2 influences neuronal network integrity and neurochemical coding controlling gastrointestinal functions.

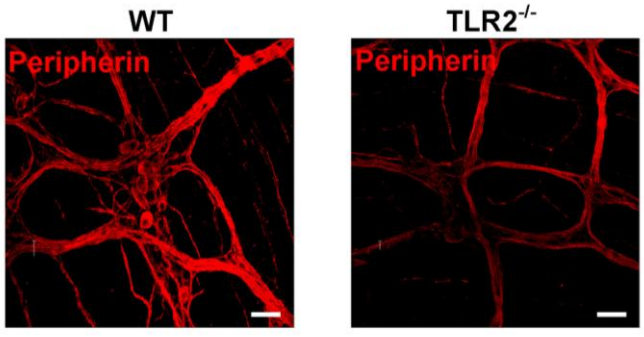

Figure 4.3. Absence of TLR2 signaling perturbs myenteric plexus structure. Confocal microphotographs showing the distribution of peripherin (red, neuronal cytoskeleton marker) in ileal LMMP whole mount preparations from TLR2$^{-/-}$ and WT mice (n=5). Scale bar = 22 µm.

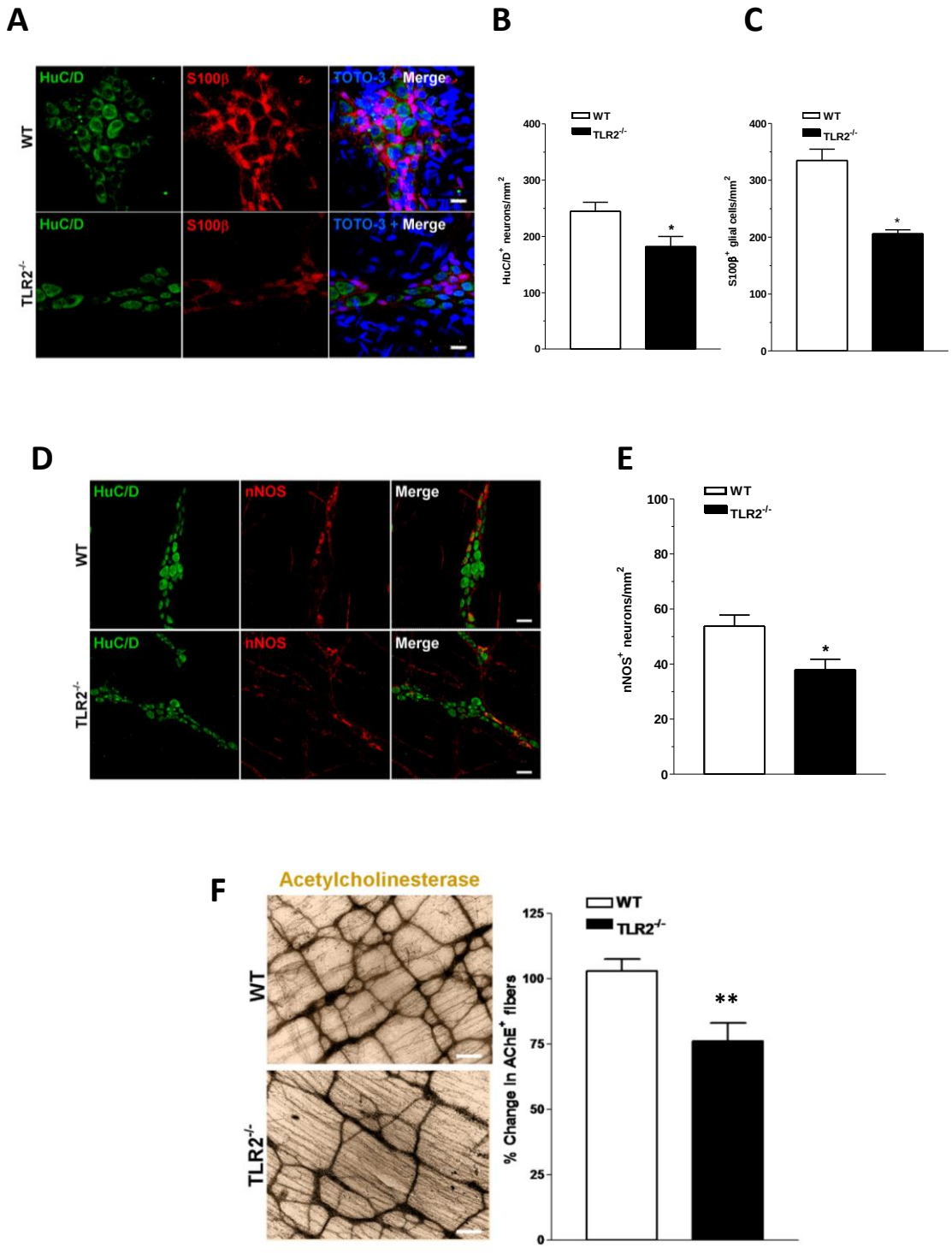

Figure 4.4. TLR2 signaling influences myenteric plexus architecture and neurochemical coding. Immunofluorescence analysis of LMMP whole mount preparations from WT and TLR2$^{-/-}$ mice double-labeled for pan-neuronal marker HuC/D (green), glial cells marker S100β, (red, Panel A), neuronal nitric oxide synthase protein (nNOS, red, panel D). Cell nuclei were stained with TOTO-3 (Panel A). Scale bars = 44 µm in panel A and 22 µm in panel D. HuC/D^{+} and nNOS^{+} neurons (Panel B and E, respectively) and S100β^{+} glial cells (Panel C) per mm^{2} were quantified in LMMP whole-mount preparations of WT and TLR2$^{-/-}$ mice (n=5). *p<0.05 vs WT. Representative photographs of AChE^{+} neurons and fibers; distribution and percentage changes in the number of the AChE^{+} fibers (Panel F) in whole mount preparations of ileum LMMPs from TLR2$^{-/-}$ and WT mice (n=4). Scale bar = 300 µm. **p<0.01 vs WT.

4.1.3 TLR2$^{-/-}$ mice experience gastrointestinal motor dysfunction

Since the enteric neural network architecture is altered in TLR2$^{-/-}$ mice, the gastrointestinal function was evaluated by performing *in vitro* contractility experiments. Isolated ileum segments of TLR2$^{-/-}$ mice, mounted vertically in organ bath, displayed abnormal excitatory neurotransmission following electric field stimulation (EFS). TLR2$^{-/-}$ mice showed a significant increase of EFS-elicited contractions (+81±8.6, $p<0.01$ at 10Hz; Figure 4.5), which up to 20 Hz is mediated by neuronal cholinergic neurotransmission as confirmed by their sensitivity to tetrodotoxin and to the muscarinic receptor blocker atropine (data not shown).

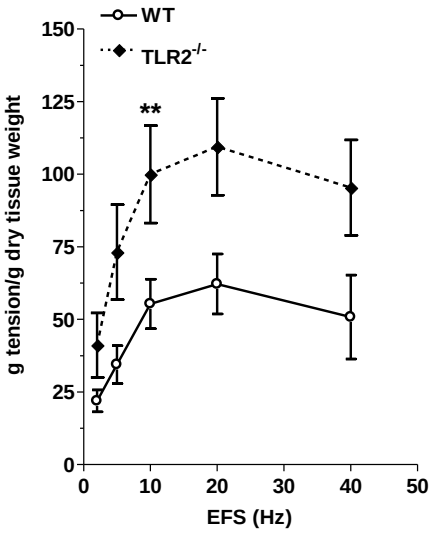

Figure 4.5. Altered gastrointestinal motor function in TLR2$^{-/-}$ mice. Electric field stimulation (EFS) elicited contractions (2-50 Hz, 40 V) in ileum segments of WT and TLR2$^{-/-}$ mice. Data are reported as mean ± SEM (n=8) and are expressed as g tension/g dry tissue weight (g/g). **p<0.01 vs WT.

In a second series of experiments, gastrointestinal transit was monitored *in vivo* using FITC-dextran, to evaluate its distribution in the intestinal tract (small and large intestine). Sixty minutes after oral administration of FITC-labeled dextran, TLR2$^{-/-}$ mice exhibited an accelerated intestinal transit compared to WT ($GC_{TLR2}^{-/-}$ =8.1, range: 7.0-8.75 vs GC_{WT} = 6.55, range: 5.8-7.15, $p<0.01$; Figures 4.6A and B). Our findings indicate that TLR2 absence in ENS induces changes in the neuromuscular function, which may partly be responsible for altered intestinal motility.

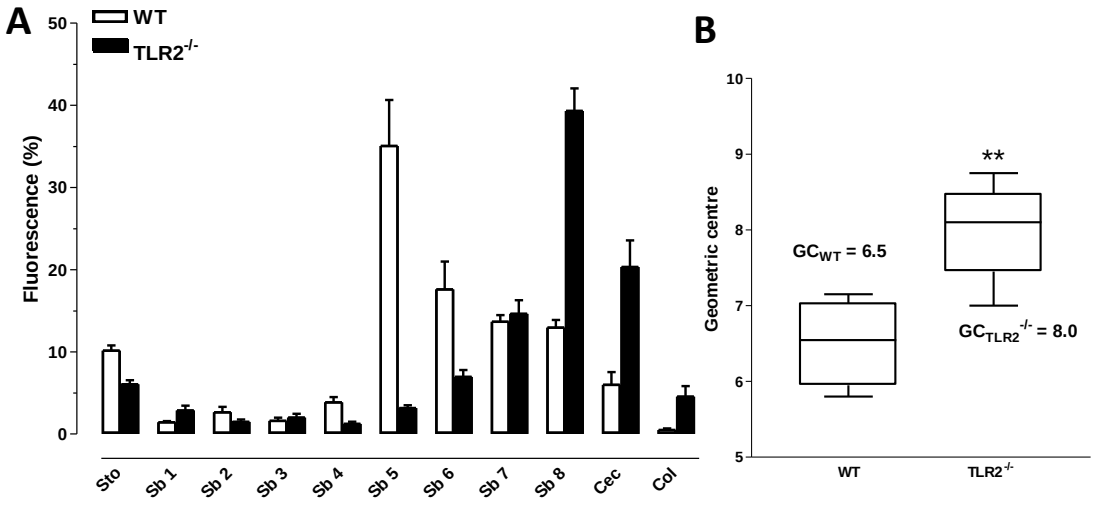

Figure 4.6. Altered gastrointestinal motor function in TLR2$^{-/-}$ mice. *Panel A:* Relative distribution of nonabsorbable fluorescein isothiocyanate (FITC) labeled dextran in the stomach (Sto), small bowel (Sb 1-8), cecum (Cec), and colon (Col) in WT mice compared with TLR2$^{-/-}$ mice. *Panel B:* Geometric centre (GC) of nonabsorbable FITC-dextran in CNTR and TLR2$^{-/-}$mice. Data are reported as the mean ± SEM (n=6) for panel A and as median, minimum, maximum, upper and lower quartiles for panel B. **p<0.01 vs WT.

4.1.4 TLR2-mediated intestinal motility changes are hematopoietic cell-independent

To verify that TLR2-dependent functional anomalies are regulated by means of a hematopoietic cell-independent mechanism, we generated bone marrow chimeric mice from WT. As shown in Figure 4.7, EFS-induced contractions were similar in WT mice given either WT or TLR2$^{-/-}$ bone marrow, as well as in TLR2 mice receiving either TLR2$^{-/-}$ or WT bone marrow, demonstrating that the absence of TLR2 signaling in non hematopoietic cells causes the structural and functional anomalies observed in the ENS.

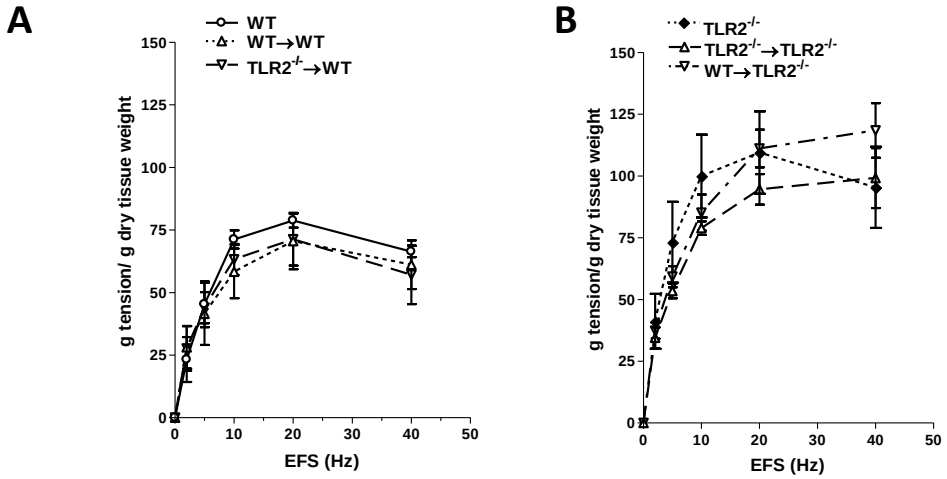

Figure 4.7. TLR2-dependent ENS anomalies are hematopoietic cell-independent. Excitatory response of ileal segments from WT, WT→WT and TLR2$^{-/-}$→WT mice (*Panel A*) and TLR2$^{-/-}$, TLR2$^{-/-}$→TLR2$^{-/-}$, WT→TLR2$^{-/-}$ mice (P*anel B*) induced by electrical field stimulation (EFS) (2-50 Hz, 40 V). Data are reported as mean ± SEM (n=8 per group) and are expressed as g tension/g dry tissue weight (g/g).

4.1.5 TLR2 signaling influences GDNF production in the *muscularis externa* layer of murine ileum

Given the well recognized role of glial derived neurotrophic factor (GDNF) in the proper development and maintenance of ENS structural and functional integrity (Gershon, 2010; Wang et al., 2010), the expression of this neurotrophic factor was examined. As shown in Figure 4.8, TLR2$^{-/-}$ mice displayed a decrement in mRNA transcripts (-70.8±0.001%, $p<0.05$; Figure 4.8A) and immunoreactivity of GDNF in ileal muscle layers (Figure 4.8B).

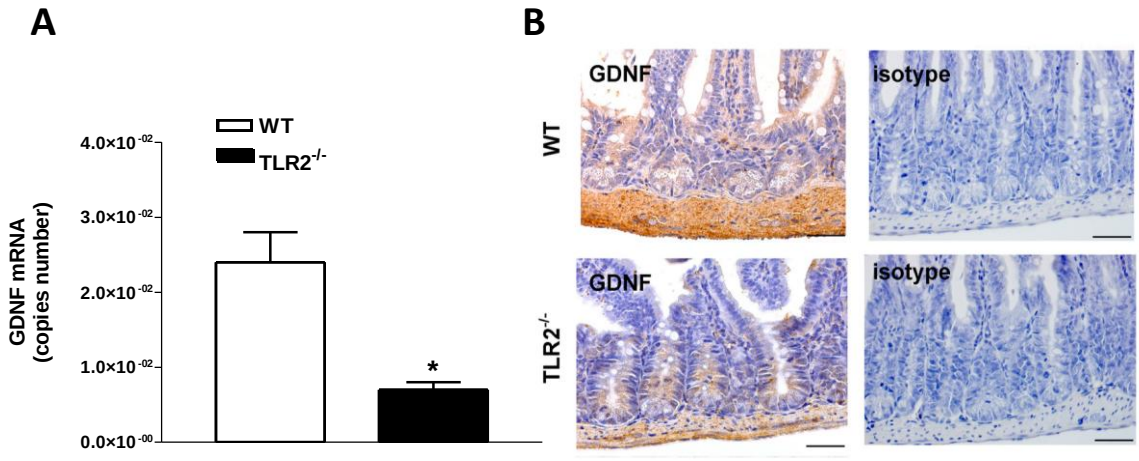

Figure 4.8. TLR2 signaling pathway influences GDNF signaling. *Panel A:* Quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis of GDNF mRNA in LMMP from WT and TLR2$^{-/-}$ mice (n = 6 per group). *p<0.05 vs WT. *Panel B:* GDNF immunostaining in ileum of WT and TLR2$^{-/-}$mice. Scale bars = 75 µm.

4.1.6 GDNF administration ameliorates ENS defects in TLR2$^{-/-}$ mice

To demonstrate that ENS abnormalities in TLR2$^{-/-}$mice are consequent to insufficient levels of GDNF, the neurotrophic factor was administered *in vivo* to TLR2$^{-/-}$ mice for 7 days. rGDNF supplementation significantly improved the contractile abnormalities in ileum segments, as demonstrated by EFS-elicited response, which was comparable between WT mice and TLR2$^{-/-}$ mice treated with rGDNF (Figure 4.9A). Administration of rGDNF also ameliorated gastrointestinal transit ($GC_{TLR2^{-/-}+GDNF}$ =6.9, range: 5.8-7.9 vs GC_{WT} = 6.55, range: 5.8-7.15, Figures 4.9B and C) and, moreover, prevented the loss of nitrergic neurons in myenteric ganglia, bringing the number of nNOS^{+} neurons related to the WT, as shown by confocal immunohistochemistry in Figures 4.10A and B.

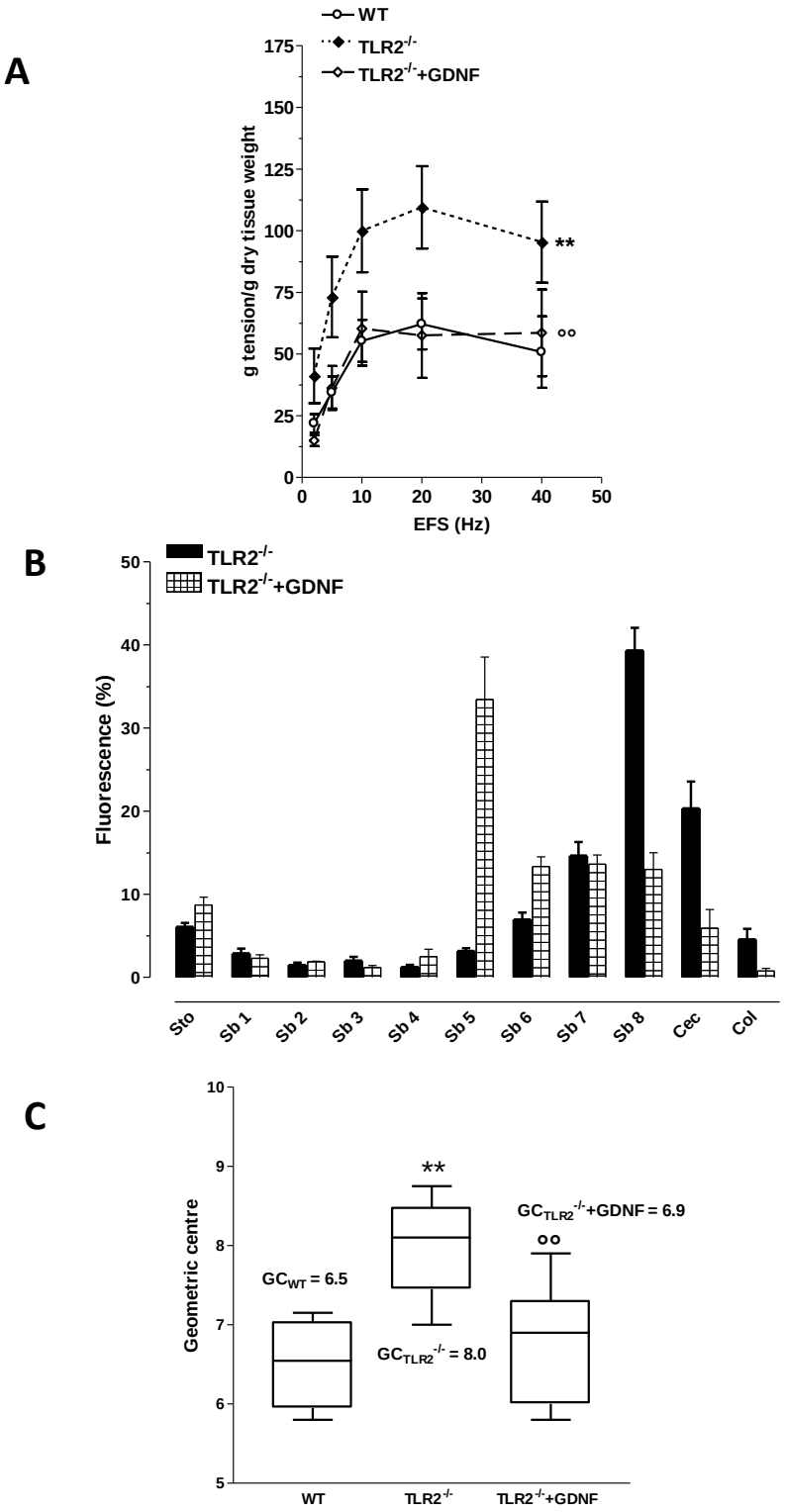

Figure 4.9. GDNF administration prevents ENS abnormalities in TLR2$^{-/-}$ mice. *Panel A:* EFS-elicited contractions in ileum segments of WT and TLR2$^{-/-}$mice treated with rGDNF or vehicle. Data are reported as mean ± SEM (n=6 per group) and are expressed as g tension/g dry tissue weight (g/g).**p<0.01 vs WT; °°p<0.01 vs TLR2$^{-/-}$. *Panel B*: Gastrointestinal distribution of non absorbable FITC-labeled dextran 60 minutes after oral administration in stomach (Sto), small bowel segments (Sb1-8), caecum (Cec) and colon (Col) of TLR2$^{-/-}$ mice treated with either rGDNF or vehicle. *Panel C:* Calculation of the geometric center (GC) of the relative FITC-dextran distribution through the GI tract of TLR2$^{-/-}$ mice treated with either rGDNF or vehicle compared to WT mice. Data are reported as the mean ± SEM (n=6) for *panels A* and *B*, and as median, minimum, maximum, upper and lower quartiles for *panel C*. **p<0.01 vs WT; °°p<0.01 vs TLR2$^{-/-}$.

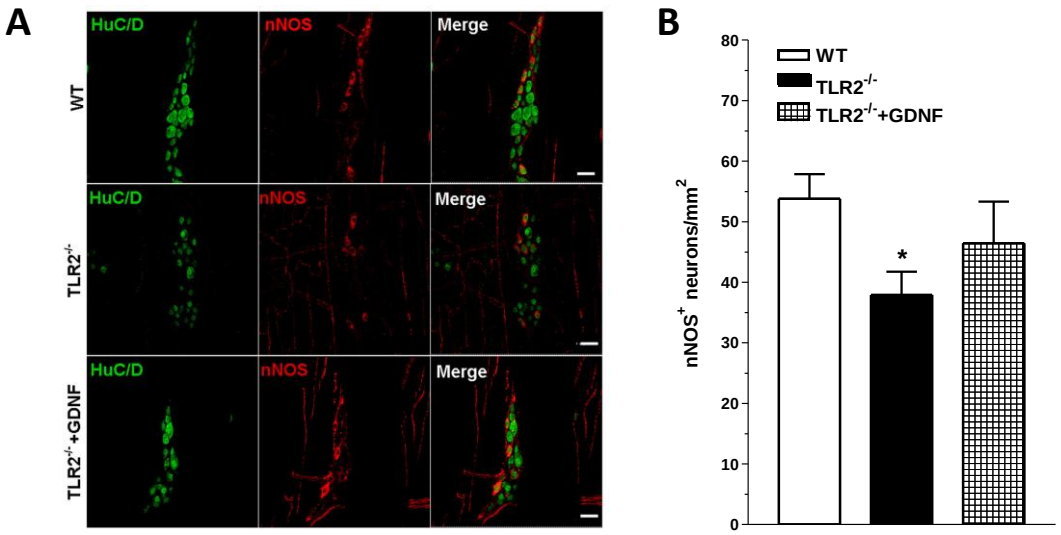

Figure 4.10. rGDNF administration prevents loss of $nNOS^+$ neurons in myenteric plexus of $TLR2^{-/-}$ mice. *Panel A:* Immunofluorescence analysis of LMMP from WT and $TLR2^{-/-}$ mice treated with either rGDNF or vehicle (n=5 per group). Myenteric ganglia were labeled for HuC/D (green) and nNOS (red) antigens. Scale bars = 44 µm. *Panel B:* Number of $nNOS^+$ neurons *per* mm^2 in LMMP of WT and $TLR2^{-/-}$ mice treated with either rGDNF or vehicle (n=5 per group). *p<0.05 vs WT.

4.2 Antibiotic-mediated microbiota depletion alters ENS structure and function in adolescent mice

The ENS network coordinates the major functions of the gastrointestinal tract. As in the case of CNS, the development of ENS takes place within a constantly changing environment which, after birth, culminates in the establishment of a dynamic and complex microbial ecosystem (Borre et al., 2014; Wopereis et al., 2014; Kabouridis et al, 2015). How such changes affect ENS development and its subsequent function throughout life is still inadequately explored. This part of the study aimed to evaluate the specific contribution of the microbiota to ENS development and gastrointestinal function by depleting gut microflora using a combinations of antibiotics administered to WT mice during their adolescence.

4.2.1 Antibiotic-induced microbiota depletion produces a *germ free*-like phenotype in adolescent mice

Mice born and raised in a *germ-free* environment possess numerous characteristics distinguishing them from mice living in a conventional microbiological environment. Macroscopically, *germ-free* mice display hypotrophic secondary lymphoid organs, enlarged ceca, and reduced epithelial cell turnover (Smith et al., 2007). Treatment with broad spectrum antibiotics for 14 days successfully depleted gut microbiota (Brun et al., 2013, data not shown),

determining a marked enlargement in caecum (+92±0.1%, p<0.01; Figures 4.11A and B) that was associated with a threefold increase of its weight (p<0.05; Figure 4.11C). These anomalies provided evidence that microbiota depletion induces in mice phenotypic characteristics similar to those found in *germ-free* mice (Reikvam et al., 2011).

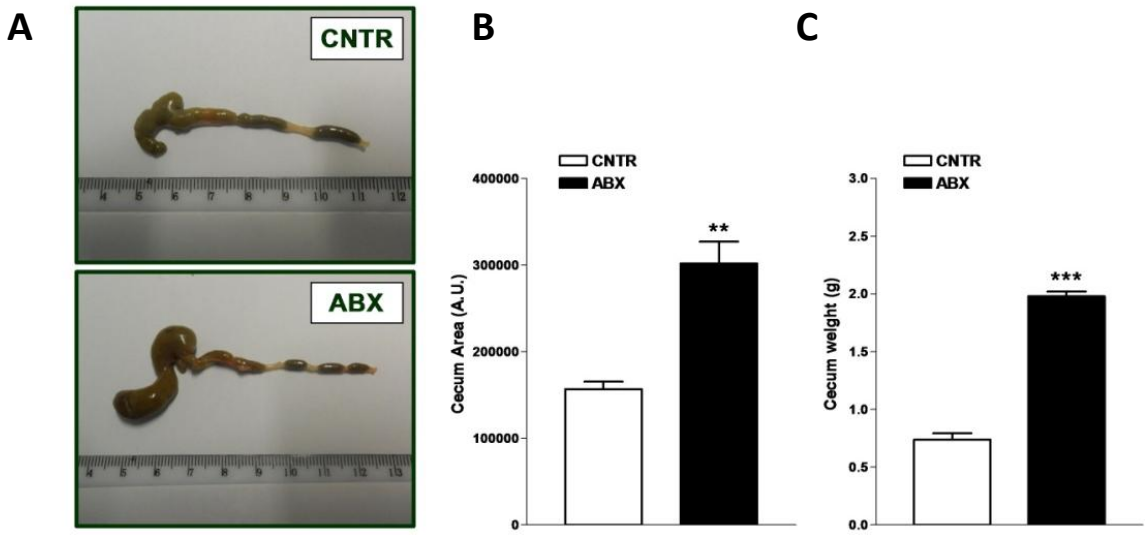

Figure 4.11. Antibiotic-induced microbiota depletion induces cecal enlargment. *Panel A:* Representative photographs of murine cecum-colon tract; cecal area (number of pixels, arbitrary units, A.U.) and weight *(Panel B e C)* in *in vivo* antibiotic-treated (ABX) and control (CNTR) mice (n=4). **p<0.05;***p<0.01 vs CNTR.

4.2.2 Depletion of gut microbiota causes morphological abnormalities in the ENS architecture

For a more comprehensive analysis of the impact of gut microbiota depletion on ENS homeostasis, the structural integrity of enteric neurons and ECGs was evaluated using confocal microscopy. The immunoreactivity for HuC/D protein, a pan-neuronal marker involved in sprouting and regenerations of neurons (De Giorgio et al., 2003) was significantly increase in antibiotic-treated (ABX) mice compared to control (CNTR) ones (+31±0.08%, p<0.01; Figures 4.12.A and B). The distribution of GFAP, the major component of intermediate filaments in the cytoskeleton of glial cells, was then analyzed. Confocal microphotographs showed a significant reduction of GFAP immunoreactivity in ileal samples of ABX mice (-50.3±0.08%, p<0.01; Figures 4.12A and C). Moreover, EGCs, which are located along myenteric ganglia, were also observed to have distorted $GFAP^+$ processes in mice after antibiotic treatment (Figure 4.13A). Changes in GFAP expression during glial cell differentiation, inflammation and injury have been associated to the functional state of glial cells (Cirillo et al., 2011a), suggesting that EGC homeostasis is dependent on microbiota health and composition.

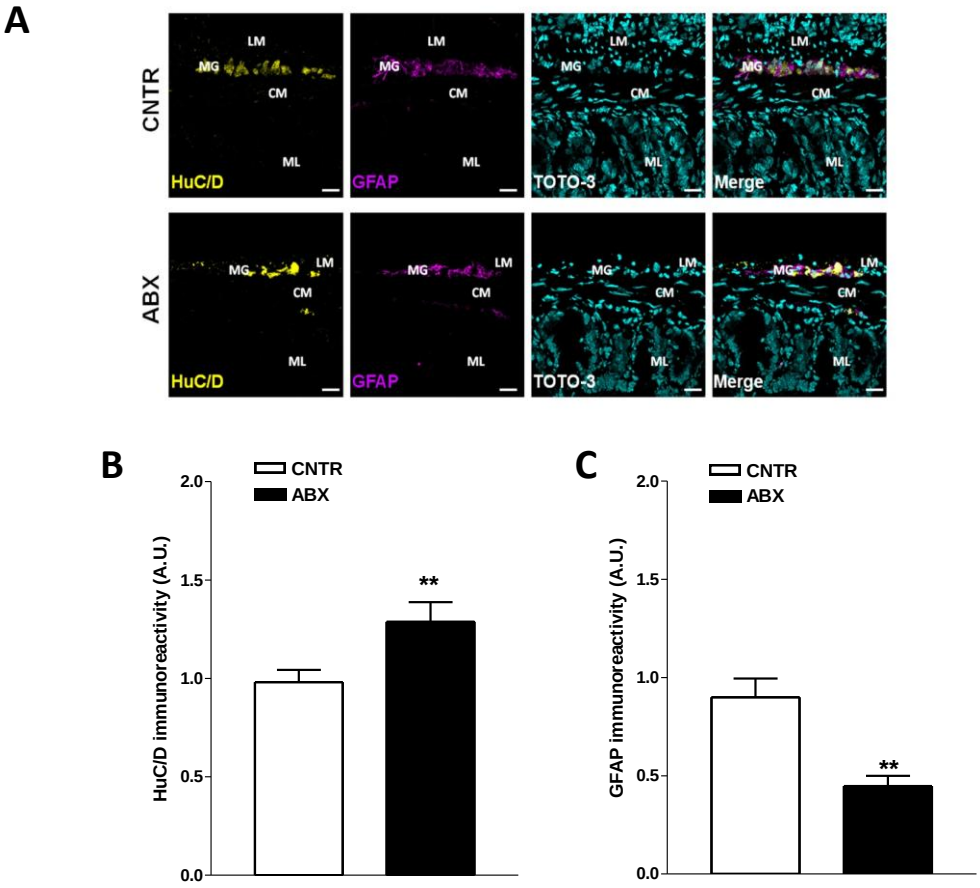

Figure 4.12. Depletion of gut microbiota causes morphological abnormalities in the ENS architecture. *Panel A:* Confocal microscopy analysis of the distribution of pan-neuronal marker HuC/D (yellow) and glial marker GFAP (magenta) in ileal cryosections from CNTR and ABX mice (n=4). Nuclei were stained with TOTO-3. Scale bar*:* 22 µm. LM, longitudinal muscle; CM, circular muscle; MG, myenteric ganglia; ML, mucosal layer. *Panel B:* Levels of HuC/D fluorescence intensity, expressed as arbitrary units (AU), in the myenteric ganglia of the ileal neuromuscular layer from ABX and CNTR mice **p < 0.01 versus CNTR. *Panel C:* Levels of GFAP fluorescence intensity, expressed as arbitrary units (AU), in the myenteric ganglia of ileum from ABX and CNTR mice **p< 0.01 versus CNTR.

Similar to the CNS astrocytes, ECGs physiologically express the protein S100β, a small diffusible calcium-binding protein, that exerts either trophic or toxic effects depending on its levels in the extracellular milieu (Cirillo et al., 2011a). S100β immunoreactivity was found to be significantly increased in the ileal myenteric plexus of ABX mice (+123.2±0.05%, p<0.001; Figures 4.13A and B). In parallel, the distribution of nNOS protein in microbiota-depleted mice was analyzed. The immunoreactivity for nNOS was significantly enhanced (+31.4±0.08%, p<0.05, Figures 4.14A and B) in ileal sections from ABX mice compared to controls. These abnormalities in the expression of both nNOS and S100β proteins, following microbiota depletion, are indicative of a possible microinflammatory state that can possibly trigger ENS neuropathy (Aubé et al., 2006; Cirillo et al., 2009).

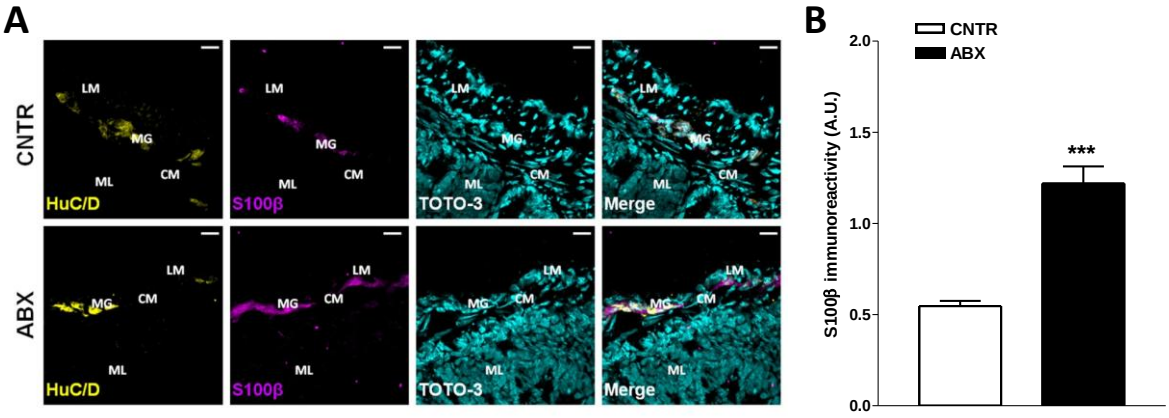

Figure 4.13. *Panel A:* Double-staining immunohistochemistry showing the distribution of S100β (magenta, glial calcium-binding protein) and HuC/D (yellow, pan-neuronal marker) proteins in ileal frozen cryosections from ABX and CNTR mice (n=5). Cell nuclei were stained with TOTO-3. For each antigen, a single optical confocal section is shown. Scale bar = 22 µm. LM, longitudinal muscle; CM, circular muscle; MG, myenteric ganglia; ML, mucosal layer. *Panel B:* Levels of S100β fluorescence intensity, expressed as arbitrary units (AU), in the myenteric ganglia of ileum from ABX and CNTR mice ***p<0.001 versus CNTR.

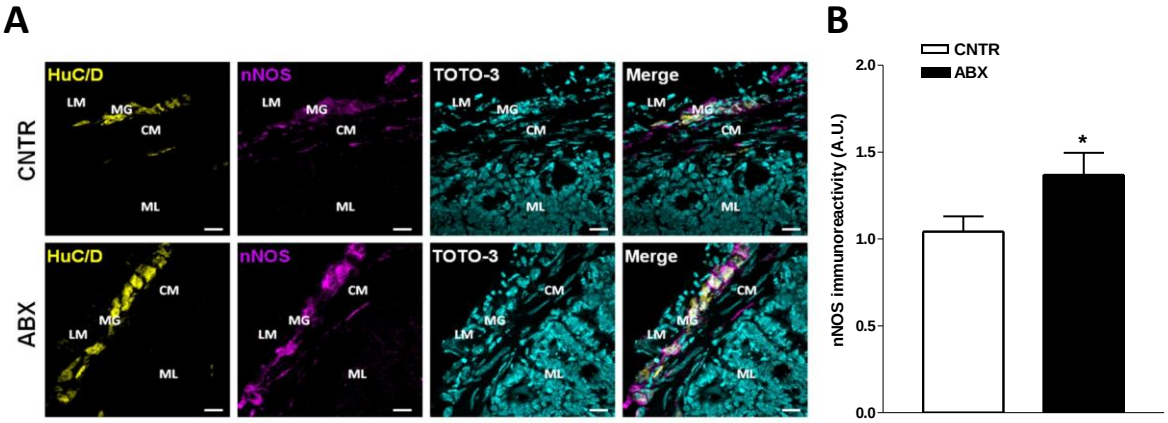

Figure 4.14. *Panel A:* Double-staining immunohistochemistry showing the distribution of neuronal nitric oxide synthase (nNOS, magenta; marker for nitrergic neurons) and HuC/D (yellow, pan-neuronal marker) proteins in ileal frozen cryosections from ABX and CNTR mice (n=5). Cell nuclei were stained with TOTO-3. For each antigen, a single optical confocal section is shown. Scale bar = 22 µm. LM, longitudinal muscle; CM, circular muscle; MG, myenteric ganglia; ML, mucosal layer. *Panel B:* Levels of nNOS fluorescence intensity, expressed as arbitrary units (AU), in the myenteric ganglia of ileum from ABX and CNTR mice *p< 0.05 versus CNTR.

4.2.3 Antibiotic-induced microbiota depletion alters ileal neuromuscular contractility

The effect of ABX treatment on intestinal neuromuscular function was examined in vitro by measuring changes in muscle tension by means of the isolated organ bath technique. To evaluate muscle responsiveness, ileal segments of ABX and CNTR mice were exposed to the non selective cholinergic receptor agonist carbachol (CCh), added cumulatively to organ baths

(0.01–100 µmol/L) in order to obtain a concentration-response curve. A significant downward shift of the concentration-response curve to CCh and a consequent decrease in Emax occurred in ABX (-52±6%, p<0.01; Figure 4.15A) compared with CNTR group. In a second series of experiments, contractions were evoked by addition of the depolarizing agent KCl (60 mM) to ileal preparations from ABX and CNTR mice. The neuromuscular response elicited by KCl-induced depolarization was significantly decreased in ABX mice (-28±8%, p<0.01; Figure 4.15B) compared with CNTR mice.

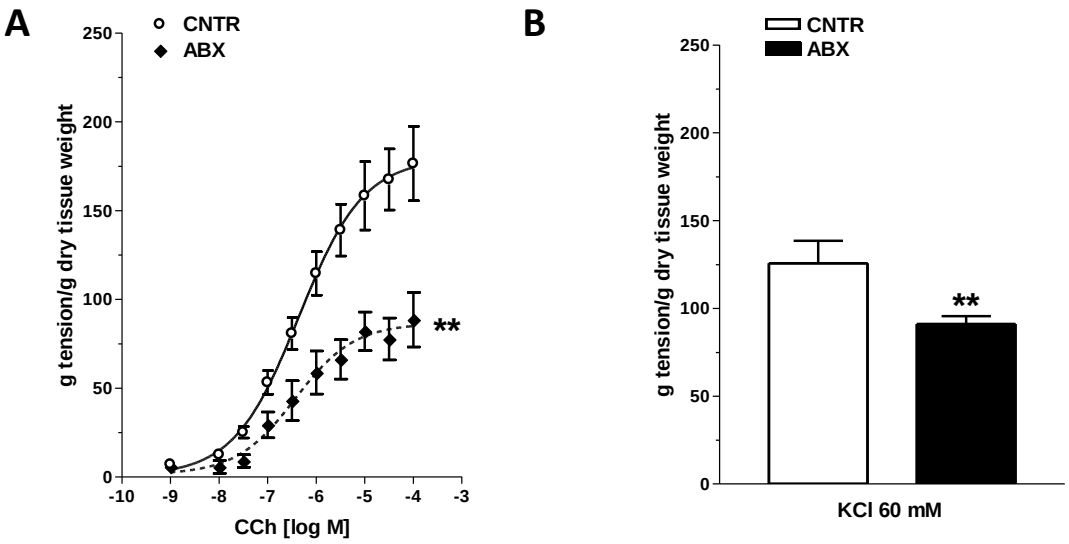

Figure 4.15. Effect of *in vivo* ABX-treatment on carbachol and KCl-induced intestinal contractions. Concentration–response curves for carbachol (0,001-100 µM) obtained in isolated ileal preparations from ABX and CNTR mice *(Panel A)* and excitatory effect elicited by KCl 60 mM (*Panel B*). Data are reported as mean ± SEM (n=8) and are expressed as g tension/g dry tissue weight (g/g). **p<0.01 vs CNTR.

To determine whether the differences in ileal contraction between ABX and CNTR mice were associated with alterations in neuromuscular function, we evaluated ileal contractility following EFS with increasing frequencies and constant voltage (2–40 Hz, 40 V). Altered excitatory neurotransmission in ABX mice was shown by decreased EFS-elicited contractions with a significant reduction of 10 Hz-neuronal cholinergic response (-62±7%, p<0.01; Figure 4.16) compared to CNTR mice.

To evaluate the impact of the main inhibitory neurotransmitter NO on intestinal motility, in a third series of experiments, atropine (1 µM) and guanethidine (1 µM) were added to Krebs solution, in order to block cholinergic and adrenergic transmission (so called NANC conditions), respectively. NANC-stimulation, evoked by EFS at 10 Hz, was performed with or without the pan-NO synthase inhibitor Nω-nitro-l-arginine methyl ester (L-NAME, 100 µM).

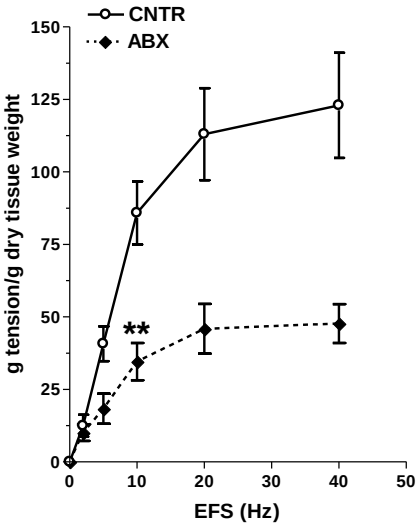

Figure 4.16. Neuromuscular excitatory response induced by EFS (2-50 Hz) in CNTR and ABX mice. Data are reported as mean ± SEM (n=8) and are expressed as g tension/g dry tissue weight (g/g). **p<0.01 vs CNTR.

EFS-induced NANC stimulation at 10 Hz caused a marked relaxation, measured as AUC, that results significantly greater in ileal preparations of ABX mice compared to CNTR mice (+144±40%, p<0.05; Figure 4.17). Addition of L-NAME significantly blocked EFS (10Hz)-evoked NANC ileal relaxation in ileal samples of CNTR mice, however, in ABX mice this response was only partially abolished by the NOS inhibitor (-64±10%, p<0.001; Figure 4.17). Our findings suggest that the depletion of intestinal microbiota influences inhibitory neurotransmission not only the one mediated by nitrergic neurotransmission but also those controlled by other inhibitory pathways (e.g. ATP or VIP), known to be involved in sustaining intestinal contractility function (Mulè and Serio, 2003; Zizzo et al., 2003).

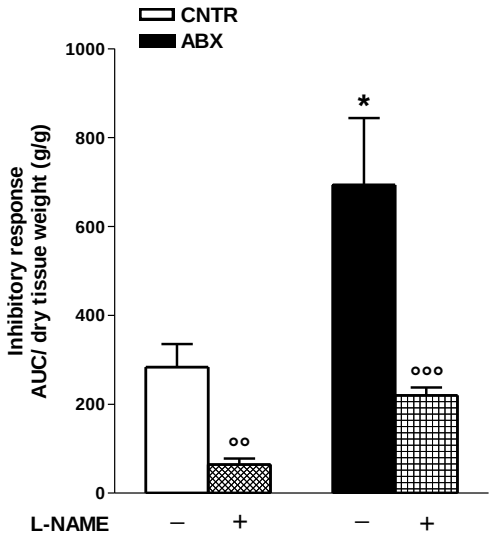

Figure 4.17. Effect of *in vivo* ABX tretament on inhibitory contractile responses. EFS (10Hz)-evoked NANC relaxation in presence or absence of L-NAME in ileal preparations from CNTR and ABX mice. Data are expressed as percentage of abolition of contraction (Area Under the Curve, AUC)/g dry tissue weight (n=8). *p<0.05 vs CNTR; °°p<0.01, °°°p<0.001 vs respective control.

4.2.4 Gastrointestinal motor function is impaired in antibiotic-treated mice

To evaluate gut motor function *in vivo*, the measurement of GI transit was performed. Antibiotic-mediated microbiota depletion determined a significant decrease of GI transit, as evidenced by the altered gut distribution of nonabsorbable FITC-dextran (Figure 4.18A). Moreover ABX mice displayed a half reduction of the geometric centre compared to CNTR mice (Figure 4.18B, GC_{ABX}=3.5, range: 2.9-4.0 vs GC_{CNTR}=7.3, range: 6.2-7.9; p<0.01).

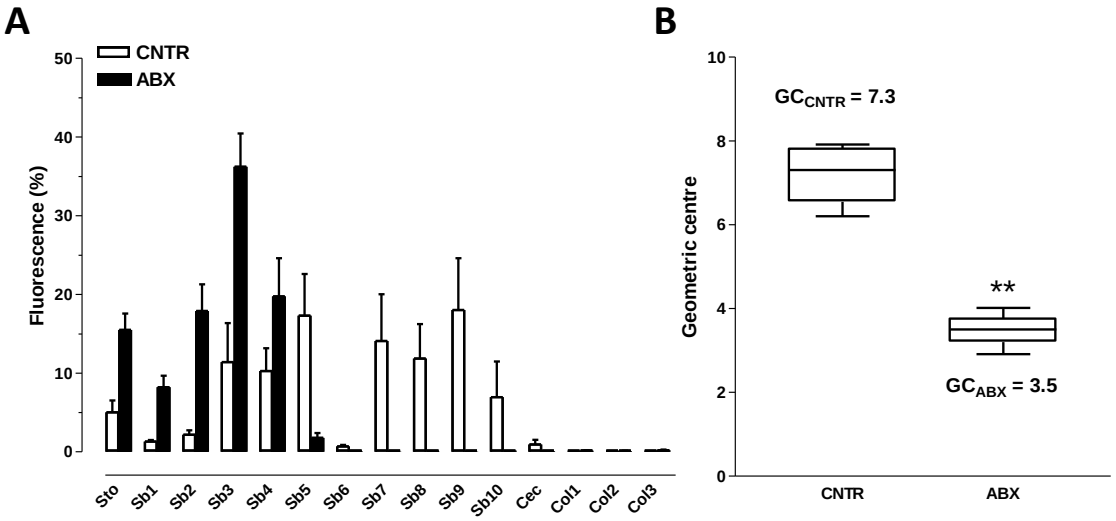

Figure 4.18. Impaired gastrointestinal transit in ABX mice. *Panel A:* Gastrointestinal (GI) transit was measured as % of nonabsorbable FITC-dextran distribution in 15 gut segments, comprising stomach (Sto), small bowel (Sb 1–10), cecum (Cec), and colon (Col 1–3), 30 minutes after oral administration in CNTR and ABX mice (n=6). *Panel B:* Calculation of the geometric center (GC) demonstrated a significant alteration of GI transit in ABX compared to CNTR mice. Data are reported as the mean ± standard error of the mean (SEM; n=6) for *panel A* and as median, minimum, maximum, upper and lower quartiles for *panel B*. **p<0.01 vs CNTR.

The delay of distribution of FITC-dextran throughout the GI tract of mice treated with ABX cocktail was reflected in corresponding changes in stool frequency and water content, which were determined from fecal pellets obtained during a 1 h-collection period. There was a significant reduction in stool frequency (-29.5±0.5%, p<0.01; Figure 4.19A) together with an increase in faecal water content (+35±7%, p<0.01; Figure 4.19B) in ABX mice compared to controls, indicating a possible state of constipation and alteration of intestinal permeability as a result of antibiotic treatment.

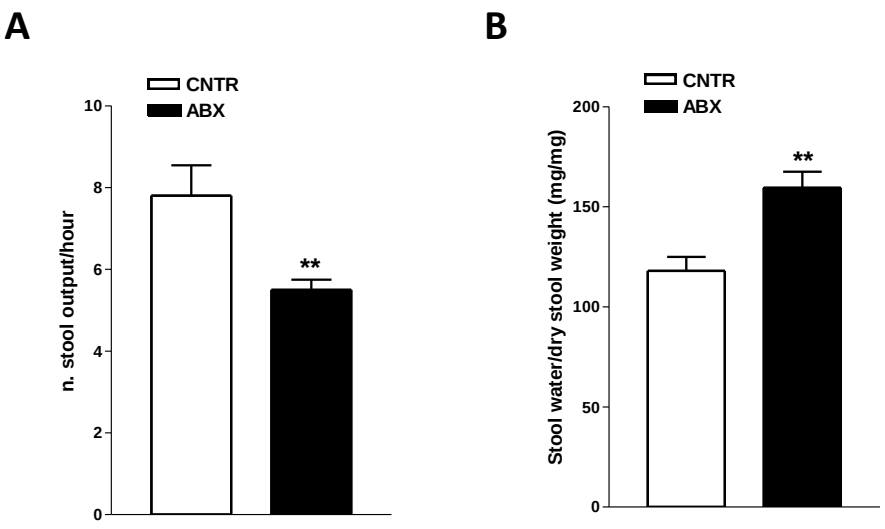

Figure 4.19. Changes in stool frequency and water content after ABX treatment. *Panel A*: Pellet frequency per hour was assessed in CNTR and ABX mice (n=10). *Panel B*: CNTR and ABX stool water content was determined by calculating the water content in faecal pellets collected over a one hour period (n=12). **p<0.01 vs CNTR.

4.2.5 Antibiotic-induced microbiota depletion influences ENS neurochemical coding

To further examine the role of gut microbiota in maintaining the ENS neurochemical code, we assessed the functional integrity of the myenteric plexus by performing acetylcholinesterase and NADPH-diaphorase biochemical staining in ileal LMMP whole mount preparations of ABX mice (Anitha et al., 2012; Brun et al., 2013).

Acetylcholinesterase (AChE) is the enzyme involved in catalyzing the hydrolysis of the excitatory neurotransmitter acetylcholine in choline and acetic acid, thus playing an essential role in cholinergic neurotransmission (Moore and Johnson, 2005; Anitha et al., 2006; Anitha et al., 2012). The number of $AChE^+$ fibers was increased in LMMP whole mount preparations of ABX (+37±9%, p<0.001; Figure 4.20) compared with CNTR mice suggesting that antibiotic-induced dysbiosis may affect intestinal excitatory neurotransmission.

The NADPH-diaphorase (NADPH-d) biochemical staining reveals the NO synthase activity in the myenteric plexus of CNTR and ABX mice (Anitha et al., 2006; Anitha et al., 2012). The localization of NADPH-d activity was detected by blue formazan precipitate that was formed by the enzyme from NBT in the presence of β-NADPH. Exposure to the ABX treatment did not affect the number of NADPH-d^+ cells that was comparable to CNTR (Figure 4.21).

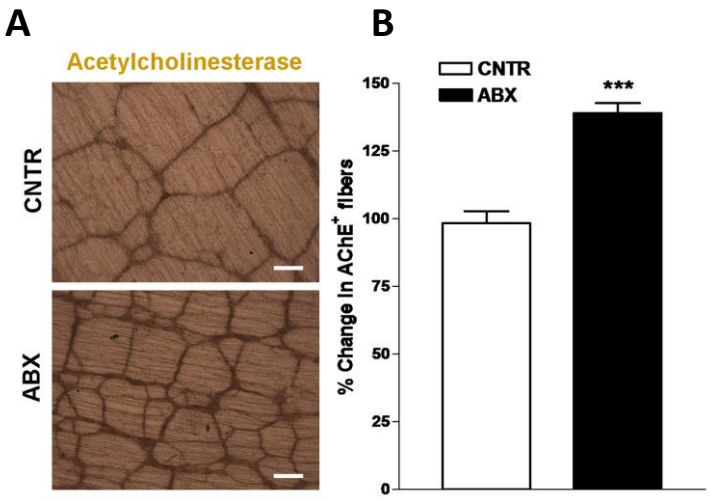

Figure 4.20. Antibiotic-induced dysbiosis alters the distribution of acetylcholinesterase (AChE)$^{+}$ neurons and fibers in the ileal ENS. Representative photographs of AChE^{+} neurons and fibers distribution (*panel A*) and percentage changes in the number of the AChE^{+} fibers *(panel B)* in whole mount preparations of ileum LMMPs from ABX and CNTR mice (n=4). Scale bar = 300 μm. ***p<0.001 vs CNTR.

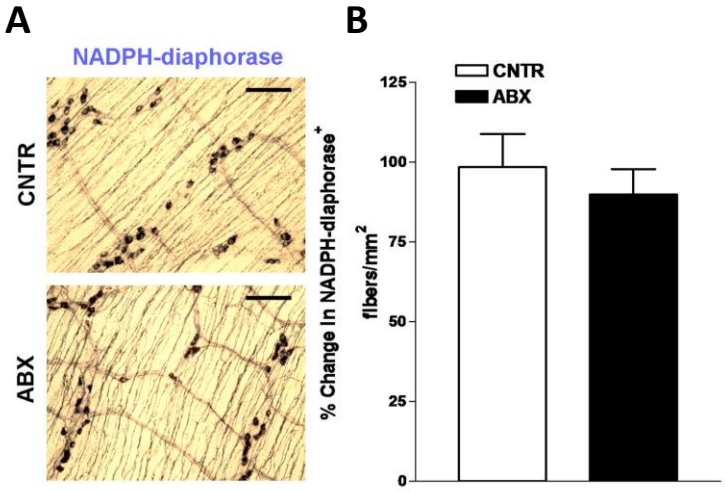

Figure 4.21 Influence of *in vivo* antibiotic-treatment on NADPH-d^{+} fibers distribution in ileal myenteric plexus. *Panel A*: Representative microphotographs showing the distribution of the NADPH-d^{+} fibers in the ENS of CNTR and ABX mice. Scale bar = 200 μm. *Panel B*: Percentage changes in the number of NADPH-d^{+} fibers in whole mount preparations of CNTR and ABX mice (n=5).

4.2.6 Antibiotic-induced microbiota depletion increases mRNA of GluN1 subunit expression

The aminoacid glutamate via the activation of the ionotropic AMPA and NMDA receptors appears to play a pivotal role in the modulation of several neurotransmitters release as well as in the control of enteric motor parameters (Kirchgessner, 2001). In particular, NMDA receptors are involved in integrating the firing of specific peripheral neurons and amplifying nociceptive

signals. This process leads to central sensitization, which is characterized by enlarged neuronal receptive fields and hyperalgesia (Willert et al., 2004). Quantitative RT-PCR analysis showed a significant increase of mRNA levels of GluN1 subunit of NMDA receptor in ileal LMMP preparations from ABX mice compared to CNTR (+35±0.04%, $p<0.01$; Figure 4.22), indicating that changes in composition of gut microbiota can impact the glutamatergic transmission in the enteric neuronal circuitries leading to visceral hypersensitivity (Zhou et al., 2009).

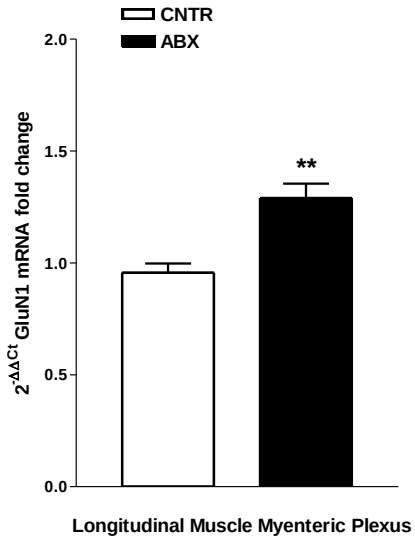

Figure 4.22. Antibiotic-induced microbiota depletion increases mRNA of GluN1 subunit expression. RT-PCR quantification of GluN1 transcripts performed in ileal LMMP preparations of ABX (black bar) and CNTR mice (white bar). Values are expressed as mean ± SEM (n=5) of the percentage variation of relative gene expression with respect to relative control. The relative gene expression was determined by comparing $2^{-\Delta\Delta Ct}$ values normalized to β-actin. **p<0.01 vs CNTR.

4.2.7 TLR2 stimulation partially corrects ENS anomalies caused by antibiotic-induced microbiota depletion

As previously reported in this study, disturbances in neurotrophic factor signaling, such as GDNF, can determine morphofunctional abnormalities of ENS and TLR2 signaling is required for adequate GDNF expression in the gut. Antibiotic-induced microbiota depletion determined a decrease of mRNA levels of GDNF (-89±0.001%, $p<0.05$; Figure 4.23A) together with a reduction of its immunoreactivity, as shown in Figure 4.23B. Administration of Pam3CSK4, a TLR2 bacterial-derived ligand, to ABX mice during the second week of ABX treatment determines a 7-fold increase of GDNF mRNA levels in LMMP preparations ($p<0.001$; Figure 4.24A). Treatment with Pam3CSK4 also partially corrected anomalies in intestinal contraction of ABX mice, as shown in Figure 4.24B, confirming that ENS integrity relies on gut microbiota-TLR2-GDNF axis.

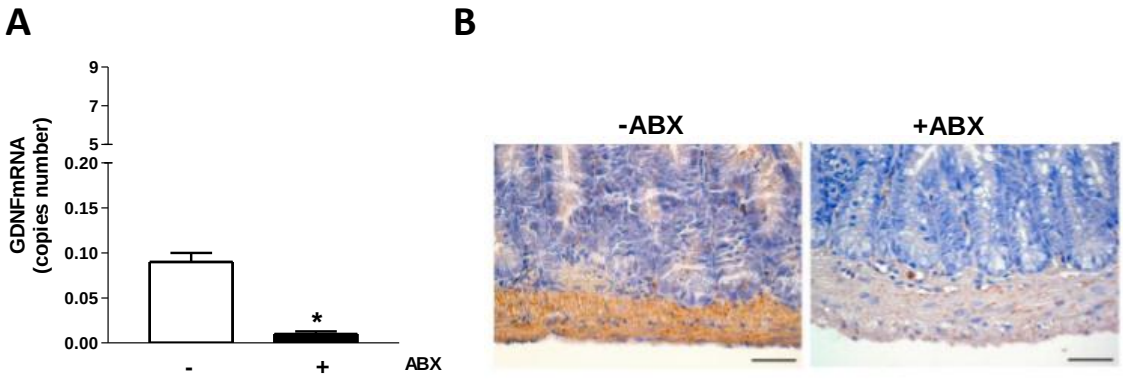

Figure 4.23: Antibiotic-induced microbiota depletion influences GDNF expression. *Panel A*: Quantitative RT-PCR analysis of GDNF mRNA in LMMP from WT mice treated for 14 days with or without antibiotics (ABX) (n=4 per group).*p<0.05 vs CNTR. *Panel B*: GDNF immunostaining in ileum of WT mice with or without antibiotics (ABX) for 14 days. Scale bar = 75 µm.

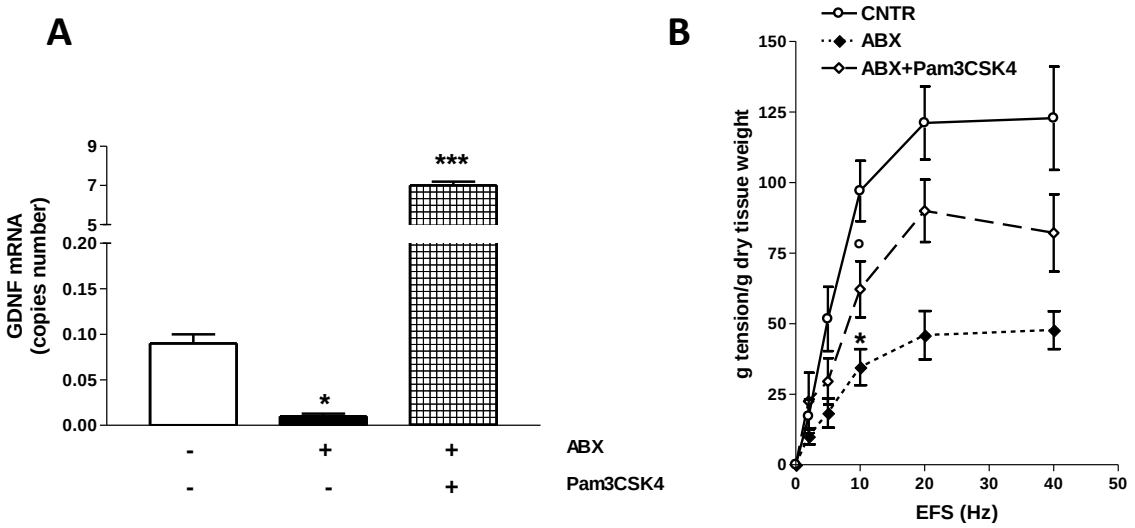

Figure 4.24. Pam3CSK4 administration improves intestinal neuromuscular contractility in ABX mice via TLR2-mediated GDNF production. *Panel A:* Quantitative RT-PCR analysis of GDNF mRNA in LMMP from WT mice treated 14 days with antibiotics (ABX) with or without Pam3CSK4 supplemented daily during the second week of treatment with antibiotics (n=4 per group). *p<0.05 vs. CNTR, ***p<0.001 vs ABX. *Panel B:* Neuromuscular excitatory response induced by EFS (2-50 Hz) in CNTR, ABX and ABX mice treated daily with Pam3CSK4. Data are reported as mean ± SEM (n=8) and are expressed as g tension/g dry tissue weight (g/g). *p<0.01 vs CNTR, °p<0.05 vs ABX.

4.2.8 OXPAPC inhibition of TLR2 and TLR4 signaling alters ileal neuromuscular contractility and influences ENS neurochemical code

To evaluate the effect of inhibition of both TLR2 and TLR4-mediated signaling on the intestinal motor responses, WT mice at postnatal day 14 (P14) were treated with OXPAPC administered

intraperitoneally once a day for 1 week. At the end of the treatment, *in vitro* contractility studies were performed. Receptor-mediated response to carbachol, added cumulatively to organ baths (0.01–100 μmol/L), resulted significantly increased in OXPAPC-treated mice (+42±3.5%, $p<0.01$; Figure 4.25A) compared to mice treated with vehicle. Moreover, frequency-response curve, obtained following electrical field stimulation (EFS) with increasing frequencies, was significantly greater in mice after OXPAPC administration (+88±10% at 10 Hz, $p<0.01$ vs CNTR; Figure 4.25B), indicating that inhibition of TLR2 and TLR4 signaling can influence intestinal neuromuscular contractility.

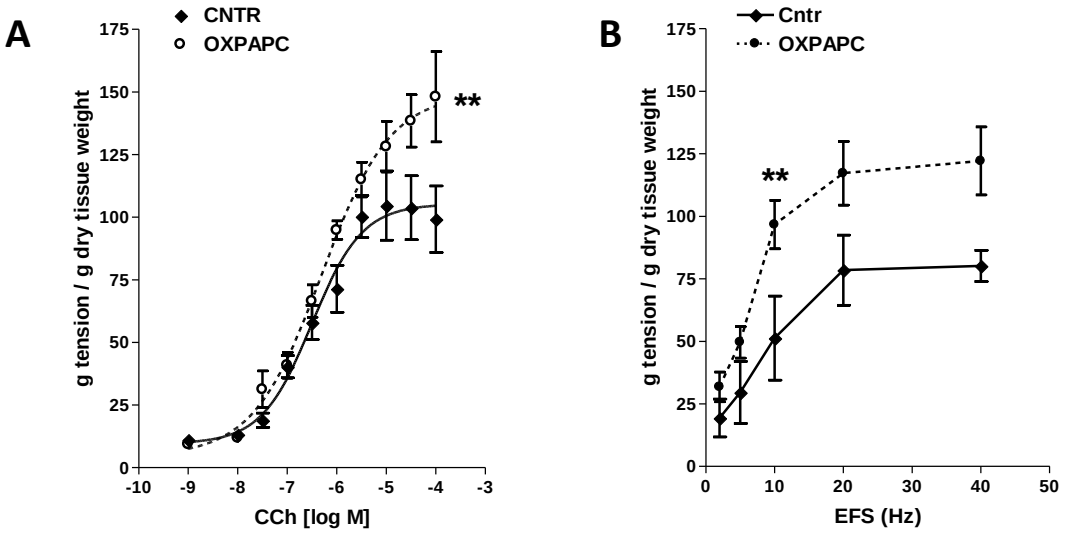

Figure 4.25. Effect of OXPAPC-mediated inhibition of TLR2 and TLR4 signaling on ileal neuromuscular contractility. Concentration-response curve to carbachol (Panel A) and excitatory response to electric field stimulation (EFS; Panel B) in isolated ileal segments from mice treated with OXPAPC and mice treated with vehicle (0.9% saline, CNTR). Data are reported as mean ± SEM (n=6 for group) and are expressed as g tension/g dry tissue weight (g/g). **p<0.01 vs CNTR.

In order to assess whether OXPAPC-mediated inhibition of TLR2 and TLR4 signaling could change the distribution of the cholinergic neurons in myenteric ganglia, the biochemical staining of AChE was performed. The number of $AChE^+$ fibers was increased in LMMP whole mount preparations of OXPAPC-treated mice (+29.5±1.2%, $p<0.001$; Figures 4.26A and B) compared to CNTR ones. These findings suggest that the blocking of the recognition of commensal microbial components by TLR2 and TLR4 can impact the integrity of ENS excitatory neuronal networks and consequently the intestinal function.

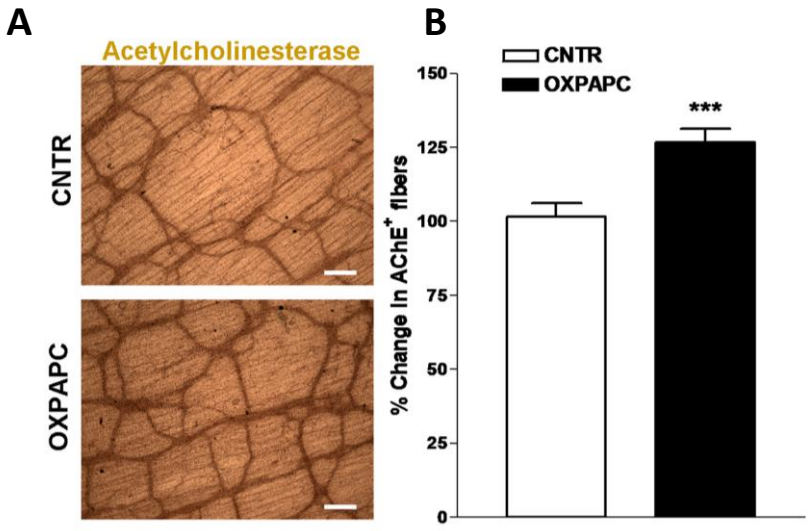

Figure 4.26. Influence of OXPAPC treatment on acetylcholinesterase^{+} fibers distribution in ileal myenteric plexus. *Panel A*: Representative microphotographs showing the distribution of acetylcholinesterase (AChE)$^{+}$ fibers in the ENS of mice treated with either OXPAPC or vehicle. Scale bar: 300 µm. *Panel B*: Percentage changes in the number of AChE^{+} fibers (n=5). ***p<0.001 vs CNTR

4.3 CATECHOL-O-METHYLTRANSFERASE GENETIC REDUCTION INFLUENCES GI MOTILITY AND ENS STRUCTURE

Intestinal functional disorders, such as irritable bowel syndrome (IBS), are characterized by abdominal discomfort more frequently in women and are often associated with a variety of psychiatric illness, ranging from anxiety and depression to obsessive-compulsive disorder. The etiology of these intestinal functional disorders is multi-factorial and has been ascribed to dysregulation of the brain-gut axis, hypothalamus-pituitary-adrenal (HPA) axis, altered gastrointestinal motility, visceral hypersensitivity, infectious factors, enhanced immunological reactivity, genetic susceptibility and psychosocial factors (Karling et al., 2011). The presence of a bidirectional communication system between CNS and ENS implies that both gut and brain are reciprocally able to influence each other via vagal and sympathetic afferent fibers (Fadgyas-Stanculete et al., 2014). The pathophysiological links between psychiatric symptoms and gut manifestation in IBS has not been yet fully understood but it has been proposed that changes in the endogenous pain modulation system, autonomic nervous system (ANS) and HPA axis together with altered catecholamine levels may each play a role (Fadgyas-Stanculete et al., 2014). Recently, genetic variation in COMT, the key enzyme responsible for catecholamine degradation (e.g. dopamine, epinephrine, and norepinephrine), has been shown to be associated with anxiety, behavior disorders, pain and IBS (Hall et al., 2015). A functional polymorphism of the COMT enzyme is the val158met. The val/val genotype results in a three to fourfold higher enzymatic activity compared with the met/met genotype, with the

val/met genotype exhibiting intermediate activity (Karling et al., 2011). Since visceral pain syndrome is associated to low COMT activity and this condition is one of the main symptoms of IBS, this part of the study aimed to explore the relationship between an altered COMT activity and gastrointestinal function using a model of genetic reduction of COMT enzyme.

4.3.1 COMT reduced expression alters ileal structure

Female mice heterozygous for COMT gene ($COMT^{+/-}$) were used for the following experiments. Phenotypic histological analysis revealed no morphological differences between ileal sections from $COMT^{+/-}$ and WT female mice. However, hematoxylin-eosin stained sections showed a significant increase in muscle layer thickness between $COMT^{+/-}$ and WT mice (+87±0.01%, $p<0.01$; Figures 4.27A and B).

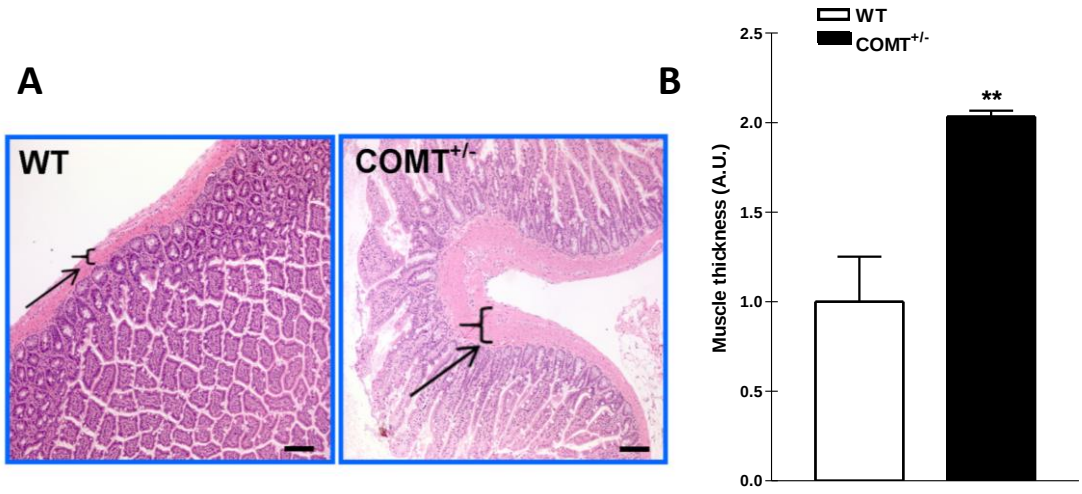

Figure 4.27. Morphological analysis of full-tickness ileal samples from $COMT^{+/-}$ and WT mice. *Panel A*: Representative ileal sections, stained with H&E, from $COMT^{+/-}$ and WT mice (n=4). Scale bar: 25 µm. *Panel B*: Changes in tickness (arrow) of ileal tunica muscularis (arbitrary units, A.U.) in $COMT^{+/-}$ mice (n=5). **p<0.01 vs WT.

4.3.2 COMT reduced expression influences ENS neurochemical coding

Along with the reported abnormalities of the ENS in various functional gastrointestinal motor disorders (Di Nardo et al., 2008), immunohistochemical analysis of LMMP whole mount preparations revealed an altered distribution of the immunoreactivity for GFAP and S100β in glial cells within the myenteric plexus of $COMT^{+/-}$ mice, whereas no difference in the βIII tubulin network was observed (Figure 4.28).

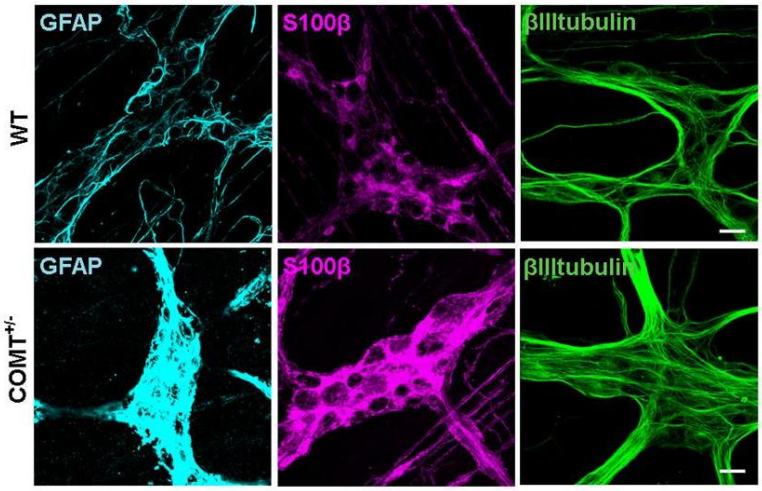

Figure 4.28. COMT genetic reduction perturbs myenteric plexus architecture. Representative confocal microphotographs showing the distribution of the glial markers GFAP (cyan) and S100β (magenta), and the neuronal marker βIII tubulin (green) in ileal whole mount preparations of WT and $COMT^{+/-}$ mice (n=4). Scale bar = 22 µm.

4.3.3 $COMT^{+/-}$ mice display abnormal gastrointestinal neuromuscular function

The effect of COMT genetic reduction on gut neuromuscular function was examined *in vivo* by assessing gastrointestinal transit and *in vitro* by measuring tension changes in ileal segments. Gastrointestinal transit was assessed by measuring the distribution of nonabsorbable FITC-labeled dextran from the stomach to the colon. COMT genetic reduction determined an altered gut distribution of the fluorescent probe, associated to a significant increase of gastrointestinal transit in $COMT^{+/-}$ mice compared to WT (Figures 4.29 A and B; $GC_{COMT^{+/-}}$=6.5, range: 5.7-7.5 vs GC_{WT} 5.8, range: 4.9-6.4; $p<0.05$).

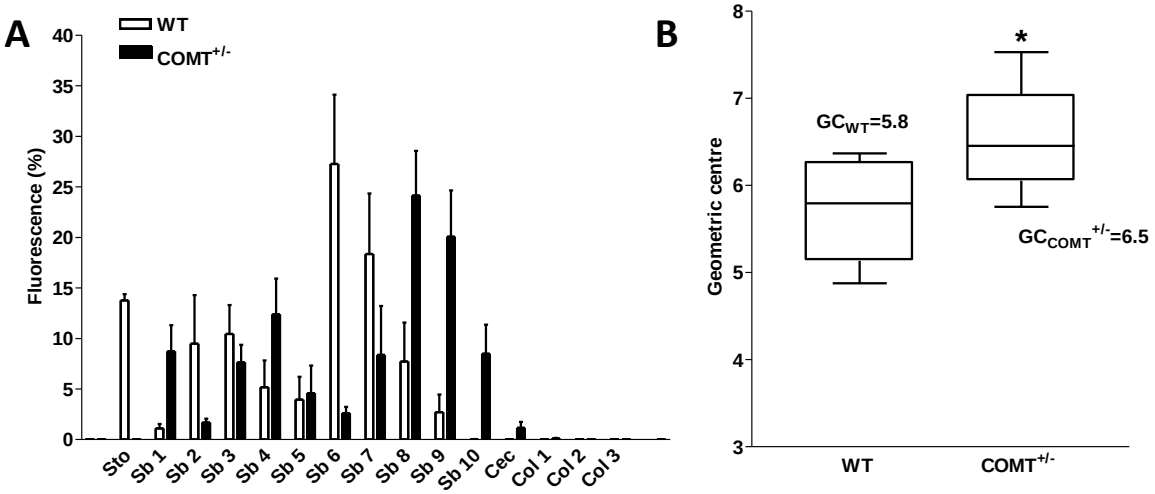

Figure 4.29. Impaired gastrointestinal transit in $COMT^{+/-}$ mice**.** *Panel A:* Percentage of the nonabsorbable FITC-dextran distribution in 15 gut segments, comprising stomach (Sto), small bowel (Sb 1–10), cecum (Cec), and colon (Col 1–3), 30 minutes after oral administration in WT and $COMT^{+/-}$mice (n=6). *Panel B:* Calculation of the geometric center (GC) demonstrated a significant alteration of GI transit in $COMT^{+/-}$ compared to WT mice (n=6). Data are reported as the mean ± SEM for panel A and as median, minimum, maximum, upper and lower quartiles for panel B (n=6). *$p<0.05$ vs WT.

In *in vitro* contractility studies, ileal preparations were subjected to increasing concentrations of carbachol (a non selective cholinergic agonist) in order to underline anomalies in the receptor-mediated response. The maximal contractile response (Emax) resulted markedly reduced in $COMT^{+/-}$ mice (Emax=-41±8%, $p<0.01$, Figure 4.30A) with respect to the value obtained in WT mice. To study the influence of COMT genetic reduction on neuromuscular activity, the ileal preparations were subjected to KCl 60 mM. The registered depolarizing effect resulted significantly reduced (-38±5%, $p<0.01$; Figure 4.30B) in $COMT^{+/-}$ mice compared with WT. To determine whether the differences in muscle contraction between WT and $COMT^{+/-}$ mice were associated with alterations in neuromuscular function, we measured changes in muscle tension in response to the EFS. COMT genetic reduction determined changes in the neuronal contractile response with a significant reduction of the neuronal excitatory cholinergic contraction up to 20 Hz (-33±6% at 10 Hz, $p<0.01$; Figure 4.30C).

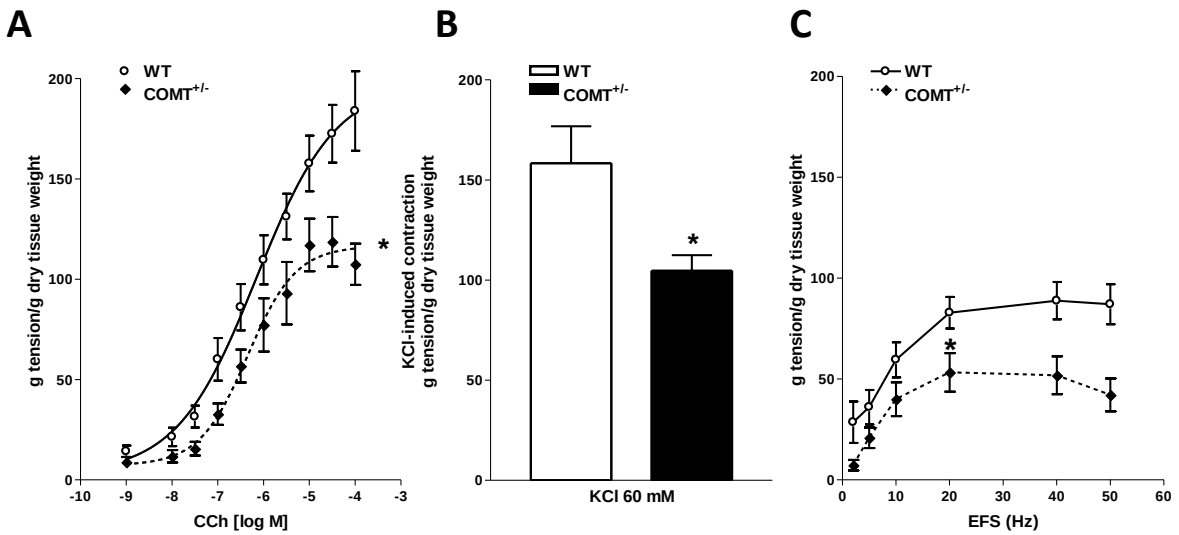

Figure 4.30. COMT genetic reduction affects ileal neuromuscular contractility. Concentration–response curves to carbachol (CCh, *Panel A*), excitatory response to KCl 60 mM (*Panel B*) and EFS-induced contractions (*Panel C*) in isolated ileal segments from WT and $COMT^{+/-}$ mice. Data are reported as mean ± SEM and are expressed as g tension/g dry tissue weight (n=8). *p<0.01 vs WT.

4.3.4 COMT reduced expression alters ileal neuromuscular inhibitory response

Non-adrenergic, non cholinergic (NANC) inhibitory neurons play an important role in the regulation of GI motility, and NO is the major NANC inhibitory neurotransmitter of the GI tract. To evaluate the influence of COMT genetic reduction on inhibitory neurotransmission, in this series of experiments atropine (1 μM) and guanethidine (1 μM) were added to Krebs solution, in order to block cholinergic and adrenergic transmission (so called NANC conditions), respectively. EFS at 10 Hz was then performed with or without the pan-NOS inhibitor L-NAME

(100 μM). EFS-induced NANC stimulation at 10 Hz caused a marked relaxation, that resulted significantly greater in ileal preparations of $COMT^{+/-}$ mice compared to WT (+114±10%, $p<0.05$; Figure 4.31). Pretreatment of ileal samples with L-NAME significantly blocked EFS (10Hz)-evoked NANC relaxation in WT mice, demonstrating its neuronal origin (Mulè and Serio, 2003). In $COMT^{+/-}$ mice the relaxation was partially block by the NOS inhibitor (-72±8%, $p<0.05$; Figure 4.31) to suggest that the disturbances of catecholaminergic transmission due to low activity of COMT can affect not only nitrergic neurotransmission but also the release of other neurotransmitters inhibitors (eg ATP, VIP, or dopamine) involved in supporting intestinal motor function (Mulè and Serio, 2003; Zizzo et al., 2003; Li et al., 2006).

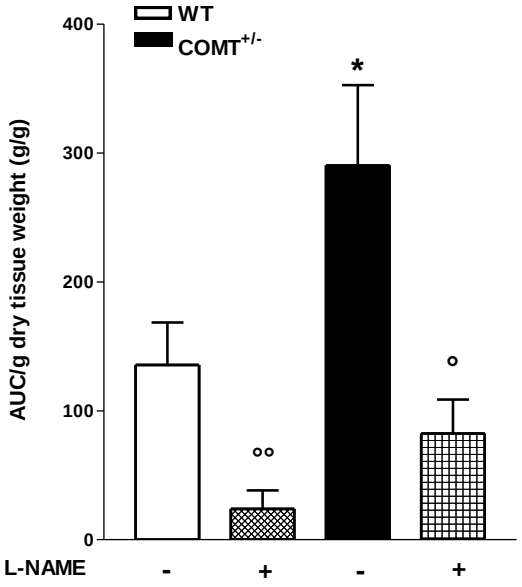

Figure 4.31. Effect of COMT genetic reduction on inhibitory contractile responses. EFS (10Hz)-evoked NANC relaxation in presence or absence of L-NAME in ileal preparations from $COMT^{+/-}$ and WT mice. Data are expressed as percentage of abolished contraction (AUC)/g dry tissue weight (n=8). *$p<0.05$ vs WT; °$p<0.05$, °°$p<0.01$ vs respective control.

4.3.6 COMT reduced expression influences ENS neurochemical coding

Given the functional alterations on both excitatory and inhibitory component of intestinal contractility in presence of a reduced COMT activity, the distribution of excitatory (i.e. cholinergic) and inhibitory (i.e. nitrergic) neurons in myenteric ganglia of $COMT^{+/-}$ mice was evaluated. Acetylcholinesterase biochemical staining revealed a significantly reduction of acetylcholinesterase^{+} neurons and fibers in whole mount preparations of LMMP of $COMT^{+/-}$ mice (-26±3%, $p<0.01$; Figure 4.32) compared with WT. Moreover, COMT genetic reduction determined an altered distribution of NADPH-d^{+} neurons with an increase of nNOS^{+} large and small fibers in transgenic mice (+41±5%, $p<0.01$; Figure 4.33), suggesting a role of the

catecholaminergic transmission in modulating ENS neurochemical code and thus gut motor function.

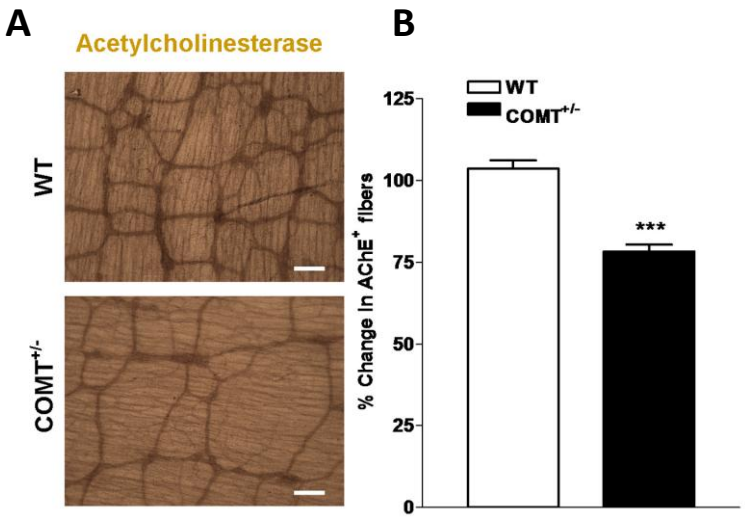

Figure 4.32. Changes in the distribution of acetylcholinesterase^{+} fibers in the ENS of COMT$^{+/-}$ mice. *Panel A:* Representative microphotographs showing the distribution of acetylcholinesterase (AChE^{+}) fibers in the ENS of WT and COMT$^{+/-}$ mice (n=4). Scale bar = 300 µm. *Panel B*: Percentage changes in the number of AChE^{+} fibers. ***p<0.01 vs WT.

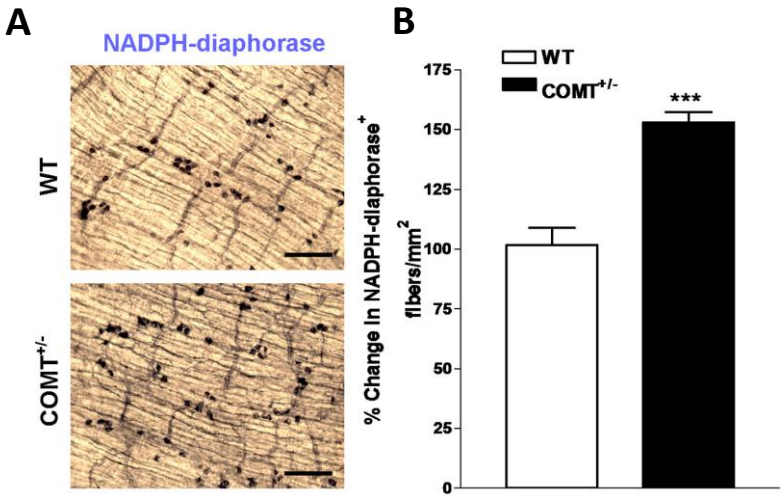

Figure 4.33. Influence of COMT genetic reduction on NADPH-d^{+} fibers distribution in ileal myenteric plexus. *Panel A*: Representative microphotographs of the distribution of NADPH d-stained neurons in ileal LMMP preparations from WT and COMT$^{+/-}$ mice (n=4). Scale bar = 200 µm. *Panel B* Percentage changes in the number of NADPH-d^{+} fibers. ***p<0.01 vs WT.

4.3.7 COMT reduced expression increases mRNA of GluN1 subunit expression

Quantitative RT-PCR analysis showed an increase of GluN1 subunit mRNA levels in ileal LMMP preparations from COMT$^{+/-}$ mice compared to WT (+72±0.2%, p<0.01; Figure 4.34). These results indicate that COMT genetic modifications can impact in the glutamatergic transmission, leading to altered visceral pain perception, a frequent IBS comorbidity (Moloney et al., 2015).

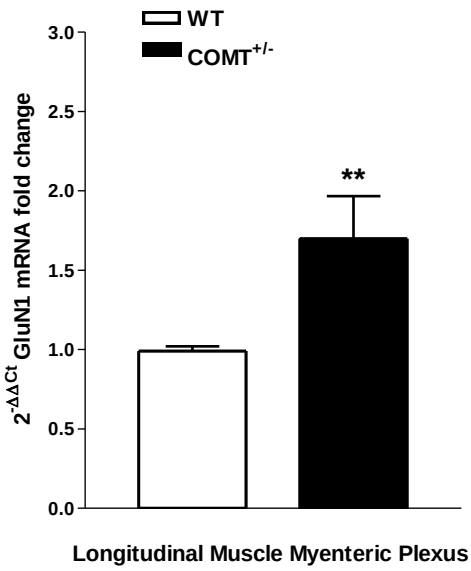

Figure 4.34. Effect of COMT genetic reduction on NMDA receptor GluN1 subunit expression in murine ileal myenteric plexus. RT-PCR quantification of GluN1 transcripts performed in ileal LMMP preparations of $COMT^{+/-}$ and WT mice. Values (n=5) are expressed as mean ± SEM of the percentage variation of relative gene expression with respect to relative control (WT). The relative gene expression was determined by comparing $2^{-\Delta\Delta Ct}$ values normalized to β-actin. **p<0.01 vs WT.

5. DISCUSSION

Homeostasis (ομοιος [similar] and στάσις [status]), a characteristic common to all living organisms, is the natural tendency of an organ to reach a relative internal stability, in terms of chemical and physical properties, in response to changes of external conditions, in order to maintain a state of equilibrium over time. Internally, the intestinal homeostasis is under the control of the ENS, the second largest nervous system of the human body, which directly coordinates gastrointestinal functions including motility, secretion, mucosal immunity and visceral perception (Brun et al, 2013). Enteric neuronal circuitries display a considerable ability to adapt to a changing microenvironment, which comprises several cellular "players", including neurons, enteric glial cells, smooth muscle cells, interstitial cells of Cajal, and immune cells (Giaroni et al., 1999; Furness, 2012). This 'second brain' of the organism is indirectly exposed to the external luminal environment, which is mainly populated by the (non pathogenic) commensal microbiota, a 'superorganism' consisting of 10-time more microbes than host somatic cells. These microorganism forms a unique relationship with its host and then are crucial in guaranteeing intestinal epithelial integrity and barrier function, promoting gut development and maturation of the mucosal immune system (Shroff et al., 1995; Collins et al., 2014). Gut microbiota is directly involved in modulating the development and function of the ENS supporting the view that changes in intestinal microbes composition, particularly in early life, induced by infections or antibiotics, perturb ENS integrity favoring the onset of gastrointestinal disorders (i.e. inflammatory bowel disease, IBD or irritable bowel syndrome, IBS; McVey Neufeld et al., 2013) Recently, the microbiota-gut-brain axis emerged as a key player also in the neurodevelopmental phases of the brain, indicating that events during initial colonization and microbiota development can impact general and mental health in later life (Diaz Heijtz et al., 2011; Borre et al., 2014). However, the molecular mechanisms by which the gut microbiota influences the development and organization of the ENS and consequently the CNS are still largely unknown. Host cells sense the presence of microorganisms throughout TLRs, an important subgroup of PRRs that are involved in the recognition and response to microbial components, derived from pathogens (PAMPs) or endogenous ligands derived from host cells damaged (DAMPs), and in detecting molecular component derived from commensal microorganisms. Thus, TLRs play a crucial role in the innate immunity protection from infection, in the control of tissue integrity and in the symbiosis between gut microbiota and the host (Kabouridis and Pachnis, 2015). Among TLRs, TLR2 is known to strengthen the intestinal epithelial cell barrier and prevent bacterial entry and inflammation (Rakoff-Nahoum

et al., 2004; Round et al., 2011) modulating immune response. Interestingly, TLR2 polymorphisms have been identified in certain cases of IBD, although the relevance of this finding to the pathogenesis of Crohn's disease or ulcerative colitis remains unclear (Pierik et al., 2006). Moreover, in the brain TLR2 is expressed in neurogenic areas of the brain. Rolls et al. demonstrated that TLR signaling play a crucial role in regulation of postnatal neuronal plasticity as it has been implicated in differentiation of neural stem/progenitor cells into neurons (Rolls et al., 2007).

In the first part of this study we demonstrated the expression of TLR2 in the outer smooth muscular layer of small intestine. In particular, throughout immunohistochemical analysis performed on murine ileal frozen sections, we revealed the presence this receptor in the myenteric neurons of the ENS, as shown by the staining of TLR2 protein in neuronal cells that express the cytoskeletal protein peripherin. In addition, flow cytometry analysis on cells dissociated from LMMP of WT mice, identified the TLR2 expression on distinct cell types in the ileal muscle layer such as ECGs and smooth muscle cells, suggesting that the presence of TLR2 in the ileal muscularis externa creates a mechanism for the gut luminal environment to communicate with the ENS without the interposition of immune or epithelial mucosal cells (Barajon et al., 2009). Given the existence of the gut-brain axis, the structural defects of regulatory proteins and neurofilaments expressed in the ENS appear to be phenotypic characteristics resembling those found in neurodegenerative disorders of the central and peripheral nervous system (Liem and Messing, 2009). Accordingly, we examined the impact of TLR2 absence on ENS development in adolescent $TLR2^{-/-}$mice. A primary histological evaluation showed that in mice lacking TLR2 there are no signs of inflammation and that the gross morphology of the intestinal wall was maintained (Brun et al., 2013). Subsequently, whole mount immunohistochemistry performed on ileal LMMP evidenced anomalies in myenteric ganglia, indicated by lower HuC/D^{+} neuronal and $S100\beta^{+}$ ECGs cells in $TLR2^{-/-}$ mice, associated with altered distribution of type III intermediate filament peripherin. The correct identification of neurons is critical to investigate and understand morphological and functional changes that may occur during health ad disease (Thacker et al., 2011; Desmet et al., 2014). HuC/D protein is used as a pan neuronal marker both in central and in the peripheral nervous system. It belongs to the family of Hu proteins, the human homologs of *Drosophila melanogaster* Embryonic Lethal Abnormal Vision (ELAV) proteins. Funtionally, HuC/D regulates the stability of specific mRNA (e.g VEGF, AChE, BDNF), modulating the precise and temporal expression of certain proteins (Desmet et al., 2014). The study of this protein allow the quantification of individual neurons and then is commonly used as a neuronal marker. However, because of its role in mRNA stabilization, HuC/D normally is located in the cytosol, but it can be also reach in

the nucleus throughout a specific transporter (Saito et al., 2004). Peripherin neuronal protein, which is expressed predominantly in the peripheral nervous system (PNS), is the major component found in inclusions of patients with amyotrophic lateral sclerosis (ALS). Moreover, peripherin expression is upregulated in ALS patients (Zhao and Liem, 2016). Similar to the CNS astrocytes, ECGs physiologically express the protein S100β, a small diffusible calcium-binding protein, that exerts either trophic or toxic effects depending on its levels in the extracellular milieu (Cirillo et al., 2011). Recently it has been demonstrated that aberrant expression and release of glial functional protein S100β correlate with the gut inflammatory status (Esposito et al., 2007; Cirillo et al., 2011). Our findings indicate that the absence of TLR2 induces anomalies in both structural and regulatory proteins of neurons and ECGs, affecting the neuronal and glial development in the ENS. Therefore, the absence of TLR2 signaling induces the presence of an underlying gut neuropathy (Brun et al., 2013). So the expression of TLR2 in the ENS could suggest a role in neuron survival, like TLR4, as demonstrated by the group of Anitha et al. (Anitha et al., 2012). The morphological abnormalities observed in the ENS of $TLR2^{-/-}$ mice were associated with impaired gut motor function. GI motility is defined by the movements of the digestive system, and the transit of the contents within it. The intestinal smooth muscle cells function as a syncytium, which is innervated by excitatory and inhibitory motor neurons. The correct equilibrium between contraction and relaxation is required for coordinate propulsive movements in the gut (Kunze and Furness, 1999). The main excitatory neurotransmitter in the ENS is acethylcoline, which act as a co-transmitter in the three broad classes of enteric neurons (motor neurons, interneurons and sensory neurons). Traditionally, cholinergic neurons were identified using a biochemical assay to detect AChE enzymatic activity (Johnson et al., 1995) at the site of active acethylcoline synthesis. AChE breaks down acethylcoline into choline and acetate once released from nerve terminals. AChE biochemical staining is used to demonstrate enlarged nerve trunks for the diagnosis of Hirschsprung's disease (Dale et al., 1979; Moore and Johnson, 2005). In the gut NO is the messenger molecule responsible for regulating motility by acting as an inhibitory neurotransmitter and is produced by the enzyme NOS, which exists in three isoforms: neuronal nNOS, inducible NOS (iNOS) and endothelial NOS (eNOS). The three isoforms are structurally related but differ from each other in their genetic origin, anatomical distribution, ion dependence for activity, and pathophysiological functions, and are responsible of producing NO, a molecule with diverse physiological functions. $TLR2^{-/-}$ mice displayed anomalies in ENS neurochemical coding, as indicated by both the decrease of myenteric $AchE^{+}$ positive nerve fibers and the reduction of the distribution and expression of neuronal nitric oxide synthase (nNOS). The morphological abnormalities in the ENS of $TLR2^{-/-}$ mice were associated with impaired small bowel function

evidenced by abnormal excitatory neurotransmission following in vitro electric field stimulation (EFS) and increased in vivo GI motility. Our findings indicate that TLR2 absence in ENS induces changes in the neurochemical coding of enteric neurons, accordingly affecting intestinal motility. Furthermore, we assessed whether TLR2-mediated functional anomalies were hematopoietic cell-independent. For this reason we generated bone-marrow chimera mice expressing TLR2 only in non-hematopoietic cells. Contractility experiments revealed no differences in neuromuscular function of WT mice given either WT or $TLR2^{-/-}$ bone marrow, as well as in that of $TLR2^{-/-}$ mice receiving either $TLR2^{-/-}$ or WT bone marrow, indicating that TLR2 signaling in nonhematopoietic cells is a main contributor to ENS health (Brun et al., 2013). Functional or organic anomalies in the ENS could be ascribable to disturbances in neurotrophic factors signaling. GDNF elsewhere in the body is a pivotal postnatal neurotrophic factor, where it can be a pro-survival factor for midbrain dopaminergic neurons and is involved in axonal growth in the peripheral nervous system (Rappold and Tieu, 2010). It has been shown the primary role of GDNF in the ENS for proper its development and maintenance of the structural and functional integrity (Gershon, 2010; Zhang et al., 2010). Lack of GDNF signaling determines aganglionosis in children (Hirschsprung's disease; Gershon, 2010) and can leads to changes in ENS architecture and function during diabetic neuropathy (Anitha et al., 2006). In line with previous reports, intestinal smooth muscle cells during postnatal development appears to be the primary source of GDNF, which is required for neuronal cell survival, growth, and innervations of target tissues (Rodriguez et al., 2011). Moreover it is now known that TLRs are able to mediate CNS neurotrophic factors production (Bsibsi et al., 2006), thus we investigated the role of TLR2 signaling in regulating GDNF expression in small intestine. Our results demonstrated a deficit in the mRNA transcripts and immunoreactivity of GDNF in ileal muscle layers of mice lacking TLR2. Given that GDNF reverses hyperglycemia-induced neuronal apoptosis and loss of nNOS-containing neurons and also improves gastrointestinal motility in diabetic mice (Anitha et al., 2006) we tested the ability of recombinant GDNF (rGDNF) administration to rescue most of the defects in ENS architecture and function observed in $TLR2^{-/-}$ mice-. rGDNF supplementation of for 1 week significantly improved the contractile abnormalities in ileum segments from juvenile $TLR2^{-/-}$ mice, as shown by EFS-elicited response, which return to levels comparable to WT mice. Administration of rGDNF also rescues GI motility and moreover, prevented the loss of nitrergic neurons in myenteric ganglia, mantaining the number of $nNOS^{+}$ neurons similar to those found in WT ileal specimens, as shown by confocal immunohistochemistry. Overall these data highlight that TLR2 is not merely a host immune sentinel but a dynamic guardian of ENS development and gut function (Brun et al., 2013).

Since it is necessary a proper expression and signaling of TLR2 in the small bowel to ensure the integrity of the ENS, this implicate that a proper commensal microbiota composition is needed. In mammalian development, the prenatal and postnatal periods are critical windows, since they are characterized by rapid changes in both neuronal and microbial organization (Borre et al., 2014). Shaping of the microbiota occurs in parallel with neurodevelopment and they have similar critical periods sensitive to damage (Wopereis et al., 2014). Importantly, childhood and adolescence are the most dynamic periods of change in relation to microbiota and nervous system development. The physicochemical conditions known to influence the composition of the intestinal microbiota include intestinal motility, pH, redox potential, nutrient supplies, host secretions (e.g. hydrochloric acid, digestive enzymes, bile and mucus), but in presence of infections or drugs can be seriously altered. In modern societies, widespread antibiotic administration is probably a major factor contributing to changes in the mucosal microbiota. Antibiotic administration, while facilitating clearance of targeted infections, also perturbs commensal microbial communities and decreases host resistance to antibiotic-resistant microbes (Ubeda and Pamer, 2012). In this regard, two recent studies showed that exposure to repeated antibiotic therapies in childhood leads to an increased risk of IBD in adulthood (Hviid et al., 2011; Kronman et al., 2012). Moreover, chronic treatment with antibiotics in mice during such critical periods can affect anxiety and cognitive behaviors as well as key neuromodulators of gut-brain communication increasing the risk of neurodevelopmental disorders and mental disorders (Borre et al., 2014; Desbonnet et al., 2015). In the second part of our study we assessed the effects of gut microbiota depletion on the structure and function of ENS in adolescent mice, by using a previously published protocol for manipulation of intestinal microbiota based on the administration of a cocktail of broad spectrum antibiotics, given to mice by oral gavage for 14 days (ABX mice; Reikvam et al., 2011; Brun et al., 2013). This protocol is based on the same composition of antibacterial agents as others have applied for ad libitum administration in drinking water. Thus, the choice of intragastric delivery is due to the fact that several published papers reported an incomplete depletion of the cultivable bacteria and an increased baseline morbidity and mortality of treated animals (Reikvam et al., 2011). Ampicillin, vancomycin, neomycin and metronidazol display activity against the full spectrum of bacteria and, especially, dual activity against both Gram positive (ampicillin and vancomycin) and Gram negative (ampicillin and neomycin) aerobic and facultative strains, which is potentially important for preventing antibiotic resistance (Kollef, 2005). The depletion of gut microbiota through the use of broad spectrum antibiotics, orally administered, is a viable alternative to the use of mice bred in sterile environment from the birth. Germ-free animals may potentially be used for such comparative studies, but they need an expensive

germ-free facilities and they require special expertise and infrastructure (Reikvam et al., 2011). Compared with animals living in a conventional microbiological environment, germ-free animals display several phenotypic characteristics, such as an immature and underdeveloped lymphoid system (Smith et al., 2007). Exposure to the broad spectrum antibiotics solution determined a significant reduction of microbial population (Brun et al.,2013, data not shown) and produced in mice a marked enlargement in caecum, a phenotypic characteristic similar to those found in adult germ-free mice (Reikvam et al., 2011). We then evaluated the structure of ileal myenteric ganglia in gut microbiota-depleted mice. The confocal microscopy analysis performed on ileal frozen sections revealed several anomalies in the expression and distribution of typical neuronal and glial proteins in the ENS of ABX mice. Firstly, the immunoreactivity for HuC/D protein in myenteric plexus of ABX mice resulted significantly greater in microbiota-depleted mice compared to controls. We also found changes in the distribution of GFAP immunoreactivity that were asscociated with glial distorted cellular process. Interestingly, S100β immunoreactivity was found to be significantly increased in the ileal myenteric plexus of ABX mice (Caputi et al., 2015a). Damage to enteric glia have detrimental consequences for enteric neurons (Thacker et al., 2011). Therefore, the perturbation of gut microbiota composition compromise in an important manner the ENS architecture with effects on neuronal and glial survival. Changes in GFAP or S100β expression during glial cell differentiation, inflammation and injury, highlight that the levels of these proteins are associated to the functional state of ECGs (Cirillo et al., 2011).

Antibiotic-induced microbiota depletion caused disturbances in ileal neuromuscular contractility, with a significant downward shift of the concentration-response curve to the non selective cholinergic receptor carbachol, along with a significant reduction to EFS at increasing frequencies (0-10 Hz), recognized by being mainly of neuronal cholinergic origin. Even the smooth muscle responsiveness was affected by antibiotic treatment as shown by the reduced Ileal contraction following high potassium-induced membrane depolarization. Given the strong impact of the main inhibitory neurotransmitter NO on intestine (Furness, 2012), intestinal contractility induced by nonadrenergic, noncholinergic nerve stimulation was assessed. EFS-induced NANC stimulation at 10 Hz induced a marked relaxation, which resulted significantly enhanced in ileal preparations of ABX mice compared to controls. Moreover, pretreatment with the pan NOS inhibitor L-NAME partially abolished this response, suggesting that the depletion of gut microbiota can influence not only the nitrergic-mediated relaxation, but also the responses induced by other inhibitory neurotransmission (e.g. ATP or VIP), known to be involved in sustaining intestinal contractility function (Zizzo et al., 2003). The alterations of both excitatory and inhibitory pathway found in ABX mice can be ascribed to changes in ENS

neurochemical coding, as revealed by cholinergic fibers positive to AChE biochemical staining in ABX mice, which outnumber those of controls. Exposure to the ABX treatment did not affect the number of NADPH-d^+ cells, however the immunoreactivity for nNOS resulted significantly increased in ABX mice (Caputi et al., 2015a). These abnormalities in the expression of both nNOS and S100β proteins, following microbiota manipulation, are indicative of the presence of a microinflammatory state causing the development of ENS neuropathy (Aubé et al., 2006; Cirillo et al., 2011). In several models of inflammatory bowel disease S100β upregulation has been shown to be accompanied by enhanced iNOS protein expression and consequent NO release (Cirillo et al., 2011), and this finding could explain the marked NO-mediated relaxation observed in ABX mice. On the other hand, the RNA-binding protein HuC/D is involved in regulating the expression of AChE during neuronal development in the CNS and the PNS (Deschenes-Furry et al., 2007), thus the increase of $AChE^+$ fibers found in ABX mice may be related to enhanced expression of HuC/D protein. Consequently, the GI motility is dramatically affected by these ENS alterations. Antibiotic-mediated microbiota depletion determined a significant delay of GI transit, which was reflected in corresponding changes in stool frequency and water content, indicating a possible state of constipation and alteration of intestinal permeability as a result of antibiotic treatment (Caputi et al., 2015a). Changes in the composition of microbiota are common features in functional GI disorders, such as IBS, where dysbiosis is hypothesized to alter several neuronal mechanisms leading to visceral hypersensitivity (Daulatzai, 2014). In the ENS, glutamatergic neurotransmitter pathway is a recognized mechanism involved in amplifying nociceptive signals, principally through ionotropic AMPA and NMDA receptors activation (Kirchgessner, 2001). The role of these receptors in the intestine is currently unknown, but it is likely that they serve to integrate and amplify signals within the network, possibly resulting in altered gut motility, secretion, and enhanced visceral nociception (Petrenko et al., 2003). In particular, changes in NMDA receptor subunits in response to inflammation may have profound implications and could be involved in the pathophysiology of chronic visceral hypersensitivity seen in patients with post-infectious IBS and other chronic visceral pain disorders (Spiller, 2003; Zhou et al., 2009). Antibiotic treatment determined a significant increase of GluN1 subunit of NMDA receptor expression in ileal LMMP preparations, suggesting a link between gut-dysbiosis and visceral hypersensitivity mediated by the glutamatergic transmission in the enteric neuronal circuitries. Therefore, gut microbiota depletion in adolescence determines complex anomalies in ENS architecture, affecting excitatory and inhibitory neurotransmission leading to constipation and impaired gut neuromuscular contractility and visceral hypersensitivity with critical effects on gut function (Caputi et al., 2015a). As reported above, anomalies in neurotrophic factor signaling, such as

GDNF, can impact the correct neurodevelopment leading to morphofunctional abnormalities of ENS. Moreover TLR2 activation by bacterial components is required to stimulate production of GDNF in the gut (Brun et al., 2013; Brun et al., 2015). Antibiotic treatment strongly alters the microbiota composition creating a deficit in TLR-bacteria derived ligands. Consequently, the levels of GDNF in ileal muscular layer of ABX mice resulted significantly reduced. The administration of Pam3CSK4, a TLR1/TLR2 bacterial-derived ligand during the second week of ABX treatment, determined a significant increase of GDNF levels in ileal smooth muscle cells and partially ameliorated the defects in intestinal neuromuscular contraction of ABX mice confirming the role of the microbiota/TLR2 axis in ensuring the expression of neurotrophic factors that are required to maintain the functional organization of the mammalian ENS (Brun et al., 2013; Brun et al., 2015). These findings argue that development and maturation of the ENS depend on the complex interplay between immune- and neuro-regulators factors (Brun et al., 2013; Brun et al., 2015).

Beside to the well-know role of TLRs in sensing molecular profiles derived from pathogens or commensal microorganisms, they also recognize endogenous molecular profiles derived from damaged cells (DAMPs), thus helping the repair of host tissue following inflammation. Oxidized phospholipids (OxPLs), an heterogeneous group of compounds that are generated during enzymatic and nonezymatic inflammatory processes and accumulate at sites of chronic inflammation, have been shown to modulate TLR-signaling in immune cells in both *in vitro* and *in vivo*. In particular, 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine (OxPAPC) has been shown to inhibit only TLR2- and TLR4-MD2-dependent signaling pathways but not those activated by other TLRs, because of its binding with serum accessory proteins such as LBP and CD14 (Bochkov et al., 2002). This interaction competitively inhibits the binding of proper bacterial-derived ligand (such as LPS) to both TLR2 and TLR4 (Erridge et al., 2007). Thus, we evaluated the effect of OXPAPC inhibition of TLR2 and TLR4 on ENS integrity and intestinal motility. In WT mice P14 treated with OXPAPC for 1 week the receptor-mediated response to carbachol together with the neuromuscular contractions elicited by EFS were significantly increased. OXPAPC inhibition of TLR2 and TLR4 strongly impacted the ileal cholinergic excitatory neurotransmission, as demonstrated by changes in the distribution of the cholinergic $AChE^{+}$ neurons in myenteric ganglia after the treatment. These findings underline the importance of a correct interaction between both TLR2/TLR4 and microbiota-derived ligands, which are required for preserving the integrity of the ENS excitatory neuronal network.

In the last years, it has been highlighted the importance of the gut-brain axis, a bidirectional communication between the GI tract and the CNS, in connecting emotional and cognitive

centers of the brain with peripheral intestinal functions (Carabotti et al., 2015). A common human polymorphism of cathecol-O-metiltranferase (COMT), the val158met, which determines low activity of the enzyme with well recognized effects on CNS neurotransmission, has been associated with the visceral pain syndrome, a main feature in the pathophysiology of IBS. In this part of the research, we evaluated structural and functional anomalies in the ENS of an animal model of psychiatric disease, characterized by COMT genetically driven reduction in female adult mice. A preliminary assessment of ileal tissue from $COMT^{+/-}$ mice did not reveal any abnormal signals of illness, however H&E staining showed a marked increase in the thickness of outer ileal muscular layer. In the ENS of $COMT^{+/-}$ mice the ECGs appeared to be in an activate state, as demonstrated by changes in the distribution of GFAP and S100β immunoreactivities (Caputi et al, 2015b). The increased levels of catecholamines, due to the reduction of the COMT gene, resulted in accelerated GI motility and visceral hypersensitivity, due to increased levels of GluN1 subunit of NMDA receptor in the ENS. $COMT^{+/-}$ mice displayed an altered ENS neurochemical coding with lower number of $AchE^{+}$ neurons and higher number of NADPH-d^{+} cells and these findings can affects intestinal contractility. Indeed, carbachol and EFS-elicited neuromuscular contractions were markedly reduced in ileal segments of $COMT^{+/-}$ mice, as well as muscle responsiveness elicited by KCl-induced depolarization (Caputi et al., 2015b). On the other hand, EFS-induced NANC stimulation showed a marked relaxation in $COMT^{+/-}$ mice, which was partially abolished by pre-treatment with the pan-NOS inhibitor L-NAME, suggesting that the disturbances of catecholaminergic transmission due to low activity of COMT can affect not only the nitrergic neurotransmission but also the involvement of other inhibitory signaling pathways (e.g. ATP, VIP, or dopamine), known to contribute in the regulation of intestinal contractility (Mulè and Serio, 2003; Zizzo et al., 2003; Li et al., 2006).

Considering that a reduced genetic expression of COMT determines increased pain perception and muscular hypertrophy/hyperplasia, accelerated total gastrointestinal transit associated to altered ENS chemical coding and dysmotility, we can hypothesized that overall these effects are depending on the higher dopamine levels, which through D1 and D2 receptors exerts a net inhibitory effect in the GI tract (Zizzo et al., 2006; Li et al., 2006; Caputi et al.,2015b).

In conclusion, our data demonstrate that TLR2 is not merely a host immune sensor, but a dynamic guardian of ENS integrity instrumental to preserve gut homeostasis. Most recently, several reports have expanded the role of gut microbiota to directly influencing gut−brain axis function, in view of the fact that alterations in microbiota are linked not just to gut inflammation, but also to changes in host behavior (Collins et al. 2012). Because stimuli delivered through TLRs, as shown by antibiotic-mediated microbiota depletion, can directly affect immunological responses and ENS integrity, it is evident that in the gut TLRs function lies

squarely at the crossroads of gut microbiota, epithelial barrier, and ENS, finely tuning beneficial and harmful insults. In the context of multifactorial pathologies, such as IBD and IBS, tuning innate immunity–ENS crosstalk might represent an attractive target for novel therapeutic strategies (Brun et al., 2013; Brun et al. 2014; Caputi et al., 2015a; Caputi et al.,2015b).

6. REFERENCES

Agans R, Rigsbee L, Kenche H, Michail S, Khamis HJ, Paliy O. Distal gut microbiota of adolescent children is different from that of adults. FEMS Microbiol Ecol. 2011 Aug;77(2):404-12.

Agreus L, Svärdsudd K, Nyrén O, Tibblin G. Irritable bowel syndrome and dyspepsia in the general population: overlap and lack of stability over time. Gastroenterology. 1995;109(3):671–680.

Akiho H, Deng Y, Blennerhassett P, Kanbayashi H, Collins SM. Mechanisms underlying the maintenance of muscle hypercontractility in a model of postinfective gut dysfunction. Gastroenterology. 2005 Jul;129(1):131-41.

Akira S, Takeda K. Toll-like receptor signalling. Nat Immunol. 2004;4: 499–511.

Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006 Feb 24;124(4):783-801.

Albert MJ, Mathan VI, Baker SJ. Vitamin B12 synthesis by human small intestinal bacteria. Nature. 1980 Feb 21;283(5749):781-2.

Andersson AF, Lindberg M, Jakobsson H, Bäckhed F, Nyrén P, Engstrand L. Comparative analysis of human gut microbiota by barcoded pyrosequencing. PLoS One. 2008 Jul 30;3(7):e2836.

Anitha M, Gondha C, Sutliff R, Parsadanian A, Mwangi S, Sitaraman SV, Srinivasan S. GDNF rescues hyperglycemia-induced diabetic enteric neuropathy through activation of the PI3K/Akt pathway. J Clin Invest. 2006 Feb;116(2):344-56.

Anitha M, Vijay-Kumar M, Sitaraman SV, Gewirtz AT, Srinivasan S. Gut microbial products regulate murine gastrointestinal motility via Toll-like receptor 4 signaling. Gastroenterology. 2012 Oct;143(4):1006-16.e4.

Aravalli RN, Peterson PK, Lokensgard JR. Toll-like receptors in defense and damage of the central nervous system. J Neuroimmune Pharmacol. 2007 Dec;2(4):297-312.

Aubé AC, Cabarrocas J, Bauer J, Philippe D, Aubert P, Doulay F, Liblau R, Galmiche JP, Neunlist M. Changes in enteric neurone phenotype and intestinal functions in a transgenic mouse model of enteric glia disruption. Gut. 2006 May;55(5):630-7.

Axelrod J, Tomchick R. Enzymatic O-methylation of epinephrine and other catechols. J Biol. Chem. 1958 233:702–705.

Balamurugan R, Janardhan HP, George S, Chittaranjan SP, Ramakrishna BS. Bacterial succession in the colon during childhood and adolescence: molecular studies in a southern Indian village. Am J Clin Nutr. 2008 Dec;88(6):1643-7.

Ball P, Knuppen R, Haupt M, Breuer H. Interactions between estrogens and catechol amines. 3. Studies on the methylation of catechol estrogens, catechol amines and other catechols by the ctechol-O-methyltransferases of human liver. J Clin Endocrinol Metab. 1972 Apr;34(4):736-46.

Barajon I, Serrao G, Arnaboldi F, Opizzi E, Ripamonti G, Balsari A, Rumio C. Toll-like receptors 3, 4, and 7 are expressed in the enteric nervous system and dorsal root ganglia. J Histochem Cytochem. 2009 Nov;57(11):1013-23.

Barbara G, Stanghellini V, De Giorgio R, Cremon C, Cottrell GS, Santini D, Pasquinelli G, Morselli-Labate AM, Grady EF, Bunnett NW, Collins SM, Corinaldesi R. Activated mast cells in proximity to colonic nerves correlate with abdominal pain in irritable bowel syndrome. Gastroenterology. 2004 Mar;126(3):693-702.

Barthel M, Hapfelmeier S, Quintanilla-Martínez L, Kremer M, Rohde M, Hogardt M, Pfeffer K, Rüssmann H, Hardt WD. Pretreatment of mice with streptomycin provides a Salmonella enterica serovar Typhimurium colitis model that allows analysis of both pathogen and host. Infect Immun. 2003 May;71(5):2839-58.

Bassotti G, Villanacci V, Fisogni S, Rossi E, Baronio P, Clerici C, Maurer CA, Cathomas G, Antonelli E. Enteric glial cells and their role in gastrointestinal motor abnormalities: introducing the neuro-gliopathies. World J Gastroenterol., 2007; 13(30):4035-41.

Bayliss WM, Starling EH. The movements and innervation of the small intestine. J Physiol. 1899 May 1. 24(2):99-143.

Belmonte L, Beutheu Youmba S, Bertiaux-Vandaële N, Antonietti M, Lecleire S, Zalar A, Gourcerol G, Leroi AM, Déchelotte P, Coëffier M, Ducrotté P. Role of toll like receptors in irritable bowel syndrome: differential mucosal immune activation according to the disease subtype. PLoS One. 2012;7(8):e42777.

Belvin MP, Anderson KV. A conserved signaling pathway: the Drosophila toll-dorsal pathway. Annu Rev Cell Dev Biol. 1996;12:393-416.

Bennett G, Thalley NJ: Irritable bowel syndrome in the elderly. Best Pract Res Clin Gastroenterol, 2002; 16: 63–76.

Bercik P, Denou E, Collins J, Jackson W, Lu J, Jury J, Deng Y, Blennerhassett P, Macri J, McCoy KD, Verdu EF, Collins SM. The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice. Gastroenterology. 2011 Aug;141(2):599-609, 609.e1-3.

Bercík P, Wang L, Verdú EF, Mao YK, Blennerhassett P, Khan WI, Kean I, Tougas G, Collins SM. Visceral hyperalgesia and intestinal dysmotility in a mouse model of postinfective gut dysfunction. Gastroenterology. 2004 Jul;127(1):179-87.

Bertolino A, Rubino V, Sambataro F, Blasi G, Latorre V, Fazio L, Caforio G, Petruzzella V, Kolachana B, Hariri A, Meyer-Lindenberg A, Nardini M, Weinberger DR, Scarabino T Prefrontal-hippocampal coupling during memory processing is modulated by COMT val158met genotype. 2006 Biol Psychiatry 60:1250 –1258

Bharucha AE, Camilleri M, Zinsmeister AR, Hanson RB. Adrenergic modulation of human colonic motor and sensory function. Am J Physiol. 1997 Nov;273(5 Pt1):G997-1006.

Biagi E, Candela M, Turroni S, Garagnani P, Franceschi C, Brigidi P. Ageing and gut microbes: perspectives for health maintenance and longevity. Pharmacol Res. 2013 Mar;69(1):11-20.

Biasucci G, Rubini M, Riboni S, Morelli L, Bessi E, Retetangos C. Mode of delivery affects the bacterial community in the newborn gut. Early Hum Dev. 2010 Jul;86 Suppl 1:13-5.

Blasi G, Mattay VS, Bertolino A, Elvevåg B, Callicott JH, Das S, Kolachana BS, Egan MF, Goldberg TE, Weinberger DR. Effect of catechol-O-methyltransferase val158met genotype on attentional control. J Neurosci. 2005 May 18;25(20):5038-45.

Bochkov VN, Kadl A, Huber J, Gruber F, Binder BR, Leitinger N. Protective role of phospholipid oxidation products in endotoxin-induced tissue damage. Nature. 2002 Sep 5;419(6902):77-81

Boesmans W, Martens MA, Weltens N, Hao MM, Tack J, Cirillo C, Vanden Berghe P. Imaging neuron-glia interactions in the enteric nervous system. Front Cell Neurosci. 2013 Oct 21;7:183.

Booijink CC, Zoetendal EG, Kleerebezem M, de Vos WM. Microbial communities in the human small intestine: coupling diversity to metagenomics. Future Microbiol. 2007 Jun;2(3):285-95.

Borre YE, O'Keeffe GW, Clarke G, Stanton C, Dinan TG, Cryan JF. Microbiota and neurodevelopmental windows: implications for brain disorders. Trends Mol Med. 2014 Sep;20(9):509-18.

Bramanti V, Tomassoni D, Avitabile M, Amenta F, Avola R. Biomarkers of glial cell proliferation and differentiation in culture. Front Biosci (Schol Ed). 2010 Jan 1;2:558-70.

Braun N, Sévigny J, Robson SC, Hammer K, Hanani M, Zimmermann H. Association of the ecto-ATPase NTPDase2 with glial cells of the peripheral nervous system. Glia. 2004 Jan 15;45(2):124-32.

Brint EK, MacSharry J, Fanning A, Shanahan F, Quigley EM. Differential expression of toll-like receptors in patients with irritable bowel syndrome. Am J Gastroenterol. 2011 Feb;106(2):329-36.

Broderick NA, Buchon N, Lemaitre B. Microbiota-induced changes in drosophila melanogaster host gene expression and gut morphology. M Bio. 2014 May 27;5(3):e01117-14.

Brun P, Giron MC, Qesari M, Porzionato A, Caputi V, Zoppellaro C, Banzato S, Grillo AR, Spagnol L, De Caro R, Pizzuti D, Barbieri V, Rosato A, Sturniolo GC, Martines D, Zaninotto G, Palù G, Castagliuolo I. Toll-like receptor 2 regulates intestinal inflammation by controlling integrity of the enteric nervous system. Gastroenterology. 2013 Dec;145(6):1323-33.

Brun P, Gobbo S, Caputi V, Spagnol L, Schirato G, Pasqualin M, Levorato E, Palù G, Giron MC, Castagliuolo I. Toll like receptor-2 regulates production of glial-derived neurotrophic factors in murine intestinal smooth muscle cells. Mol Cell Neurosci. 2015 Sep;68:24-35.

Bsibsi M, Persoon-Deen C, Verwer RW, Meeuwsen S, Ravid R, Van Noort JM. Toll-like receptor 3 on adult human astrocytes triggers production of neuroprotective mediators. Glia. 2006 May;53(7):688-95.

Buchon N, Broderick NA, Chakrabarti S, Lemaitre B. Invasive and indigenous microbiota impact intestinal stem cell activity through multiple pathways in Drosophila. Genes Dev. 2009 Oct 1;23(19):2333-44.

Burokas A, Moloney RD, Dinan TG, Cryan JF. Microbiota regulation of the Mammalian gut-brain axis. Adv Appl Microbiol. 2015;91:1-62.

Camilleri M, Katzka DA. Irritable bowel syndrome: methods, mechanisms, and pathophysiology. Genetic epidemiology and pharmacogenetics in irritable bowel syndrome. Am J Physiol Gastrointest Liver Physiol. 2012 May 15;302(10):G1075-84.

Camilleri M. Management of the irritable bowel syndrome. Gastroenterology, 2001; 120: 652–68

Canavan C, West J, Card T. The epidemiology of irritable bowel syndrome. Clin Epidemiol. 2014 Feb 4;6:71-80

Carabotti M, Scirocco A, Maselli MA, Severi C. The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems. Ann Gastroenterol. 2015 Apr-Jun;28(2):203-209.

Cario E, Gerken G, Podolsky DK. Toll-like receptor 2 controls mucosal inflammation by regulating epithelial barrier function. Gastroenterology. 2007 Apr;132(4):1359-74.

Caputi V, Marsilio I, Marinelli F, Filpa V, Lante I, Galuppini F, Dall'Acqua S, Debetto P, Rugge M, Orso G, Giaroni C, Giron MC. Gut microbiota depletion alters the stucture and the function of the enteric nervous system in adolescent mice. United European Gastroenterology Journal 3(5S) A100. (UEG week, 24-28 October 2015, Barcelona; *Presented as oral communication*). (a)

Caputi V, Marsilio I, Cavallo L, Frizzo S, Marinelli F, Mereu M, Lante I, Orso G, Papaleo F, Giron MC. Involvement of catechol-O-methyltransferase genetic reduction in murine intestinal dysmotility: a possible link between psychiatric disorders and irritable bowel syndrome. Gastroenterology 2015; Vol. 148, Issue 4, S-774–S-775. DDW, 17-19 May 2015, Washington DC; *Presented as poster*). (b)

Chang L, Adeyemo M, Karagiannides I, Videlock EJ, Bowe C, Shih W, Presson AP, Yuan PQ, Cortina G, Gong H, Singh S, Licudine A, Mayer M, Tache Y, Pothoulakis C, Mayer EA. Serum and colonic mucosal immune markers in irritable bowel syndrome. Am J Gastroenterol. 2012 Feb;107(2):262-72.

Chen J, Lipska BK, Halim N, Ma QD, Matsumoto M, Melhem S, Kolachana BS, Hyde TM, Herman MM, Apud J, Egan MF, Kleinman JE, Weinberger DR. Functional analysis of genetic variation in catechol-O-methyltransferase (COMT): effects on mRNA, protein, and enzyme activity in postmortem human brain. Am J Hum Genet. 2004 Nov;75(5):807-21.

Cirillo C, Sarnelli G, Esposito G, Grosso M, Petruzzelli R, Izzo P, Calì G, D'Armiento FP, Rocco A, Nardone G, Iuvone T, Steardo L, Cuomo R. Increased mucosal nitric oxide production in ulcerative colitis is mediated in part by the enteroglial-derived S100B protein. Neurogastroenterol Motil. 2009 Nov;21(11):1209-e112.

Cirillo C, Sarnelli G, Esposito G, Turco F, Steardo L, Cuomo R. S100B protein in the gut: the evidence for enteroglial-sustained intestinal inflammation. World J Gastroenterol. 2011 Mar 14;17(10):1261-6. (a)

Cirillo C, Sarnelli G, Turco F, Mango A, Grosso M, Aprea G, Masone S, Cuomo R. Proinflammatory stimuli activates human-derived enteroglial cells and induces autocrine nitric oxide production. Neurogastroenterol Motil. 2011 Sep;23(9):e372-82. (b)

Claesson MJ, Cusack S, O'Sullivan O, Greene-Diniz R, de Weerd H, Flannery E, Marchesi JR, Falush D, Dinan T, Fitzgerald G, Stanton C, van Sinderen D, O'Connor M, Harnedy N, O'Connor K, Henry C, O'Mahony D, Fitzgerald AP, Shanahan F, Twomey C, Hill C, Ross RP, O'Toole PW. Composition, variability, and temporal stability of the intestinal microbiota of the elderly. Proc Natl Acad Sci U S A. 2011 Mar15;108 Suppl 1:4586-91.

Claesson MJ, Jeffery IB, Conde S, Power SE, O'Connor EM, Cusack S, Harris HM, Coakley M, Lakshminarayanan B, O'Sullivan O, Fitzgerald GF, Deane J, O'Connor M, Harnedy N, O'Connor K, O'Mahony D, van Sinderen D, Wallace M, Brennan L, Stanton C, Marchesi JR, Fitzgerald AP, Shanahan F, Hill C, Ross RP, O'Toole PW. Gut microbiota composition correlates with diet and health in the elderly. Nature. 2012 Aug 9;488(7410):178-84.

Collins J, Borojevic R, Verdu EF, Huizinga JD, Ratcliffe EM. Intestinal microbiota influence the early postnatal development of the enteric nervous system. Neurogastroenterol Motil. 2014 Jan;26(1):98-107.

Collins JS, Goldsmith TH. Spectral properties of fluorescence induced by glutaraldehyde fixation. J Histochem Cytochem. 1981 Mar;29(3):411-4.

Collins SM, Surette M, Bercik P. The interplay between the intestinal microbiota and the brain. Nat Rev Microbiol. 2012 Nov;10(11):735-42.

Conly JM, Stein K, Worobetz L, Rutledge-Harding S. The contribution of vitamin K2 (menaquinones) produced by the intestinal microflora to human nutritional requirements for vitamin K. Am J Gastroenterol. 1994 Jun;89(6):915-23.

Cornet A, Savidge TC, Cabarrocas J, Deng WL, Colombel JF, Lassmann H, Desreumaux P, Liblau RS. Enterocolitis induced by autoimmune targeting of enteric glial cells: a possible mechanism in Crohn's disease? Proc Natl Acad Sci U S A. 2001 Nov 6;98(23):13306-11.

Costa M, Brookes SJ, Hennig GW. Anatomy and physiology of the enteric nervous system. Gut. 2000 Dec;47 Suppl 4:iv15-9; discussion iv26.

Costedio MM, Hyman N, Mawe GM. Serotonin and its role in colonic function and in gastrointestinal disorders. Dis Colon Rectum. 2007 Mar;50(3):376-88.

Creedon DJ, Tansey MG, Baloh RH, Osborne PA, Lampe PA, Fahrner TJ, Heuckeroth RO, Milbrandt J, Johnson EM Jr. Neurturin shares receptors and signal transduction pathways with glial cell line-derived neurotrophic factor in sympathetic neurons. Proc Natl Acad Sci U S A. 1997 Jun 24;94(13):7018-23.

Cryan JF, Dinan TG. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. Nat Rev Neurosci. 2012 Oct;13(10):701-12

Dale G, Bonham JR, Lowdon P, Wagget J, Rangecroft L, Scott DJ. Diagnostic value of rectal mucosal acetylcholinesterase levels in Hirschsprung's disease. Lancet. 1979 Feb 17;1(8112):347-9.

D'Amato M. Genes and functional GI disorders: from casual to causal relationship. Neurogastroenterol Motil. 2013 Aug;25(8):638-49.

Daulatzai MA. Chronic functional bowel syndrome enhances gut-brain axis dysfunction, neuroinflammation, cognitive impairment, and vulnerability to dementia. Neurochem Res. 2014 Apr;39(4):624-44

De Giorgio R, Bovara M, Barbara G, Canossa M, Sarnelli G, De Ponti F, Stanghellini V, Tonini M, Cappello S, Pagnotta E, Nobile-Orazio E, Corinaldesi R. Anti-HuD-induced neuronal apoptosis underlying paraneoplastic gut dysmotility. Gastroenterology. 2003 Jul;125(1):70-9.

Dekaban AS. Changes in brain weights during the span of human life: relation of brain weights to body heights and body weights. Ann Neurol. 1978 Oct;4(4):345-56.

Desbonnet L, Clarke G, Traplin A, O'Sullivan O, Crispie F, Moloney RD, Cotter PD, Dinan TG, Cryan JF. Gut microbiota depletion from early adolescence in mice: Implications for brain and behaviour. Brain Behav Immun. 2015 Aug;48:165-73.

Deschênes-Furry J, Mousavi K, Bolognani F, Neve RL, Parks RJ, Perrone-Bizzozero NI, Jasmin BJ. The RNA-binding protein HuD binds acetylcholinesterase mRNA in neurons and regulates its expression after axotomy. J Neurosci. 2007 Jan 17;27(3):665-75.

Desmet AS, Cirillo C, Vanden Berghe P. Distinct subcellular localization of the neuronal marker HuC/D reveals hypoxia-induced damage in enteric neurons. Neurogastroenterol Motil. 2014 Aug;26(8):1131-43.

Dethlefsen L, Eckburg PB, Bik EM, Relman DA. Assembly of the human intestinal microbiota. Trends Ecol Evol. 2006 Sep;21(9):517-23.

Di Nardo G, Blandizzi C, Volta U, Colucci R, Stanghellini V, Barbara G, Del Tacca M, Tonini M, Corinaldesi R, De Giorgio R. Review article: molecular, pathological and therapeutic features of human enteric neuropathies. Aliment Pharmacol Ther. 2008 Jul;28(1):25-42.

Diatchenko L, Slade GD, Nackley AG, Bhalang K, Sigurdsson A, Belfer I, Goldman D, Xu K, Shabalina SA, Shagin D, Max MB, Makarov SS, Maixner W. Genetic basis for individual variations in pain perception and the development of a chronic pain condition. Hum Mol Genet. 2005 Jan 1;14(1):135-43.

Diaz Heijtz R, Wang S, Anuar F, Qian Y, Björkholm B, Samuelsson A, Hibberd ML, Forssberg H, Pettersson S. Normal gut microbiota modulates brain development and behavior. Proc Natl Acad Sci U S A. 2011 Feb 15;108(7):3047-52.

Diez-Fraile A, Van Hecke N, Guérin CJ, D'Herde K. 2012. Optimizing Multiple Immunostaining of Neural Tissue, Applications of Immunocytochemistry, Dr. Hesam Dehghani (Ed.), ISBN: 978-953-51-0229-8, InTech, Available from: http://www.intechopen.com/books/applications-ofimmunocytochemistry/ optimizing-multiple-immunostaining-of-neural-tissue.

Dinan TG, Stilling RM, Stanton C, Cryan JF. Collective unconscious: how gut microbes shape human behavior. J Psychiatr Res. 2015 Apr;63:1-9.

Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, Knight R. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc Natl Acad Sci U S A. 2010 Jun 29;107(26):11971-5.

Domschke K, Deckert J, O'donovan MC, Glatt SJ. Meta-analysis of COMT val158met in panic disorder: ethnic heterogeneity and gender specificity. Am J Med Genet B Neuropsychiatr Genet. 2007 Jul 5;144B(5):667-73.

Drabant EM, Hariri AR, Meyer-Lindenberg A, Munoz KE, Mattay VS, Kolachana BS, Egan MF, Weinberger DR. Catechol O-methyltransferase val158met genotype and neural mechanisms related to affective arousal and regulation. Arch Gen Psychiatry. 2006 Dec;63(12):1396-406.

Drabant EM, Hariri AR, Meyer-Lindenberg A, Munoz KE, Mattay VS, Kolachana BS, Egan MF, Weinberger DR. Catechol O-methyltransferase val158met genotype and neural mechanisms related to affective arousal and regulation. 2006 Arch Gen Psychiatry 63:1396 –1406.

Drossman DA, Camilleri M, Mayer E, Whitehead WE: AGA technical review on irritable bowel syndrome. Gastroenterology. 2002; 123: 2108–31

Drossman DA. The functional gastrointestinal disorders and the Rome III process. Gastroenterology. 2006 Apr;130(5):1377-90.

Dunlop SP, Coleman NS, Blackshaw E, Perkins AC, Singh G, Marsden CA, Spiller RC. Abnormalities of 5-hydroxytryptamine metabolism in irritable bowel syndrome. Clin Gastroenterol Hepatol. 2005 Apr;3(4):349-57.

Eastwood C, Grundy D. Opioid-receptor-mediated excitation of rat mesenteric afferent fibres supplying the rat jejunum. Neurogastroenterol Motil. 2000 Dec;12(6):517-22.

Echchannaoui H, Frei K, Schnell C, Leib SL, Zimmerli W, Landmann R. Toll-like receptor 2-deficient mice are highly susceptible to Streptococcus pneumonia meningitis because of reduced bacterial clearing and enhanced inflammation. J Infect Dis. 2002 Sep 15;186(6):798-806.

Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE, Relman DA. Diversity of the human intestinal microbial flora. Science. 2005 Jun 10;308(5728):1635-8.

Egan MF, Goldberg TE, Kolachana BS, Callicott JH, Mazzanti CM, Straub RE, Goldman D, Weinberger DR. Effect of COMT Val108/158 Met genotype on frontal lobe function and risk for schizophrenia. Proc Natl Acad Sci U S A. 2001 Jun 5;98(12):6917-22.

Eisenberg J, Mei-Tal G, Steinberg A, Tartakovsky E, Zohar A, Gritsenko I, Nemanov L, Ebstein RP. Haplotype relative risk study of catechol-O-methyltransferase (COMT) and attention deficit hyperactivity disorder (ADHD): association of the high-enzyme activity Val allele with ADHD impulsive-hyperactive phenotype. Am J Med Genet. 1999 Oct 15;88(5):497-502.

Eng LF, Ghirnikar RS, Lee YL. Glial fibrillary acidic protein: GFAP-thirty-one years (1969-2000). Neurochem Res. 2000 Oct;25(9-10):1439-51.

Erridge C, Kennedy S, Spickett CM, Webb DJ. Oxidized phospholipid inhibition of toll-like receptor (TLR) signaling is restricted to TLR2 and TLR4: roles for CD14, LPS-binding protein, and MD2 as targets for specificity of inhibition. J Biol Chem. 2008 Sep 5;283(36):24748-59.

Escherich T. Die darmbakterien des s€auglings und ihre beziehungen zur physiologie der Verdauung. Stuttgart: F. Enke, 1886.

Esposito G, Cirillo C, Sarnelli G, De Filippis D, D'Armiento FP, Rocco A, Nardone G, Petruzzelli R, Grosso M, Izzo P, Iuvone T, Cuomo R. Enteric glial-derived S100B protein stimulates nitric oxide production in celiac disease. Gastroenterology. 2007 Sep;133(3):918-25.

Fadgyas-Stanculete M, Buga AM, Popa-Wagner A, Dumitrascu DL. The relationship between irritable bowel syndrome and psychiatric disorders: from molecular changes to clinical manifestations. J Mol Psychiatry. 2014 Jun 27;2(1):4.

Fan W, Huo G, Li X, Yang L, Duan C. Impact of diet in shaping gut microbiota revealed by a comparative study in infants during the six months of life. J Microbiol Biotechnol. 2014 Feb 28;24(2):133-43.

Farhadi A, Fields JZ, Keshavarzian A. Mucosal mast cells are pivotal elements in inflammatory bowel disease that connect the dots: stress, intestinal hyperpermeability and inflammation. World J Gastroenterol. 2007 Jun 14;13(22):3027-30.

Faure C, Chalazonitis A, Rhéaume C, Bouchard G, Sampathkumar SG, Yarema KJ, Gershon MD. Gangliogenesis in the enteric nervous system: roles of the polysialylation of the neural cell adhesion molecule and its regulation by bone morphogenetic protein-4. Dev Dyn. 2007 Jan;236(1):44-59.

Ferri GL, Probert L, Cocchia D, Michetti F, Marangos PJ, Polak JM. Evidence for the presence of S-100 protein in the glial component of the human enteric nervous system. Nature. 1982 Jun 3;297(5865):409-10.

Fletcher EL, Clark MJ, Furness JB. Neuronal and glial localization of GABA transporter immunoreactivity in the myenteric plexus. Cell Tissue Res. 2002 Jun;308(3):339-46.

Fox, C.H.; Johnson, F.B.; Whiting, J. & Roller, P.P. Formaldehyde fixation. The Journal of Histochemistry and Cytochemistry. 1985. Vol. 33, No. 8; pp. 845-853.

Furness JB, Costa M. The enteric nervous system. New York: Churchill Livingstone, 1987.

Furness JB. Types of neurons in the enteric nervous system. J Auton Nerv Syst. 2000; 81(1-3):87-96.

Furness JB. The organisation of the autonomic nervous system: peripheral connections. Auton Neurosci., 2006; 130(1-2):1-5.

Furness JB. The enteric nervous system and neurogastroenterology. Nat Rev Gastroenterol Hepatol. 2012 Mar 6;9(5):286-94.

Garakani A, Win T, Virk S, Gupta S, Kaplan D, Masand PS. Comorbidity of irritable bowel syndrome in psychiatric patients: a review. Am J Ther. 2003 Jan-Feb;10(1):61-7.

Garnett WR: Management of irritable bowel syndrome. J Am Soc Consult Pharmac 1999; 14(Suppl. 8): 1–12

Garris PA, Collins LB, Jones SR, Wightman RM. Evoked extracellular dopamine in vivo in the medial prefrontal cortex. J Neurochem. 1993 Aug;61(2):637-47.

Gasbarrini A, Lauritano EC, Garcovich M, et al. New insights into the pathophysiology of IBS: intestinal microflora, gas production and gut motility. Eur Rev Med Pharmacol Sci 2008; 12 Suppl 1:111-7.

Gerritsen J, Smidt H, Rijkers GT, de Vos WM. Intestinal microbiota in human health and disease: the impact of probiotics. Genes Nutr. 2011 Aug;6(3):209-40.

Gershon MD, Ratcliffe EM. Development of the enteric nervous system. In: Johnson LR, Barrett K, Ghishan FK, Merchant JL, Said HM, Wood JD, eds. Physiology of the Gastrointestinal Tract, vol. 1, 4th edn. Burlington, MA: Elsevier Academic Press, 2006: 499–521.

Gershon MD. Nerves, reflexes, and the enteric nervous system: pathogenesis of the irritable bowel syndrome. J Clin Gastroenterol. 2005 May-Jun;39(5 Suppl 3):S184-93.

Gershon MD. Developmental determinants of the independence and complexity of the enteric nervous system. Trends Neurosci. 2010 Oct;33(10):446-56.

Giaroni C, De Ponti F, Cosentino M, Lecchini S, Frigo G. Plasticity in the enteric nervous system. Gastroenterology. 1999 Dec;117(6):1438-58

Gibson MK, Pesesky MW, Dantas G. The yin and yang of bacterial resilience in the human gut microbiota. J Mol Biol. 2014 Nov 25;426(23):3866-76.

Giros B, Jaber M, Jones SR, Wightman RM, Caron MG Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. Nature 1996 379:606–612.

Gomes P, Chevalier J, Boesmans W, Roosen L, van den Abbeel V, Neunlist M, Tack J, Vanden Berghe P. ATP-dependent paracrine communication between enteric neurons and glia in a primary cell culture derived from embryonic mice. Neurogastroenterol Motil. 2009 Aug;21(8):870-e62.

Gonzalez-Martinez T, Perez-Piñera P, Díaz-Esnal B, Vega JA. S-100 proteins in the human peripheral nervous system. Microsc Res Tech. 2003 Apr 15;60(6):633-8.

Goyal RK, Hirano I. The enteric nervous system. N Engl J Med. 1996 Apr 25;334(17):1106-15.

Gribar SC, Anand RJ, Sodhi CP, Hackam DJ. The role of epithelial Toll-like receptor signaling in the pathogenesis of intestinal inflammation. J Leukoc Biol. 2008 Mar;83(3):493-8. Epub 2007 Dec 26.

Griffin WS, Stanley LC, Ling C, White L, MacLeod V, Perrot LJ, White CL 3rd, Araoz C. Brain interleukin 1 and S-100 immunoreactivity are elevated in Down syndrome and Alzheimer disease. Proc Natl Acad Sci U S A. 1989 Oct;86(19):7611-5.

Grossman MH, Creveling CR, Rybczynski R, Braverman M, Isersky C, Breakefield XO. Soluble and particulate forms of rat catechol-O-methyltransferase distinguished by gel electrophoresis and immune fixation. J Neurochem. 1985 Feb;44(2):421-32.

Gui XY. Mast cells: a possible link between psychological stress, enteric infection, food allergy and gut hypersensitivity in the irritable bowel syndrome. J Gastroenterol Hepatol. 1998 Oct;13(10):980-9.

Guigoz Y, Doré J, Schiffrin EJ. The inflammatory status of old age can be nurtured from the intestinal environment. Curr Opin Clin Nutr Metab Care. 2008 Jan;11(1):13-20.

Gulbransen BD, Sharkey KA. Novel functional roles for enteric glia in the gastrointestinal tract. Nat Rev Gastroenterol Hepatol. 2012 Nov;9(11):625-32.

Gulbransen BD, Sharkey KA. Purinergic neuron-to-glia signaling in the enteric nervous system. Gastroenterology. 2009 Apr;136(4):1349-58.

Guldberg HC, Marsden CA. Catechol-O-methyl transferase: pharmacological aspects and physiological role. Pharmacol Rev. 1975 Jun;27(2):135-206.

Gürsoy S, Erdal E, Herken H, Madenci E, Alaşehirli B, Erdal N. Significance of catechol-O-methyltransferase gene polymorphism in fibromyalgia syndrome. Rheumatol Int. 2003 May;23(3):104-7.

Hagen K, Pettersen E, Stovner LJ, Skorpen F, Zwart JA. The association between headache and Val158Met polymorphism in the catechol-O-methyltransferase gene: the HUNT Study. J Headache Pain. 2006 Apr;7(2):70-4.

Hall KT, Tolkin BR, Chinn GM, Kirsch I, Kelley JM, Lembo AJ, Kaptchuk TJ, Kokkotou E, Davis RB, Conboy LA. Conscientiousness is modified by genetic variation in catechol-O-methyltransferase to reduce symptom complaints in IBS patients. Brain Behav. 2015 Jan;5(1):39-44.

Halvorson HA, Schlett CD, Riddle MS. Postinfectious irritable bowel syndrome--a meta-analysis. Am J Gastroenterol. 2006 Aug;101(8):1894-9.

Hanani, M. & Reichenbach, A. Morphology of horseradish peroxidase (HRP)-injected glial cells in the myenteric plexus of the guinea-pig. Cell Tissue Res. 1994, 278, 153–160.

Hansen MB. The enteric nervous system I: organisation and classification. Pharmacol Toxicol. 2003 Mar;92(3):105-13. (a)

Hansen MB. The enteric nervous system II: gastrointestinal functions. Pharmacol Toxicol. 2003 Jun;92(6):249-57. (b)

Hébuterne X. Gut changes attributed to ageing: effects on intestinal microflora. Curr Opin Clin Nutr Metab Care. 2003 Jan;6(1):49-54.

Herschkowitz N, Kagan J, Zilles K. Neurobiological bases of behavioral development in the first year. Neuropediatrics. 1997 Dec;28(6):296-306.

Heuckeroth RO, Lampe PA, Johnson EM, Milbrandt J. Neurturin and GDNF promote proliferation and survival of enteric neuron and glial progenitors in vitro. Dev Biol. 1998 Aug 1;200(1):116-29.

Hickey JG, Myers SM, Tian X, Zhu SJ, V Shaw JL, Andrew SD, Richardson DS, Brettschneider J, Mulligan LM. RET-mediated gene expression pattern is affected by isoform but not oncogenic mutation. Genes Chromosomes Cancer. 2009 May;48(5):429-40.

Hoff S, Zeller F, von Weyhern CW, Wegner M, Schemann M, Michel K, Rühl A. Quantitative assessment of glial cells in the human and guinea pig enteric nervous system with an anti-Sox8/9/10 antibody. J Comp Neurol. 2008 Aug 1;509(4):356-71.

Holzer P. Sensory neurone responses to mucosal noxae in the upper gut: relevance to mucosal integrity and gastrointestinal pain. Neurogastroenterol Motil. 2002 Oct;14(5):459-75.

Hopkins MJ, Sharp R, Macfarlane GT. Variation in human intestinal microbiota with age. Dig Liver Dis. 2002 Sep;34 Suppl 2:S12-8.

Huber JD, Egleton RD, Davis TP. Molecular physiology and pathophysiology of tight junctions in the blood-brain barrier. Trends Neurosci. 2001 Dec;24(12):719-25.

Husain N, Chaudhry IB, Jafri F, Niaz SK, Tomenson B, Creed F. A population-based study of irritable bowel syndrome in a non-Western population. Neurogastroenterol Motil. 2008;20(9):1022–1029.

Huttenlocher PR. Synaptic density in human frontal cortex – developmental changes and effects of aging. Brain Res. 1979 Mar 16;163(2):195-205.

Hviid A, Svanström H, Frisch M. Antibiotic use and inflammatory bowel diseases in childhood. Gut. 2011 Jan; 60(1):49-54.

Jacquemont S, Coe BP, Hersch M, Duyzend MH, Krumm N, Bergmann S, Beckmann JS, Rosenfeld JA, Eichler EE. A higher mutational burden in females supports a "female protective model" in neurodevelopmental disorders. Am J Hum Genet. 2014 Mar 6;94(3):415-25.

Jakobsson HE, Abrahamsson TR, Jenmalm MC, Harris K, Quince C, Jernberg C, Björkstén B, Engstrand L, Andersson AF. Decreased gut microbiota diversity, delayed Bacteroidetes colonisation and reduced Th1 responses in infants delivered by caesarean section. Gut. 2014 Apr;63(4):559-66.

Janssens S, Beyaert R. Role of Toll-like Receptors in Pathogen Recognition. Clin. Microbiol. Rev. 2003; 637-46.

Jeffery DR, Roth JA. Characterization of membrane-bound and soluble catechol-O-methyltransferase from human frontal cortex. J Neurochem. 1984 Mar;42(3):826-32.

Jessen KR, Mirsky R. Glial cells in the enteric nervous system contain glial fibrillary acidic protein. Nature. 1980 Aug 14;286(5774):736-7.

Jessen, K. R. & Mirsky, R. Astrocyte-like glia in the peripheral nervous system: an immunohistochemical study of enteric glia. J. Neurosci. 3, 2206–2218 (1983).

Jiménez E, Marín ML, Martín R, Odriozola JM, Olivares M, Xaus J, Fernández L, Rodríguez JM. Is meconium from healthy newborns actually sterile? Res Microbiol. 2008 Apr;159(3):187-93.

Jones G, Zammit S, Norton N, Hamshere ML, Jones SJ, Milham C, Sanders RD, McCarthy GM, Jones LA, Cardno AG, Gray M, Murphy KC, Owen MJ. Aggressive behaviour in patients with schizophrenia is associated with catechol-O-methyltransferase genotype. Br J Psychiatry. 2001 Oct;179:351-5

Jones R, Lydeard S. Irritable bowel syndrome in the general population. BMJ. 1992;304(6819):87–90.

Jones WJ, Roe BA, Affourtit JP, Egholm M, Henrissat B, Heath AC, Knight R, Gordon JI. A core gut microbiome in obese and lean twins. Nature. 2009 Jan 22;457(7228):480-4.

Joseph NM, He S, Quintana E, Kim YG, Núñez G, Morrison SJ. Enteric glia are multipotent in culture but primarily form glia in the adult rodent gut. J Clin Invest. 2011 Sep;121(9):3398-411.

Kabouridis PS, Lasrado R, McCallum S, Chng SH, Snippert HJ, Clevers H, Pettersson S, Pachnis V. The gut microbiota keeps enteric glial cells on the move; prospective roles of the gut epithelium and immune system. Gut Microbes. 2015 Nov 11:0. [Epub ahead of print]

Kabouridis PS, Pachnis V. Emerging roles of gut microbiota and the immune system in the development of the enteric nervous system. J Clin Invest. 2015 Mar 2;125(3):956-64.

Kanazawa M, Palsson OS, van Tilburg MA, Gangarosa LM, Fukudo S, Whitehead WE. Motility response to colonic distention is increased in postinfectious irritable bowel syndrome (PI-IBS). Neurogastroenterol Motil. 2014 May;26(5):696-704.

Karling P, Norrback KF, Adolfsson R, et al. Gastrointestinal symptoms are associated with hypothalamic-pituitary-adrenal axis suppression in healthy individuals. Scand J Gastroenterol 2007; 42(11):1294-301.

Karling P, Danielsson Å, Wikgren M, Söderström I, Del-Favero J, Adolfsson R, Norrback KF. The relationship between the val158met catechol-O-methyltransferase (COMT) polymorphism and irritable bowel syndrome. PLoS One. 2011; 6(3):e18035.

Kashyap PC, Marcobal A, Ursell LK, Larauche M, Duboc H, Earle KA, Sonnenburg ED, Ferreyra JA, Higginbottom SK, Million M, Tache Y, Pasricha PJ, Knight R, Farrugia G, Sonnenburg JL. Complex interactions among diet, gastrointestinal transit, and gut microbiota in humanized mice. Gastroenterology. 2013 May;144(5):967-77.

Katsanos AH, Giannopoulos S, Tsivgoulis G. The brain-gut axis in the pathophysiology of irritable bowel syndrome. Immuno-Gastroenterology. 2012. 1(1): 23-26.

Kawai T, Akira S. TLR signalling. Seminars Immunol. 2007; 19: 24-32.

Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol. 2010 May;11(5):373-84.

Kennedy PJ, Clarke G, Quigley EM, et al. Gut memories: towards a cognitive neurobiology of irritable bowel syndrome. Neurosci Biobehav Rev 2012; 36(1):310-40.

Kim HJ, Camilleri M, Carlson PJ, Cremonini F, Ferber I, Stephens D, McKinzie S, Zinsmeister AR, Urrutia R. Association of distinct alpha(2) adrenoceptor and serotonin transporter polymorphisms with constipation and somatic symptoms in functional gastrointestinal disorders. Gut. 2004 Jun;53(6):829-37.

Kim S, Takahashi H, Lin WW, Descargues P, Grivennikov S, Kim Y, Luo JL, Karin M. Carcinoma-produced factors activate myeloid cells through TLR2 to stimulate metastasis. Nature. 2009 Jan 1;457(7225):102-6.

Kirchgessner AL, Gershon MD. Innervation of the pancreas by neurons in the gut. J Neurosci 1990; 10:1626-42.

Kirchgessner AL. Glutamate in the enteric nervous system. Curr Opin Pharmacol. 2001 Dec;1(6):591-6.

Kirkup AJ, Brunsden AM, Grundy D. Receptors and transmission in the brain-gut axis: potential for novel therapies. I. Receptors on visceral afferents. Am J Physiol Gastrointest Liver Physiol. 2001 May;280(5):G787-94.

Kollef MH. Bench-to-bedside review: antimicrobial utilization strategies aimed at preventing the emergence of bacterial resistance in the intensive care unit. Crit Care. 2005 Oct 5;9(5):459-64.

Kramer MS, Aboud F, Mironova E, Vanilovich I, Platt RW, Matush L, Igumnov S, Fombonne E, Bogdanovich N, Ducruet T, Collet JP, Chalmers B, Hodnett E, Davidovsky S, Skugarevsky O, Trofimovich O, Kozlova L, Shapiro S; Promotion of Breastfeeding Intervention Trial (PROBIT) Study Group. Breastfeeding and child cognitive development: new evidence from a large randomized trial. Arch Gen Psychiatry. 2008 May;65(5):578-84.

Kroenke K, Mangelsdorff AD. Common symptoms in ambulatory care: incidence, evaluation, therapy, and outcome. Am J Med. 1989 Mar;86(3):262-6.

Kronman MP, Zaoutis TE, Haynes K, Feng R, Coffin SE. Antibiotic exposure and IBD development among children: a population-based cohort study. Pediatrics. 2012 October 130(4): e794–e803.

Kumar H, Kawai T, Akira S. Toll-like receptors and innate immunity. Biochem Biophys Res Commun. 2009 Oct 30;388(4):621-5.

Kunze WA, Furness JB. The enteric nervous system and regulation of intestinal motility. Annu Rev Physiol. 1999;61:117-42.

Lachman HM, Papolos DF, Saito T, Yu YM, Szumlanski CL, Weinshilboum RM. Human catechol-O-methyltransferase pharmacogenetics: description of a functional polymorphism and its potential application to neuropsychiatric disorders. Pharmacogenetics. 1996 Jun;6(3):243-50.

Lake JI, Heuckeroth RO. Enteric nervous system development: migration, differentiation, and disease. Am J Physiol Gastrointest Liver Physiol. 2013 Jul 1; 305(1):G1-24.

Land WG. The Role of Damage-Associated Molecular Patterns in Human Diseases: Part I - Promoting inflammation and immunity. Sultan Qaboos Univ Med J. 2015 Feb;15(1):e9-e21.

Laranjeira C, Sandgren K, Kessaris N, Richardson W, Potocnik A, Vanden Berghe P, Pachnis V. Glial cells in the mouse enteric nervous system can undergo neurogenesis in response to injury. J Clin Invest. 2011 Sep;121(9):3412-24.

Laranjeira, C. & Pachnis, V. Enteric nervous system development: recent progress and future challenges. Auton. Neurosci. 2009, 151, 61–69.

Lavoie EG, Gulbransen BD, Martín-Satué M, Aliagas E, Sharkey KA, Sévigny J. Ectonucleotidases in the digestive system: focus on NTPDase3 localization. Am J Physiol Gastrointest Liver Physiol. 2011 Apr;300(4):G608-20.

Le Douarin NM, Teillet MA. Experimental analysis of the migration and differentiation of neuroblasts of the autonomic nervous system and of neurectodermal mesenchymal derivatives, using a biological cell marking technique. Dev Biol 1974; 41: 162–84.

Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell. 1996 Sep 20;86(6):973-83.

Letiembre M, Liu Y, Walter S, Hao W, Pfander T, Wrede A, Schulz-Schaeffer W, Fassbender K. Screening of innate immune receptors in neurodegenerative diseases: a similar pattern. Neurobiol Aging. 2009 May;30(5):759-68.

Lewis DA, Melchitzky DS, Sesack SR, Whitehead RE, Auh S, Sampson A. Dopamine transporter immunoreactivity in monkey cerebral cortex: regional, laminar, and ultrastructural localization. J Comp Neurol. 2001 Mar 26;432(1):119-36.

Li ZS, Schmauss C, Cuenca A, Ratcliffe E, Gershon MD. Physiological modulation of intestinal motility by enteric dopaminergic neurons and the D2 receptor: analysis of dopamine receptor expression, location, development, and function in wild-type and knock-out mice. J Neurosci. 2006 Mar 8;26(10):2798-807.

Liem RK, Messing A. Dysfunctions of neuronal and glial intermediate filaments in disease. J Clin Invest 2009;119:1814–1824.

Locke GR, III, Pemberton JH, Phillips SF. AGA technical review on constipation. Gastroenterology 2000;119:1766–1778.

Loftus EV Jr, Sandborn WJ. Epidemiology of inflammatory bowel disease. Gastroenterol Clin North Am. 2002 Mar;31(1):1-20.

Lomax AE, Fernández E, Sharkey KA. Plasticity of the enteric nervous system during intestinal inflammation. Neurogastroenterol Motil. 2005 Feb;17(1):4-15.

Longstreth GF, Thompson WG, Chey WD, Houghton LA, Mearin F, Spiller RC. Functional bowel disorders. Gastroenterology 2006; 130: 1480–91.

Lotta T, Vidgren J, Tilgmann C, Ulmanen I, Melén K, Julkunen I, Taskinen J. Kinetics of human soluble and membrane-bound catechol O-methyltransferase: a revised mechanism and

description of the thermolabile variant of the enzyme. Biochemistry. 1995 Apr 4;34(13):4202-10.

Lovell RM, Ford AC. Global prevalence of and risk factors for irritable bowel syndrome: a meta-analysis. Clin Gastroenterol Hepatol. 2012;10(7):712–721. e4.

Lowen MB, Mayer E, Tillisch K, et al. Deficient habituation to repeated rectal distensions in irritable bowel syndrome patients with visceral hypersensitivity. Neurogastroenterol Motil 2015;27:646–655.

Lundström K, Salminen M, Jalanko A, Savolainen R, Ulmanen I. Cloning and characterization of human placental catechol-O-methyltransferase cDNA. DNA Cell Biol. 1991 Apr;10(3):181-9.

Lundström K, Tenhunen J, Tilgmann C, Karhunen T, Panula P, Ulmanen I. Cloning, expression and structure of catechol-O-methyltransferase. Biochim Biophys Acta. 1995 Aug 16;1251(1):1-10.

Lupp C, Robertson ML, Wickham ME, Sekirov I, Champion OL, Gaynor EC, Finlay BB. Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. Cell Host Microbe. 2007 Sep 13;2(3):204.

Mach T. The brain-gut axis in irritable bowel syndrome--clinical aspects. Med Sci Monit. 2004 Jun;10(6):RA125-31.

Mandal RS, Saha S, Das S. Metagenomic surveys of gut microbiota. Genomics Proteomics Bioinformatics. 2015 Jun;13(3):148-58.).

Manning AP, Thompson WG, Heaton KW, Morris AF. Towards positive diagnosis of the irritable bowel. Br Med J. 1978 Sep 2;2(6138):653-4.

Männistö PT, Kaakkola S. Catechol-O-methyltransferase (COMT): biochemistry, molecular biology, pharmacology, and clinical efficacy of the new selective COMT inhibitors. Pharmacol Rev. 1999 Dec;51(4):593-628

Marbach JJ, Levitt M. Erythrocyte catechol-O-methyltransferase activity in facial pain patients. J Dent Res. 1976 Jul-Aug;55(4):711.

Martin R, Nauta AJ, Ben Amor K, Knippels LM, Knol J, Garssen J. Early life: gut microbiota and immune development in infancy. Benef Microbes. 2010 Nov;1(4):367-82.

Martínez I, Kim J, Duffy PR, Schlegel VL, Walter J. Resistant starches types 2 and 4 have differential effects on the composition of the fecal microbiota in human subjects. PLoS One. 2010 Nov 29;5(11):e15046.

Mattay VS, Goldberg TE, Fera F, Hariri AR, Tessitore A, Egan MF, Kolachana B, Callicott JH, Weinberger DR. Catechol O-methyltransferase val158-met genotype and individual variation in the brain response to amphetamine. Proc Natl Acad Sci U S A. 2003 May 13;100(10):6186-91.

Mayer EA, Craske M, Naliboff BD. Depression, anxiety, and the gastrointestinal system. J Clin Psychiatry. 2001;62 Suppl 8:28-36; discussion 37.

Mayer EA. Gut feelings: the emerging biology of gut-brain communication. Nat Rev Neurosci. 2011 Jul 13;12(8):453-66.

McHenry J, Carrier N, Hull E, Kabbaj M. Sex differences in anxiety and depression: role of testosterone. Front Neuroendocrinol. 2014 Jan;35(1):42-57.

McLean PG, Borman RA, Lee K. 5-HT in the enteric nervous system: gut function and neuropharmacology. Trends Neurosci. 2007 Jan;30(1):9-13.

McVey Neufeld KA, Mao YK, Bienenstock J, Foster JA, Kunze WA. The microbiome is essential for normal gut intrinsic primary afferent neuron excitability in the mouse. Neurogastroenterol Motil. 2013 Feb;25(2):183-e88.

Medzhitov R. Recognition of microorganisms and activation of the immune response. Nature. 2007 Oct 18;449(7164):819-26.

Mertz H, Naliboff B, Munakata J, Niazi N, Mayer EA. Altered rectal perception is a biological marker of patients with irritable bowel syndrome. Gastroenterology 1995;109:40–52.

Mier D, Kirsch P, Meyer-Lindenberg A. Neural substrates of pleiotropic action of genetic variation in COMT: a meta-analysis. Mol Psychiatry. 2010 Sep;15(9):918-27.

Moloney RD, Johnson AC, O'Mahony SM, Dinan TG, Greenwood-Van Meerveld B, Cryan JF. Stress and the Microbiota-Gut-Brain Axis in Visceral Pain: Relevance to Irritable Bowel Syndrome. CNS Neurosci Ther. 2016 Feb;22(2):102-17.

Moore SW, Johnson G. Acetylcholinesterase in Hirschsprung's disease. Pediatr Surg Int. 2005 Apr;21(4):255-63.

Mucke L, Eddleston M. Astrocytes in infectious and immune-mediated diseases of the central nervous system. FASEB J. 1993 Oct;7(13):1226-32.

Mulè F, Serio R. NANC inhibitory neurotransmission in mouse isolated stomach: involvement of nitric oxide, ATP and vasoactive intestinal polypeptide. Br J Pharmacol. 2003 Sep;140(2):431-7.

Muller-Lissner SA, Collins SM, Vatnm H: Pathophysiology. In: Stockbrugger R, Pace F, editors. The irritable bowel syndrome. London: Mosby-Wolfe; 1999; 25–42.

Muller PA, Koscsó B, Rajani GM, Stevanovic K, Berres ML, Hashimoto D, Mortha A, Leboeuf M, Li XM, Mucida D, Stanley ER, Dahan S, Margolis KG, Gershon MD, Merad M, Bogunovic M. Crosstalk between muscularis macrophages and enteric neurons regulates gastrointestinal motility. Cell. 2014 Jul 17;158(2):300-13.

Mulligan LM. RET revisited: expanding the oncogenic portfolio. Nat Rev Cancer. 2014 Mar;14(3):173-86.

Munakata J, Naliboff B, Harraf F, Kodner A, Lembo T, Chang L, et al. Repetitive sigmoid stimulation induces rectal hyperalgesia in patients with irritable bowel syndrome. Gastroenterology 1997;112:55–63.

Murakami M, Ohta T, Ito S. Lipopolysaccharides enhance the action of bradykinin in enteric neurons via secretion of interleukin-1beta from enteric glial cells. J Neurosci Res. 2009 Jul;87(9):2095-104.

Myöhänen TT, Schendzielorz N, Männistö PT. Distribution of catechol-O-methyltransferase (COMT) proteins and enzymatic activities in wild-type and soluble COMT deficient mice. J Neurochem. 2010 Jun;113(6):1632-43.

Nackley AG, Shabalina SA, Tchivileva IE, Satterfield K, Korchynskyi O, Makarov SS, Maixner W, Diatchenko . Human catechol-Omethyltransferase haplotypes modulate protein expression by altering mRNA secondary structure. 2006 Science 314:1930 –1933

Nagahama M, Semba R, Tsuzuki M, Aoki E. L-arginine immunoreactive enteric glial cells in the enteric nervous system of rat ileum. Biol Signals Recept. 2001 Sep-Oct;10(5):336-40.

Neunlist M, Van Landeghem L, Bourreille A, Savidge T. Neuro-glial crosstalk in inflammatory bowel disease. J Intern Med. 2008 Jun;263(6):577-83.

Nguyen MD, Julien JP, Rivest S. Innate immunity: the missing link in neuroprotection and neurodegeneration? Nat Rev Neurosci. 2002 Mar;3(3):216-27.

Nissinen E, Männistö PT. Biochemistry and pharmacology of catechol-O-methyltransferase inhibitors. Int Rev Neurobiol. 2010; 95:73-118.

Nissinen E, Tuominen R, Perhoniemi V, Kaakkola S. Catechol-O-methyltransferase activity in human and rat small intestine. Life Sci. 1988;42(25):2609-14.

Ohman L, Simrén M. New insights into the pathogenesis and pathophysiology of irritable bowel syndrome. Dig Liver Dis. 2007 Mar;39(3):201-15.

Ohman L, Simren M. Pathogenesis of IBS: role of inflammation, immunity and neuroimmune interactions. Nat Rev Gastroenterol Hepatol 2010;7:163e73.

Öhman L, Törnblom H, Simrén M. Crosstalk at the mucosal border: importance of the gut microenvironment in IBS. Nat Rev Gastroenterol Hepatol. 2015 Jan;12(1):36-49.

Okun E, Griffioen KJ, Lathia JD, Tang SC, Mattson MP, Arumugam TV. Toll-like receptors in neurodegeneration. Brain Res Rev. 2009 Mar;59(2):278-92.

Okun E, Griffioen KJ, Mattson MP. Toll-like receptors signaling in neural plasticity and disease. Trends in Neurosci. 2011; 34: 1-13.

O'Mahony SM, Clarke G, Borre YE, Dinan TG, Cryan JF. Serotonin, tryptophan metabolism and the brain-gut-microbiome axis. Behav Brain Res. 2015 Jan 15;277:32-48.

Oshiumi H, Sasai M, Shida K, Fujita T, Matsumoto M, Seya T. TIR-containing adapter molecule (TICAM)-2, a bridging adapter recruiting to toll-like receptor 4 TICAM-1 that induces interferon-beta. J Biol Chem. 2003 Dec 12;278(50):49751-62.

Ozinsky A, Underhill DM, Fontenot JD, Hajjar AM, Smith KD, Wilson CB, Schroeder L, Aderem A. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. Proc Natl Acad Sci U S A. 2000 Dec 5;97(25):13766-71.

Palmatier MA, Kang AM, Kidd KK. Global variation in the frequencies of functionally different catechol-O-methyltransferase alleles. Biol Psychiatry. 1999 Aug 15;46(4):557-67.

Palmer C, Bik EM, DiGiulio DB, Relman DA, Brown PO. Development of the human infant intestinal microbiota. PLoS Biol. 2007 Jul;5(7):e177.

Papaleo F, Crawley JN, Song J, Lipska BK, Pickel J, Weinberger DR, Chen J. Genetic dissection of the role of catechol-O-methyltransferase in cognition and stress reactivity in mice. J Neurosci. 2008 Aug 27;28(35):8709-23.

Papaleo F, Erickson L, Liu G, Chen J, Weinberger DR. Effects of sex and COMT genotype on environmentally modulated cognitive control in mice. Proc Natl Acad Sci U S A. 2012 Dec 4;109(49):20160-5.

Papolos DF, Veit S, Faedda GL, Saito T, Lachman HM. Ultra-ultra rapid cycling bipolar disorder is associated with the low activity catecholamine-O-methyltransferase allele. Mol Psychiatry. 1998 Jul;3(4):346-9.

Parkes GC, Brostoff J, Whelan K, Sanderson JD. Gastrointestinal microbiota in irritable bowel syndrome: their role in its pathogenesis and treatment. Am J Gastroenterol. 2008 Jun;103(6):1557-67.

Paus T, Keshavan M, Giedd JN. Why do many psychiatric disorders emerge during adolescence? Nat Rev Neurosci. 2008 Dec;9(12):947-57.

Petanjek Z, Judaš M, Šimic G, Rasin MR, Uylings HB, Rakic P, Kostovic I. Extraordinary neoteny of synaptic spines in the human prefrontal cortex. Proc Natl Acad Sci U S A. 2011 Aug 9;108(32):13281-6.

Petrenko AB, Yamakura T, Baba H, Shimoji K. The role of N-methyl-D-aspartate(NMDA) receptors in pain: a review. Anesth Analg. 2003 Oct;97(4):1108-16.

Pierik M, Joossens S, Van Steen K, Van Schuerbeek N, Vlietinck R, Rutgeerts P, Vermeire S. Toll-like receptor-1, -2, and -6 polymorphisms influence disease extension in inflammatory bowel diseases. Inflamm Bowel Dis. 2006 Jan;12(1):1-8.

Pooley EC, Fineberg N, Harrison PJ. The met(158) allele of catechol-O-methyltransferase (COMT) is associated with obsessive-compulsive disorder in men: case-control study and meta-analysis. 2007 Mol Psychiatry 12:556–561.

Qin J, Li R, Raes J, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature 2010: 464: 59–65.

Quigley EM. Current concepts of the irritable bowel syndrome. Scand J Gastroenterol Suppl. 2003;(237):1-8.

Rakic, P. and Nowakowski, R.S. (1981) The time of origin of neurons in the hippocampal region of the rhesus monkey. J. Comp. Neurol. 196, 99–128.

Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell. 2004 Jul 23;118(2):229-41.

Rao SS, Read NW, Davison PA, Bannister JJ, Holdsworth CD. Anorectal sensitivity and responses to rectal distention in patients with ulcerative colitis. Gastroenterology. 1987 Dec;93(6):1270-5.

Rappold PM, Tieu K. Astrocytes and therapeutics for Parkinson's disease. Neurotherapeutics. 2010 Oct;7(4):413-23.

Rauch U, Klotz M, Maas-Omlor S, Wink E, Hänsgen A, Hagl C, Holland-Cunz S, Schäfer KH. Expression of intermediate filament proteins and neuronal markers in the human fetal gut. J Histochem Cytochem. 2006 Jan;54(1):39-46.

Reikvam DH, Erofeev A, Sandvik A, Grcic V, Jahnsen FL, Gaustad P, McCoy KD, Macpherson AJ, Meza-Zepeda LA, Johansen FE. Depletion of murine intestinal microbiota: effects on gut mucosa and epithelial gene expression. PLoS One. 2011 Mar 21;6(3):e17996.

Rhee SH, Pothoulakis C, Mayer EA. Principles and clinical implications of the brain-gut-enteric microbiota axis. Nat Rev Gastroenterol Hepatol. 2009 May;6(5):306-14.

Ridley EV, Wong AC, Westmiller S, Douglas AE. Impact of the resident microbiota on the nutritional phenotype of Drosophila melanogaster. PLoS One. 2012;7(5):e36765.

Ringel-Kulka T, Cheng J, Ringel Y, Salojärvi J, Carroll I, Palva A, de Vos WM, Satokari R. Intestinal microbiota in healthy U.S. young children and adults—a high throughput microarray analysis. PLoS One. 2013 May 23;8(5):e64315.

Rivett AJ, Francis A, Roth JA. Localization of membrane-bound catechol-O-methyltransferase. J Neurochem. 1983 May;40(5):1494-6.

Robinette ML, Colonna M. GI motility: microbiota and macrophages join forces. Cell. 2014 Jul 17;158(2):239-40.

Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF. A family of human receptors structurally related to Drosophila Toll. Proc Natl Acad Sci U S A. 1998 Jan 20;95(2):588-93.

Rodrigues DM, Li AY, Nair DG, Blennerhassett MG. Glial cell line-derived neurotrophic factor is a key neurotrophin in the postnatal enteric nervous system. Neurogastroenterol Motil. 2011 Feb;23(2):e44-56.

Rolls A, Shechter R, London A, Ziv Y, Ronen A, Levy R, Schwartz M. Toll-like receptors modulate adult hippocampal neurogenesis. Nat Cell Biol. 2007 Sep;9(9):1081-8.

Roth JA. Membrane-bound catechol-O-methyltransferase: a reevaluation of its role in the O-methylation of the catecholamine neurotransmitters. Rev Physiol Biochem Pharmacol. 1992;120:1-29.

Round JL, Lee SM, Li J, Tran G, Jabri B, Chatila TA, Mazmanian SK. The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. Science. 2011 May 20;332(6032):974-7

Ruan HZ, Burnstock G. The distribution of P2X5 purinergic receptors in the enteric nervous system of mouse. Cell Tissue Res. 2005;319:191-200.

Rühl A, Franzke S, Collins SM, Stremmel W. Interleukin-6 expression and regulation in rat enteric glial cells. Am J Physiol Gastrointest Liver Physiol. 2001 Jun;280(6):G1163-71.

Saffrey MJ. Cellular changes in the enteric nervous system during ageing. Dev Biol. 2013 Oct 1;382(1):344-55.

Saito K, Fujiwara T, Katahira J, Inoue K, Sakamoto H. TAP/NXF1, the primary mRNA export receptor, specifically interacts with a neuronal RNA-binding protein HuD. Biochem Biophys Res Commun. 2004 Aug 20;321(2):291-7

Salminen M, Lundström K, Tilgmann C, Savolainen R, Kalkkinen N, Ulmanen I. Molecular cloning and characterization of rat liver catechol-O-methyltransferase. Gene. 1990 Sep 14;93(2):241-7.

Sanders CJ, Moore DA 3rd, Williams IR, Gewirtz AT. Both radioresistant and hemopoietic cells promote innate and adaptive immune responses to flagellin. J Immunol. 2008 Jun 1;180(11):7184-92. Erratum in: J Immunol. 2008 Aug 15;181(4):2933.

Satokari R, Grönroos T, Laitinen K, Salminen S, Isolauri E. Bifidobacterium and Lactobacillus DNA in the human placenta. Lett Appl Microbiol. 2009 Jan;48(1):8-12.

Savidge TC, Newman P, Pothoulakis C, Ruhl A, Neunlist M, Bourreille A, Hurst R, Sofroniew MV. Enteric glia regulate intestinal barrier function and inflammation via release of S-nitrosoglutathione. Gastroenterology. 2007 Apr;132(4):1344-58.

Schäfer KH, Hänsgen A, Mestres P. Morphological changes of the myenteric plexus during early postnatal development of the rat. Anat Rec. 1999 Sep 1;256(1):20-8.

Scheggia D, Sannino S, Scattoni ML, Papaleo F. COMT as a drug target for cognitive functions and dysfunctions. CNS Neurol Disord Drug Targets. 2012 May;11(3):209-21.

Schwandner R, Dziarski R, Wesche H, Rothe M, Kirschning CJ. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. J Biol Chem. 1999 Jun 18;274(25):17406-9.

Schwartz S, Friedberg I, Ivanov IV, Davidson LA, Goldsby JS, Dahl DB, Herman D, Wang M, Donovan SM, Chapkin RS. A metagenomic study of diet-dependent interaction between gut microbiota and host in infants reveals differences in immune response. Genome Biol. 2012 Apr 30;13(4):r32.

Schwiertz A, Taras D, Schäfer K, Beijer S, Bos NA, Donus C, Hardt PD. Microbiota and SCFA in lean and overweight healthy subjects. Obesity (Silver Spring). 2010 Jan;18(1):190-5.

Scully P, McKernan DP, Keohane J, Groeger D, Shanahan F, Dinan TG, Quigley EM. Plasma cytokine profiles in females with irritable bowel syndrome and extra-intestinal co-morbidity. Am J Gastroenterol. 2010 Oct;105(10):2235-43.

Sengupta JN. Visceral pain: the neurophysiological mechanism. Handb Exp Pharmacol. 2009;(194):31-74.

Shroff KE, Meslin K, Cebra JJ. Commensal enteric bacteria engender a self-limiting humoral mucosal immune response while permanently colonizing the gut. Infect Immun. 1995 Oct;63(10):3904-13.

Simrén M, Barbara G, Flint HJ, Spiegel BM, Spiller RC, Vanner S, Verdu EF, Whorwell PJ, Zoetendal EG; Rome Foundation Committee. Intestinal microbiota in functional bowel disorders: a Rome foundation report. Gut. 2013, Jan; 62(1):159-76.

Simsek I (2011) Irritable bowel syndrome and other functional gastrointestinal disorders. J Clin Gastroenterol 45 Suppl: S86-S88.

Smith K, McCoy KD, Macpherson AJ. Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. Semin Immunol. 2007 Apr;19(2):59-69.

Sofroniew MV, Vinters HV. Astrocytes: biology and pathology. Acta Neuropathol. 2010 Jan;119(1):7-35.

Sowell ER, Peterson BS, Thompson PM, Welcome SE, Henkenius AL, Toga AW. Mapping cortical change across the human life span. Nat Neurosci. 2003 Mar;6(3):309-15.

Spiller RC. Postinfectious irritable bowel syndrome. Gastroenterology. 2003 May;124(6):1662-71.

Spiller R, Garsed K. Postinfectious irritable bowel syndrome. Gastroenterology. 2009 May;136(6):1979-88.

Stark PL, Lee A. The microbial ecology of the large bowel of breast-fed and formula-fed infants during the first year of life. J Med Microbiol 1982;15:189e203.

Steinkamp M, Geerling I, Seufferlein T, von Boyen G, Egger B, Grossmann J, Ludwig L, Adler G, Reinshagen M. Glial-derived neurotrophic factor regulates apoptosis in colonic epithelial cells. Gastroenterology. 2003 Jun;124(7):1748-57.

Storelli G, Defaye A, Erkosar B, Hols P, Royet J, Leulier F. Lactobacillus plantarum promotes Drosophila systemic growth by modulating hormonal signals through TOR-dependent nutrient sensing. Cell Metab. 2011 Sep 7;14(3):403-1

Taguchi T, Mitcham JL, Dower SK, Sims JE, Testa JR. Chromosomal localization of TIL, a gene encoding a protein related to the Drosophila transmembrane receptor Toll, to human chromosome 4p14. Genomics. 1996 Mar 15;32(3):486-8.

Takahashi M. The GDNF/RET signaling pathway and human diseases. Cytokine Growth Factor Rev. 2001 Dec;12(4):361-73.

Takeuchi O, Hoshino K, Akira S. Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to Staphylococcus aureus infection. J Immunol. 2000 Nov 15;165(10):5392-6.

Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, Takeda K, Akira S. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity. 1999 Oct;11(4):443-51.

Talley NJ, Phillips SF, Melton LJ, Mulvihill C, Wiltgen C, Zinsmeister AR. Diagnostic value of the Manning criteria in irritable bowel syndrome. Gut 1990; 31(1): 77-81

Tenhunen J, Salminen M, Lundström K, Kiviluoto T, Savolainen R, Ulmanen I. Genomic organization of the human catechol O-methyltransferase gene and its expression from two distinct promoters. Eur J Biochem. 1994;1;223(3):1049-59.

Thabane M, Kottachchi DT, Marshall JK. Systematic review and meta-analysis: The incidence and prognosis of post-infectious irritable bowel syndrome. Aliment Pharmacol Ther. 2007 Aug 15;26(4):535-44.

Thacker M, Rivera LR, Cho HJ, Furness JB. The relationship between glial distortion and neuronal changes following intestinal ischemia and reperfusion. Neurogastroenterol Motil. 2011 Nov;23(11):e500-9.

Thompson WG, Irvine EJ, Pare P, Ferrazzi S, Rance L. Functional gastrointestinal disorders in Canada: first population-based survey using Rome II criteria with suggestions for improving the questionnaire. Dig Dis Sci. 2002;47(1):225–235.

Thompson WG, Longstreth GF, Drossman DA, Heaton KW, Irvine EJ, Muller-Lissner SA. Functional bowel isorders and functional abdominal pain. Gut 1999; 45(2): II43–7

Thum C, Cookson AL, Otter DE, McNabb WC, Hodgkinson AJ, Dyer J, Roy NC. Can nutritional modulation of maternal intestinal microbiota influence the development of the infant gastrointestinal tract? J Nutr. 2012 Nov;142(11):1921-8.

Ubeda C, Pamer EG. Antibiotics, microbiota, and immune defense. Trends Immunol. 2012 Sep;33(9):459-66.

Uematsu S, Akira S. The role of Toll-like receptors in immune disorders. Expert Opin Biol Ther. 2006 Mar;6(3):203-14.

Valanne S, Wang JH, Rämet M. The Drosophila Toll signaling pathway. J Immunol. 2011 Jan 15;186(2):649-56.

Van Eldik LJ, Griffin WS. S100 beta expression in Alzheimer's disease: relation to neuropathology in brain regions. Biochim Biophys Acta. 1994 Sep 29;1223(3):398-403.

Van Landeghem L, Mahé MM, Teusan R, Léger J, Guisle I, Houlgatte R, Neunlist M. Regulation of intestinal epithelial cells transcriptome by enteric glial cells: impact on intestinal epithelial barrier functions. BMC Genomics. 2009 Nov 2;10:507.

Verdú EF, Bercik P, Verma-Gandhu M, Huang XX, Blennerhassett P, Jackson W, Mao Y, Wang L, Rochat F, Collins SM. Specific probiotic therapy attenuates antibiotic induced visceral hypersensitivity in mice. Gut. 2006 Feb;55(2):182-90.

Verma-Gandhu M, Verdu EF, Bercik P, Blennerhassett PA, Al-Mutawaly N, Ghia JE, Collins SM. Visceral pain perception is determined by the duration of colitis and associated neuropeptide expression in the mouse. Gut. 2007 Mar;56(3):358-64.

Vijayraghavan S, Wang M, Birnbaum SG, Williams GV, Arnsten AF. Inverted-U dopamine D1 receptor actions on prefrontal neurons engaged in working memory. Nat Neurosci. 2007 Mar;10(3):376-84.

Viramontes BE, Malcolm A, Camilleri M, Szarka LA, McKinzie S, Burton DD, Zinsmeister AR. Effects of an alpha(2)-adrenergic agonist on gastrointestinal transit, colonic motility, and sensation in humans. Am J Physiol Gastrointest Liver Physiol. 2001 Dec;281(6):G1468-76.

Wang H, Hughes I, Planer W, Parsadanian A, Grider JR, Vohra BP, Keller-Peck C, Heuckeroth RO. The timing and location of glial cell line-derived neurotrophic factor expression determine enteric nervous system structure and function. J Neurosci. 2010 Jan 27;30(4):1523-38.

Wasserman SA. A conserved signal transduction pathway regulating the activity of the rel-like proteins dorsal and NF-kappa B. Mol Biol Cell. 1993 Aug;4(8):767-71.

Wayment HK, Schenk JO, Sorg BA. Characterization of extracellular dopamine clearance in the medial prefrontal cortex: role of monoamine uptake and monoamine oxidase inhibition. J Neurosci. 2001 Jan 1;21(1):35-44.

Wehner S, Behrendt FF, Lyutenski BN, Lysson M, Bauer AJ, Hirner A, Kalff JC. Inhibition of macrophage function prevents intestinal inflammation and postoperative ileus in rodents. Gut. 2007 Feb;56(2):176-85.

Whitehead WE, Palsson O, Jones KR. Systematic review of the comorbidity of irritable bowel syndrome with other disorders: what are the causes and implications? Gastroenterology. 2002 Apr;122(4):1140-56.

Willert RP, Woolf CJ, Hobson AR, Delaney C, Thompson DG, Aziz Q. The development and maintenance of human visceral pain hypersensitivity is dependent on the N-methyl-D-aspartate receptor. Gastroenterology. 2004;126:683–692.

Willing BP, Russell SL, Finlay BB. Shifting the balance: antibiotic effects on host-microbiota mutualism. Nat Rev Microbiol. 2011 Apr; 9(4):233-43.

Winterer G, Egan MF, Kolachana BS, Goldberg TE, Coppola R, Weinberger DR. Prefrontal electrophysiologic "noise" and catechol-O-methyltransferase genotype in schizophrenia. Biol Psychiatry. 2006 Sep 15;60(6):578-84.

Wood JD, Alpers DH, Andrews PL. Fundamentals of neurogastroenterology. Gut. 1999 Sep;45 Suppl 2:II6-II16.

Wood JD. Neuropathophysiology of irritable bowel syndrome. J Clin Gastroenterol. 2002 Jul;35(1 Suppl):S11-22.

Wopereis H, Oozeer R, Knipping K, Belzer C, Knol J. The first thousand days - intestinal microbiology of early life: establishing a symbiosis. Pediatr Allergy Immunol. 2014 Aug;25(5):428-38.

Workman AD, Charvet CJ, Clancy B, Darlington RB, Finlay BL. Modeling transformations of neurodevelopmental sequences across mammalian species. J Neurosci. 2013 Apr 24;33(17):7368-83.

Wrase J, Reimold M, Puls I, Kienast T, Heinz A. Serotonergic dysfunction: brain imaging and behavioral correlates. Cogn Affect Behav Neurosci. 2006 Mar;6(1):53-61.

Yamamoto M, Sato S, Mori K, Hoshino K, Takeuchi O, Takeda K, Akira S. Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. J Immunol. 2002 Dec 15;169(12):6668-72.

Yang R, Puranam RS, Butler LS, Qian WH, He XP, Moyer MB, Blackburn K, Andrews PI, McNamara JO. Autoimmunity to munc-18 in Rasmussen's encephalitis. Neuron. 2000 Nov;28(2):375-83.

Yano JM, Yu K, Donaldson GP, Shastri GG, Ann P, Ma L, Nagler CR, Ismagilov RF, Mazmanian SK, Hsiao EY. Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. Cell. 2015 Apr 9;161(2):264-76.

Yavich L, Forsberg MM, Karayiorgou M, Gogos JA, Männistö PT. Site-specific role of catechol-O-methyltransferase in dopamine overflow within prefrontal cortex and dorsal striatum. J Neurosci. 2007 Sep 19;27(38):10196-209.

Yiangou Y, Facer P, Dyer NH, Chan CL, Knowles C, Williams NS, Anand P. Vanilloid receptor 1 immunoreactivity in inflamed human bowel. Lancet. 2001 Apr 28;357(9265):1338-9.

Zhang DK, He FQ, Li TK, Pang XH, Cui de J, Xie Q, Huang XL, Gan HT. Glial-derived neurotrophic factor regulates intestinal epithelial barrier function and inflammation and is therapeutic for murine colitis. J Pathol. 2010 Oct;222(2):213-22.

Zhang Z, Schluesener HJ. Mammalian toll-like receptors: from endogenous ligands to tissue regeneration. Cell Mol Life Sci. 2006 Dec;63(24):2901-7.

Zhou Q, Price DD, Caudle RM, Verne GN. Spinal NMDA NR1 subunit expression following transient TNBS colitis. Brain Res 2009; 1279:109-20.

Zimmer DB, Cornwall EH, Landar A, Song W. The S100 protein family: history, function, and expression. Brain Res Bull. 1995;37(4):417-29.

Zimmer DB, Chaplin J, Baldwin A, Rast M. S100-mediated signal transduction in the nervous system and neurological diseases. Cell Mol Biol (Noisy-le-grand). 2005 Sep 5;51(2):201-14.

Zizzo MG, Mulè F, Serio R. Duodenal contractile activity in dystrophic (mdx) mice: reduction of nitric oxide influence. Neurogastroenterol Motil. 2003 Oct;15(5):559-65.

Zoetendal EG, Rajilic-Stojanovic M, de Vos WM. High-throughput diversity and functionality analysis of the gastrointestinal tract microbiota. Gut. 2008 Nov;57(11):1605-15.

Zubieta JK, Heitzeg MM, Smith YR, Bueller JA, Xu K, Xu Y, Koeppe RA, Stohler CS, Goldman D. COMT val158met genotype affects mu-opioid neurotransmitter responses to a pain stressor. Science. 2003 Feb 21;299(5610):1240-3.