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Functional and Molecular Studies of the Crosstalk between Intestinal Microbioma and Enteric Nervous System and Potential Effects on the Gut-Brain Axis

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RIASSUNTO

L'interazione fra i costituenti della parete intestinale e la microflora commensale costituisce il principale artefice del mantenimento della barriera mucosale, della promozione dello sviluppo del tratto gastrointestinale (GI) e della modulazione delle funzioni GI, quali motilità, secrezione, immunità mucosale e sensibilità viscerale. Un'alterata microflora è stata associata a disordini GI (malattia infiammatoria cronica intestinale, MICI e 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 sistema nervoso enterico (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 (TLRs), un sofisticato sistema di proteine che attivano la risposta immunitaria innata contro agenti patogeni e mediano segnali benefici al fine di assicurare l'integrità funzionale e strutturale sia in condizioni fisiologiche che patologiche. Polimorfismi nei geni che codificano i TLRs sono stati associati a fenotipi diversi di malattia in pazienti affetti da disordini GI. In questo studio sono state caratterizzate le alterazioni strutturali e funzionali del SNE murino indotte da: i) cambiamenti nel segnale dell'immunità innata, mediato dal recettore TLR4, ii) una miscela di fosfolipidi ossidati (OxPAPC), implicati nel blocco del segnale generato dai recettori TLR2 e TLR4 al fine di eliminare parzialmente gli effetti mediati dalla flora intestinale batterica e iii) anomalie nella composizione del microbiota.

Data l'importanza di un corretto segnale TLRs-dipendente 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 WT e $TLR4^{-/-}$ di pari età (9 ± 1 settimane). Queste analisi hanno evidenziato anomalie nell'attività contrattile neuromuscolare associate ad un'eccessiva modulazione inibitoria controllata da ossido nitrico ed ATP, a sostegno della presenza di un dialogo tra TLR4, SNE e microflora, fondamentale per la modulazione della funzione neuromuscolare. Studi strutturali su preparati di ileo provenienti da topi $TLR4^{-/-}$ hanno dimostrato un'alterata architettura del SNE determinata da un'anomala distribuzione 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 indicano un coinvolgimento del recettore TLR4 nel mantenimento dell'integrità della rete gliale enterica mediato dalla produzione di ATP e nell'attivazione della trasmissione purinergica e pertanto evidenziano il ruolo primario di questo recettore nella conservazione dell'omeostasi strutturale e funzionale del SNE. Inoltre, in tale modello, è stato approfondito il ruolo del recettore TLR4 nell'asse 'intestino-cervello' attraverso la valutazione strutturale del sistema nervoso centrale in particolare a livello dell'ippocampo, area deputata all'apprendimento. Negli animali $TLR4^{-/-}$ è stato dimostrato che la mancanza del recettore TLR4 determina nell'ippocampo, come a livello del SNE, una compromessa neuroplasticità caratterizzata da alterazioni nella densità neuronale associata a variazioni della distribuzione della rete gliale, a confermare un ruolo fondamentale del segnale TLRs anche a livello centrale.

In parallelo, è stato ulteriormente indagato il ruolo primario del segnale mediato dai recettori TLRs nell'asse microbiota-TLRs-SNE, saggiando l'effetto di una somministrazione in acuto per 3 giorni consecutivi con OxPAPC, inibitore del segnale mediato da entrambi i recettori TLR2 e TLR4, in topi adolescenti (3 ± 1 settimane). Il trattamento con OxPAPC ha causato un'alterazione significativa della risposta neuromuscolare sia recettore-mediata che non, nei topi trattati rispetto al controllo, associata a modifiche della rete neuro-gliale del SNE, confermando l'importanza del segnale mediato da tali recettori nell'assicurare l'integrità funzionale e strutturale del SNE durante l'adolescenza. Recenti studi riportano un ruolo primario nel dialogo tra i recettori TLRs e il sistema serotoninergico, spesso coinvolto in disturbi intestinali, pertanto è stato valutato se la somministrazione di OxPAPC per via intraperitoneale influenzasse tale sistema. È stato evidenziato come l'inibizione in acuto del segnale TLR2 e TLR4 comporti iperesponsività alla serotonina, alterazioni nella distribuzione recettoriale serotoninergica associata a variazioni nel metabolismo del triptofano, amminoacido coinvolto nella produzione di serotonina, a sostegno della presenza di un dialogo tra immunità innata e sistema serotoninergico.

Al fine di approfondire il ruolo dell'asse microbiota-intestino nell'omeostasi del SNE 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 C57BL/6J adolescenti (3 ± 1 settimane; topi ABX). Da una prima valutazione il trattamento antibiotico ha determinato un fenotipo simil *germ-free*, come già dimostrato da altri autori, ed alterazioni della motilità intestinale e dell'integrità della

rete neuronale e gliale enterica. A tal proposito, 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 e della subunità β della proteina S-100. Data l'importanza di una corretta composizione del microbiota commensale sia nel mantenimento della rete nervosa e del codice neurochimico del SNE che nella produzione di neurotrasmettitori a livello enterico, sono state studiate le vie di neurotrasmissione coinvolte nella sensibilità viscerale in tale modello di disbiosi intestinale. È stato osservato un incremento dei livelli di mRNA di GluN1 e TRPV1 nel plesso mienterico dei preparati di ileo provenienti dai topi ABX, evidenziando gli effetti di un'alterata composizione del microbiota intestinale sulla sensibilità viscerale. Infine, è stato valutato l'effetto di uno stato di disbiosi indotto da antibiotici sul sistema serotoninergico, sistema le cui funzioni sono modulate da serotonina, metabolita la cui produzione è influenzata dall'azione di specifiche spore batteriche. Il trattamento antibiotico riporta anomalie nella risposta neuromuscolare alla serotonina accompagnate da una compromessa rete recettoriale serotoninergica e del metabolismo del triptofano, sottolineando l'importanza di una corretta composizione del microbiota nel mantenimento delle funzioni mediate dal sistema serotoninergico.

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) changes in innate immunity response, mediated by TLR4, ii) a mixture of oxidized phospholipids (OxPAPC) that blocks both TLR2 and TLR4 signaling to partially avoid the recognition of gut commensal microflora and iii) anomalies in the composition of the microbiota.

Highlighted the role of proper TLRs signaling in the maintenance of neuronal network and neurochemical coding of the ENS, intestinal contractility was evaluated in isolated ileal segments from WT e TLR4$^{-/-}$ mice (9±1 weeks) using organ bath technique. Functional studies reported significant alterations of intestinal contractility associated to an increased inhibitory neurotransmission via the combined action of nitric oxide (NO) and adenosine-5′-triphosphate (ATP), suggested a crosstalk between TLR4, ENS and microflora in the fine-tuning of ileal contractility. Furthermore, the absence of TLR4 affects ENS architecture characterized by abnormalities in the distribution and expression of the pan-neuronal marker HuC/D and induced a reactive gliosis state with alterations in the glial structural protein GFAP (glial fibrillary acidic protein) and the cytoplasmatic and nuclear glial calcium-binding protein S100β in the ileal myenteric plexus. Once demonstrated that TLR4 signaling is involved in the control of purinergic pathways in

enteric neural-glial communication and highlighted its role in tuning structural and functional integrity of ENS, we assessed the role of TLR4 receptors in the central nervous system (CNS), in particular in the hippocampus assessing few architectural proteins expressed in neurons or astrocytes or microglial cells. The absence of TLR4 receptor determines neuroplasticity in the hippocampus, as well as in the ENS, characterized by a reduction of neuronal density associated with altered glial networks, to underline a key role of TLRs also in the CNS.

In parallel, to investigate the importance of TLRs-dependent signaling in modulating ENS-microbiota axis, juvenile male C57BL/6J mice (3±1 weeks old) were treated intraperitoneally with OxPAPC, that blocks both TLR2 and TLR4 signaling, twice a day for 3 days. In vivo inhibition of both TLR2 and TLR4 determined a significant alteration of receptor and non-receptor-mediated neuromuscular responses and affected myenteric plexus integrity, providing evidence that TLR2 and TLR4 signaling is essential in ensuring the structural and functional integrity of the ENS during adolescence. Recent studies demonstrated the role of TLRs in modulating intestinal serotonergic system and given that this system is involved in many GI functions, we evaluated the effect of OxPAPC treatments in this context. OxPAPC-mediated TLR2 and TLR4 inhibition affects serotonin-mediated response, in term of hyperresponsivity, and alters both serotonergic receptor distributions and tryptophan (TRP) metabolism during adolescence suggesting a cross-talk between innate immunity and serotonergic system.

To investigate the role of the microbiota-gut axis in the homeostasis of ENS 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 3 ± 1 weeks, ABX). Mice after antibiotic treatment displayed a phenotype-like *germ-free* mice, as already reported by other Authors, and reveled an impairment in intestinal motility and in the neuro-glia integrity. Immunohistochemical analysis of ileal preparations from ABX mice showed abnormalities in the distribution and expression of the pan-neuronal marker HuC/D, the glial proteins GFAP and S100β. Given the importance of proper composition of commensal microbiota in the maintenance of neuronal network and neurochemical coding of the ENS and in the influencing neurotransmitter content, it has been investigated enteric neurotransmission involved in the control of central sensitization. Increased mRNA levels of GluN1 and TRPV1 in the myenteric plexa of ABX mice was found, suggesting that commensal microbiota is involved in modulating visceral

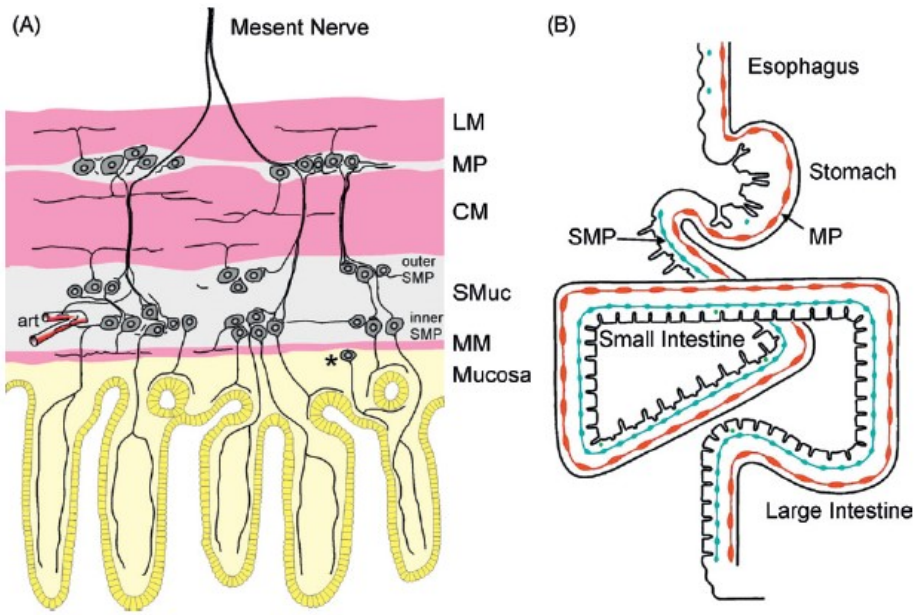
sensitivity. Finally, the effect of antibiotic mediated microbiota dysbiosis in serotonergic system was evaluated. The concept of a direct communication between commensals and the enteric nervous system was suggested by different Authors; specifically, indigenous spore-forming bacteria from mouse and human microbiota have been shown to promote serotonin biosynthesis. The antibiotic treatment affects serotonin-mediated response associated with impairments of serotonergic pathways and TRP metabolism, to evidence an involvement of microbiota in serotonin-mediated functions and potentially in microbiota-gut axis.

1. INTRODUCTION

1.1 Enteric Nervous System

The enteric nervous system (ENS) has received special attention in the last years since it is the only limb of the peripheral nervous system (PNS) which has the ability to function independently from the central nervous system (CNS) and as such it has often been referred to as the "second brain" or the "little brain" (Goyal & Hirano, 1996).

The ENS is a complex tissue, extending from the esophagus to the anal sphincter within the gastrointestinal (GI) system walls, and is composed of ganglia with neuronal fibers innervating the effector tissues (Furness et al., 2014). The human ENS contains 200–600 million neurons, the same number of neurons that is found in the human spinal cord (Furness & Costa, 1987a; Furness, 2006). 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 have been detected in the mucosa, close to the *muscularis mucosae* (mucous plexus) (Hansen, 2003).



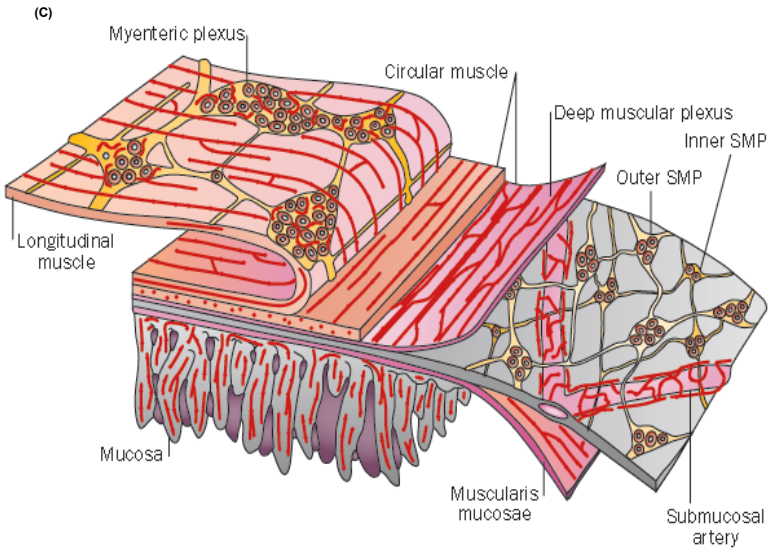


Figure 1.1. Anatomy of ENS. (**A**) In the small and large intestines, neurons are confined in ganglia of the myenteric plexus (MP), localized between the longitudinal (LM) and circular muscle (CM) layers, and in ganglia distributed between the circular muscle and the *muscularis mucosa* (MM) within the submucosa (SMuc), depicted in the transverse section of the gut wall. The ganglia and fibers in the submucosa form inner and outer submucosal plexus (SMP). (**B**) The distribution of ganglia along the gastrointestinal tract. (**C**) Neuromuscular layers along the small and large intestines (modified from Furness, 2012).

The myenteric plexus is positioned between the outer longitudinal and inner circular muscle layers, where forms a continuous network of ganglia 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 & Gershon, 1990). Moreover, a substantial number of projections from the myenteric neurons are connected to the sympathetic ganglia (Goyal & Hirano, 1996; Hansen, 2003; **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, 2003). In large mammals, the submucous plexus is located in the submucosa and composed by an inner network located at the serosal side of the muscularis mucosae (*Meissner's plexus*) and an outer layer (*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, 2003). 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**).

The ENS is in continuous communication with autonomic nervous system (ANS) through sympathetic and parasympathetic afferent and efferent neurons. The ANS drives both afferent signals, arising from the lumen and transmitted through enteric, spinal and vagal pathways to CNS, and efferent signals from CNS to the intestinal wall. In the GI tract, sympathetic, parasympathetic, and spinal afferent nerve fibers are extrinsically innervating the ENS and ensure the bidirectional communication with the CNS through intimate connections with the spinal cord. The gut vast innervations and connections between intrinsic and extrinsic fibers guarantee the CNS monitoring of a number of gut parameters, from chemical sensing in the lumen, to sensing mechanical stress along the gut wall (Furness, 2000). Along the GI tract, the vagus nerve has three afferent endings within the gut wall: intraganglionic laminar endings within the myenteric plexus, intramuscular arrays within the smooth muscle layers and mucosal fibers within the mucosa. The stomach has the highest density of the vagal afferent ending and the density deceases towards the distal regions of the GI (Powley & Phillips, 2002). The sympathetic neurons (effector branch of the ANS) have axons that extend along the mesenteric nerves deep into the gut wall to the myenteric, submucosal and mucosal plexuses of the ENS (Lomax et al., 2010). The terminals of these axons are responsible of releasing numerous neurotransmitters, mainly norepinephrine (NE) and tyrosine hydroxylase (TH). In the other side, vagal efferent neurons of the motor pathways are parasympathetic preganglionic neurons (Hansen, 2003). A variety of central effects, primarily on the upper GI tract, are mediated through these neurons, including relaxation of the proximal stomach, enhancement of gastric peristalsis, and promotion of gastrin secretion. Transmission from vagal input neurons to enteric neurons is mediated principally by acetylcholine (ACh) acting on nicotinic cholinergic receptors, but several other transmitters are involved in these processes (Hansen, 2003). This bidirectional connection, the so-called gut-brain axis, provides neural control of all functions of the GI tract (Goyal & Hirano, 1996). The ENS is endowed with a wide array of restorative, maintenance and adaptive functions. Motility patterns, gastric secretion, transport of fluid across the epithelium, blood flow, nutrient handling, interaction with the immune and endocrine systems of the gut are function under the control of the ENS (Furness, 2012; Wood, 2012).

According to neurons morphology, neurochemical coding, cell physiology, projections to targets and functional roles, approximately 20 distinct types of neurons have been

described (Costa et al., 2000). The enteric neuronal circuits are composed by intrinsic primary afferents neurons, sensory neurons which detect mechanical distortion of the mucosa, mechanical forces in the external musculature (tension of the gut wall) or the presence of chemical luminal stimuli and initiate appropriate reflex control of functions including motility, secretion and blood flow (Clerc et al., 2002). Along the whole GI tract, the longitudinal and circular smooth muscle layers and the muscularis mucosae are innervated by uni-axonal excitatory and inhibitory motor neurons (Dogiel type I morphology), which receive prominent fast excitatory synaptic potentials (Wood, 2012). The primary neurotransmitters for excitatory motor neurons are ACh and tachykinins. Several neurotransmitters have been identified in inhibitory motor neurons, including nitric oxide (NO), vasoactive intestinal peptide (VIP) and adenosine triphosphate (ATP)-like transmitters, although NO is considered the primary transmitter (Furness et al., 2014; **Table 1.1**).

NEURON TYPE	CODE	SHAPE	PROPORTION	ELECTROPHYSIOLOGICAL SIGNATURES
Intrinsic primary afferent neurons	ACh/NF/CGRP/ calbindin +/- calretinin	Dogiel Type II	26%	AH-neurons
Inhibitory circular muscle motor neuron	NOS/VIP +/- NPY	Dogiel Type I	23%	S-neurons
Inhibitory longitudinal muscle motor neuron	NOS/VIP	Small, no obvious dendrites	3%	S-neurons
Excitatory circular muscle motor neuron	ACh/TK +/- calretinin	Small/medium, no obvious dendrites	21%	S-neurons
Excitatory longitudinal muscle motor neuron	ACh/calretinin +/- TK	Small, no obvious dendrites	13%	S-neurons
Descending interneurons	ACh/NOS	Dogiel Type I	3%	S-neurons
Descending interneurons	ACh/5-HT	Dogiel Type I	1%	S-neurons
Descending interneurons	ACh/SOM/calretinin	Filamentous	4%	S-neurons
Ascending interneurons	ACh/TK +/- calretinin	Dogiel Type I	4% (estimated)	S-neurons
Intestinofugal neurons	Not identified	Not known	Not known	S-neurons
Tyrosine hydroxylase neurons	TH	Dogiel Type I	<0.5%	S-neurons

Table 1.1. Proportions of all neurons attributed to different functional classes in myenteric ganglia of mouse small intestine (modified from Qu et al., 2008; Hao et al., 2013). Abbreviations: AH, after-hyperpolarizing; S, synaptic.

Another important class of enteric neurons is represented by secretomotor and secretomotor/vasodilator neurons regulating the electrolyte and water transport across the intestinal mucosa (Vanner & Macnaughton, 2004).

In the ENS, in parallel with neuron population, it is possible to identify another cell population that is represent by enteric glial cells (EGCs). In the last couple of years, the role of EGCs in ENS function has gained significant attention (Sharkey, 2015). EGCs constitute a major population of peripheral glia that is located within the ganglia of the myenteric and submucosal plexus of the ENS and in extraganglionic sites, such as the smooth muscle layers and the mucosa (Gershon & Rothman, 1991; Gulbransen & Sharkey, 2012; Ruhl et al., 2004). The EGCs 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 (Gulbransen & Sharkey, 2012; **Figure 1.2**). EGCs 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). Different types of EGCs have been identified (Boesmans et al., 2015; Hanani & Reichenbach, 1994) and are subdivided into four subtypes which correspond to unique locations within the plexus and extraganglionic spaces and to specific phenotypic properties (Boesmans et al., 2015). The EGCs '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 fibers within myenteric and submucosal ganglia (intraganglionic EGCs). Enteric glia 'type II' represents the elongated glial cells within interganglionic fiber 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' EGCs. The fourth type of enteric gliocytes are distributed between smooth muscle cells, running with neuronal fibers in the musculature, thus these cells are 'intramuscular' or 'type IV' EGCs (Hanani & Reichenbach, 1994; **Figure 1.2**). Traditionally, EGCs were thought to contribute primarily to the structural integrity and nourishment of the ENS. However, during the last decades several studies have confuted the concept of a merely supportive function of EGCs and ascribed to them a wide variety of roles that are essential for proper GI function (Boesmans et al., 2015). In addition to be a scaffold for neurons, EGCs are involved in most gut functions such as mucosal integrity, neuroprotection, adult neurogenesis, neuro-

immune interactions, and synaptic transmission (De Giorgio et al., 2012; Gulbransen & Sharkey, 2012; Neunlist et al., 2013; Ruhl et al., 2004).

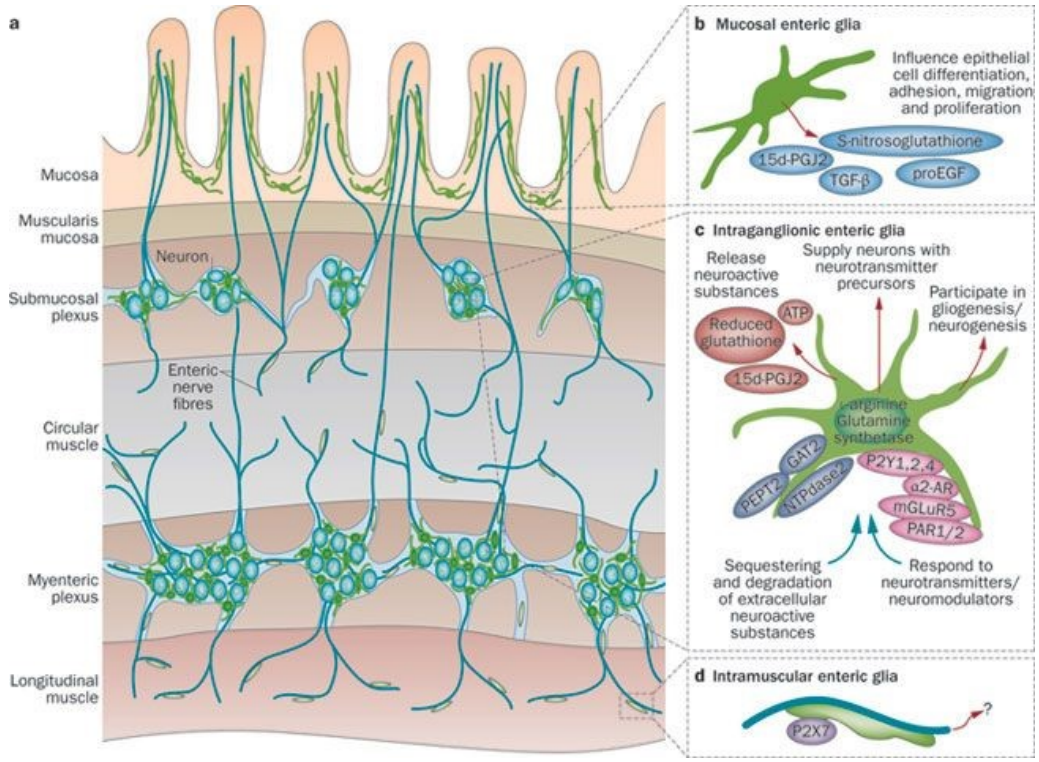


Figure 1.2. Subpopulations of enteric glia. (**A**) Several subpopulations of enteric glia located within the gut wall with different proposed physiological functions and signaling mechanisms. (**B**) Mucosal enteric glia lies in the mucosa directly beneath the epithelial cells. (**C**) Intraganglionic glia surround neurons (blue) within the enteric nerve plexuses (submucosal and myenteric plexus). (**D**) Intramuscular glia is associated with enteric nerve fibers innervating the smooth muscle layers (circular muscle and longitudinal muscle). Abbreviations: α2-AR, α2 adrenergic receptor; 15d-PGJ2, 15-deoxy-Δ12,14-prostaglandin J2; GAT2, sodium- and chloride-dependent GABA transporter 2; mGluR5, metabotropic glutamate receptor 5; NTPdase2, ectonucleoside triphosphate diphosphohydrolase 2; PAR1/2, protease-activated receptor 1/2; PEPT2, peptide transporter 2 (also known as solute carrier family 15 member 2); proEGF, proepidermal growth factor; P2X7, P2X7 receptor; P2Y1,2,4, P2Y1,2,4 receptor; TGF-β, transforming growth factor β. (modified from Gulbransen & Sharkey, 2012).

Although at present EGCs are the least-studied peripheral glial cells in mammals, there is an increasing interest in understanding the complex roles of these cells in GI physiology. Clonal cultures of ENS progenitors have shown that EGCs originate from common neuro-glial progenitors (Bondurand et al., 2003), but the presence of bi-potential or committed neurogenic and gliogenic progenitors in vivo has not been documented so far. Moreover, it remains unclear the role of individual progenitors in generating distinct subtypes of enteric neurons and glial cells. In rodents, a significant fraction of enteric neurons and EGCs develops during the early postnatal period, and it would be very interesting to explore how changes associated with feeding or the establishment of luminal microflora and the maturation of the mucosal immune system after birth affect

ENS development (Kabouridis & Pachnis, 2015). Recently, emerging evidence suggests that gut microflora can have dramatic effects on the development and function of the nervous system, both at the local as well as at the systemic level (Obata & Pachnis, 2016). The role of microbiota on ENS organization is highlighted by the reduced number of enteric neurons and the associated deficits in gut motility observed in germ-free (GF) mice (Anitha et al., 2013). Furthermore, the development and continuous homeostatic influx of EGCs into the intestinal mucosa is defective in GF mice or in antibiotic-treated mice (Kabouridis et al., 2015). These findings reveal the complex and intricate relationship between the microbiota and EGCs as regulators of neuroimmune control of host defense in the intestinal mucosa (Sharkey et al., 2018) and essential for the assembly of intestinal neural-glial circuits. Interestingly, reconstitution of GF mice with conventional microbiota normalized the density of EGCs network and gut physiology (Kashyap et al., 2013; Kabouridis et al., 2015) raising interesting questions relating to the cellular plasticity of the ENS and the mechanisms by which microbiota influence its homeostasis. Furthermore, the potential role of the microbiota and the mucosal immune system in the activation of glial progenitors and the homeostasis of EGCs is currently unclear, but it is interesting that glial cells are capable to direct influence immune responses (Turco et al., 2014). To this concern, an upon bacterial stimulation, EGCs upregulate expression of MHC class II, which suggests that they actively respond to the colonization of the gut lumen by microbiota and participate in antigen presentation to the adaptive immune system (Turco et al., 2014). Taken together, these observations highlight that dynamic host–microbe interactions are a key element for EGCs development, suggesting that an improved understanding of this mechanism will provide important insights into the pathophysiology of GI diseases.

1.2 Toll-like Receptors

All living organisms are constantly exposed to environmental microorganisms and 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 defense 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 & 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 non-self, such as 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 1.3**). 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 & Beyaert, 2003; **Figure 1.3**).

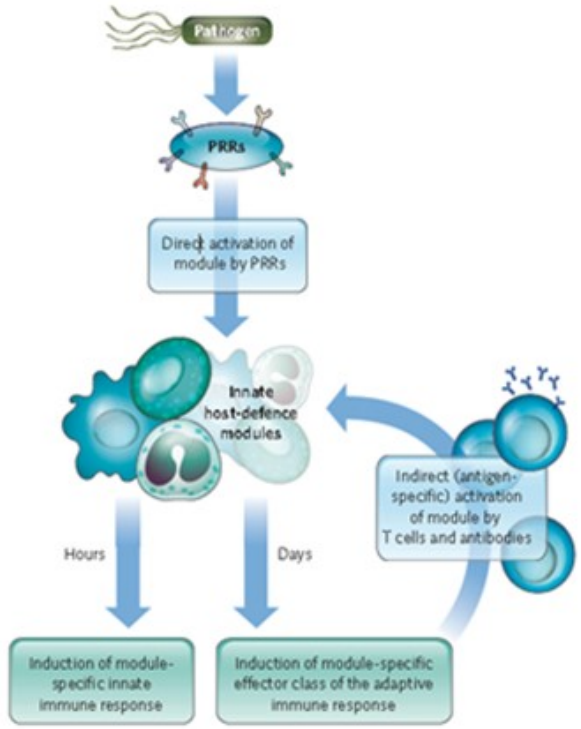


Figure 1.3. Pathways of host-defense mechanisms (modified from Medzhitov, 2007). Abbreviation: PRRs, pattern recognition receptors.

Toll like receptors (TLRs) are one of the most important family of the PRRs. 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 & 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 & 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 the 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 the 1997, the first mammalian TLRs 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

& Akira, 2010; **Table 1.2**). Studies in mice deficient in each single TLRs type have demonstrated that every TLR has a distinct function in terms of PAMPs 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 & 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 1.2**). 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).

TLR	Location of TLR	PAMPs recognized by TLR	Co-receptor (s)	Signaling adaptor		Effector cytokines induced	Endogenous ligands
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.)	Serum amyloid A, Snapin A, HMGB-1, biglycan, endoplasmin, hyaluronan, and monosodium urate crystals
TLR3	Endosome	ssRNA virus (WNV), dsRNA virus(Reovirus), RSV, MCMV		TRIF	NFκB, IRF3,7	Inflammatory cytokines (TNF-α, IL-6 etc.), type I IFNs	mRNA
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	S100A8/9, tenascin C, surfactant protein A, high-mobility group box protein-1, fibrinogen, heat shock protein (-20, -60, -70, and -96), extra domein A of fibronectin, byglican, CD138, βdefensin, heparan sulfate, and resistin
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.)	heat shock protein (-20, -60, -70, and -96, andsoluble tuberculosis factor
TLR7	Endosome	ssRNA viruses (VSV, Influenza virus)		MyD88	NFκB, IRF7	Inflammatory cytokines (TNF-α, IL-6 etc.), type I IFNs	ssRNA-containing ICs, siRNA
TLR8	Endosome	ssRNA from RNA virus		MyD88	NFκB, IRF7	Inflammatory cytokines (TNF-α, IL-6 etc.), type I IFNs	ssRNA-containing ICs, human cardiac myosin
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	DNA-containing ICs
TLR11	Plasma membrane (cell surface)	Uropathogenic bacteria, profillin-like molecule (*Toxoplasma gondii*)		MyD88	NFκB	Inflammatory cytokines (TNF-α, IL-6 etc.)	-

Table 1.2. Descriptions of TLR location and characteristics (modified from Kumar et al., 2009; Duffy & O'Reilly, 2016).

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 signaling (Kawai & 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 1.4**).

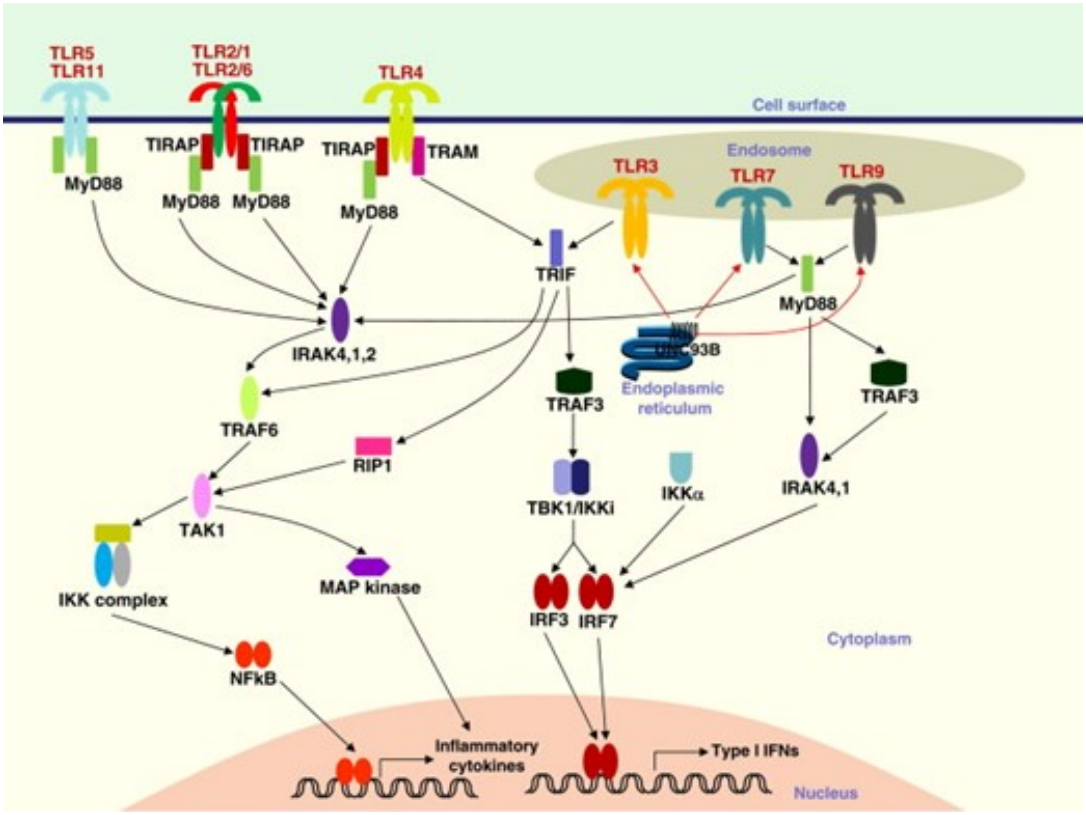


Figure 1.4. TLR signaling in conventional dendritic cells, macrophages and plasmatic dendritic cells. Abbreviations: IKK complex, Inhibitor of nuclear factor kappa-B kinase complex; IKKα, Inhibitor of nuclear factor kappa-B kinase subunit alpha; IRAK4,1,2, Interleukin-1 receptor-associated kinase 4, 1, 2; IRF3, 7, Interferon regulatory factor 3, 7; MAP kinase, Mitogen-activated protein kinase; MyD88, myeloid differentiation factor 88; NFkB, Nuclear-factor kappa B; RIP1, receptor-interacting protein 1; TLR, Toll-like receptor; TAK1, Transforming growth factor beta-activated kinase 1; TBK1/KKi, kinase binding domain; TIRAP, Toll/interleukin-1 receptor domain-containing adapter protein; TRAM, Translocating chain-associated membrane protein 1; TRAF3,6, TNF receptor-associated factor 3; TRIF, TIR-domain-containing adapter-inducing interferon-β (modified from Kumar et al., 2009).

The differential responses mediated by distinct TLRs 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 TLRs 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 & 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 1.4**).

1.2.1 Toll-like Receptors in the Nervous System

In the gut, resident bacteria confer many benefits to intestinal physiology and have a truly mutualistic relationship with the host (Hooper & Gordon, 2001). However, inappropriate activation of the immune system by commensal bacteria or pathogens appears to play crucial importance in the pathogenesis of intestinal disease (Podolsky, 2002). Intriguingly, GF animal studies demonstrate that the microbiota is necessary for the development of gut mucosal immunity (Macpherson & Harris, 2004). TLRs play a role in the cross-talk between the intestinal microbiota and the host, as they specifically recognize conserved microbial molecular structures, called MAMPs (Martin et al., 2010) and protect against pathogenic microorganisms. Furthermore, microbiota-driven immune response can prevent the development of inappropriate inflammatory response to commensal microbiota and establish a host-microbial homeostasis. A breakdown of this gut homeostasis due to microbial dysbiosis could cause immune-related disorders (Manichanh et al., 2006), diabetes (Wen et al., 2008), allergies (Penders et al., 2007) and obesity (Ley et al., 2005). The composition of intestinal microbiota has an important role in shaping host immunity, including cytokine expression, development of GALT and mucosal barrier. GF mice have numerous immune abnormalities, including failure of secondary lymphoid development, lower levels of antimicrobial peptides and smaller numbers of intraepithelial lymphocytes (Sauza et al., 2004). Colonization of GF mice can restore the proper organization of the intestinal immune system (Hooper, 2004). Thus, microbiota can enhance innate immunity through mucous secretion and production of antimicrobial peptides. These data show that the precise composition of the intestinal

microbiota can qualitatively and quantitatively influence host immune responses, which in turn have the capacity to affect gut function.

The expression of TLRs in both CNS and ENS (Barajon et al., 2009) has suggested that TLRs are not only involved in regulating host immune responses but, also, they may have a role in central aspects of neuroinflammation, neurodevelopment and neuroplasticity (Aravalli et al., 2007; Okun et al., 2011). Among all TLRs the most important bacteria-sensor proteins are TLR2 and TLR4; since they are expressed by enteric neurons and glia, which suggest that ENS lineages can directly sense microbial microbiota. TLR2 recognizes large variety of PAMPs, in particular different ligand such as porins, lipoprotein, LTA, bacterial PG, viral hemagglutinin and glycoproteins (component of Gram-positive bacteria) and their interaction leads to the activation of MyD88-dependent signaling pathways (Takeda et al., 2003). 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 (Akira & Takeda, 2004). On the other hand, TLR4 has been found to detect LPS, a major component of Gram-negative bacteria cell wall (Takeda et al., 2003). The stimulation of TLR4 by LPS results 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, respectively (Akira & Takeda, 2004). Several studies advocate for a role of TLRs in ENS homeostasis (Kabouridis & Pachnis, 2015). Recently TLR2 and TLR4 signaling seems to be fundamental for ensuring intestinal integrity and protecting from harmful injuries, in fact changes in their expression have been reported in functional and/or inflammatory bowel disease (IBD, Rakoff-Nahoum et al., 2006). Changes in the architecture and neurochemical coding of ENS lead to gut dysmotility and to higher IBD susceptibility in a model of $TLR2^{-/-}$ mice highlighting TLR2 as major player in gut homeostasis (Brun et al., 2013). Furthermore, myenteric ganglia of $TLR2^{-/-}$ mice contained fewer neurons compared with their wild-type mice, with reduction in inhibitory $nNOS^{+}$ neurons being the most notable phenotype (Brun et al., 2013). The reduction in $nNOS^{+}$ neurons is accompanied by intestinal dysmotility and impaired chloride secretion in ileum. Administration of GDNF can correct many of the ENS deficiencies in $TLR2^{-/-}$ mice and

in antibiotic-treated animals, suggesting that one of the roles of the microbiota-TLR2 axis is to promote the expression of neurotrophic factors that are required to maintain the functional organization of the mammalian ENS (Brun et al., 2013). Some studies reported also, that the absence of TLR2 increases susceptibility to intestinal injury and inflammation (Cario et al., 2007). Anitha, and colleagues, showed that GF and antibiotic-treated mice exhibited reduced motility and fewer $nNOS^{+}$ neurons (Anitha et al., 2012). This effect was mediated, at least partly, via TLR4, since $TLR4^{-/-}$ mice exhibited similar deficits in intestinal motility and a reduced number of nitrergic neurons as GF mice. This phenotype was reproduced in mice with ENS specific MyD88 knockout, suggesting that TLR4 signaling is critical for the nitrergic neurons within ENS lineages. The same study demonstrated that LPS promoted the survival of cultured enteric neurons in an NF-κB–dependent manner (Anitha et al., 2012). TLR4 is the best characterized pathogen-recognition receptor and recently recognized to modulate ENS phenotype and function (Anitha et al., 2012; Caputi et al., 2017a). Our group recently demonstrated the role of TLR4 in controlling small bowel contractility through nitrergic-purinergic neurotransmission (Caputi et al., 2017a) and in modulating the distribution of EGCs in the ileum and the concomitant release of two signaling molecules, NO and ATP involved in controlling GI motility (Caputi et al., 2017a). Thus, the cross-talk between TLR4 and nitrergic/purinergic pathways in neural-glial communication is likely to be a prerequisite for understanding normal gut physiology and the pathology. Polymorphisms in TLRs genes or in general a defective immune response, appear to be involved in the initiation and perpetuation of chronic inflammation in 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 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 (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 & 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). Several authors have recently highlighted the involvement of TLR4 in CNS plasticity, learning and memory, and behavior such as novelty seeking and social interaction (Okun et al., 2012; Li et al., 2016). Zhu et al (2016) showed various cerebellum-related motor defects in TLR4-deficient mice, due to the loss of Purkinje cells. Furthermore, TLR4 is involved in modulating the self-renewal and the cell-fate of neuronal stem/progenitor cells (Rolls et al., 2007). These findings suggest that TLR4 signaling is essential for neuron development and plasticity in the CNS. Other "pivotal" players in the innate regulation of inflammatory responses in the CNS are microglial cells, once activated by 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 peripheral tissues, leading to the degeneration of the neuronal structure (Nguyen et al., 2002). In this case the condition is critical, because alterations of BBB are possibly linked to increased vulnerability of CNS cells and excessive innate immune responses together with the production of cytokines, 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 present 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).

1.3 Microbiota-Gut-Brain axis

In the last decade, emerging evidence has revealed the presence of an intense dialogue between the brain and the GI system, the so-called brain-gut axis, and furthermore that microbiota can influence not only the immune and metabolic systems, but also the nervous system (Collins et al., 2012). The 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 whereas 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. Lately, it is becoming increasingly clear that a third player, such as the gut microbiota, can significantly influence the gut-brain crosstalk, having a marked impact on digestive processes, immune responses, emotional status, perception and cognitive functions (Felice et al., 2016). The microbiota-gut-brain axis has attracted much attention regarding the pathogenesis of different central neurodegenerative disease, in which GI dysfunction appears many years before of degenerative state. In addition, the enteric microbiota is a huge antigenic load resident in the gut and confers marked potential danger if not kept under continuous surveillance, such as under TLRs sensing (Caputi & Giron, 2018). However, the enteric commensal microbiota is required for the constant stimulation of the immune system and TLR-mediated sensing of these microorganisms may play a dual role in disease development as a source of both inflammatory and regulatory signals. In this respect, it is important to take into account that the microbiota is also a source of biological active signaling molecules, immune mediators and gut hormones. Some of those, including serotonin, purines, GABA and neurotrophic factors, among others, have been shown to be involved in TLRs signaling (Brun et al., 2013, 2015; Latorre et al., 2016; Caputi et al 2017a, b). The emerging evidence described above suggests the existence of a multifaceted TLR signaling network that influences neural circuits and immune-mediated processes both in the gut and in the brain. Further studies focused on discovering the enteric microbiota-derived factors responsible of TLRs engagement and the consequent signaling outcomes of TLR activation in both the ENS and the CNS will provide novel insights into the

complex dialogue between the host and the microbiota in neurodegenerative disorders (Caputi & Giron, 2018).

1.4 Enteric Neurotransmission

Neural networks for the control of digestive functions are positioned at many levels: the CNS, spinal cord, prevertebral sympathetic ganglia, and in the wall of the specialized tissues that composed the digestive system. The major actors of this system are the neurons, which usually express a combination of different neurotransmitters, a phenomenon known as *chemical coding*. The chemical code depends on the type of neuron, the species and the intestinal segment (Hansen, 2003). The resting membrane potential of enteric neurons is normally less negative than in the CNS (-40 to -70 mV) and is largely determined by potassium channels. Hyperpolarization induces inhibition, whereas depolarization induces excitation of the neuron (Furness et al., 2000). Action potentials are mostly carried by sodium (extrinsic nerves) and calcium (intrinsic nerves) (Vanden Berghe et al., 2001). The general mechanism of chemically mediated synaptic transmission in the ENS is complex as in the CNS. More than 30 neurotransmitters have been identified in the ENS (Galligan, 1998; Furness et al., 2000). Enteric neurotransmitters are either small molecules (e.g. norepinephrine and serotonin), larger molecules (peptides) or gases (NO and carbon monoxide).

The last classification of enteric neurons relies on their functional proprieties and three types of neurons can be distinguished.

- ✓ SENSORY NEURONS: these neurons are divided into two categories:
  - Intrinsic primary afferent neurons (IPANs) belong to AH type (AFTER HYPERPOLARISATION), present an oval or rounded soma and their prolongations contact different neurons in the mucosal and sub-mucosal plexus. Their activation after mechanical or chemical stimuli applied to the intestinal mucosa allow the ENS to generate appropriate reflex responses. Chemosensitive IPANs respond to chemical stimuli applied on the luminal surface of the small intestine's mucosa while mechanoreceptor myenteric IPANs react to intestinal wall musculature's contraction through mechanosensitive ion channels (Kunze et al., 2000). Sub-mucosal IPANs

instead response indirectly to mucosal distortions through enterochromaffin cells that release serotonin after mechanical stimulus.

- Extrinsic primary afferent neurons (EPANs) can be vagal or spinal afferents where the first involved in physiological events while the second primary response to physiological stimuli.

Sensory neurons can be also nociceptors because their activation after a nociceptive stimulus evokes protective responses (Furness et al., 2014).

- ✓ INTERNEURONS: usually these neurons belong to Dogiel type II and could be S- (synaptic) or AH type. They are interposed between the primary afferent neurons and the motor or secretomotor neurons. Four types of interneurons constitute chains extended along the intestine: one ascendant interneuron is cholinergic and is the conduct for the ascendant path that compose the projectile reflexes. Three descendant interneurons have a complex chemical distinction and studies performed on the connections established by neurons revealed that each type perform different actions (Costa et al., 2000).
  - ChAT (choline acetyltransferase)/NOS (nitric oxide synthase)/VIP (vasoactive intestinal peptide) interneurons are involved in local motor reflexes (Costa et al., 2000).
  - ChAT/SOM (somatostatin) interneurons play a key role in the conduction of migrating myoelectric complexes in the small intestine (Costa et al., 2000).
  - ChAT/5HT (serotonin) interneurons are associated in secreto-motor but not directly in motor reflexes (Costa et al., 2000).
- ✓ MOTONEURONS: these neurons belong to Dogiel type I and S-type (Hansen, 2003).
  - Muscular motoneurons innervate the muscularis mucosae, longitudinal and circular musculature of the entire digestive tract. They mediate cholinergic and tachykinergic excitatory stimulation but also inhibitory arousal (Hansen, 2003).
  - Secretomotor and vasomotor neurons control secretion and blood flow and are directly regulated by IPANs through the release of ACh and VIP. The soma of the majority of secretomotor and vasomotor neurons is in the sub-mucosal plexus and some of these neurons project in the myenteric plexus

while other in the muscularis mucosae. These neurons can be either cholinergic or non-cholinergic: ACh released by cholinergic neurons acts on muscarinic receptors in the mucosal epithelium, VIP instead is released by non-cholinergic neurons as transmitter (Hansen, 2003). Sympathetic afferents modulate local reflexes loop that regulate secretion and blood flow (Hansen, 2003).

1.4.1 Cholinergic Neurotransmission

The major excitatory transmission within the ENS are mediated by cholinergic transmission, with ACh, producing excitatory potentials in post-synaptic effectors. The cholinergic circuitry of the ENS is extensive and mediates motility (muscular) and secretory (mucosal) reflexes, in addition to intrinsic sensory and vascular reflexes. ACh is a co-transmitter of the greater population of the enteric neurons and is synthesized in nerve terminals from choline and acetyl-CoA by choline acetyltransferase (ChAT) and is then translocated to synaptic vesicles by the vesicular acetylcholine transporter (VAChT; Eiden, 1998). ACh is then stored in the vesicles until it is released on demand (Wessler et al., 2003).

There are two types of receptors mediating cholinergic transmission with the ENS. ACh binds to nicotinic (nAChRs) and muscarinic receptors (mAChRs). nAChR are ligand-gated ion channels, whereas mAChR are G-protein-coupled receptors (Caulfield & Birdsall, 1998). Thus, ACh binding generates variable postsynaptic potentials depending on the receptor present on the cell membrane, with nAChRs mediating rapid excitatory transmission and mAChRs mediating slow excitatory transmission (Harrington et al., 2010). At the cholinergic synapse both classes of receptors are present on effector cells (post- synaptic receptors) or on nerve terminals (pre-synaptic receptors) where they act as autoreceptors regulating release of ACh from nerve terminals. Within the ENS nAChRs are required for rapid neurotransmission, in order to propagate reflexes quickly and produce fast responses to stimuli (Galligan, 2002). nAChR activation is the predominant mechanism for cholinergic neurotransmission in enteric ascending reflex pathways. mAChR mediate responses to ACh by activating second messenger cascades and intracellular signalling pathways (Caulfield & Birdsall, 1998). The importance of mAChR to determining the multiple roles ACh has in intestinal physiology is reflected in their diverse distribution and different subtypes coupling to various G-proteins, thus

eliciting variable intracellular responses upon activation. ACh binding to mAChR either depolarizes the cell membrane, resulting in the initiation of another action potential, or hyperpolarizes the cell membrane, inhibiting additional action potentials.

1.4.2 Tachykinergic Neurotransmission

Neuropeptides are small molecules used by neurons for communication. However, they are not only important for neurotransmission, but they also have effects on tissue growth and differentiation, inflammation, immunomodulation and tumor growth. The production of neuropeptides occurs in the cell body of the neurons, and they are then transported to the varicosities and are released after stimulation.

A neuropeptide frequently discussed in inflammatory situations, including those of the intestine, is substance P (SP), a tachykinin expressed throughout the nervous and immune systems, that regulates an extraordinarily diverse range of physiological processes. This peptide consists of 11 amino acids and belongs to the tachykinin family of peptides, interacts with three neurokinin receptors (NKRs) encoded by three *Tacr genes*. SP has been discovered in extracts of horse brain and intestine with effects on intestinal contractility and blood pressure. The finding of SP was the first discovery of many other "brain-gut neuropeptides," which are present in enteric neurons and enteroendocrine cells as well as in neurons of the brain (Euler & Gaddum, 1931).

The preferred receptor for SP is the neurokinin-1 receptor (NK-1R) (Nakanishi, 1991; Regoli & Nantel, 1991), but SP can also bind to NK-2R with low affinity (Hershey & Krause, 1990). NK-1R is a member of the superfamily of guanin nucleotide binding-coupled receptors with seven membrane-spanning domains, three extracellular and intracellular loops, and extracellular NH2 and intracellular COOH termini, that couple with G-proteins to promote high-affinity binding and signal transduction (Hershey & Krause, 1990). Binding of SP to the NK-1R mediates rapid endocytosis and internalization of the receptor (O'Connor et al., 2004).

SP, classically, is a peptide produced in sensory neurons, is a pain mediator and is involved in vaso-regulation and so-called neurogenic inflammation (Foreman, 1987; Gamse et al., 1987). SP is also involved in immunomodulatory activities and has long been considered to play a key role in IBD (Gross & Pothoulakis, 2007). SP has profound effects for intestinal physiology and it is involved in the regulation of motility and

transmural and electrolyte transport as well as in the regulation of blood flow in the intestine (Holzer & Holzer, 1997; Riegler et al., 1999). SP has excitatory effects in the GI canal, mediating smooth muscle contractions (Bartho & Holzer, 1985). SP is present in enteric efferent neurons but also in sensory innervation. It is synthesized by enteric cholinergic motor neurons, and hence, SP containing nerve fibers are frequent in the smooth musculature but are also present in the submucous plexus, blood vessel walls and lamina propria (Brodin et al., 1983).

1.4.3 Serotonergic Neurotransmission

In 1937, Vittorio Erspamer extracted a factor, derived from the enterochromaffin (ECs) cells of the GI epithelium, and identified it as an amine, called 'enteramine'. 'Enteramine' was unknown to Maurice Rapport when, in 1948, he isolated serotonin (5-HT) as a serum vasoconstrictor and demonstrated its chemical structure (Rapport et al., 1948). 5-HT has thus been known to posterity as serotonin, not as 'enteramine'.

Unfortunately, after 5-HT was discovered to be present and synthesized in the CNS (Twarog & Page, 1953), peripheral 5-HT, whether circulating or enteric, was found in high levels in several peripheral tissues, including the nervous system. However, not only is 95% of the body's 5-HT located in the gut, but also enteric 5-HT plays vital roles as a growth factor, a hormone, a paracrine factor and a neurotransmitter (Gershon, 2013). The majority of 5-HT in the body is synthesized, stored, and released from a subset of enteroendocrine cells, the ECs cells in the intestinal mucosa (Gershon & Tack, 2007). But also 10% of gut 5-HT is present in enteric neurons and is synthesized in the ENS (Dreyfus & Bornstein, 1977). Serotonergic neurons constitute about 2% of all myenteric neurons (Furness & Costa, 1987b).

5-HT mediates many GI functions through activation of a diverse family of 5-HT receptors (5-HTRs) present on enteric neurons, ECs, GI smooth muscle, and possibly on enterocytes and immune tissues (Gebauer et al., 1993). There are several neuronal and muscular 5-HTRs subtypes in the gut. Seven types or families and multiple subtypes of 5-HTRs have now been identified. These receptors are coupled by G proteins to the inositol triphosphate and diacyl glycerol (IP3/DAG) pathway of cellular excitation (Schmidt & Peroutka, 1989), only 5-HT_3 receptor is uniquely a ligand-gated cationic channel that is prone to rapid desensitization (Derkach et al., 1989). An important

property of intercellular signaling is the termination of the signal by local enzymatic degradation, or by uptake into nearby cells or nerve terminals for recycling or degradation. In the case of 5-HT, there are no intercellular degradative enzymes and it requires a specialized transport mechanism to cross the plasma membrane where it can be degraded by intracellular enzymes such as monoamine oxidase. The serotonin-selective reuptake transporter (SERT), which is also responsible for 5-HT uptake in the brain, is expressed by essentially all epithelial cells of the intestinal mucosa (Wade et al., 1996). The SERT is a member of the neurotransmitter/sodium symporter (NSS) family, that internalizes 5-HT via a sodium- and chloride-dependent mechanism (Murphy et al., 2004; Mawe & Hoffman, 2013). Since all of the epithelial cells in the lining of the intestines appear to express SERT, it serves as a selective sponge to remove 5-HT from the interstitial space following release by ECs cells. By inactivating 5-HT through rapid reuptake into epithelial cells of the intestinal mucosa, SERT serves as a critical protein in the local regulation of 5-HT availability and intestinal function (Kim & Camilleri, 2000).

1.4.4 Nitrergic Neurotransmission

NO is a major inhibitory neurotransmitter in the GI tract and has been proposed as a neuromuscular neurotransmitter of nonadrenergic noncholinergic (NANC) inhibitory nerves in the parasympathetic system (Toda & Okamura, 2003; Toda & Herman, 2005).

In mammals NO can be produced by three different isoforms of the NO synthase enzyme (NOS). These enzymes are the neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). All the NOS isoforms have been localized in myenteric neurons of different species (Vannucchi et al., 2002; Talapka et al., 2011) and nNOS seems to represent the main source of NO involved in the physiological modulation of NANC inhibitory motor responses of the gut. All NOS isoforms utilize L-arginine as substrate and molecular oxygen and reduced nicotinamide-adenine-dinucleotide phosphate (NADPH) as co-substrates. Flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and (6R-)5,6,7,8-tetrahydro-L-biopterin (BH4) are cofactors of all isoenzymes. All NOS proteins are homodimer. In orders to synthesize NO, the NOS enzyme goes through two steps. In the first step NOS hydroxylates L-arginine to Nω-hydroxy-L-arginine; in the second step, NOS oxidizes Nω-hydroxy-L-arginine to L-citrulline and NO (Stuehr et al., 2001). All NOS isoforms bind calmodulin. In the nNOS and eNOS, calmodulin binding is brought by an increase in intracellular Ca^{2+}. In the iNOS isoform

calmodulin binds at extremely low intracellular Ca^{2+} concentrations (below 40nM) (Forstermann & Sessa, 2012).

In the ENS, NO, in physiologic conditions, plays a pivotal role in the inhibitory regulation of peristalsis (Furness, 2000). The intracellular mechanisms of relaxation induced by NO are mediated via NO-sensitive guanylyl cyclase (NO-GC), expressed in several GI cell types, including smooth muscle cells (SMC), interstitial cells of Cajal (ICC), and fibroblast-like cells. Up to date, the interplay between neuronally released NO and these cells to initiate a nitrergic inhibitory junction potential (IJP) is biphasic: a fast ICC-mediated and a slower SMC-mediated phase. Conceivably, NO-GC in ICC is responsible for initiation of the nitrergic IJP, whereas NO-GC in SMC is important for its maintenance (Lies et al., 2014). The expression of iNOS, but not of nNOS and eNOS, prevails during diseases states, such as intestinal inflammation (Miampamba et al., 1999) and I/R injury (Giaroni et al., 2013). In these conditions, large amounts of NO can cause damages in different cellular populations, such as neurons, by the formation of peroxynitrite and nitrotyrosine (Rivera et al., 2011). This phenomenon reflects a functional plasticity of myenteric neurons which activate different NOS isoforms depending on either physiological or pathological conditions (Robinson et al., 2011; Giaroni et al., 2013).

1.4.5 Purinergic Neurotransmission

It is well known that ATP has a prominent role in metabolic processes as well as in intracellular energy store for living cells. It has also been known for a long time that it takes part in the synthesis of genetic material as a nucleic acid precursor. Long and complex story taking part with a proposal in 1970 that “adenosine triphosphate or a related nucleotide” was released by inhibitory nerves supplying the external layers of smooth muscle in the GI tract (Burnstock et al., 1970). Thereafter, the term “purinergic” was created by Burnstock and entered in the lexicon of neurotransmission in 1971 (Burnstock, 1971). Several articles confirmed that ATP, adenosine and other purine nucleotides and/or nucleosides play an important role as neurotransmitters, co-transmitters or neuromodulators in the regulation of the GI tract.

ATP is a neurotransmitter of inhibitory junction potentials, released by NANC nerves (Burnstock et al., 1970). ATP and related compounds activate purinoceptors (P2) include P2X ligand gated cation channels, P2Y G-protein-linked receptors and adenosine G-

protein-coupled receptors localized in the neurons of ENS. A primary role for P2X receptors has been found in mediating fast synaptic transmission while P2Y receptors predominately mediate slow synaptic transmission and adenosine receptors mediate presynaptic inhibition (Kin, 2015). Together, these receptors affect many enteric reflexes and motor patterns in GI tract (Galligan, 2008). Adenosine is thought to be liberated from cells as a consequence of metabolic stress or as a breakdown product of released ATP. In contrast, ATP is thought to be primarily released from neuronal sources as a neurotransmitter, although there is evidence of ATP release from nonneuronal cells such as working muscle (Ren & Bertrand, 2008). Changes in purinergic signaling in the ENS may contribute to some pathological mechanisms in the GI tract. Recent evidence points toward an alteration in purinergic synaptic transmission in inflamed tissue.

1.4.6 Others Neurotransmission

Calcitonin gene-related peptide (CGRP) belongs to the calcitonin family of peptides (Juaneda et al., 2000). CGRP is shown to be co-localized with SP in a majority of sensory nerve fibers (Gibbins et al., 1958). However, in the intestine, there are also intramural neurons that contain CGRP (Juaneda et al., 2000). CGRP is, similar to SP, involved in the regulation of blood flow (Holzer, 1988), in the modulation of intestinal motility (Holzer et al., 1989), and in wound healing (Luger & Lotti, 1998).

Vasoactive intestinal peptide (VIP) belongs to the VIP-glucagon peptide family, was originally isolated from small intestine by Said and Mutt (Said & Mutt, 1970). VIP is mainly present in parasympathetic neurons (Lundberg et al., 1980; Domeij et al., 1991), and is frequently expressed in the enteric neurons. In contrast to SP, VIP has anti-inflammatory properties (Kim et al., 2000) and it is involved in the regulations of intestinal motility and blood flow (Grider & Jin, 1993), and the secretion of electrolytes and water (Polak & Bloom, 1979). VIP effects on the intestinal smooth muscle are inhibitory. As well as in neurons, VIP is also reported to be produced by inflammatory cells in the intestine (Delgado, 2003). VIP has effects on class II family of G-protein-coupled receptors named VPAC1 and VPAC2 (Harmar et al., 1998). VPAC1 and VPAC2 do not discriminate between VIP and another peptide, pituitary adenylate cyclase-activating polypeptide (PACAP) (Laburthe et al., 2007). PACAP binds to the PAC1 receptor, while VIP has a low affinity for the PAC1 receptor. The VIP/PAC receptor

reported to dominate in the human colonic mucosa is the VPAC1 receptor (Schulz et al., 2004). The VPAC1 receptor is also called VIP receptor 1 (VIPR1).

Somatostatin is a tetradecapeptide and it has been found in virtually every organ of the body, and it is particularly abundant in the GI tract. Somatostatin exerts an inhibitory action on numerous physiological functions, acting as a classical endocrine hormone, a local (paracrine) regulator, or a neurotransmitter (Vinik et al., 2000). Somatostatin actions are mediated through five distinct receptor subtypes (sst1–5). sst2 and sst5 are the main mediators of the gastrointestinal effects of the peptide. Within the GI tract, somatostatin exerts a broad range of physiological and pharmacological effects, mostly inhibitory, of which suppression of gastric acid secretion is a landmark response. Several diseases of the GI tract imply disturbances in the somatostatin production or an altered expression of somatostatin receptors (Kloppel & Anlauf, 2005).

1.5 Oxidized Phospholipids

Phospholipids (PL) are ubiquitously found throughout the body and as such constitute an integral part of the lipid bilayer of cell membranes. Glycerophospholipids comprise a glycerol backbone with three carbon residues. The first two carbon residues are connected to fatty acid chains, which form the hydrophobic tails, and the third carbon residue is linked to a negatively charged phosphate group, forming the polar headgroup. Binding of choline, serine, ethanolamine, or inositol to the polar head group at the sn-3 position of the glycerol backbone differentiates PL into distinct classes. The most abundant phospholipid is phosphatidylcholine (PCs), which is located mainly in the extracytosolic leaflet of the plasma membrane, whereas phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, or phosphatidic acid are found in the cytosolic leaflet (Ikeda et al., 2006).

Oxidation of these PL occurs during physiological processes such as metabolism or in the course of inflammatory responses, notoriously so during chronic inflammatory diseases like atherosclerosis or diabetes (Matt et al., 2015). Lipid modification through oxidation renders PL biologically active, hence influencing inflammatory processes. Oxidation of PL can occur either enzymatically by, e.g., 12/15 lipoxygenase, or non-enzymatically by reactive oxygen species (ROS) that are produced by macrophages and neutrophils to aid the anti-bacterial defense (Hampton et al., 1998; Babior, 2000; Zhang et al., 2002). Other

sources of non-enzymatic oxidation are air pollution, UV radiation, and smoking (Bochkov et al., 2010). Following the primary peroxidation reaction that can be enzymatic or non-enzymatic, intermediates such as peroxyl radicals and hydroperoxides are formed, which then undergo additional oxidation steps by an enzyme-independent process, leading to the formation of a variety of different PL oxidation products (Bochkov et al., 2010). Oxidative fragmentation of PL molecules (OxPLs) generates several biologically active products, that demonstrate multiple biological activities. Products of OxPLs can lead to the generation of immunogenic molecules leading to production of antiphospholipid antibodies that are found in autoimmune diseases, that are associated with increased oxidative stress (Bochkov et al., 2010). OxPLs can influence the functions of membrane proteins by variations in chemical structure determining changes in the physical properties of these important membrane and lipoprotein components, in term of ion transport, mobility and diffusion (Kinnunen, 1991). Recent evidence report that OxPLs showed potential relevance in the development of several chronic disease such as atherosclerosis, for their role in the formation of atherosclerotic plaque (Berliner et al., 2001). OxPLs, also, were shown to play a role in inflammatory state by accumulation in inflamed sites in condition of sepsis, acute lung injury or neurodegenerative disease (Qin et al., 2007). However, some research has reported a potential role of OxPLs in anti-inflammatory events, such as endotoxemia, as a result of endogenous feedback mechanism that serves to limit the potential damage caused by inflammation-induced oxidants (Bochkov et al., 2002).

OxPLs have been identified as endogenous DAMPs, characteristic of oxidatively damaged tissue (Serbulea et al., 2018) and are increasingly recognized for their ability to modulate function of TLRs. OxPLs can inhibit inflammation induced by TLRs through various mechanisms including inhibition of intracellular inflammatory signaling through electrophilic mechanisms or selective inactivation of TLR4 and TLR2 and their accessory proteins according to a receptor antagonism mechanism (Mauerhofer et al., 2016). Indeed, in addition to recognition of bacterial- and viral-derived molecules, TLRs have recently been suggested to be activated by some various endogenous ligands that are produced in response to stress or tissue injury (Tsan & Gao, 2004). A prototypic mixture of OxPLs is the oxidized 1-palmitoyl-2-arachidonoyl-sn-phosphatidylcholine (OxPAPC), which is generated upon oxidation of 1-palmitoyl-2-arachidonoyl-sn-phosphatidylcholine (PAPC) and contains different oxidized PCs that include 1-

palmitoyl-2-(5,6)-epoxyisoprostane E2-sn-glycero-3-phosphocholine (PEIPC) and 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC), and smaller chemical fragments (Bochkov et al., 2010). High concentrations of OxPAPC are known to induce proinflammatory effects in endothelial cells including upregulation of cell adhesion molecules for mononuclear leukocytes and secretion of chemokines and are unlikely to accumulate in tissues (Oskolkova et al., 2010). The intracellular anti-inflammatory action of OxPLs at least partially is mediated by electrophilic mechanisms suppressing NFκB and other inflammatory mechanisms. However, this is clearly not the only mechanism of anti-inflammatory action of OxPLs because at low concentrations this mixture of OxPLs, by acting as DAMPs, was also able to inhibit TLR2 and TLR4 signaling by competitively interfering with extra-cellular accessory proteins such as CD14, LPS-binding protein (LBP), and MD2, and act as receptor antagonist (**Figure 1.5**; Erridge et al., 2008; Mauerhofer et al., 2016).

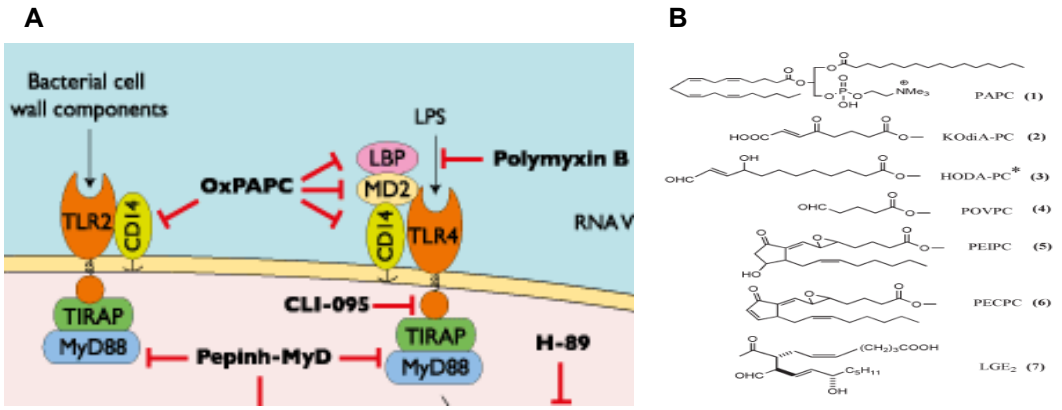


Figure 1.5. (**A**) Mechanism of blocking the signaling of TLR2 and TLR4 by the composition of mixture of oxidized phospholipids, indicated as OxPAPC. (**B**) Structure of selected bioactive phospholipid oxidation products (modified from Berliner et al., 2009). Abbreviations: CD14, cluster of differentiation 14; LBP, lipopolysaccharide binding protein; MD2, myeloid differentiation factor-2; TIRAP, adapter molecule associated with toll-like receptors.

Evidence that TLRs are involved in the recognition of OxPLs are provided by functional studies in TLR or TLR-adaptor-deficient mice showing that OxPAPC-mediated lung inflammation is attenuated in TLR4, myeloid differentiation primary response gene 88 (MyD88) and TIR domain-containing adaptor inducing interferon-beta (TRIF) null mice compared to their WT counterparts (Imai et al., 2008). Furthermore, another report showed that OxPAPC-induced inflammation is reduced in TLR2-deficient bone marrow-derived macrophages, implicating a role for TLR2 in OxPLs-mediated signaling (Kadl et al., 2011). Recent data show that OxPLs are markers of disease of the 'modified-self' type that are recognized by natural antibodies and TLRs, but increasing evidence shows

that OxPLs are not only markers of disease but also 'makers' that are likely to play an active role in variable pathological states (Bochkov et al., 2010). The exact contribution of these co-receptors to OxPLs-mediated inflammation requires further investigation especially given the potent and well-described anti-inflammatory effects of OxPLs on PAMP-mediated response.

1.6 Intestinal Microbiota

The human GI tract represents one of the largest interfaces (250–400 m^2) between the host, environmental factors and antigens in the human body. The community of commensal microorganisms, including a collection of bacteria, archaea and eukarya, colonizing in or passing through the GI tract is referred to as the intestinal microbiota (Dethlefsen et al., 2006). The gut microbiota has co-evolved with the host over thousands of years to confer many benefits to intestinal physiology and form a truly mutualistic relationship with the host (Hooper & Gordon, 2001). The mutualistic co-evolution of microbes and the human body, composed of more than 90% microbial cells and 10 million microbial genes (the so-called microbiome) has led to the collective being described as a "superorganism" (Nicholson et al., 2005).

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 feces (Dethlefsen et al., 2006; Booijink et al., 2007; Gerritsen et al., 2011), indicating that the greater microbial amount of the human microbiota is located in the large intestine. In terms of bacterial phyla found in the gut, *Firmicutes* (species such as *Lactobacillus, Clostridium, Enterococcus*) and *Bacteroidetes* (species such as *Bacteroides*) account for the majority (Dethlefsen et al., 2007), though the other phyla such as *Actinobacteria* (*Bifidobacteria*), *Proteobacteria* (*Escherichia coli*), *Fusobacteria*, *Verrucomicrobia* and *Cyanobacteria* are also present (Eckburg et al., 2005; Qin et al., 2010).

The intestinal microbiota is known to confer a number of health benefits relating to, for example, pathogen protection, nutrition, host metabolism and immune modulation (Guinane & Cotter, 2013). 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 function 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).

The developmental trajectory of the gut microbiota is compatible with concepts of the early-life period as a vulnerable phase for the subsequent emergence of psychopathology in adulthood (O'Mahony et al., 2017). Different group have only recently revealed 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*) can result from the translocation of the mother's gut bacteria via the bloodstream and placenta (Jimenez et al., 2008). Interestingly, this challenge to the prevailing sterile womb paradigm brings into focus the possible role of maternal microbiome transmission in the modulation of immune activation associated with maternal stress and the implications of this on fetal programming (Howerton & Bale, 2012). 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). During the first days of life, the infant gut microbiota shows a low diversity and 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 1.6**). Other factors such as gestational age (Barrett et al., 2013), feeding mode (Koenig et al., 2011), antibiotic use (Persaud et al., 2014), and exposure to family members and pets (Dominguez-Bello et al., 2010; Marques et al., 2010) also influence the trajectory of microbiota acquisition (**Figure 1.6**). The relative importance of these factors in determining the eventual stable microbiota profile has not been fully elucidated. A critical function of the microbiota is to prime the development of the neuroimmune system (Olszak et al., 2012; Chistiakov et al., 2014; Francino, 2014).

Alterations in the gut microbiota signature early in life can predispose to immune disorders (Penders et al., 2007).

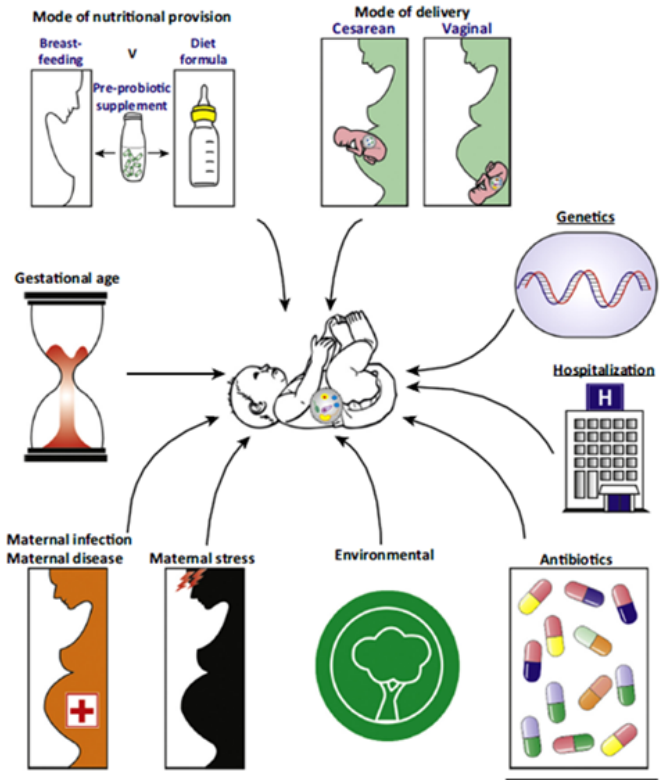


Figure 1.6. Factors influencing the development of the infant microbiota. In addition to mode of birth, mode of early nutrition, environment, other factors such as gestational age, genetics, and hospitalization, also influence the microbial composition of the infant. Infections and antibiotic usage influence the development of microbial landscape as does the selective transient enrichment by probiotics and prebiotics (modified from Borre et al., 2014).

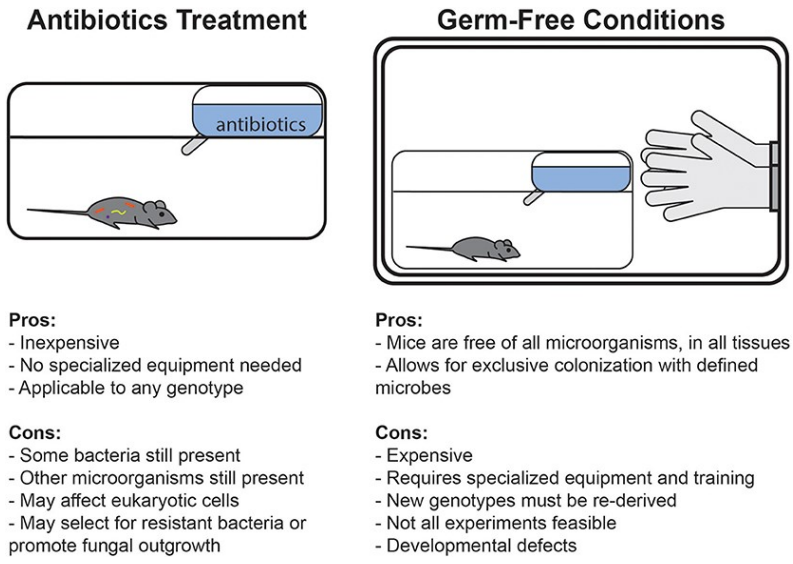


Figure 1.7. Comparison of the advantages and disadvantages of germ-free and antibiotics-treated mouse model systems (Kennedy et al., 2018).

Two main methods have emerged to explore the effects of the microbiota on physiology and disease of the gut, these are GF models and antibiotics treatment regimens (**Figure 1.7**). GF mice are bred in isolators which fully block exposure to microorganisms, with the intent of keeping them free of detectable bacteria, viruses, and eukaryotic microbes. GF mice allow for study of the complete absence of microbes or for the generation of gnotobiotic animals exclusively colonized by known microbes (Yi & Li, 2012). However, generating and maintaining these mice requires specialized facilities, and the cost, labor, and skills required to maintain them can make these models inaccessible to many researchers. An alternate method that has emerged to avoid some of these complications has been the use of antibiotics treatment. Treatment with broad-spectrum antibiotics is commonly used to deplete the gut microbiota of mice and can be readily applied to any genotype or condition of mouse. Unlike germ-free conditions, under which complete sterility is maintained throughout life, antibiotics can deplete bacterial populations in mice which were normally colonized since birth. Due to differences in mechanism of action, antibiotics can selectively deplete different members of the microbiota. Individual antibiotics can be used to shift the composition of the gut microbiota in order to identify classes of bacteria relevant to different phenotypes (Schubert et al., 2015; Zackular et al., 2016). In contrast, a cocktail of different classes of antibiotics can be used to broadly deplete the gut microbiota (Caputi et al., 2017b).

ANTIBIOTIC	EFFECT ON THE MICROBIOTA
Amoxicillin	Lactobacillus spp. depletion in SI ↓aerobic and anaerobic bacterial numbers in the colon
Metronidazole, neomycin and vancomycin	↓ bacterial numbers in SI and LI Multiple effects on composition, including: ↓ Bacteroidetes ↑ Enterobacteriaceae
Ampicillin, neomycin, metronidazole, vancomycin	Microbiota depletion ↓ peptidoglycan levels in serum
Ampicillin, gentamicin, metronidazole, neomycin, vancomycin	↓ bacterial numbers in LI Multiple effects on composition, including: ↓ luminal Firmicutes in LI ↓ mucosal associate Lactobacillus in LI

Table 1.3. Antibiotic-induced changes in the microbiota composition (modified from Ubeda & Pamer, 2012) Abbreviations: SI, small intestine; LI, large intestine.

Mice on antibiotics are not completely cleared of bacteria, but significant reductions in bacterial load are associated with shifts in microbial populations, immunity signaling pathways, and gut morphology and functions, with results often paralleling what is seen in GF mice (**Table 1.3**; Kennedy et al., 2018).

The gut microbiota patterns also change during human ageing. Several recent studies have demonstrated 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). Then, the microbiota continues to evolve until adulthood, 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). 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 health, leading to development of gut disorders later in life (**Figure 1.6**; Hviid et al., 2011; Kronman et al., 2012; Borre et al., 2014; Desbonnet et al., 2015). Recently, an increasing awareness of the role of microbiota in a large number of intestinal and extra-intestinal diseases has become steadily clear (Thursby & Juge, 2017).

As mentioned above, a multitude of factors influence the composition of the microbiota, although the composition of the gut microbiota, in the absence of insults remains relatively stable during adulthood, even if there are significant interpersonal differences (Hamady & Knight, 2009; Lozupone et al., 2012). 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 (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). Although adulthood does not appear to be a critical or vulnerable phase like adolescence however it remains a period of time during which alterations in the microbiota can influence gut health. Therefore, maintaining a healthy gut microbiota is an important aspect in possible prevention or attenuation different disorders associated with aging.

1.7 Serotonin in the Gut and Tryptophan Metabolism

As mentioned above, 5-HT is a critical signaling molecule in the brain-gut-microbiota axis (O'Mahony et al., 2015) and is involved in a wide range of physiological functions. 5-HT is synthesized from the essential amino acid tryptophan (TRP) by two enzymatic steps. First, hydroxylation of TRP by the enzyme tryptophan hydroxylase (TPH, the activity of which is rate-limiting) produces 5-hydroxytryptophan (5-HTP). The second enzymatic step is decarboxylation of 5-HTP by the enzyme aromatic L-amino acid decarboxylase producing 5-HT (Tyce, 1990; **Figure 1.8**).

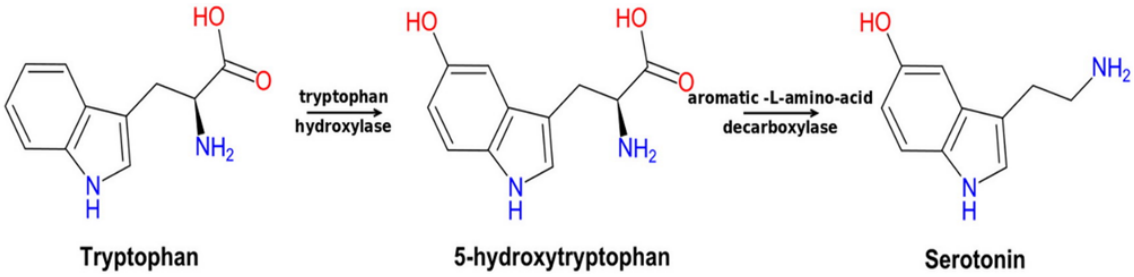


Figure 1.8. Tryptophan metabolism to 5-HT.

There are two TPH isoforms, TPH1 and TPH2; TPH1 is peripheral and critical for 5-HT biosynthesis in ECs cells, while TPH2 is critical for 5-HT biosynthesis in neurons (Cote et al., 2003; Walther & Bader, 2003). The two 5-HT compartments are kept apart, the disparity in the sizes of the two 5-HT compartments creates a temptation to ignore enteric neuronal 5-HT; however, the size of a package does not necessarily determine its importance. In fact, the large amount of 5-HT in ECs cells does not affect constitutive motility of the bowel whereas the small amount of neuronal 5-HT not only does so but is indispensable (Gershon, 2012) and it is also possible that the very large amount of 5-HT in ECs cells serves purposes that are not related to GI motility, such as peristalsis, secretion, vasodilation and perception of pain or nausea (Mawe & Hoffman, 2013).

Emerging evidence also suggests that the serotonergic system may be under the influence of gut microbiota, especially, but not limited, to periods prior to the emergence of a stable adult-like gut microbiota (El Aidy et al., 2012; Clarke et al., 2013). Metabolomics studies demonstrated that the gut microbiota has a significant impact on blood metabolites and recently showed a key role of gut microbiota in influencing 5-HT content (Spohn & Mawe, 2017). Specifically, distinct microbial metabolites produced by spore forming bacteria from mouse and human microbiota have been shown to promote 5-HT

biosynthesis in colonic ECs cells (Yano et al., 2015). Marked alterations in TRP and 5-HT expression in GF mice support the gut microbial regulation of TRP metabolism and 5-HT signaling. GF mice have increased levels of plasma TRP and hippocampal 5-HT concentrations. Microbial colonization of GF mice normalized plasma TRP levels and anxiety-like behaviors but failed to normalize 5-HT levels in the hippocampus (Clarke et al., 2013). Some investigators have reported a reduction of the 5-HT serum concentration associated with a morphologically enlargement of intestinal ECs cells in GF animals, to suggest that microbes could impact intestinal serotonergic system (Uribe et al., 1994; Wikoff et al., 2009). The concept that gut bacteria can influence colonic motility by regulating mucosal 5-HT is further supported by the study of *Yano et al.*, who demonstrated that indigenous gut bacteria upregulate the TPH1 activity of ECs cells to increase 5-HT levels both in the colon and in the blood, resulting in increased myenteric plexus stimulation and, therefore, gut motility in mice.

TRP metabolism depends on three major pathways in the GI tract (**Figure 1.9**).

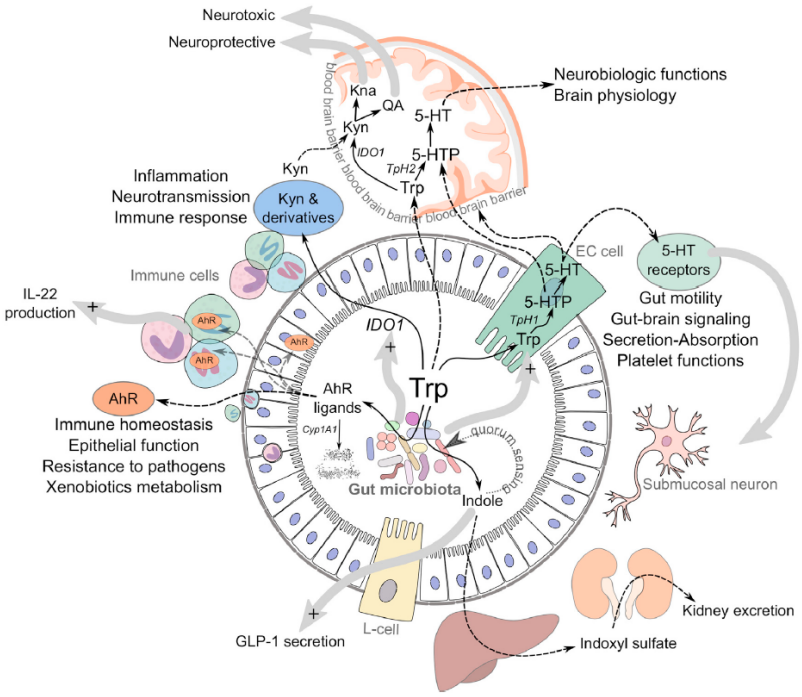


Figure 1.9. Integrated Trp Metabolism under the Control of the Gut Microbiota in Host Physiology. Dietary TRP can be directly converted by gut microbiota into AhR ligands that are able to tune local and distant host functions, including immune homeostasis and barrier physiology. Gut microbiota also influences the kynurenine-producing IDO pathway, which plays a critical role in inflammatory mechanisms, immune responses, and neurobiological functions. Peripheral production of serotonin by enterochromaffin cells is also under the influence of the gut microbiota. Gut-produced serotonin has many local effects, such as stimulating gut motility, and, even if it does not cross the blood-brain barrier, gut microbiota indirectly affects central serotoninergic pathways by modulating TRP and tryptamine availability. Abbreviations: 5-HTP, 5-hydroxytryptophan; IL, interleukin; QA, quinolinic acid. (modified from Agus et al., 2018).

These three metabolic enteric pathways for TRP are: (1) the direct transformation of TRP by the gut microbiota into an array of molecules, including ligands of the aryl hydrocarbon receptor (AhR); (2) the kynurenine pathway (KYN) in both immune and epithelial cells via indoleamine 2,3-dioxygenase (IDO) 1; and (3) the 5-HT production pathway in enterochromaffin cells via TpH1 (Agus et al., 2018).

The majority of TRP is metabolized along the kynurenine (KYN) pathway. The rate of TRP metabolism through the KYN pathway is dependent on the expression of the tryptophan-2,3-dioxygenase (TDO) enzyme found in the liver or the indoleamine-2,3-dioxygenase (IDO) enzyme found in all tissues. TDO can be induced by TRP or glucocorticoids, whereas IDO is influenced by the action of inflammatory cytokines particularly IFN-γ and TNF-α52. Alteration of the KYN:TRP ratios (recognized as an index of IDO or TDO activity) has been found in GF mice in terms of reduction of IDO activity compared to conventional animals, which was normalized following bacterial colonization post-weaning (Clarke et al., 2013). These studies have spurred interest in whether targeting microbiota might be a viable strategy to influence circulating TRP availability for KYN metabolism. In fact, administration of *Lactobacillus johnsonii* in rats decreased serum KYN concentration possibly via the inhibition of IDO activity (Valladares et al., 2013). Recently, IDO expression has been proposed as a biomarker of GI diseases such as IBD, where it reflects mucosal inflammation and colon cancer (Ciorba, 2013). IBS patients reported an increase of IDO activity and low-grade inflammation which could drive an increase production of KYN metabolites leading to altered gut functions (Clarke et al., 2012). Once KYN is produced by TDO or IDO, it is further metabolized into kynurenic and quinolinic acids which are NMDA antagonist and agonist, respectively. In the CNS, kynurenic acid has long been viewed as neuro-protective whereas quinolinic acid has been considered excitotoxic (Stone et al., 2012). Less is understood regarding the functions of kynurenic acid and quinolinic acid in the GI tract; however, both appear to be involved in immunoregulation (Keszthelyi et al., 2009).

This complex crosstalk is an important example of the interface between the gut microbiota, TRP pathway metabolism and the gut physiology. It is clear that the major influence on circulating TRP availability in the host is the utilization and metabolism of

TRP by the microbiota, and that this is one of the mechanisms by which gut bacteria can influence the gut function.

1.8 Gut Barrier and Visceral Hypersensitivity

There has been recent interest in the role of intestinal barrier and visceral pain in functional GI disorders.

The intestinal barrier is a functional entity separating the gut lumen from the inner host, and consisting of mechanical elements (e.g. mucus, epithelial layer), humoral elements (e.g. defensins, IgA), immununological elements (e.g. lymphocytes, innate immune cells), muscular and neurological elements. Other structures such as blood vessels, smooth muscle cell layers and components of the ENS contribute to the intestinal barrier by regulating the mucosa and by their capacity to initiate specific defense programs in case of danger (Groschwitz & Hogan, 2009; Wada et al., 2013). The intestinal barrier covers a surface of about 400 m^2 and requires approximately 40% of the body's energy expenditure. It prevents the loss of water and electrolytes and the entry of antigens and microorganisms into the body (Brandtzaeg, 2011), while allowing exchange of molecules between host and environment and absorption of nutrients in the diet. Specialized adaptations of the mammalian intestinal mucosa fulfill two seemingly opposing functions: firstly, the establishment of a peaceful co-existence with intestinal symbionts without eliciting chronic inflammation and, secondly, the maintenance of a measured inflammatory and defensive response according to the threat from pathogens (Hooper et al., 2012; Maynard et al., 2012). It is a complex multilayer system, consisting of an external "physical" barrier and an inner "functional" immunological barrier. The interaction of these two barriers enables equilibrated permeability to be maintained (Scaldaferri et al., 2012). Intestinal barrier dysfunction has been found to play a pathogenic role in IBS (Brandtzaeg, 2011). Most importantly, there is evidence now that increased intestinal permeability is related to low-grade inflammation, visceral hypersensitivity and pain in IBS patients (Camilleri et al., 2012).

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 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 ECs (Sengupta, 2009). Hypersensitivity and sensitization may occur through altered receptor sensitivity at the gut mucosa, at the submucous and myenteric plexus, which may be enabled by mucosal inflammation, degranulation of mast cells close to enteric nerves, or increased 5-HT 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. 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. Although there is no direct evidence for the participation of glutamate to visceral pain transmission, the ability of NMDA receptor antagonist to reduce pelvic and splanchnic afferent stimulation from the colon to mechanical stimuli is highly indicative of an endogenous glutamatergic modulation of mechanosensitive pathways. GluN1 subunit of the glutamatergic NMDA receptor is localized in the cell bodies of the dorsal root ganglion (DRG) and in peripheral terminals of primary afferent innervating the rat colon and mediates the local release of neuropeptides, such as CGRP and SP, which play an important role in neurogenic inflammation and hyperalgesia (McRoberts et al., 2001; Filpa et al., 2016).

The transient receptor potential vanilloid-1 (TRPV1) cation channel is the receptor to which many proalgesic pathways converge. It is activated by heat (>42°C), acidosis (pH<6) and a variety of chemicals such as the well-known capsaicin. In the last 30 years, TRPV1 has emerged as a polymodal nocisensor of nociceptive afferent neurons, although some non-neuronal cells and neurons in the brain also express TRPV1. The activity of TRPV1 is influenced by a variety of regulatory mechanisms that may cause sensitization or desensitization of the channel. Since this nocisensor is upregulated and sensitized by inflammation and injury, TRPV1 is thought to be a central transducer of hyperalgesia and a prime target for the pharmacological control of pain (Holzer, 2008a).

2. AIM

Our knowledge and understanding of the influence of intestinal microbiota on host development and function has come a long way, however, we are still far from understanding how gut bacteria influence these systems throughout life. It is evident that gut microbiota and the ENS are able to communicate with each other to maintain a homeostatic relationship. Although intestinal microbiota influences host physiology directly through their metabolic products or indirectly interacting with microbial associated molecular patterns receptors such as TLRs. TLRs play a pivotal role in the homeostatic microflora-host crosstalk. In particular, TLR4-mediated modulation of both motility and enteric neuronal survival has been reported mainly for colon with limited information on the role of TLR4 in tuning structural and functional integrity of ENS and in controlling small bowel motility. Moreover, TLRs appears to be involved in the low-grade inflammation and immunological alterations found in patients with irritable bowel syndrome (IBS). 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 parallel, gut microbiota seems to be directly involved in modulating the development and function of ENS, supporting the concept that changes in commensal microbiome composition, induced by infections or antibiotics, can perturb ENS integrity and activity. The relationship between indigenous gut microbes and their host can shift from commensalism towards pathogenicity in diseases, such as IBD. Increasing evidence points to a crucial role for the gut microbiota in modulating signaling pathways and neurotransmitters content. Based on this, some investigators have reported the presence of a balanced crosstalk between the microbiota and the host in TRP utilization and metabolism, TRP synthesis and 5-HT production that cumulatively may also determine implications in ENS neurotransmission.

The overall goal of the present PhD final thesis is to characterize the role of intestinal microbiota in the control of gut homeostasis, and potentially of the CNS, directly through its metabolic products or indirectly by interaction with TLRs. Thus, the study aims to evaluate the impact of:

- ✓ TLR4 in tuning structural and functional integrity of ENS and in controlling small bowel contractility for identifying the signaling pathways involved in neuroimmune crosstalk;
- ✓ TLR4 in hippocampus homeostasis compared to the ileal ENS;
- ✓ OxPAPC-mediated *in vivo* inhibition of TLRs in small intestine contractility and serotonergic neurotransmission in juvenile mice and consequently in GI homeostasis;
- ✓ dysbiosis induced with a broad-spectrum antibiotic cocktail in visceral perception, ileal serotonergic pathway integrity as well as TRP metabolism to evidence potential consequences in the microbiota-gut-brain axis.

3. MATERIALS and METHODS

3.1 Mice

For characterizing the role of TLR4 in tuning structural and functional integrity of ENS, male TLR4$^{-/-}$ (B6.B10ScN-Tlr4lps-del/JthJ; 9±1 weeks old) and age-matched wild-type (WT) C57BL/6J mice (Charles River Laboratories, Italy) were used. Male juvenile C57BL/6J mice (CNTR, 3±1 weeks old) were used for investigating the effect of in vivo OxPAPC administration or antibiotic-induced gut dysbiosis on ENS architecture and small intestine serotonergic pathway. Treatment at early age it is important to prevent a compensatory mechanism that arises during adulthood. All these animals were housed in individually ventilated cages at the animal facility of the Department of Pharmaceutical and Pharmacological Sciences, University of Padova. 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 under controlled environmental conditions (temperature 22° ± 2°C; relative humidity 60–70%). All animals were specific pathogen-free and given standard chow diet and tap water *ad libitum* and maintained at a regular 12/12-h light/dark cycle. All experimental protocols were approved by the Animal Care and Use Ethics Committee, University of Padova and Italian Ministry of Health and were in compliance with national and European guidelines for the handling and use of experimental animals.

3.2 Mice Treatments

To induce inhibition of both TLR2 and TLR4 receptors, CNTR mice were subjected to an intraperitoneally administration for 3 days, twice a day with a mixture of oxidized phospholipid, 1-palmitoyl-2-arachidonyl-snglycero-3-phosphorylcholine (OxPAPC; Invivogen, 1.5 µg/g animal in 0.9% saline, OxPAPC mice). CNTR mice were injected intraperitoneally with saline solution.

To induce gut dysbiosis, we used a previously established pharmacological model, which reproduces a *germ-free* like phenotype as shown by us and others (Reikvam et al., 2011; Brun et al., 2013; Caputi et al., 2017b). CNTR mice were treated with 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). Briefly, the antibiotics cocktails

were administered every 12 hours for 14 days by oral gavage (100 µL per mouse), using a stainless-steel feeding tube without prior sedation of the animal (ABX-treated group). Control mice (CNTR group) were treated with vehicle (tap water). Animals were randomized to treatment groups. At the end of procedures, animals were killed by cervical dislocation. All the following experimental procedures were conducted blindly.

3.3 Confocal Immunohistochemistry

3.3.1 Immunohistochemistry on Frozen Sections

Immunohistochemical analysis was performed on ileal tissues embedded in optimal cutting temperature (OCT, Kaltek; Padua, Italy) Mounting Medium and frozen in dry ice (-80°C), as previously described (Caputi et al., 2017a; Caputi et al., 2017b). In parallel, brain was dissected, postfixed at 4°C in 4% paraformaldehyde (PFA) in PBS for 4 hours. Brains were then cryoprotected for 72 h in sucrose 30% v/v in PBS, and frozen in OCT. Ileal tissues were sectioned at 7 µm-thick, instead brain was cut sagittally, to obtain 8-µm-thick sections, using a Leica cryostat-microtome (CM 1850 UV, Milan, Italy), mounted onto Superfrost Plus slides, which were stored at -80°C until use. Ileal and brain sections were fixed for 15 min with 4% PFA in PBS, washed in Tris-buffer saline (TBS) and incubated with 0.05 M NH_4Cl. Fixed sections were permeabilized and blocked with TBS containing 2% bovine serum albumin (BSA) and 0.3% Triton X-100 for 1 h. The sections were incubated overnight with primary antibodies (**Table 3.1**), diluted in TBS with 0.5% BSA at room temperature. Immunocomplexes were visualized by incubating samples for 2 h at room temperature with the corresponding secondary antibodies (**Table 3.2**). Nuclei were stained with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) (dilution 1:1000; Thermo Fisher Scientific, Milan, Italy), added together with the secondary antibodies. Negative controls were obtained by incubating sections with isotype-matched control antibodies at the same concentration as primary antibody and/or pre-incubating each antibody with the corresponding control peptide (final concentration as indicated by manufacturer's instructions). Sections were washed, mounted on glass slides using a Mowiol Mounting Medium (Sigma Aldrich, Milan, Italy) and visualized under a Zeiss LSM 800 confocal microscope.

3.3.2 Immunohistochemistry on Ileal Whole Mount Preparations

Distal ileum, isolated after cervical dislocation from animals, was washed with Krebs solution, to remove any contents and filled with fixative solution (4% PFA in PBS) for 2 hours at room temperature. Tissues were then subjected to two subsequent 30-minutes washes with PBS and stored at 4°C in PBS containing 5% sodium merthiolate (Thimerosal, Sigma-Aldrich, Italy). Using a dissecting microscope, whole-mount preparations consisting of the longitudinal muscle with the myenteric plexus attached (LMMPs) were prepared as previously described (Brun et al., 2013). 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 LMMPs (Ruan & Burnstock, 2005). LMMPs were gently stretched and pinned down on a wax support and washed in PBT (PBS with 0.2% Triton X-100) for 45 minutes with gentle shaking. After blocking nonspecific sites with PBT containing 2% BSA for 1 hour at room temperature, LMMPs were incubated with primary antibodies (**Table 3.1**) diluted in PBT/BSA 2% overnight at room temperature. The following day, LMMPs were washed for 45 minutes in PBT and incubated for 2 hours at room temperature with the secondary antibodies (**Table 3.2**). After three subsequent 15-minutes washes with PBT, LMMPs were mounted on glass slides using a Mowiol Mounting Medium. Negative controls were obtained by incubating sections with isotype-matched control antibodies at the same concentration as primary antibody and/or pre-incubating each antibody with the corresponding control peptide (final concentration as indicated by manufacturer's instructions).

ANTIGEN (HOST)	CLONE	SOURCE	DILUTION	APPLICATION
HuC/D (mouse)	Biotin conjugated 16A11	Life Technologies	1:100	IHC-fr/ IHC-WM
GFAP (rabbit)	polyclonal	Merk Millipore	1:200	IHC-fr/ IHC-WM
S100β (rabbit)	EP1576Y	Merk Millipore	1:100	IHC-fr/ IHC-WM
Iba-1 (rabbit)	polyclonal	Wako Corporation	1:800	IHC-fr/ IHC-WM
Substance P (guinea pig)	Polyclonal	Abcam	1:100	IHC-fr
nNOS (rabbit)	polyclonal	Life Technologies	1:100	IHC-WM
VIP (rabbit)	polyclonal	GenWay Biotech	1:100	IHC-WM
Choline acetyltransferase (ChAT, goat)	polyclonal	Merk Millipore	1:50	IHC-WM
iNOS (rabbit)	polyclonal	Santa Cruz Biotechnology	1:50	IHC-WM
P2X7 receptor-ATTO-488 (rabbit)	polyclonal	Alomone Labs	1:100	IHC-WM
P2Y1 receptor (rabbit)	polyclonal	Alomone Labs	1:100	IHC-WM
5-HT_{2A} receptor (rabbit)	polyclonal	Alomone Labs	1:50	IHC-WM
5-HT_3 receptor (rabbit)	polyclonal	Alomone Labs	1:50	IHC-WM
5-HT_4 receptor (rabbit)	polyclonal	Alomone Labs	1:50	IHC-WM
SERT (rabbit)	polyclonal	Alomone Labs	1:50	IHC-WM

Table 3.1. Characteristics of the primary antibodies used in immunofluorescence analysis. IHC-fr = immunohistochemistry in frozen tissue sections; IHC-WM = immunohistochemistry in whole mount preparations.

SECONDARY ANTIBODY	SOURCE	DILUTION	APPLICATION
goat anti-rabbit IgG Alexa Fuor 555 conjugate	Life Technologies	1:1000	IHC-fr/ IHC-WM
goat anti-rabbit IgG Alexa Fuor 488 conjugate	Life Technologies	1:1000	IHC-fr/ IHC-WM
Streptavidin Alexa Fuor 555 conjugate	Life Technologies	1:1000	IHC-fr/ IHC-WM
Streptavidin Alexa Fuor 488 conjugate	Life Technologies	1:1000	IHC-fr/ IHC-WM
goat anti-guinea pig IgG Alexa Fluor 488 conjugate	Life Technologies	1:1000	IHC-fr
donkey anti-goat IgG Dy Light 549 conjugate	Jackson ImmunoResearch	1:1000	IHC-WM

Table 3.2. Characteristics of the secondary antibodies used in immunofluorescence analysis. IHC-fr = immunohistochemistry in frozen tissue sections; IHC-WM = immunohistochemistry in whole mount preparations.

3.3.3 Acquisition and Analysis of Images

Images were acquired with a Zeiss LSM 800 confocal imaging system (Oberkoken, Germany) equipped with a 40× objectives (NA 0.95) and an oil-immersion 63× objectives (NA 1.4). Z-series images, composed of 10 to 15 plane forming Z-stack of 1024 × 1024 pixels were captured and processed as maximum intensity projections. Z-stacks of 8-µm or 10-µm depth were obtained for LMMPs whole-mount preparations or for ileal and brain sections of 4-6 animals per group. All microscope settings were set to collect images below saturation and were kept constant for all images. In LMMPs whole mount preparations we analyzed total neuron population through images of myenteric ganglia by counting HuC/D$^+$ cells in 10 randomly-chosen images per mouse (N = 8 mice/group). The total number of HuC/D$^+$ neurons was recorded in each image and normalized to the total area of 4.05 mm^2. To evaluate the distribution of nitrergic and VIPergic neurons in ileal myenteric plexus, the number of nNOS$^+$ and VIP$^+$ enteric neurons was blindly counted and normalized as described before (Caputi et al., 2017a). glial calcium-binding protein S100β, glial fibrillary acidic protein (GFAP), Iba-1, SP, P2X7 receptors (P2X7Rs), P2Y1 receptors (P2Y1Rs), iNOS, 5-HT$_{2A}$ receptors, 5-HT$_3$ receptors, 5-HT$_4$ receptors and SERT immunoreactivities were determined in LMMPs whole mount preparation or ileal frozen sections by measuring the fluorescent intensity for each antigen in 20 images captured randomly in the ileal neuromuscular compartment per mouse (N = 5 mice/group), as previously reported (Arqués et al., 2014). Fluorescence values were expressed as mean values in arbitrary fluorescence units (A.U.). Fluorescence intensity of GFAP$^+$ fibers was determined by applying the skeleton analysis method developed to quantify brain microglia morphology as previously described (Morrison et al., 2013). Briefly, for skeleton analysis, the maximum intensity projection of the GFAP$^+$ channel was enhanced to image all enteric glial processes, followed by noise de-speckling to eliminate single-pixel background fluorescence. After converting the resulting images to binary, they were skeletonized using ImageJ software and then analyzed by the AnalyzeSkeleton plugin to determine the number of endpoints per frame and process length. These data were normalized to a total area of 13.14 mm^2, obtained from 20 images per mouse (N = 5 mice/group) in order to assess changes in EGCs.

In brain slices, total neuron population analysis was performed by counting HuC/D$^+$ cells in twenty images *per* mouse (four animals *per* group), taken bilateral along the entire area of the hippocampus (West & Gundersen, 1990). The number of HuC/D$^+$ neurons gathered

in each image was normalized to the total area of hippocampus (40X magnification, total area = 2.5 mm^2). In brain sections changes in the immunoreactivity for GFAP, S100β and Iba-1 were assessed by analyzing the density index of labelling *per* tissue area (N = 4 mice/group, 20 images taken bilateral along the hippocampus area at 40× magnification, total area = 2.5 mm^2). Images were captured at identical acquisition exposure-time conditions and with a threshold to allow ImageJ software (version 1.50a) to select and measure only stained segments (Caputi et al., 2017a, b).

3.3.4 Acetylcholinesterase and NADPH-diaphorase Biochemical Staining in Ileal Whole Mount Preparations

For acetylcholinesterase staining, whole mount preparations described above, 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 (AChE) and NADPH diaphorase–stained (NADPH-d) 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 ($AChE^+$, $NADPH\text{-}d^+$, respectively).

3.4 In Vitro Contractility Studies

Intestinal contractility was examined *in vitro* by measuring tension changes on ileal samples with the isolated organ bath technique as previously described (Giron et al., 2008; Caputi et al., 2017a, b). Experiments were performed on full-thickness distal ileum segments isolated from $TLR4^{-/-}$, OxPAPC, ABX, and respective control mice. 1-cm segments were mounted along the longitudinal axis in organ baths containing 10 ml of

oxygenated (95% O_2 and 5% CO_2) and heated (37°C) Krebs solution (NaCl 118 mM, KCl 4.7 mM, $CaCl_2 \cdot 2H_2O$ 2.5 mM, $MgSO_4 \cdot 7H_2O$ 1.2 mM, K_2HPO_4 1.2 mM, $NaHCO_3$ 25 mM, $C_6H_{12}O_6$ 11 mM). 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. The tension of the segments was set to 1 g and changes in ileum mechanical activity were recorded by isometric transducers (World Precision Instruments, Berlin, Germany) connected to a quad bridge amplifier and PowerLab 4/30 data acquisition system using LabChart6 software (ADInstruments, Besozzo, VA, Italy). After 45 min equilibration, ileal segments were stretched passively to an initial tension of 0.5 g and brought to their optimal point of length-tension relationship using 1 µM carbachol (CCh). At first, the ileal segments were exposed to CCh stimulations of the following concentrations (0.01-100 µM) to obtain a concentration-response curve. To evaluate the smooth muscle response, we exposed the segments to 60 mM KCl. Then, neuronal-mediated contractions were obtained through electrical field stimulation (EFS, 0-40 Hz; 1-ms pulse duration; 10-s pulse-trains, 40 V) using platinum electrodes connected to an S88 stimulator (Grass Instrument, Quincy, MA, USA) in basal conditions or in NANC conditions, obtained by adding 1 µM guanethidine and 1 µM atropine to the organ bath. To evaluate nitrergic-mediated neurotransmission, ileal preparations were incubated with 100 µM N_ω-nitro-l-arginine methyl ester (L-NAME), a pan-NOS inhibitor, or 10 µM 1400W, iNOS inhibitor, within the same NANC conditions for an additional 20 minutes and 10 Hz-EFS-mediated NANC responses were evaluated. Ten hertz-EFS-mediated tachykinergic responses were recorded in NANC conditions with 100 µM L-NAME. Then to assess the purinergic-mediated response in NANC condition, 100 µM theophylline, a P1-purinoceptor antagonist, 100 µM suramin, a P2-purinoceptor antagonist, 1 µM MRS2500, a P2Y1 receptor antagonist or 0.1 µM A804598, a P2X7 receptor antagonist, were added in organ bath and adenosine diphosphate (ADP; 0.001–1 µM) dose-response curves were obtained cumulatively. Concentration-response curves to 5-HT (0.3–30 µM) were obtained through addition in organ bath, in a non-cumulative manner (Forcèn et al., 2015). In order to study the role of the 5-HT receptors on the responses evoked by EFS or 5-HT, ileum segments were incubated for 30 min with the 5-HT receptors antagonist for: i) 5-HT_{2A}, 1 µM ketanserin, ii) 5-HT_3, 0.1 µM ondansetron, iii) 5-HT_4, 0.1 µM GR113808, before electrical stimulation or addition of 5-HT. The concentrations used were based on the pKi described in literature (Alexander

et al., 2011). Contractile responses were expressed as gram tension/gram dry tissue weight of ileal segments. 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 (AUC) defined as the integrated area under the contractile response and normalized per g dry tissue weight to allow comparison between tissue samples (Caputi et al., 2017a, b).

3.5 Gastrointestinal Transit Analysis

The 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. FITC-dextran dissolved in 0.9% saline (100 µl of 25 mg/ml FITC-dextran solution for each mouse) were administered to TLR4$^{-/-}$ and WT mice. Then 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. Luminal contents from each part (both tissue and fecal 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):

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

3.6 Intestinal Paracellular Permeability

Intestinal paracellular permeability was assessed as previously described (Aubé et al., 2006). Briefly, WT and TLR4$^{-/-}$ mice were gavaged orally with absorbable fluorescein isothiocyanate (FITC)-dextran (4 kDa molecular weight; 200 µl, 600 mg/kg body weight). Preliminary experiments at various time points (0, 0.5, 1, 2, 4, and 6 h) showed that the

appearance in blood of low molecular weight FITC-dextran peaked at 4 h following oral administration in WT and TLR4$^{-/-}$ mice. After 4 h, FITC-dextran serum concentration was determined using a fluorimeter (PerkinElmer, Milan, Italy) at 490/530 nm.

3.7 Pellet Frequency and Fecal Water Content

Fecal pellet output and water content was assessed in WT and TLR4$^{-/-}$ mice. Fecal water content provides an indication of constipation, diarrhea or malabsorption. The 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 minutes 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).

3.8 RNA Isolation and Quantitative RT-PCR

For evaluation of the mRNA relative abundance of SERT, 5-HT$_{2A}$, 5-HT$_3$ and 5-HT$_4$ receptors in CNTR and OxPAPC mice, real-time PCR was performed as previously described (Forcèn et al., 2015), in collaboration with Prof. J. Mesonero of the Department of Pharmacology and Physiology, University of Zaragoza, Spain. RNA extractions were carried out with the RNeasy mini kit (Qiagen, Hilden, Germany) from LMMPs preparations and the cDNA was synthesized using the AffinityScript Multiple Temperature cDNA synthesis kit (Stratagene, La Jolla, CA, USA) according to the supplier's protocol. cDNAs obtained were used to measure 5-HT receptor mRNA expression levels by SYBR Green and specific primers (**Table 3.3**). Reactions were run using the StepOne Plus Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). The reaction mixture (10 μL) was comprised of 4.5 μL FastStart Universal SYBR Green Master (Roche, Mannheim, Germany), 0.5 μL of each primer 30 μM, 2.5 μL of sterile distilled water, and 2 μL of cDNA template (200 ng). Each sample was run in triplicate, and the mean Ct was determined from the three runs. Relative 5-HT receptors' mRNA expression in each group of animals (CNTR or OxPAPC-treated) was expressed as ΔCt = Ct5-HTreceptor − Ctcalibrator. GAPDH and HPRT housekeeping genes' expressions

were used as calibrators after verification of their stability under our experimental conditions. Relative 5-HT receptors' mRNA expression was then calculated as ΔΔCt = ΔCtOxPAPC − ΔCtCNTR. Finally, the relative gene expression levels were converted and expressed as fold difference (= 2−ΔΔCt). GluN1 and TRPV1 expressions in ABX experimental groups were 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 LMMPs 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. β-actin was used as housekeeping gene. For quantitative RT-PCR a final concentration of 500 nmol/L for each primer was used.

Gene	*Sense and antisense primers*
SERT	GGCCTGGAAGGTGTGATCA GCGCTTGGCCCAGATGT
5-HT_{2A}	TGCCGTCTGGATTTACCTGGATGT TACGGATATGGCAGTCCACACCAT
5-HT_{3A}	TCTTGCTGCCCAGTATCTTCCTCA TTATGCACCAGCCGCACAATGAAG
5-HT_{4}	AATGCAAGGCTGGAACAACATCGG TGTATCTGCTGGGCATGCTCCTTA
HPRT	CTGGTGAAAAGGACCTCTCGAA CTGAAGTACTCATTATAGTCAAGGGCAT
GAPDH	AACGACCCCTTCATTGAC TCCACGACATACTCAGCAC
NR1	CAGGAGCGGGTAAACAACAGCAAC GCAGCCCCACCAGCAGCCACAGT
TRPV1	CCCATTGTGCAGATTGAGCAT TTCCTGCAGAAGAGCAAGAAGC
β-actin	ACCAGAGGCATACAGGGACA CTAAGGCCAACCGTGAAAAG

Table 3.3. Primers sequences 5'-3' used in mouse intestine by real-time PCR.

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 LMMPs preparations from ABX mice were expressed as the percentage variation versus values obtained in control preparations.

3.9 HPLC Analysis of Tryptophan Metabolites

Plasma samples were stored at -80°C. For 5-HT metabolites analysis plasma samples were properly diluted with milliQ water and then centrifugated at 13,000 g for 2 minutes. TRP metabolites were analyzed on ileal homogenates by high-performance liquid chromatography (HPLC) as previously described (Giron et al., 2008; Bertazzo et al., 2016). Briefly, freshly isolated ileal segments were immersed in liquid nitrogen, and pulverized in a cooled stainless mortar containing 1N $HClO_4$ (0.5 mL). The homogenates were then sonicated with Elmasonic S30 sonicator (Elma, Singer, Germany; four 15-second bursts, 60W). After centrifugation (13,000 g for 30 minutes at 4°C), the supernatants were stored at −80°C until HPLC analysis whereas the samples pellets were dissolved in 1N NaOH and boiled for 20 minutes at 60°C and then centrifugate at 15,000 g for 10 minutes at 4°C. The isolated supernatants were used for protein determination (Lowry et al., 1951). The supernatants were brought to about pH 4-5 with 1 N NaOH and analyzed using a HPLC system (Shimadzu LC-10AD, Kyoto, Japan) equipped with a fluorometric detector (Shimadzu RF-10AXL) set at the excitation and emission wavelengths of 285 and 345 nm, respectively. Briefly, chromatographic separation of TRP metabolites was performed using an analytical Apollo EPS C18 100A column (5 μm; 250 mm × 4.6 mm; Grace, Deerfield, IL, USA) and an Alltech guard column with stationary phase RP-8 (25-40 μm Lichroprep, Merck Darmstadt, Germany). KYN analysis was carried out on an analytical Grace Smart RP-18 column (5 μm; 250 mm × 4.6 mm; Grace) using a UV-VIS (ultraviolet-visible) detector (SPD-10A, Shimadzu), set at 360 nm. The mobile phases were as follows: Phase A, 95% acetonitrile – 5% water, and Phase B, 90% water – 5% methanol (pH 3.8). The analytes elution was performed with an isocratic gradient (5% Phase A and 95% Phase B, v/v) at 1ml/min flow rate.

3.10 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 or 2-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).

3.11 Materials and Reagents

SUBSTANCE	SOURCE
A804598	Tocris Bioscience
Adenosine diphosphate	Sigma-Aldrich, Italy
Ampicillin	Sigma-Aldrich, Italy
Atropine sulfate	Sigma-Aldrich, Italy
Bovine serum albumin	Sigma-Aldrich, Italy
$CaCl_2 \cdot 2H_2O$	Merck, Italy
Carbamoylcholine chloride	Sigma-Aldrich, Italy
D-glucose anhydrous	Sigma-Aldrich, Italy
Fluorescein isothiocyanate–dextran 70.000 Da	Sigma-Aldrich, Italy
Guanethidine	Sigma-Aldrich, Italy
GR113808	Tocris Bioscience
KCl	Carlo Erba, Italy
Ketanserin	Tocris Bioscience
KH_2PO_4	Carlo Erba, Itay
$MgSO_4 \cdot 7H_2O$	Sigma-Aldrich, Italy
Metronidazol	Sigma-Aldrich, Italy
Mounting Medium	Sigma-Aldrich, Italy
MRS2500	Tocris Bioscience
Na_2HPO_4	Merck, Italy
NaCl	Carlo Erba, Italy
$NaHCO_3$	Carlo Erba, Italy
Neomycin	Sigma-Aldrich, Italy
NH_4Cl	Sigma-Aldrich, Italy
Nω-nitro-L-arginina metil estere cloridrato	Sigma-Aldrich, Italy
Ondansetron	Tocris Bioscience
OxPAPC	InvivoGen, Italy
Paraformaldehyde 8% Aqueous solution	Electron Microscopy Sciences, Italy
Serotonin	Sigma-Aldrich, Italy
Sucrose	Sigma-Aldrich, Italy
Suramin	Sigma-Aldrich, Italy
Theophylline	Sigma-Aldrich, Italy

4. RESULTS

4.1 Toll-Like Receptor 4 in Murine Small Intestine

The innate immune system acts as the first line of host defense against pathogens and depends on the family of PRRs (Janssens & Beyaert, 2003). Among all the PRRs identified until now, the TLRs represent the most extensively studied class of innate immune receptors involved in the recognition of MAMPs and DAMPs (Rakoff-Nahoum et al., 2004). TLRs are expressed on a variety of different cells, including immune and non-immune cell types, such as epithelial cells and smooth muscle cells as well as neurons and glial cells. Furthermore, certain TLRs, such as TLR1, 2, 4, 5, and 6, are expressed on the cell membrane, whereas others (e.g. TLR3, 7, 8, and 9) are found exclusively in internal compartments (Akira et al., 2006). The finding of their expression in both CNS and ENS (Barajon et al., 2009) has suggested that TLRs are not only involved at regulating host immune responses but they may also have a role in neuroinflammation, neurodevelopment and neuroplasticity (Aravalli et al., 2007; Okun et al., 2011). Among all TLRs, TLR4 was recently discovered to be expressed in the ENS where has been shown to regulate murine GI motility and to promote the survival of enteric neurons (Anitha et al., 2012).

4.1.1 TLR4 Influences Ileal Morphology and ENS Architecture

Since TLR4 expression is required for normal growth (including villus height) of the small intestine (Riehl et al., 2015), we sought to determine whether the absence of TLR4 influences structural architecture by examining hematoxylin and eosin-stained sections. In agreement with previous findings (Riehl et al., 2015), $TLR4^{-/-}$ ileal morphology was comparable to WT, except for villi height, which was significantly diminished (349 ± 9 μm in WT mice vs. 306 ± 15 μm in $TLR4^{-/-}$ mice; N = 20 animals/group, **Figure 4.1**). No significant differences in serum levels of absorbable FITC-dextran were found between $TLR4^{-/-}$ and WT mice (0.42 ± 0.1 μg/ml and 0.32 ± 0.1 μg/ml, respectively; N = 12 animals/group), to indicate no alterations in intestinal permeability. Considering that TLR4 is expressed in ENS (Rumio et al., 2006; Barajon et al., 2009), the impact of TLR4 absence on ENS integrity was evaluated by immunohistochemistry.

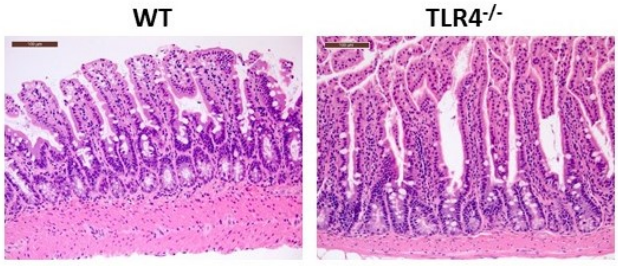


Figure 4.1. TLR4 deficiency alters villus height. Representative microphotographs of the ileal architecture in hematoxylin/eosin-stained full-thickness cross sections obtained from WT and TLR4$^{-/-}$ mice. (bars = 100 µm; N = 20 mice/group).

In ileal cryosections, a 1.84-fold increase in the immunoreactivity of the glial marker S100β was found in TLR4$^{-/-}$ myenteric plexus (**Figures 4.2A, B**). These increases in S100β immunoreactivity were associated to a 3.1-fold increase in process length of GFAP^{+} gliofilaments in TLR4$^{-/-}$ ENS (**Figures 4.2C–E**).

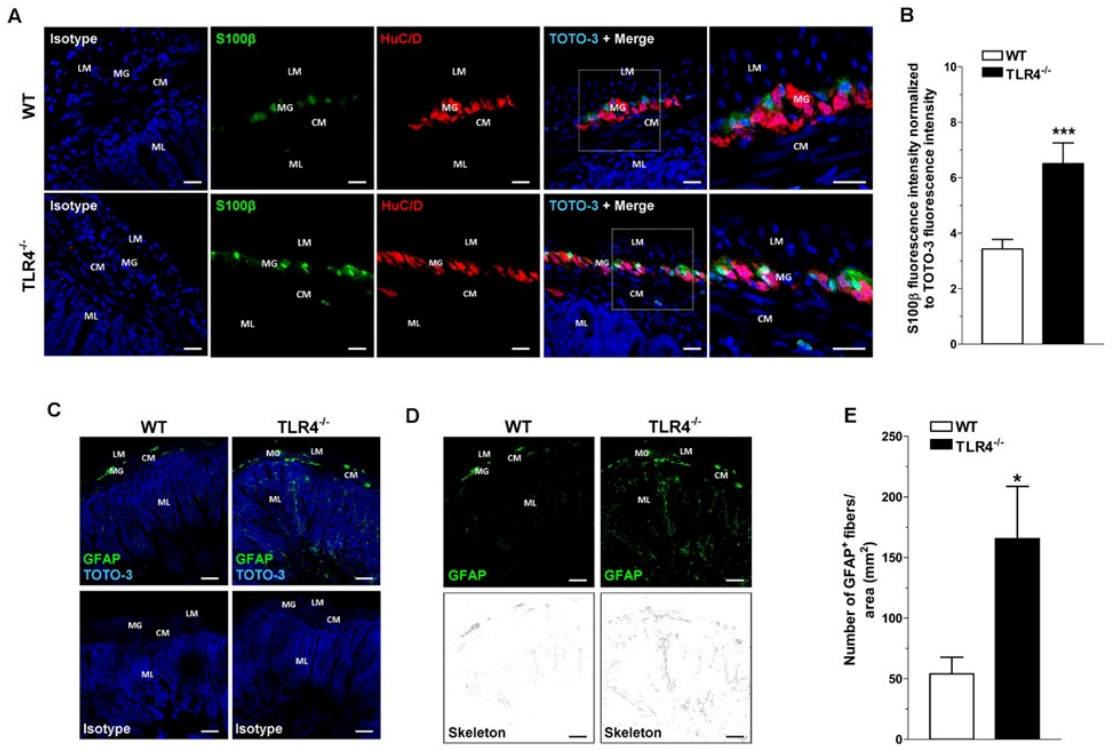


Figure 4.2. TLR4 deficiency alters glial phenotype. Representative confocal microphotographs (**A**) of HuC/D (red) and S100β (green) distribution in wild-type (WT) and TLR4$^{-/-}$ cryosections and quantification (**B**) of S100β fluorescence intensity (bars = 22 µm). (**C**) Representative confocal microphotographs of GFAP distribution in WT and TLR4$^{-/-}$ cryosections (bars = 75 µm). (**D**) Representative confocal maximum intensity projection of GFAP^{+} channel with the corresponding skeleton images. (**E**) Number of GFAP^{+} glial processes/area (mm^2). Cell nuclei were stained with TOTO-3 (blue). *P < 0.05, ***P < 0.001 vs. WT (N = 5 mice/group). LM, longitudinal muscle; CM, circular muscle; MG, myenteric ganglia; ML, mucosal layer.

4.1.2 Absence of TLR4 Impairs Gastrointestinal Motility

Previous studies have shown a reduced GI transit 4 h after non-absorbable FITC-dextran administration in C3H/HeJ mice with a spontaneous point mutation in Tlr4 gene (Tlr4Lps-d) (Anitha et al., 2012). We hypothesized that the same functional impairment could be present in TLR4$^{-/-}$ mice (B6.B10ScN-Tlr4lps-del), which are homozygous for a null mutation of Tlr4 gene. In WT mice, the non-absorbable FITC-dextran transited through the GI tract over a 30-min period and localized at the terminal ileum (**Figure 4.3A**). Conversely, a significant reduction of GC and gastric emptying was observed in TLR4$^{-/-}$ mice ($GC_{TLR4}^{-/-}$ = 6.52 ± 0.21 vs GC_{WT} = 7.05 ± 0.16; **Figures 4.3B, C**). The number of fecal pellets/hour and stool water content were significantly lower in TLR4$^{-/-}$ mice compared to WT mice (**Figures 4.3D, E**).

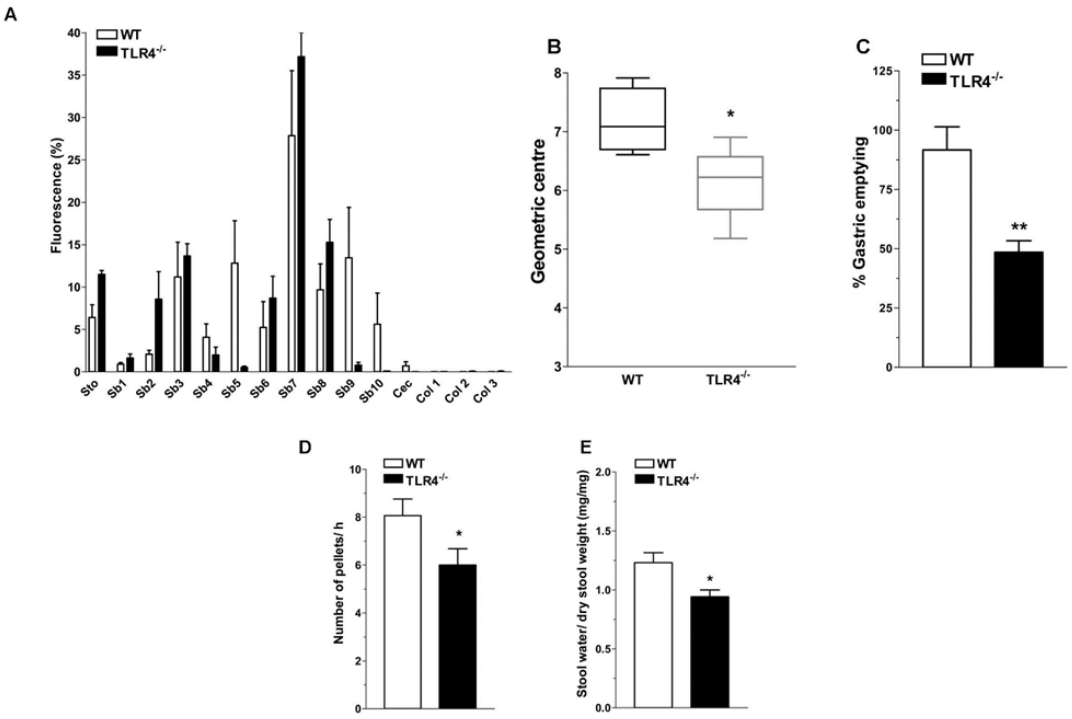


Figure 4.3. TLR4 signaling influences GI transit and gastric emptying. (**A**) Percentage of FITC-dextran distribution along the GI tract (stomach, Sto; small bowel, Sb 1–10; caecum, Cec; and colon, Col 1–3), (**B**) geometric center, (**C**) percentage of gastric emptying, (**D**) pellet frequency per hour, (**E**) stool water content in WT and TLR4$^{-/-}$ mice. *P < 0.05, **P < 0.01 vs. WT (N = 12 mice/group).

4.1.3 TLR4 Deficiency Affects Excitatory Neurotransmission

Based on the delayed GI transit, we assessed spontaneous contractility that resulted comparable in frequency and amplitude in both genotypes (**Figures 4.4A, B**). To evaluate excitatory responses, cumulative concentration-response curves to the non-selective

cholinergic receptor agonist CCh were performed. Ileal segments from TLR4$^{-/-}$ mice showed a significant downward shift of the concentration-response curve to CCh and a significant related decrease in the maximum response compared to WT (Emax = -25.6 ± 7.5%; **Figure 4.4C**). However, the response to high potassium-induced depolarization was similar in both genotypes (**Figure 4.4D**).

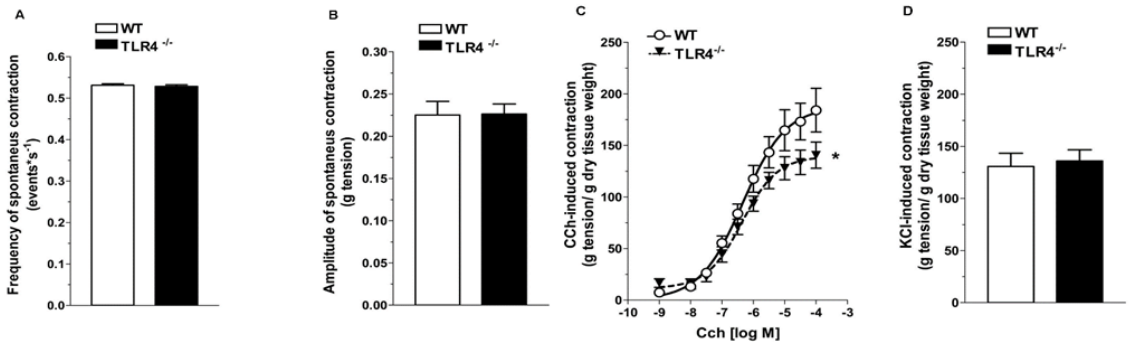


Figure 4.4. TLR4 deficiency impairs contractile responses to carbachol but not to high KCl. Frequency (**A**) and amplitude (**B**) of spontaneous contraction in WT and TLR4$^{-/-}$ ileal preparations (N = 15 mice/group). Concentration–response curves to CCh (**C**) and KCl-mediated excitatory response (**D**) in WT and TLR4$^{-/-}$ preparations (N = 8 mice/group). *P < 0.05 vs. WT.

Since ENS structural abnormalities have been described in Tlr4Lps-d mice (Anitha et al., 2012), we sought to test neuromuscular function by analyzing frequency-response curves to EFS. Altered neurotransmission in TLR4$^{-/-}$ ileal segments was reflected by reduced EFS-elicited contractions (by 28.5 ± 9.7% at 10 Hz; **Figure 4.5A**). The EFS-induced contractions up to 10 Hz were of neuronal cholinergic origin as confirmed by their sensitivity to tetrodotoxin (TTX) and to the muscarinic receptor blocker atropine, as previously shown (Brun et al., 2013) and also confirmed in both mouse (data not shown). However, no changes in the number of AChE^{+} fibers were found in TLR4$^{-/-}$ whole-mount preparations (**Figures 4.5B, C**).

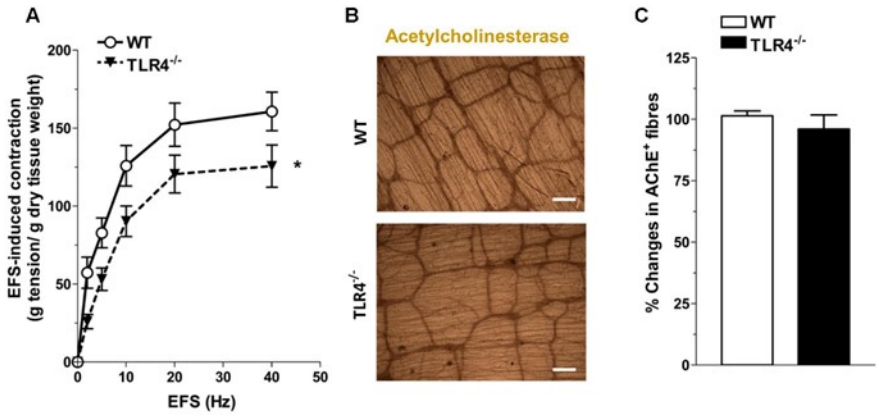


Figure 4.5. TLR4 deficiency alters ileal excitatory contractility. (**A**) EFS-induced excitatory responses in WT and TLR4$^{-/-}$ preparations (N = 8 mice/group). Representative microphotographs showing the distribution (**B**) and % changes (**C**) of AChE^{+} fibers in WT and TLR4$^{-/-}$ preparations (N = 5 mice/group). Bars = 200 µm. *P < 0.05 vs. WT.

To evaluate the contribution of other excitatory neurotransmitters besides ACh, we evaluated the post-stimulus excitatory responses in NANC conditions, which are determined by tachykinergic neurotransmission (Lecci et al., 2002). In WT mice, NANC responses evoked by EFS determined a transient relaxation of ileal preparations, followed by TTX-sensitive excitatory responses (**Figures 4.6A, B**) (Zizzo et al., 2003). These excitatory responses were significantly reduced in TLR4$^{-/-}$ ileal segments (by 30 ± 8.3%; **Figures 4.6A, B**). Upon addition of L-NAME, a tachykinin-mediated excitatory response (Lecci et al., 2002) was found in TLR4$^{-/-}$ preparations comparable to WT (**Figure 4.6C**). Accordingly, no differences were observed in the immunofluorescence distribution of SP in WT and TLR4$^{-/-}$ frozen sections (**Figure 4.6D**).

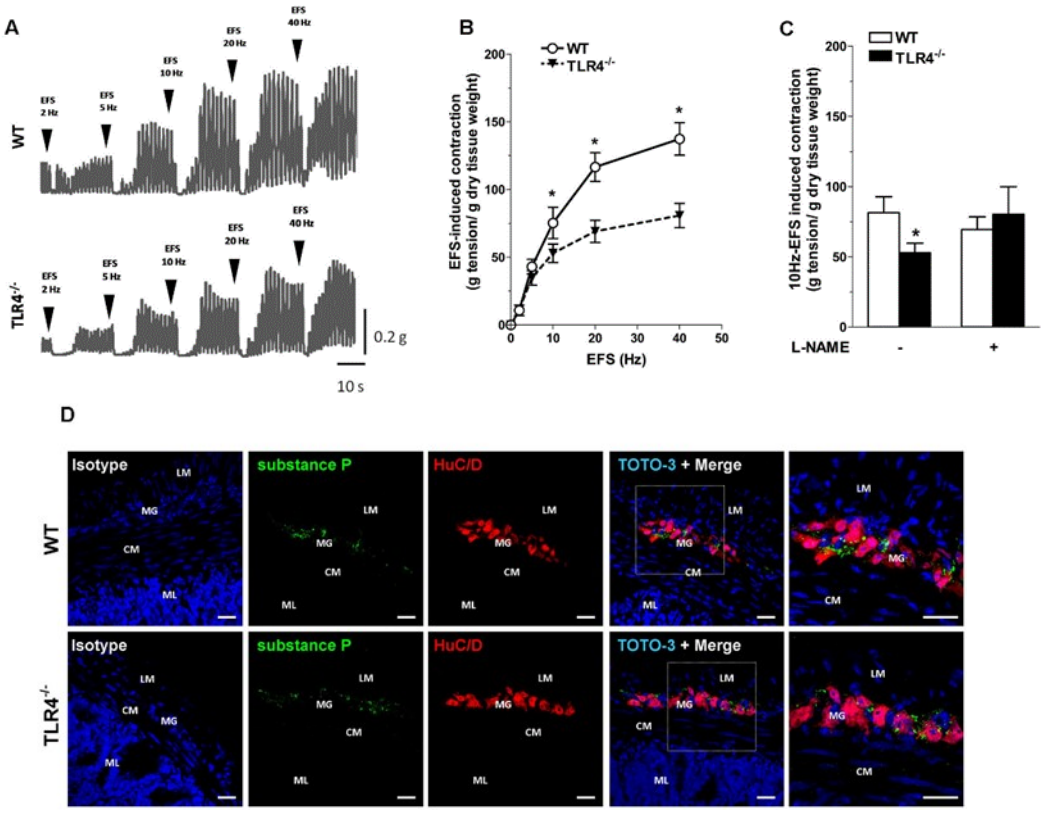


Figure 4.6. Tachykinergic neurotransmission is not affected by TLR4 deficiency. (**A**) Representative traces of contractile responses to increasing EFS frequencies in WT and TLR4$^{-/-}$ segments under NANC conditions. (**B**) NANC responses evoked by EFS. (**C**) Tachykinergic nerve-evoked contractions induced by 10 Hz-EFS in NANC condition with or without L-NAME in WT and TLR4$^{-/-}$ preparations (N = 8 mice/group). (**D**) Representative confocal microphotographs of HuC/D (red) and SP (green) distribution in WT and TLR4$^{-/-}$ cryosections. Cell nuclei were stained with TOTO-3 (blue; N = 5 mice/group). Bars = 22 µm. LM, longitudinal muscle; CM, circular muscle; MG, myenteric ganglia; ML, mucosal layer. *P < 0.05 vs. WT.

4.1.4 TLR4 Modulates Inhibitory Neurotransmission

Considering that nitrergic neurotransmission (Zizzo et al., 2004; Lomax et al., 2010), the primary inhibitory pathway in the gut, is affected by dysbiosis (Kabouridis & Pachnis, 2015), we tested whether the reduced excitatory contraction could be the result of an increase of the inhibitory component. Consistent with this prediction, a reduction of nitrergic neurons stained with NADPH-d or anti-nNOS was observed in $TLR4^{-/-}$ preparations (**Figure 4.7**). These changes were accompanied by a significant reduction of the total number of HuC/D^{+} neurons in $TLR4^{-/-}$ myenteric plexus (**Figures 4.7C–E**). The reduction in $nNOS^{+}$ neurons was associated with a proportional increase of VIP^{+} neurons in $TLR4^{-/-}$ mice (**Figure 4.7**).

In NANC conditions EFS at 10 Hz caused a 1.48-fold increase in relaxation in $TLR4^{-/-}$ mice (**Figure 4.8A**). Pretreatment with 1400W, a selective inhibitor of iNOS, significantly reduced the NANC-mediated relaxation in $TLR4^{-/-}$ mice (by 25.2 ± 0.5%; **Figure 4.8A**), whereas a slight but not significant relaxation (13.8 ± 0.9%; **Figure 4.8A**) was recorded in WT. These findings support an involvement of iNOS in NO-mediated relaxation in the absence of TLR4. Furthermore, iNOS immunoreactivity increased by 5.7-fold in $TLR4^{-/-}$ myenteric neurons and EGCs compared to WT (**Figures 4.8B, C**). Pretreatment with the pan-NOS inhibitor L-NAME almost completely blocked EFS-evoked NANC relaxation in WT mice. Conversely, in $TLR4^{-/-}$ mice, this response was only partially abolished by L-NAME (**Figure 4.8A**) suggesting an influence of TLR4 in nitrergic-mediated relaxation and possibly in other inhibitory pathways (e.g., purinergic or VIPergic), known to sustain intestinal contractility (Zizzo et al., 2004). Accordingly, we evaluated the role of adenosine and ATP in modulating relaxation in NANC conditions. Pretreatment with L-NAME and theophylline, a non-selective adenosine receptor antagonist, partially reduced the amplitudes of NANC-mediated relaxation in both genotypes whereas the addition of L-NAME and suramin, a non-selective ATP receptor antagonist, resulted in a significant reduction of the inhibitory response (by 75.3 ± 1.5%; **Figure 4.8D**) in $TLR4^{-/-}$ mice, reaching a relaxation amplitude comparable to WT.

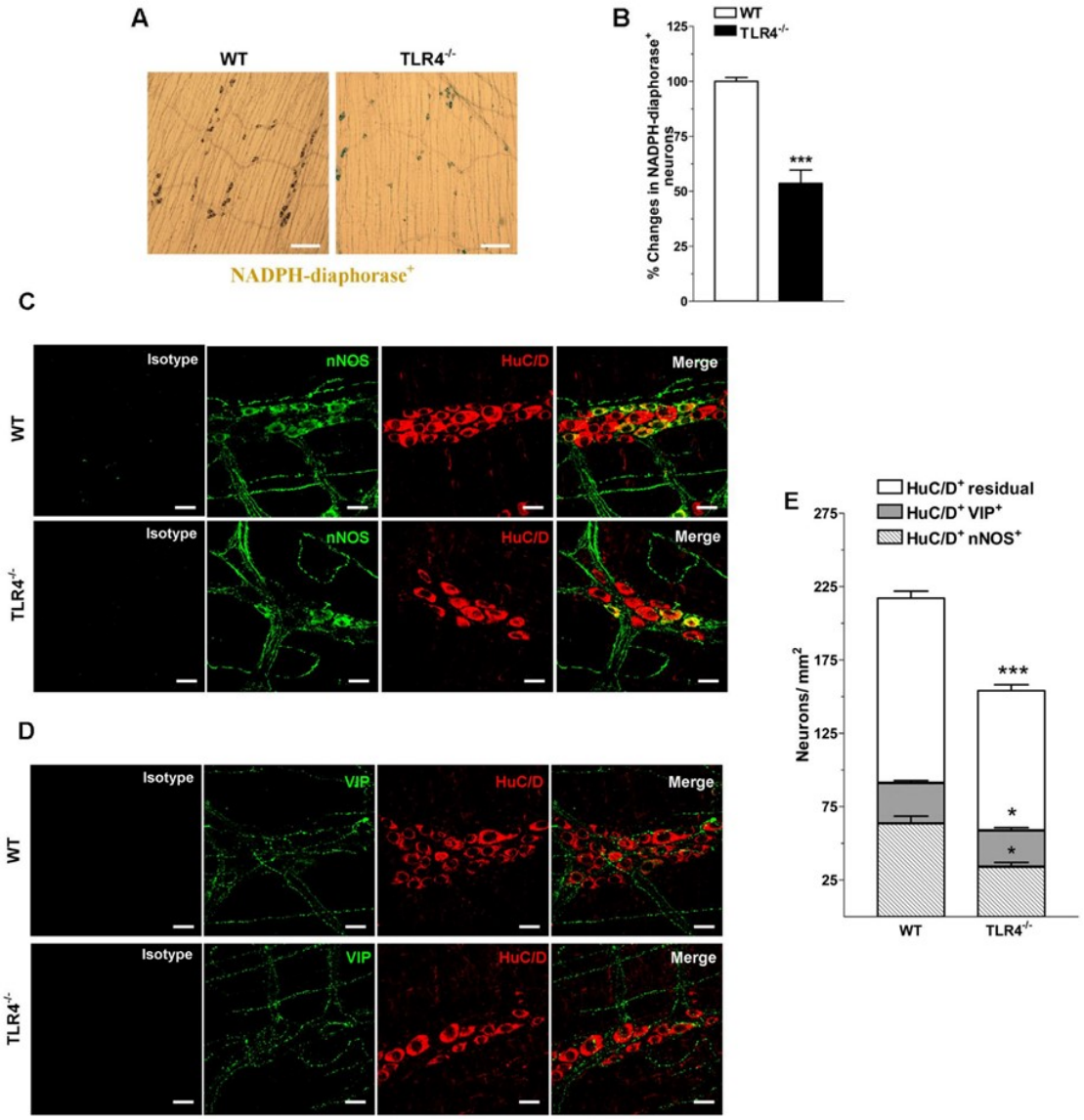


Figure 4.7. TLR4 signaling modulates nitrergic and VIPergic neuron distribution. Representative microphotographs showing the distribution (**A**) and density (**B**) of NADPH-d^{+} neurons in WT and TLR4$^{-/-}$ LMMP preparations (bars = 300 µm). Representative confocal microphotographs showing the distribution of nNOS (**C**; green), VIP (**D**; green) and HuC/D (**C**, **D**; red) in WT and TLR4$^{-/-}$ LMMP preparations (bars = 22 µm). (**E**) Number of HuC/D^{+}nNOS^{+}, HuC/D^{+}VIP^{+}, and residual HuC/D^{+} neurons in WT and TLR4$^{-/-}$ LMMP preparations (N = 8 mice/group). *P < 0.05, ***P < 0.001 vs. WT.

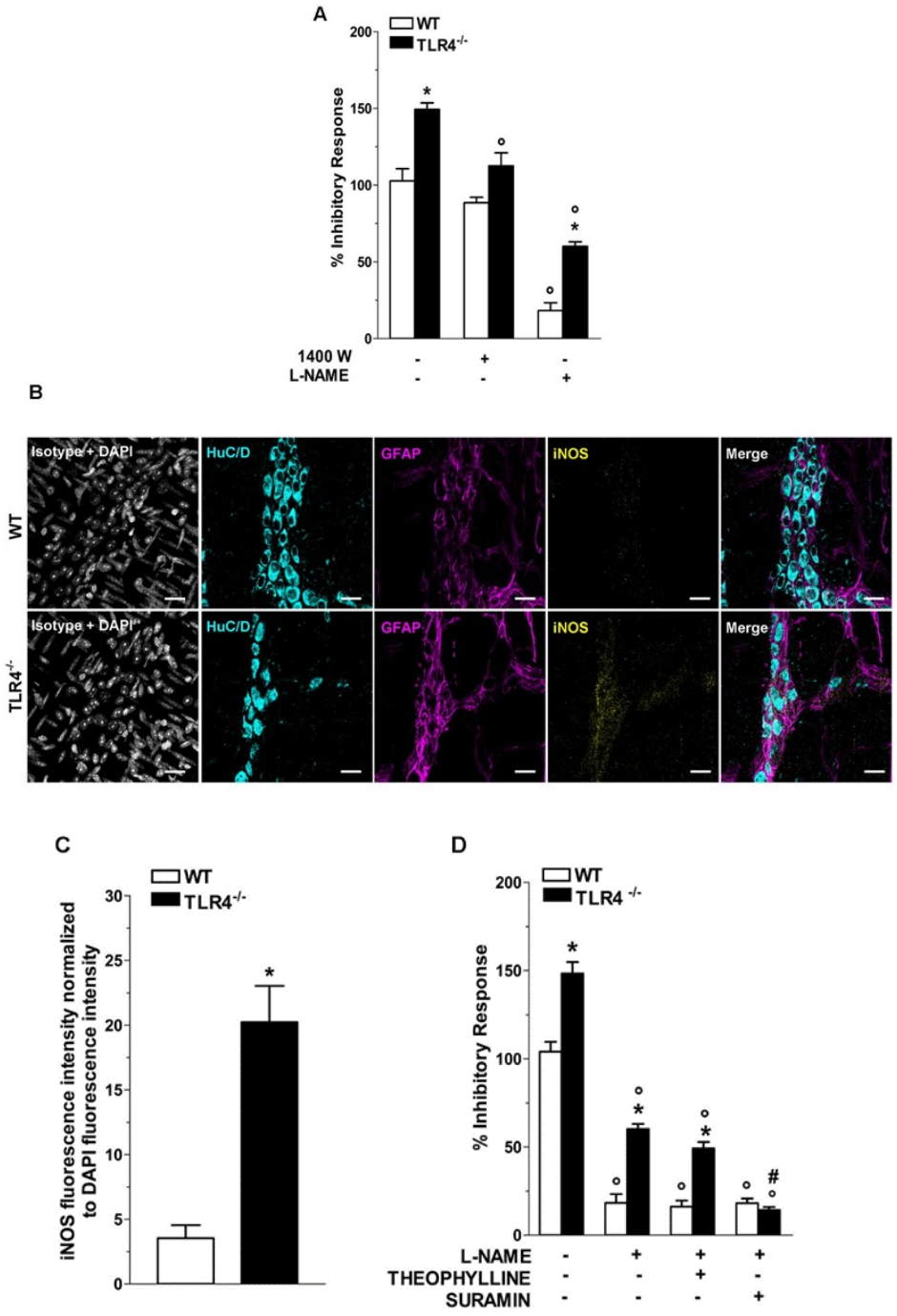


Figure 4.8. TLR4 signaling modulates NO and P2 receptor-mediated relaxation. (**A**) 10 Hz-EFS-evoked NANC relaxation responses with or without 1400 W or L-NAME in WT and TLR4$^{-/-}$ preparations (N = 8 mice/group). (**B**) Representative confocal microphotographs showing HuC/D (cyan), GFAP (magenta) and iNOS (yellow) distribution (bars = 22 µm). (**C**) Analysis of changes in iNOS fluorescence intensity in WT and TLR4$^{-/-}$ LMMP preparations (N = 5 mice/group). (**D**) 10 Hz-EFS-evoked NANC relaxation responses with or without L-NAME or L-NAME+theophylline or L-NAME+suramin in WT and TLR4$^{-/-}$ preparations (N = 8 mice/group). *P < 0.05 vs. WT; °P < 0.05 vs. respective control without L-NAME; $^{\#}$P < 0.001 vs. respective control with L-NAME.

4.1.5 TLR4 Absence Affects Purinergic Inhibitory Neurotransmission

Considering that inhibitory neurotransmission in TLR4$^{-/-}$ mice depends on both nitrergic and ATP-mediated relaxation, we evaluated the modulatory effect of ADP, the endogenous agonist of P2Y1Rs. TLR4$^{-/-}$ mice showed a 1.43-fold increase in relaxation amplitude following addition of ADP in the organ bath with a significant shift to the left of the dose-response curve to ADP compared to WT mice (**Figure 4.9A**). Since also enteric P2X7Rs respond to ATP by mediating inhibitory neurotransmission and are involved in neuronal death during intestinal inflammation (Antonioli et al., 2014; Brown et al., 2015), we examined the influence of P2Y1Rs and P2X7Rs in NANC-mediated relaxation in the absence of TLR4. In ileal tissues from TLR4$^{-/-}$ mice, P2Y1Rs blockade with MRS2500 in presence of L-NAME markedly reduced the amplitudes of NANC-mediated relaxation (by 45.1 ± 3%; **Figure 4.9B**) whereas the addition of A804598 (a selective P2X7Rs antagonist) determined a reduction of the inhibitory response (by 24.2 ± 1.5%; **Figure 4.9B**), suggesting an involvement of both receptors in modulating relaxation in NANC conditions in the absence of TLR4.

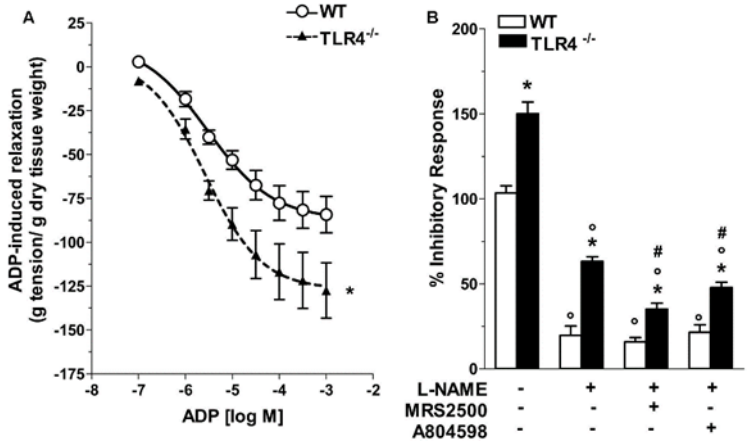


Figure 4.9. Involvement of TLR4 signaling in purinergic neurotransmission. (**A**) Concentration-response curve to ADP in WT and TLR4$^{-/-}$ preparations (N = 8 mice/group). (**B**) 10 Hz-EFS-evoked NANC relaxation responses with or without L-NAME or L-NAME+MRS2500 (a P2Y1Rs antagonist) or L-NAME+A804598 (a P2X7Rs antagonist) in WT and TLR4$^{-/-}$ preparations (N = 8 mice/group). *P < 0.05 vs. WT; °P < 0.05 vs. respective control without L-NAME; $^{\#}$P < 0.001 vs. respective control with L-NAME.

Immunohistochemical analysis revealed a 1.79-fold increase of P2Y1Rs staining in myenteric ganglia, in both neurons and EGCs (**Figure 4.10**) and a 2.4-fold increase in P2X7Rs immunoreactivity in TLR4$^{-/-}$ myenteric neurons, underlining the involvement of TLR4 in ensuring ENS homeostasis (**Figure 4.11**).

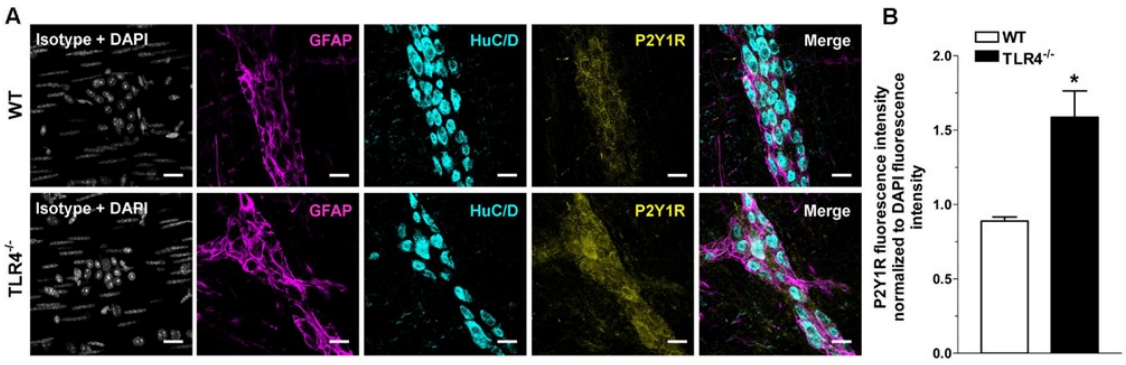


Figure 4.10. TLR4 signaling influences P2Y1 receptor distribution. Representative confocal microphotographs showing (**A**) GFAP (magenta), P2Y1Rs (yellow) and HuC/D (cyan) distribution. (**B**) Analysis of P2Y1Rs fluorescence intensities in WT and TLR4$^{-/-}$ LMMP preparations (N = 5 mice/group). Cell nuclei were stained with DAPI (gray). Bars = 22 µm. *P < 0.05 vs. WT.

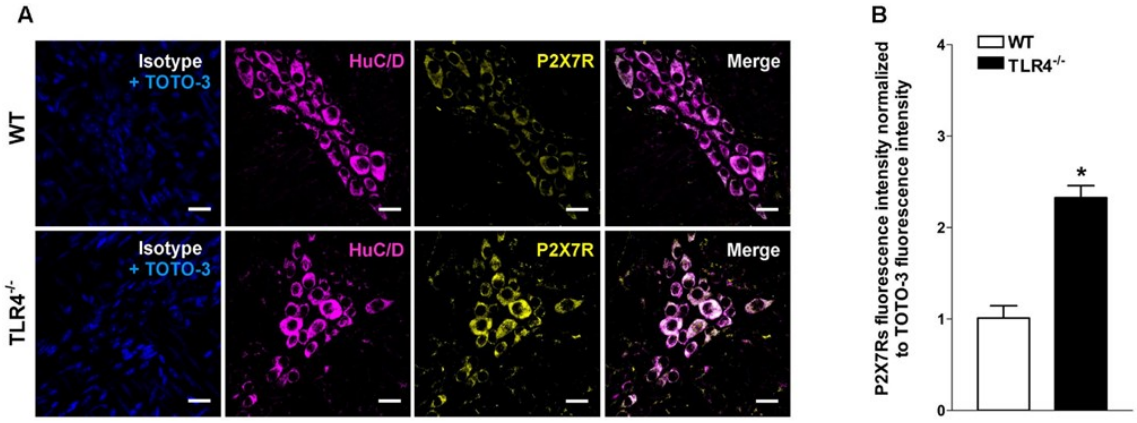


Figure 4.11. TLR4 signaling influences P2X7 receptor distribution. Representative confocal microphotographs showing (**A**) HuC/D (magenta) and P2X7Rs (yellow) distribution. (**B**) Analysis of P2X7Rs fluorescence intensities in WT and TLR4$^{-/-}$ preparations (N = 5 mice/group). Cell nuclei were stained with TOTO-3 (blue). Bars = 22 µm. *P < 0.05 vs. WT.

4.2 TLR4 in Mouse Central Nervous System

Several authors have recently highlighted the involvement of TLR4 in CNS plasticity, learning and memory, behavior and cognitive decline in pathological conditions (Pascual et al., 2011; Okun et al., 2012; Li et al., 2015). Zhu *et al.* (2016) showed the presence of diverse cerebellum-related motor defects in TLR4-deficient mice and found that the loss of TLR4 was associated with a reduction in the number of Purkinje cells and in the thickness of the molecular layer of the cerebellum, suggesting that TLR4 is essential for maintaining Purkinje cell survival and functioning. Furthermore, TLR4 was also recognized as involved in neuronal survival in the brain during sterile injuries, stroke, other neurodegenerative conditions or infections (Trotta et al., 2014; Su et al., 2016) and in modulating the self-renewal and the cell-fate of neuronal stem/progenitor cells (Rolls et al., 2007). These findings highlight that TLR4 signaling is essential for

neurodevelopment and neuroplasticity of both ENS and CNS (Okun et al., 2011; Anitha et al., 2012).

4.2.1 TLR4 is Required for Sustaining Neuron and Glia Network in Murine Hippocampus

To determine whether TLR4 may influence neuronal and glia network, we evaluated the distribution of the pan-neuronal marker HuC/D and both glial markers, S100β and GFAP, in the hippocampus of male WT and TLR4$^{-/-}$ mice (9 ± 1 weeks old). In hippocampal brain sections, the number of HuC/D^{+} neurons from TLR4$^{-/-}$ mice was significantly lower than in WT mice (by 30 ± 0.08%; **Figure 4.12A, B**). Even if no difference in S100β density index was found in the two genotypes (**Figure 4.12C, D**), a significant increase of density index (by 27 ± 0.6%; **Figure 4.12E, F**) and process length of GFAP^{+} gliofilaments (by 70 ± 12%; **Figure 4.12G**) was revealed in TLR4$^{-/-}$ mice to underline an involvement of TLR4 signaling in shaping neuroglia network and a differential response of GFAP and S100β, two astrocyte associated proteins, in the murine hippocampus.

In parallel microglia activation was evaluated through the analysis of Iba-1 distribution, frequently an up-regulation of this marker is associated with a state of neuroinflammation, characterized by alteration in microglia morphology and functional properties (Marshall et al., 2013). The Iba-1 staining intensity in the hippocampus was found comparable in both genotypes (**Figure 4.13A, B**)

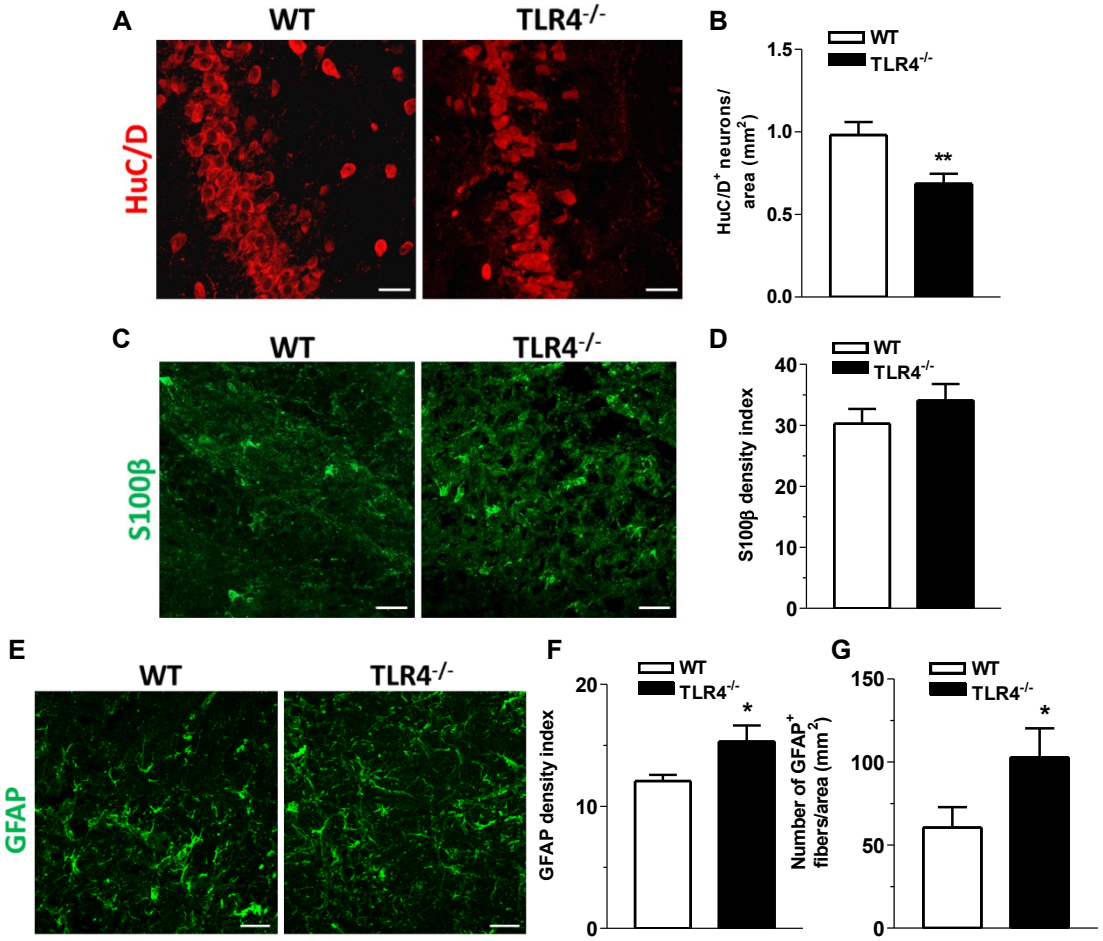


Figure 4.12. TLR4 signaling influences neuronal and glial network. Representative confocal microphotographs showing HuC/D (red; **A**), S100β (green; **C**) and GFAP (green; **E**) distribution in WT and TLR4$^{-/-}$ brain cryosections (bars = 22 µm). Number of HuC/D^{+} neurons per area (**B**) and relative analysis of S100β (**D**) and GFAP (**F**) density index in hippocampus from WT and TLR4$^{-/-}$. (**G**) Number of GFAP^{+} glial processes. (N = 4 mice/group). *P < 0.05, **P< 0.01 vs. WT.

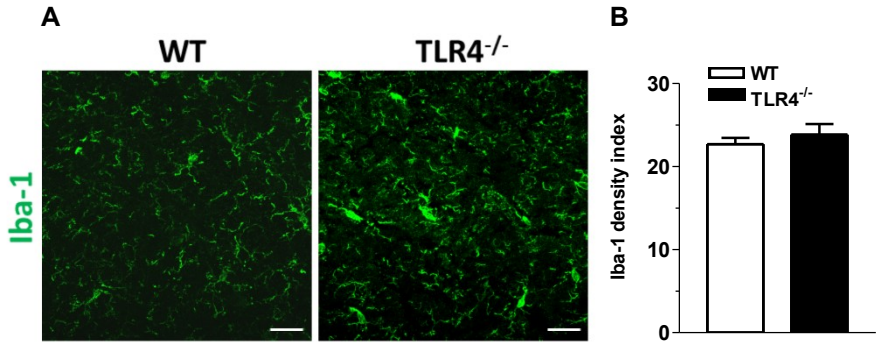


Figure 4.13. Involvement of TLR4 signaling in microglial activation. Representative confocal microphotographs showing Iba-1 (green; **A**) distribution in WT and TLR4$^{-/-}$ brain cryosections (bars = 22 µm). Analysis of Iba-1 (**B**) density index in hippocampus from WT and TLR4$^{-/-}$. (N = 4 mice/group).

However, in parallel we analyzed Iba-1 immunofluorescence in the ENS of ileum, to determine the distribution of muscularis macrophages (MM), which are tissue resident

macrophages localized within and between the circular and longitudinal muscle layers of the GI wall. MM interact closely with enteric neurons in the myenteric plexus (Gabanyi et al., 2016) and represent a unique population of bowel macrophages that differs morphologically, transcriptionally, and likely functionally from neighboring macrophages in bowel mucosa and lamina propria (Avetisyan et al., 2018). Recent studies of MM in bowel muscle layers reported a tight interaction between MM and enteric neurons and suggested that MM regulates functional activity of neurons in the control of neuronal development (Muller et al., 2014). To this end, we evaluated the number of Iba-1 expressing cells in ENS and revealed a significant increase in Iba1^{+} cells (by 22 ± 2%; **Figure 4.14B**) together with higher Iba1 staining area (by 20.9 ± 0.3%; **Figure 4.14C**).

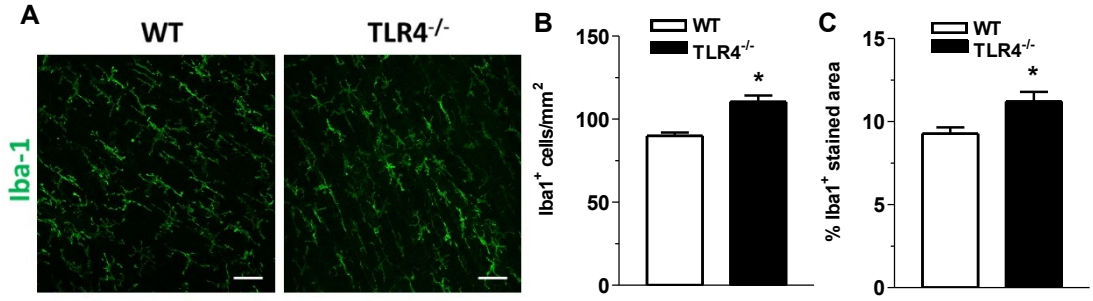


Figure 4.14. TLR4 signaling modulates muscularis macrophages. Representative confocal microphotographs showing Iba1 (green; **A**) distribution in WT and TLR4$^{-/-}$ ileal preparations (bars = 22 µm). Analysis of Iba-1 (**B**) positive cells and stained area (**C**) in LMMP preparation from WT and TLR4$^{-/-}$ (N = 4 mice/group). *P < 0.05 vs. WT.

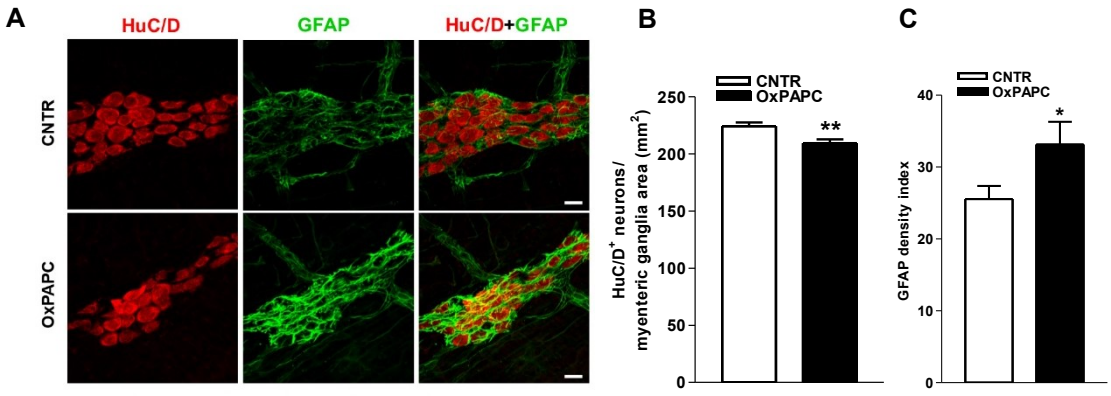
4.3 TLR2 and TLR4 Signaling Modulates Small Intestine Function

Among all TLRs the most important microbial-sensor proteins are TLR2 and TLR4. TLR2 recognizes different ligand such as porins, lipoprotein, LTA, bacterial PG, viral hemagglutinin and glycoproteins and this interaction leads to the activation of MyD88-dependent signaling pathways (Takeda et al., 2003). On the other hand, TLR4 has been found to detect LPS, a major component of Gram-negative bacteria cell wall (Takeda et al., 2003). Several studies advocate for a role of TLRs in ENS homeostasis (Kabouridis & Pachnis, 2015). TLR2 and TLR4 signaling seems to be fundamental for ensuring intestinal integrity and protecting from harmful injuries, in fact changes in their expression have been reported in functional and/or inflammatory bowel disease (IBD, Rakoff-Nahoum et al., 2006). Variations in the architecture and neurochemical coding of ENS leading to gut dysmotility and to higher IBD susceptibility were shown in a model of TLR2$^{-/-}$ mice highlighting TLR2 as major player in gut homeostasis (Brun et al., 2013).

In parallel, TLR4 is the best characterized PRRs and recently recognized to modulate ENS phenotype and function (Anitha et al., 2012; Caputi et al., 2017a). Furthermore, in a model of double knockout (KO) mice for TLR2 and 4 receptors (TLR2/4 DKO) *Latorre* and colleagues have demonstrated that both these receptors are involved in modulating intestinal motor responses by influencing 5-HT-mediated pathways in mouse ileum (Forcén et al., 2015). To evaluate the impact of acute inhibition of both TLR2 and TLR4 on gut integrity, we treated the mice with a mixture of oxidized chain-shortened phospholipids and oxygenated phospholipids, termed OxPAPC.

4.3.1 OxPAPC-mediated TLR2 and TLR4 Inhibition Alters the Architecture of the Myenteric Plexus of Juvenile Mice

Considering that changes in TLR2 or/and TLR4 expression affects ENS integrity (Anitha et al., 2012; Brun et al., 2013; Forcén et al., 2015; Caputi et al., 2017a, b), we sought to determine the impact of acute pharmacological inhibition of both these receptors on ENS architecture by confocal immunofluorescence. In the myenteric plexus of OxPAPC-treated mice, the total number of HuC/D$^+$ neurons were found significantly lower (by 10 ± 2%; **Figures 4.15A, B**) compared to that found in CNTR mice. This change was accompanied by a marked increase of GFAP immunoreactivity (by 30 ± 2%; **Figures 4.15A, C**) together with changes in the immunofluorescence of the other glial marker S100β (by an increase of 13 ± 2%; **Figures 4.15D, E**).



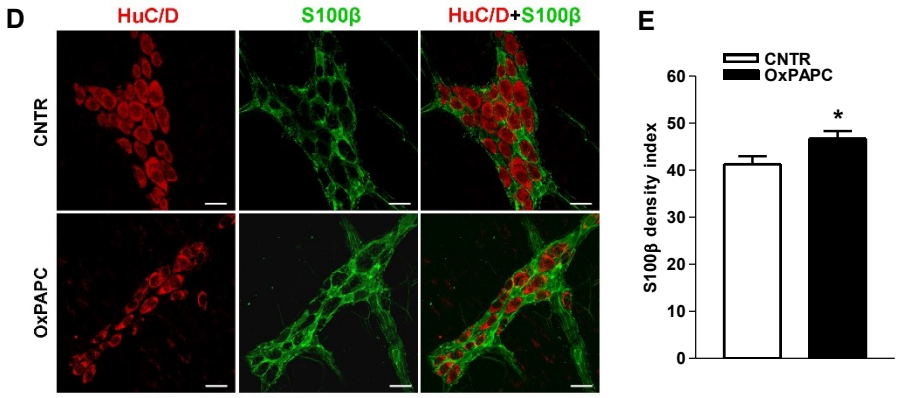


Figure 4.15. OxPAPC treatment alters the architecture of the ileal myenteric plexus. Representative confocal microphotographs showing the distribution of GFAP (green; **A**), S100β (green; **B**) and HuC/D (red; **A**, **B**) in LMMP preparations from CNTR and OxPAPC-treated mice (bars = 22 μm). Number of HuC/D^+ neurons per myenteric ganglia area (**B**) and relative analysis of GFAP (**C**) and S100β (**E**) density index. *P < 0.05, **P < 0.01 vs. CNTR (N = 6 mice/group).

4.3.2 OxPAPC Treatment Increases Excitatory Neuromuscular Contractility

Since mice deficient for TLR2 or TLR4 receptors show altered GI motility (Anitha et al., 2012; Brun et al., 2013; Caputi et al., 2017a; Caputi et al., 2017b), we thus examined in vitro neuromuscular function by measuring tension changes in isolated ileal preparations following cumulative addition of the non-selective cholinergic agonist, CCh. Ileal segments from OxPAPC-treated mice showed a significant upward shift of the concentration–response curve to carbachol and a consequent increase in maximum response, Emax rising to about 50% of the CNTR value (**Figure 4.16A**). To verify whether the contraction changes in OxPAPC-treated mice were determined by alterations in neuromuscular function, we assessed the effect of increasing frequencies of stimulation on ileal preparations. Altered neurotransmission in OxPAPC-treated ileal segments was reflected by increased frequency-response curves to EFS (by 75 ± 12% at 10 Hz; **Figure 4.16B**). We previously confirmed that in mouse ileum, EFS-mediated responses to frequencies up to 10 Hz are of neuronal cholinergic origin, being sensitive to both TTX and atropine (Brun et al., 2013). However, no changes in ChAT immunoreactivity was found in ileal whole mount preparations of OxPAPC mice (**Figures 4.16C, D**).

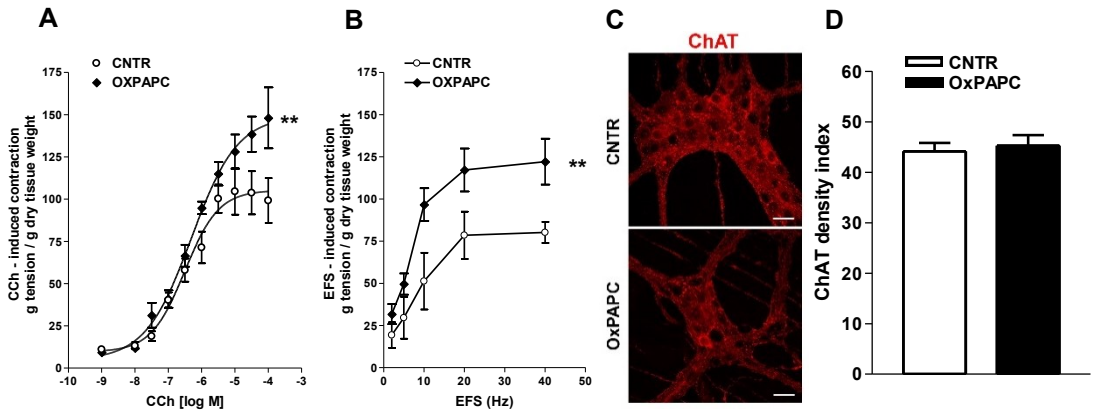


Figure 4.16. Effect of OxPAPC-mediated inhibition of TLR2 and TLR4 signaling on ileal neuromuscular contractility and cholinergic neurochemical coding. Concentration-response curve to CCh (**A**) and excitatory response to EFS (**B**) in isolated ileal segments from CNTR and OxPAPC-treated mice. Data are reported as mean ± SEM (N=8 mice/ group) and are expressed as g tension/g dry tissue weight (g/g). **P < 0.01 vs. CNTR. Representative confocal microphotographs showing the distribution of ChAT (red; **C**) in CNTR and OxPAPC-treated LMMP preparations (bars = 22 μm) and relative analysis of ChAT (**D**) density index. (N = 5 mice/group).

4.3.3 OxPAPC Treatment Affects Inhibitory Neurotransmission

To evaluate the contribution of inhibitory neurotransmission in the control of intestinal motility and considering that the increased excitatory contractions could be due to a reduction of the inhibitory component, we tested NO-mediated relaxation responses in presence of guanethidine and atropine. EFS NANC-relaxations at 10 Hz caused a significant reduction (by 41 ± 25%; **Figure 4.17A**) of the response in OxPAPC mice compared with CNTR animals. The pretreatment with L-NAME induced an EFS-relaxation comparable between the two different groups. To better characterize the effect of both TLR2 and TLR4 inhibition in the nitrergic neurotransmission, we analyzed the distribution of nitrergic neurons in the myenteric plexus. Even if no difference was observed in the total number of $nNOS^+$ neurons in the ileum of the two different groups (**Figures 4.17B, C**), the reduction in HuC/D^+ neurons was associated with a proportional increase of $nNOS^+$ neurons in OxPAPC-treated mice (**Figure 4.17D**).

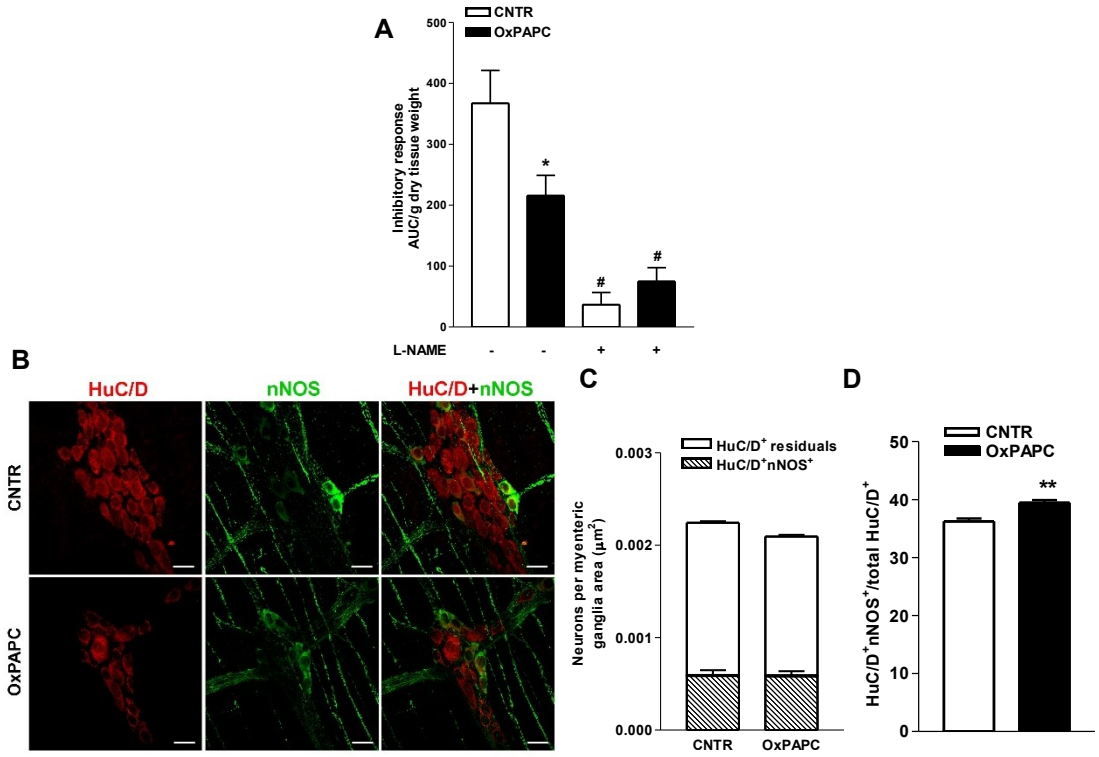


Figure 4.17. OxPAPC-mediated inhibition of TLR2 and TLR4 signaling affects nitrergic neurotransmission. 10-Hz-EFS induced NANC relaxation responses (**A**) in presence or absence of L-NAME in ileal preparations from CNTR and OxPAPC-treated mice. Data are expressed as AUC/g dry tissue weight. *P < 0.05 vs. CNTR, #P < 0.05 vs. respective control in absence of L-NAME. (**B**) Representative confocal photomicrographs showing the distribution of nNOS (green) and HuC/D (red). (**C**) Number of HuC/D$^+$ nNOS$^+$ and residual HuC/D$^+$ neurons per myenteric ganglia area (bar = 22 µm); (**D**) percentage of HuC/D$^+$ nNOS$^+$ neurons respect to total HuC/D$^+$ neurons in ileal LMMP whole-mount preparations of CNTR and OxPAPC-treated mice. **P < 0.01 vs. CNTR. (N = 6 mice/ group).

4.3.4 OxPAPC Treatment Influences Ileal SERT and 5-HT Receptors Expression

Intestinal 5-HT plays an important role in stimulating the ENS and gut function (Gershon & Tack, 2007) and 5-HT-mediated effects appear to be modulated by TLR2 and TLR4 signaling (Forcèn et al., 2015, 2016). In order to gain an in-depth knowledge of both TLR2 and 4 influences on the serotonergic system, SERT expression was analyzed by measuring immunoreactivity in LMMPs and ileal SERT mRNA levels. A 1.2-fold increase in the mRNA expression of SERT (**Figure 4.18A**) together with a marked increase in the immunoreactivity of SERT (by 38 ± 2%; **Figures 4.18B, C**) in LMMPs was found after treatment with OxPAPC.

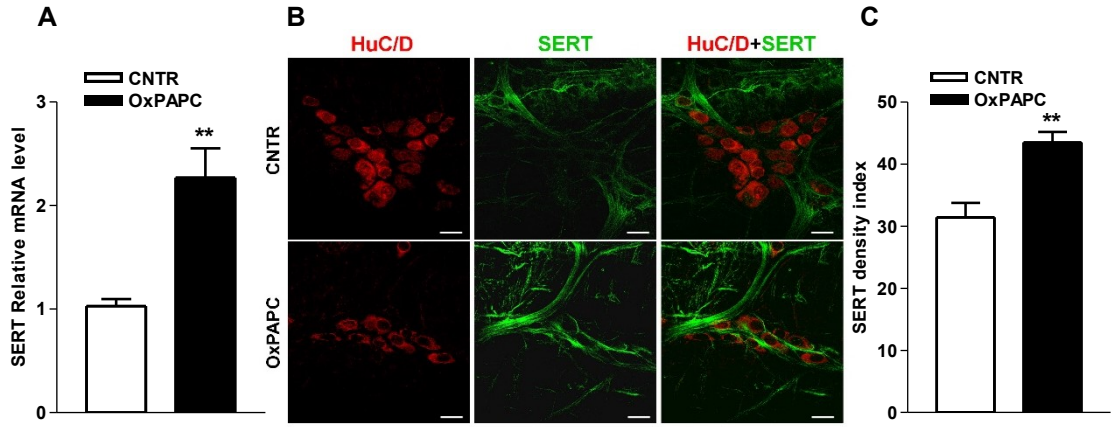
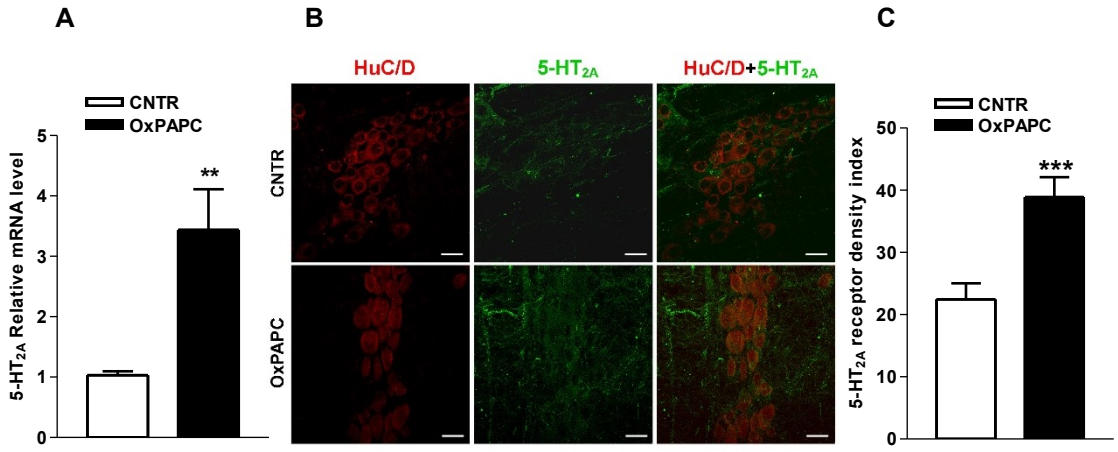


Figure 4.18. OxPAPC treatment alters serotonin transporter expression. (**A**) Real-time PCR analysis of SERT mRNA levels in ileal LMMPs from CNTR and OxPAPC-treated mice. (**B**) Representative confocal microphotographs showing SERT (green) and HuC/D (red) distribution in LMMP preparations from CNTR and OxPAPC-treated mice (bars = 22 µm). (**C**) Analysis of SERT density index in ileal LMMP whole-mount preparations of CNTR and OxPAPC-treated mice. **P < 0.01 vs. CNTR. (N = 5 mice/group).

Considering that the actions of 5-HT on GI motility are transduced by a large family of 5-HT receptor subtypes, real-time quantitative PCR and confocal immunofluorescence were performed to characterize the involvement of 5-HT_{2A}, 5-HT_3, 5-HT_4 receptors in OxPAPC-mediated hypercontractility. In LMMPs of OxPAPC-treated mice, the mRNA levels of 5-HT_{2A} and 5-HT_4 receptors were increased by 3.3- and 2-fold, respectively (**Figures 4.19A, G**), with no changes in 5-HT_3 receptor mRNA levels, when compared with CNTR preparations (**Figure 4.19D**). The enhanced expression levels of 5-HT_{2A} receptors was then confirmed by confocal immunofluorescence and density index analysis in whole mount preparation of LMMPs (by 73 ± 3% for 5-HT_{2A} receptor; **Figure 4.19B, C**), together with a significant increase of density index for 5-HT_3 receptor (by 45 ± 2%; **Figure 4.19E, F**).



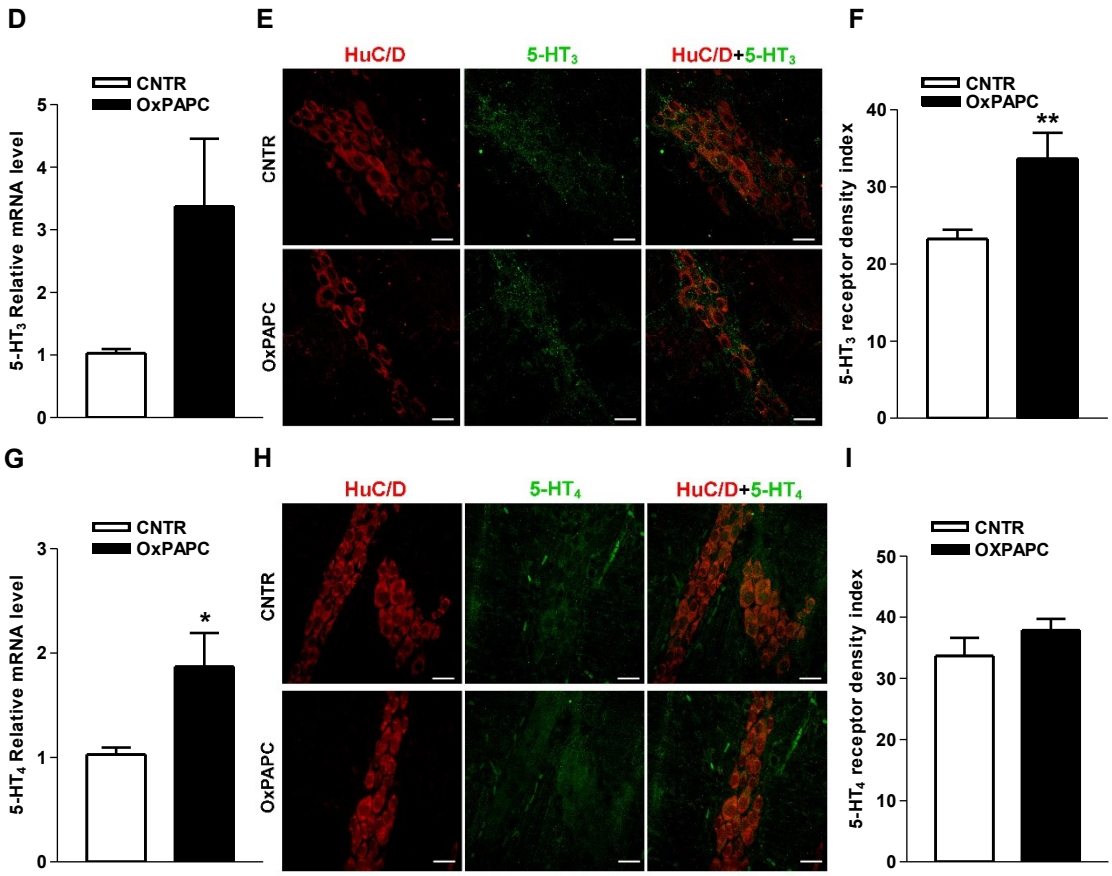


Figure 4.19. OxPAPC-mediated inhibition of TLR2 and TLR4 signaling affects serotonergic neurochemical coding. Real-time PCR analysis of 5-HT_{2A} (**A**), 5-HT_3 (**D**) and 5-HT_4 (**G**) receptors mRNA expression levels in ileum LMMPs from CNTR and OxPAPC-treated mice. Representative confocal microphotographs showing 5-HT_{2A} (green; **B**), 5-HT_3 (green; **E**) and 5-HT_4 (green; **H**) receptors and HuC/D (red) distribution in CNTR and OxPAPC-treated LMMP preparations (bars = 22 μm). Analysis of 5-HT_{2A} (**C**), 5-HT_3 (**F**) and 5-HT_4 (**I**) receptors density index in ileal LMMP whole-mount preparations of CNTR and OxPAPC-treated mice. *P < 0.05, **P < 0.01, ***P < 0.001 vs. CNTR. (N = 5 mice/group).

4.3.5 OxPAPC Treatment Modifies Serotonergic Neurotransmission

Since 5-HT activates both intrinsic excitatory and inhibitory enteric motor neurons, we analyzed the influence of OxPAPC-mediated inhibition of TLR2 and TLR4 on the contractile response evoked by the non-cumulative addition of exogenous 5-HT in isolated ileal segments. 5-HT evoked a concentration-dependent contractile response that was significantly higher in OxPAPC-treated mice compared to CNTR animals (by 23 ± 9% Emax; **Figure 4.20A**). To further investigate which of these receptor subtypes is responsible for OxPAPC-induced neuromuscular hypercontractility to 5-HT, we evaluated the effect of ketanserin (an antagonist of 5-HT_{2A} receptors), ondansetron (an antagonist of 5-HT_3 receptors) and GR113808 (an antagonist of 5-HT_4 receptors) on 5-HT-mediated contraction. The incubation of OxPAPC-treated ileal preparations with all

three antagonists didn't affect the non-cumulative concentration-curve to 5-HT (**Figures 4.20B, C, D**). However, the presence of ketanserin modified the response to 5-HT of CNTR ileal segments which increased significantly reaching a tension comparable to OxPAPC-treated preparations, indicating that 5-HT_{2A} receptor mediates relaxation in mouse ileum and this response was affected by OxPAPC treatment (Wang et al., 2011; **Figure 4.20B**).

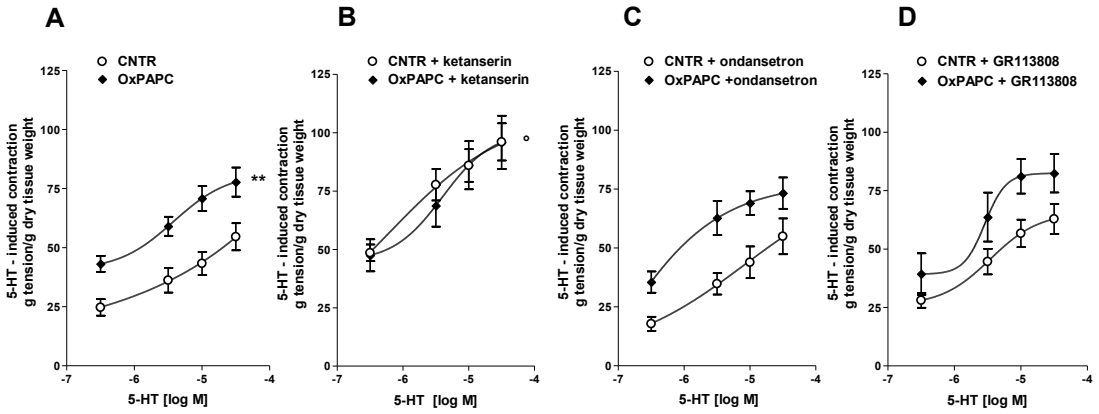


Figure 4.20. OxPAPC treatment alters serotonergic response. Graphs showing the concentration-response curves to 5-HT (0.3–30 μM; **A**) in isolated ileal preparations from CNTR and OxPAPC-treated mice. Graphs showing the concentration-response curves to 5-HT (0.3–30 μM) in presence of different 5-HT receptors antagonist, ketanserin (**B**), ondansetron (**C**) and GR113808 (**D**) in CNTR and OxPAPC-treated preparations. *P < 0.05 vs. CNTR mice; °P < 0.05 vs. CNTR in absence of ketanserin. (N = 6 mice/group).

4.3.6 OxPAPC-mediated Inhibition of TLR2 and TLR4 Impairs Tryptophan Metabolism

In view of altered pattern of expression of 5-HT receptors as well as the changes in ileal motor response to 5-HT we tested whether the pharmacological blockade of TLR2 and TLR4 signaling affects TRP metabolism. After OxPAPC treatment, TRP levels in ileal tissue were found to be comparable to CNTR specimens (**Figure 4.21A**). However, along the 5-HT pathways of TRP metabolism, the metabolite 5-HTP was significant increased by 2.2-fold (**Figure 4.21B**) and was associated with a reduction of 5-HT levels (by 56 ± 0.0005%; **Figure 4.21C**) in ileal tissue after OxPAPC treatment. However, the dominant physiological pathway for TRP is actually along the KYN pathway (Le Floc'h N et al., 2011). In this concern, we found a slight but not significant increase of KYN levels in ileal tissue of OxPAPC-treated mice (**Figure 4.21D**), to highlight that OxPAPC-mediated inhibitory effects on TLR2 and TLR4 signaling may influences 5-HT availability versus KYN metabolism.

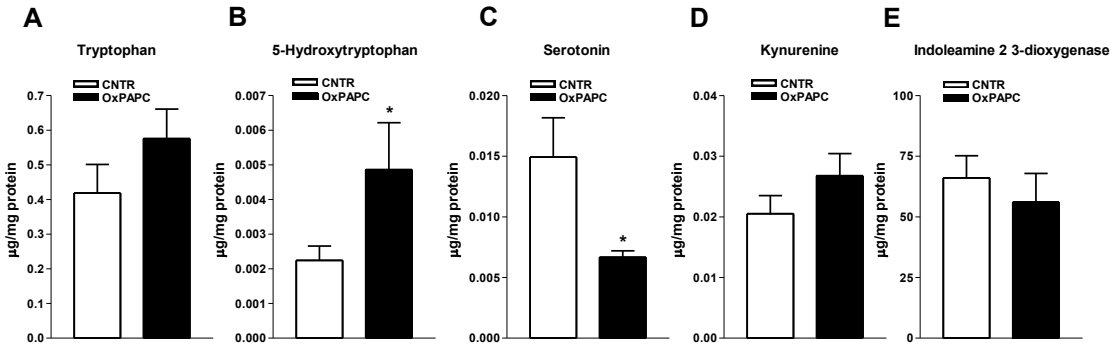


Figure 4.21. OxPAPC-mediated inhibition of TLR2 and TLR4 signaling affects TRP metabolism. TRP levels (**A**), 5-HTP levels (**B**), 5-HT levels (**C**), KYN levels (**D**) and IDO activity (**E**) in ileal tissue measured by HPLC analysis in ileal specimens from CNTR and OxPAPC-treated mice (N=8 mice per group). *P < 0.05 vs. CNTR mice. Data are reported as mean ± SEM. IDO activity was measured by assessing the ratio KYN/TRP expressed in percentage.

4.4 Microbiota-Gut Axis Regulates Serotonergic Neurotransmission

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 & Pachnis, 2015). During the first years of life a disruption or a perturbation of microbial milieu affects the development pattern with consequent adverse health outcomes in adulthood (O'Mahony et al., 2014). Antibiotic treatment can affect the gut microflora integrity and its use has been related to the development of GI symptoms in intestinal functional disorders such as IBS. Several observations report that patients treated with antibiotics for non-GI complaints are three times more likely to acquire functional bowel symptoms (Alun-Jones et al., 1984).

4.4.1 Antibiotic-induced Microbiota Dysbiosis Affects Visceral Sensitivity

The IBS pathophysiology is still not well-defined, and symptoms change over time in terms of intensity and character; visceral perception is one of the most important symptoms in IBS patients with direct effects on gut fitness (Verdù et al., 2006). Considering that gut microbiota influences the functional GI symptoms; the impact of antibiotic treatments on visceral hypersensitivity was evaluated. The amino acid glutamate via the activation of the ionotropic NMDA receptors is 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). Upregulation of TRPV1 in afferent nerve fibers has also been observed in the absence of overt inflammation as is typical of patients with IBS (Holzer, 2008b). Following antibiotic-induced microbiota dysbiosis, quantitative RT-PCR analysis showed a significant increase of mRNA levels of GluN1 subunit of NMDA receptor in ileal LMMPs preparations from ABX-treated mice compared to CNTR (by 35 ± 0.04%; **Figure 4.22A**), 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). In parallel, a significant increase of TRPV1 subunit mRNA levels was found in LMMPs preparations obtained from ABX-treated mice compared to CNTR (by 2.4-fold increase; **Figure 4.22B**).

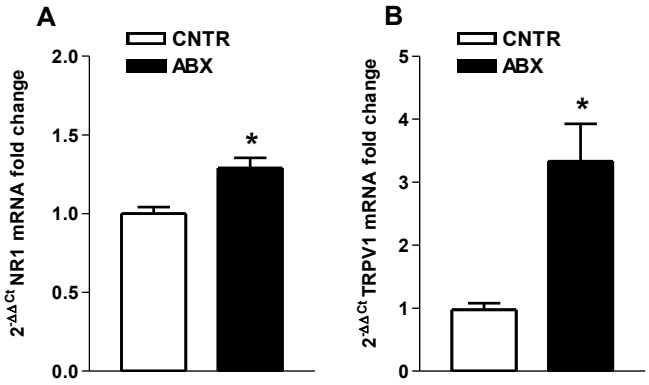


Figure 4.22. Antibiotic-induced microbiota dysbiosis affects visceral sensitivity. RT-PCR quantification of GluN1 (**A**) and TRPV1 (**B**) transcripts performed in ileal LMMP preparations of ABX and CNTR mice. Values are expressed as mean ± SEM (N = 5 mice per group) of the percentage variation of relative 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 mice.

4.4.2 Antibiotic-induced Microbiota Dysbiosis Affects Tachykinergic Neurotransmission

To assess the contribution of tachykinergic neurotransmission to neuromuscular function, we evaluated post-stimulus excitatory off-responses under NANC conditions in the presence of L-NAME, to unmask tachykinergic nerve-evoked contractions (Lecci et al., 2006). The tachykinin-mediated response was increased in ABX-treated mice (by 37 ± 6%; **Figure 4.23**) and this off-response was significantly reduced after selective blockade of NK1 receptors with L732138 (**Figure 4.23**).

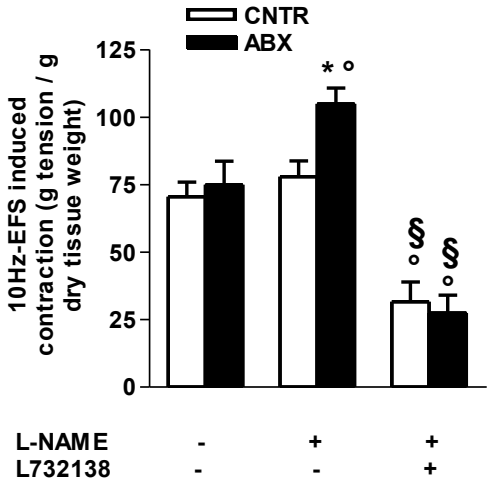


Figure 4.23. Effect of antibiotic treatments in the tachykinergic-mediated response. 10 Hz EFS evoked response under NANC conditions, in absence or presence of L-NAME or L732138 in ileal preparations from CNTR and ABX-treated mice. (N = 5 mice per group). Data are reported as mean ± SEM. *P < 0.05 vs. CNTR; °P < 0.05 vs. respective control without L-NAME; §P < 0.05 vs. respective control without L732138.

In parallel, immunostaining for SP, a member of the tachykinin family of neuropeptides, with higher affinity for NK1 than for NK2 or NK3 receptors (Lecci et al., 2006) was evaluated. A significant increase of SP^+ neurons was observed in ileal myenteric plexus of ABX-treated mice (by 35 ± 0.5%; **Figures 4.24A, B, C**) relative to preparations obtained from CNTR animals.

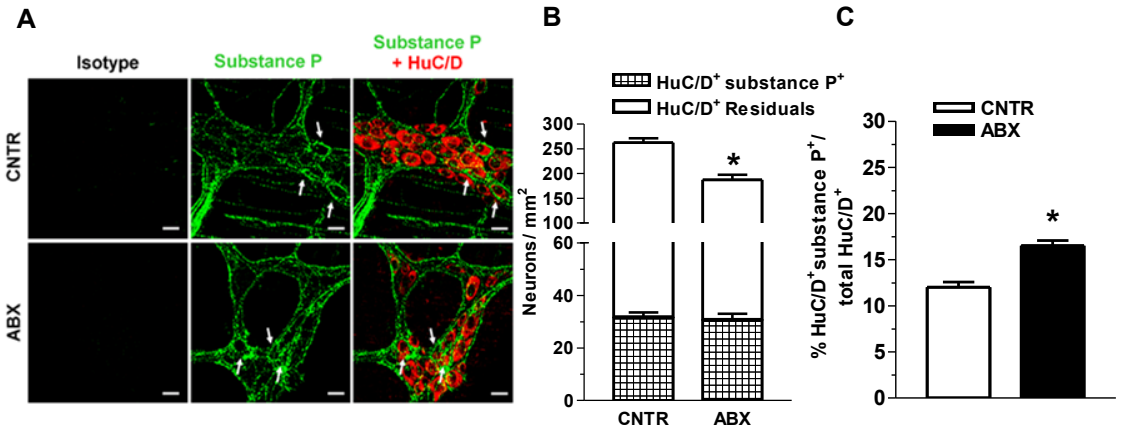


Figure 4.24. Antibiotic-induced microbiota dysbiosis increases SP immunostaining. (**A**) Representative microphotographs showing the distribution of SP (green) and HuC/D (red) and (**B**) number of HuC/D^+SP^+ and residual HuC/D^+ neurons per myenteric ganglia area and (**C**) percentage of SP^+ neurons respect to total HuC/D^+ neurons in ileal LMMP whole-mount preparations from CNTR and ABX mice (N = 5 mice per group). (bars = 22 µm). Arrows indicate SP^+HuC/D^+ neurons in myenteric plexus of CNTR and ABX mice. Data are reported as mean ± SEM. *P < 0.05 vs. CNTR mice.

4.4.3 Serotonin Neurotransmission Involves Ileum Relaxation-Response following Antibiotic-induced Microbiota Dysbiosis

Until now, we showed that the antibiotic treatment induced change in sensory neurotransmitter pattern, however, we cannot exclude the possibility that bacteria may directly influence neurotransmitter content. Recent studies have suggested that gut microbes were able to modulates the amount of neurotransmitter directly or indirectly. Thus, we evaluated the effect of antibiotic-induced dysbiosis on ileal response to 5-HT. The contractile response induced by higher concentration of exogenous 5-HT addition to isolated ileal segments evoked a relaxation response significantly higher in ABX-treated mice compared to CNTR animals (by 29 ± 5% Emax; **Figure 4.25A**). The 5-HT neuromuscular response is mediated by a large family of 5-HT receptors, to further investigate which of these receptor subtypes is responsible 5-HT-induced relaxation, we analyzed the effect of ketanserin (an antagonist of 5-HT_{2A} receptors) and ondansetron (an antagonist of 5-HT_3 receptors) on 5-HT-mediated relaxation. Incubation of isolated ileal preparations with $5HT_{2A}$ or $5HT_3$ antagonists almost blocked the 5-HT-mediated relaxation in CNTR and ABX-treated mice indicating that these receptors are implicated in 5-HT-mediated response. However, only the inhibition of 5-HT_3 with ondansetron determined response curves to 5-HT comparable between CNTR and ABX-treated mice (**Figure 4.25C**), to suggest an involvement of these receptors in the increased neuromuscular relaxation determined by antibiotic-induced dysbiosis.

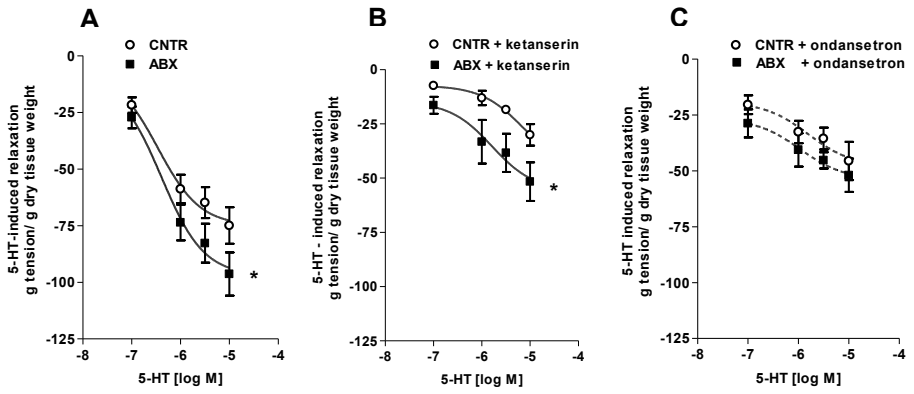


Figure 4.25. Antibiotic-induced microbiota dysbiosis influences serotonergic response. (**A**) Concentration-response curves to 5-HT (0.3–30 μM) in isolated ileal preparations from CNTR and ABX-treated mice (N=5 mice per group) in absence and presence of ketanserin (**B**) and ondansetron (**C**). Data are reported as mean ± SEM. *P < 0.05 vs. CNTR mice.

Since 5-HT response was found to be altered after antibiotic treatment, we sought to test neuromuscular function by analyzing frequency-response curves to EFS in presence of 5-HT_{2A} or 5-HT_3 receptor antagonists. Altered neurotransmission in ABX-treated ileal segments was reflected by reduced EFS-elicited contractions (by 40 ± 8% at 10 Hz; **Figure 4.26**). Incubation of isolated ileal preparations with ketanserin modified the motor responses in ABX ileum which increased significantly (by 29 ± 8% at 10 Hz; **Figure 4.26**) reaching a tension comparable to that obtained from preparations of CNTR mice, to confirm that 5-HT_{2A} mediated contraction is affected by gut dysbiosis.

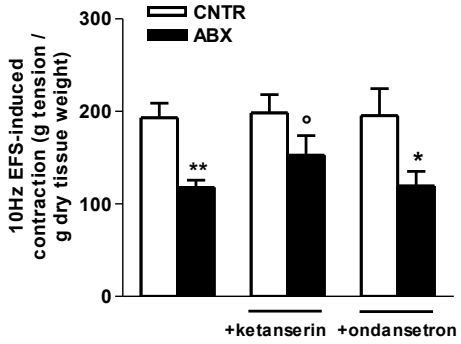


Figure 4.26. Antibiotic treatment affects neuromuscular response. EFS-induced excitatory responses in CNTR and ABX-treated preparations in absence and presence of ketanserin and/or ondansetron. Data are reported as mean ± SEM. *P < 0.05, **P < 0.01 vs. CNTR mice; °P < 0.05 vs ABX in absence of ketanserin. (N=5 mice per group).

4.4.4 Antibiotics Treatment Influences Ileal SERT and 5-HT Receptors Expression

Since we showed an involvement of 5-HT in mediating contractility response in a state of dysbiosis, we next asked whether perturbation of gut microbiota after antibiotic treatment affected the pattern of distribution of 5-HT receptors. In ABX-treated mice, a significant increase (by 42 ± 2%; **Figures 4.27A, B**) of immunoreactivity of 5-HT_{2A} receptor was found in whole mount preparation of myenteric plexus, however no difference in the immunoreactivity of 5-HT_3 receptor was detected (**Figures 4.27C, D**).

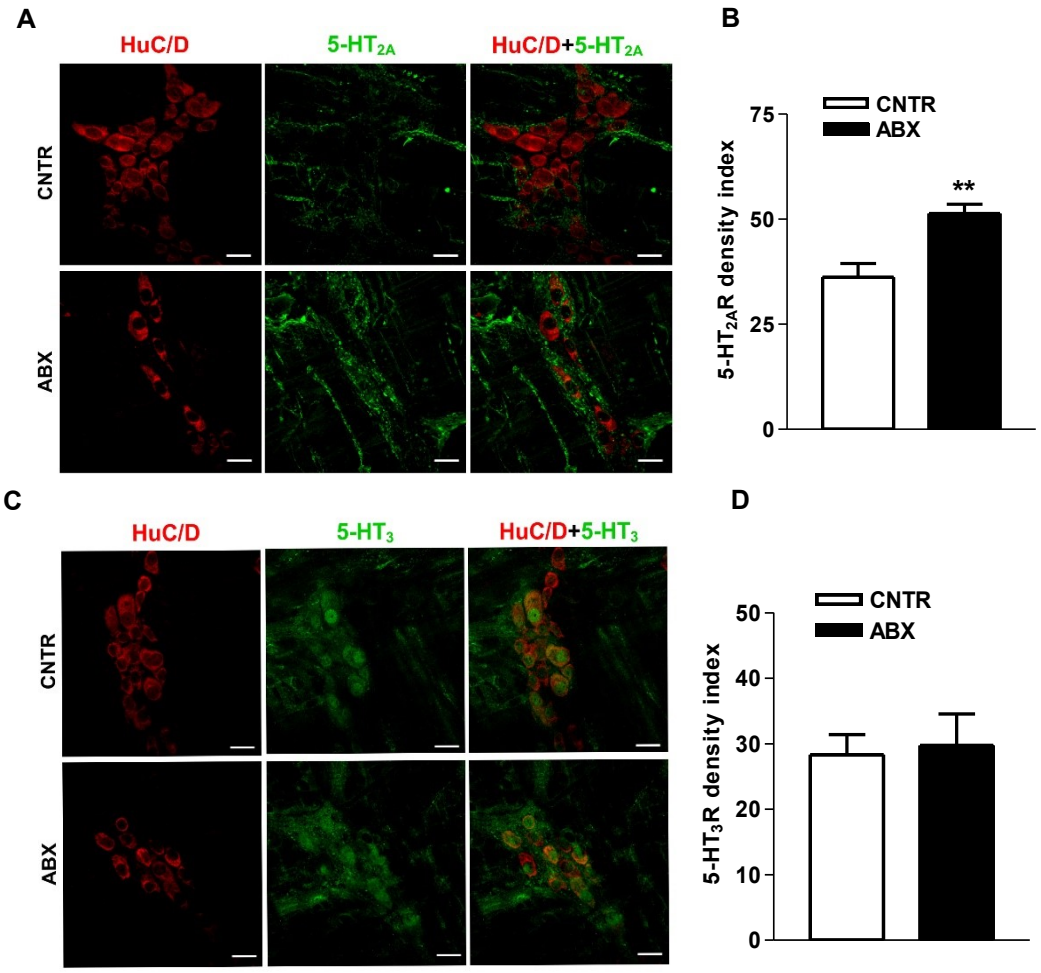


Figure 4.27. Antibiotic-induced microbiota dysbiosis affects serotonergic neurochemical coding. (**A**) Representative confocal microphotographs showing 5-HT_{2A} (green) and HuC/D (red) distribution. (**B**) Analysis of 5-HT_{2A}R fluorescence intensities in CNTR and ABX-treated ileal LMMPs. (**C**) Representative confocal microphotographs showing 5-HT_3 (green) and HuC/D (red) distribution. (**D**) Analysis of 5-HT_3R fluorescence intensities in CNTR and ABX-treated ileal LMMPs. **P < 0.01 vs. CNTR mice. (N=5 mice per group).

In order to gain an in-depth knowledge of microbiota-mediated effects in serotonergic system, SERT expression was analyzed by measuring SERT immunoreactivity in ileal whole mount preparations. The results showed a significant reduction of immunoreactivity of SERT after antibiotic-treatment (by 27 ± 3%; **Figures 4.28A, B**). This finding suggests that gut microbiota seems to be involved in the modulation of SERT expression.

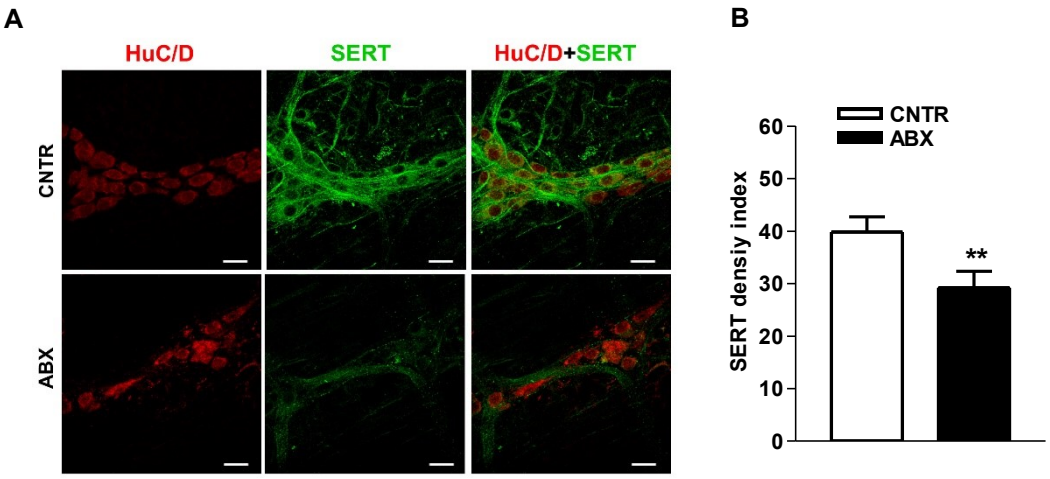


Figure 4.28. Antibiotic treatment alters SERT expression. (**A**) Representative confocal microphotographs showing SERT (green) and HuC/D (red) distribution. (**B**) Analysis of SERT fluorescence intensities in CNTR and ABX-treated LMMPs. (N=5 mice per group). **P < 0.01 vs. CNTR mice.

4.4.5 Antibiotics-induced Microbiota Dysbiosis Affects Tryptophan Metabolism

The involvement of microbiota in the control of the serotonergic neurotransmitter pathways has been proposed until now. To this concern we tested if microbial dysbiosis affects TRP metabolism, a potential key point in the control of serotonergic signaling. As such, emerging data reported the ability of gut microbes to regulate host 5-HT biosynthesis to control GI homeostasis (Yano et al., 2015). The amino acid TRP together with its metabolite 5-HT is essential metabolite in the control of gut homeostasis (O'Mahony et al., 2015). After antibiotic treatment, TRP levels were significant increase in plasma (by 33 ± 0.4%; **Figure 4.29A**) whereas only a slight increase was detected in ileal tissue that did not reach statistical significance (**Figure 4.29A**). Interestingly, ex vivo analysis of TRP metabolism showed a significant reduction (by 45 ± 0.0003%; **Figure 4.29B**) of the 5-HTP in ileal tissue after antibiotic treatment, to suggest a reduced availability for 5-HT synthesis. To this end, a significant increase of 5-HT turnover was found in ileal tissue (by 42 ± 0.001%; **Figure 4.29C**) of antibiotic-treated mice, whereas in the plasma of treated mice, only a tendency of reduction of 5-HT levels was detected (**Figure 4.29C**).

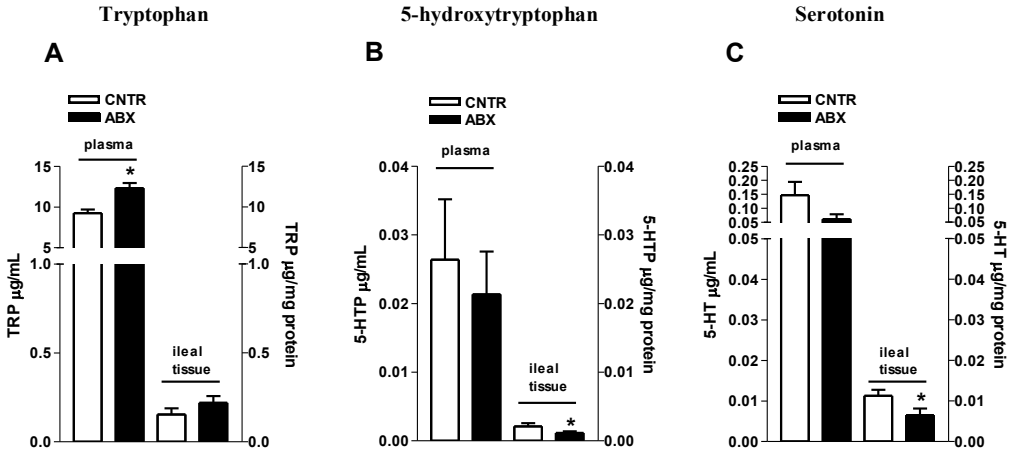


Figure 4.29. Antibiotic treatment alters TRP metabolism. TRP levels (**A**), 5-HTP levels (**B**) and 5-HT levels (**C**) in plasma or ileal tissue measured by HPLC analysis in CNTR and ABX-treated mice (N=5 mice per group). Data are reported as mean ± SEM. *P < 0.05 vs. CNTR mice.

However, the dominant physiological pathway for TRP is actually along the KYN pathway (Le Floc'h N et al., 2011). KYN is produced from TRP by the action of the largely hepatic-based enzyme, TDO or the ubiquitous IDO (Badawy, 2017). The influence of the microbiota on TRP availability and 5-HT synthesis have been also reported by some indirect mechanisms via the enzymes responsible for TRP degradation along the KYN pathway. In this concern, we found no significant difference in KYN levels in plasma and ileal tissue in the two groups of mice (**Figure 4.30A**) even if a lower IDO enzymatic activity was detected in ileal tissue of ABX-treated mice compared to CNTR mice (by 52 ± 10%; **Figure 4.30B**).

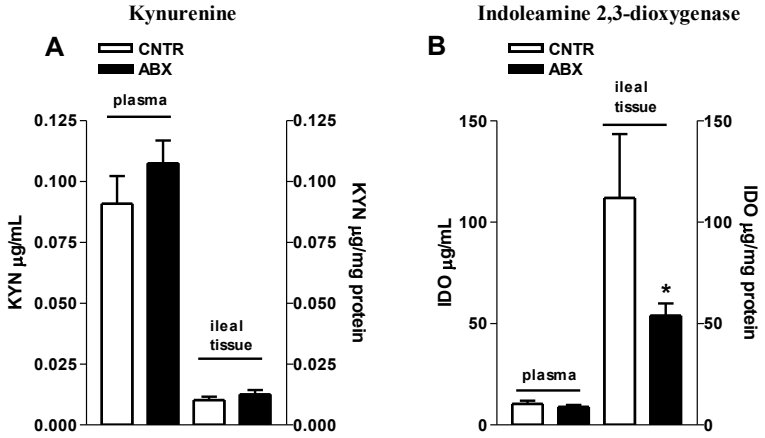


Figure 4.30. Antibiotic-induced microbiota dysbiosis affects kynurenine pathways. KYN levels (**A**) and IDO activity (**B**) in plasma and ileal tissue measured by HPLC analysis in CNTR and ABX-treated mice (N=5 mice per group). *P < 0.05 vs. CNTR mice. Data are reported as mean ± SEM. IDO activity was assessed by analyzing the ratio KYN/TRP expressed in percentage.

4.4.6 Antibiotic-induced Microbiota Dysbiosis Causes Morphological Abnormalities in the Architecture of the Myenteric Plexus

Changes in microbiota composition and/or signaling are known to influence ENS morphology (Brun et al., 2013; Hyland & Cryan, 2016). In the myenteric plexus of ABX-treated mouse ileum, the total number of HuC/D-stained neurons per myenteric ganglia resuted significantly lower (by 29 ± 10%; **Figures 4.31A, B**) compared that one measured in CNTR mice.

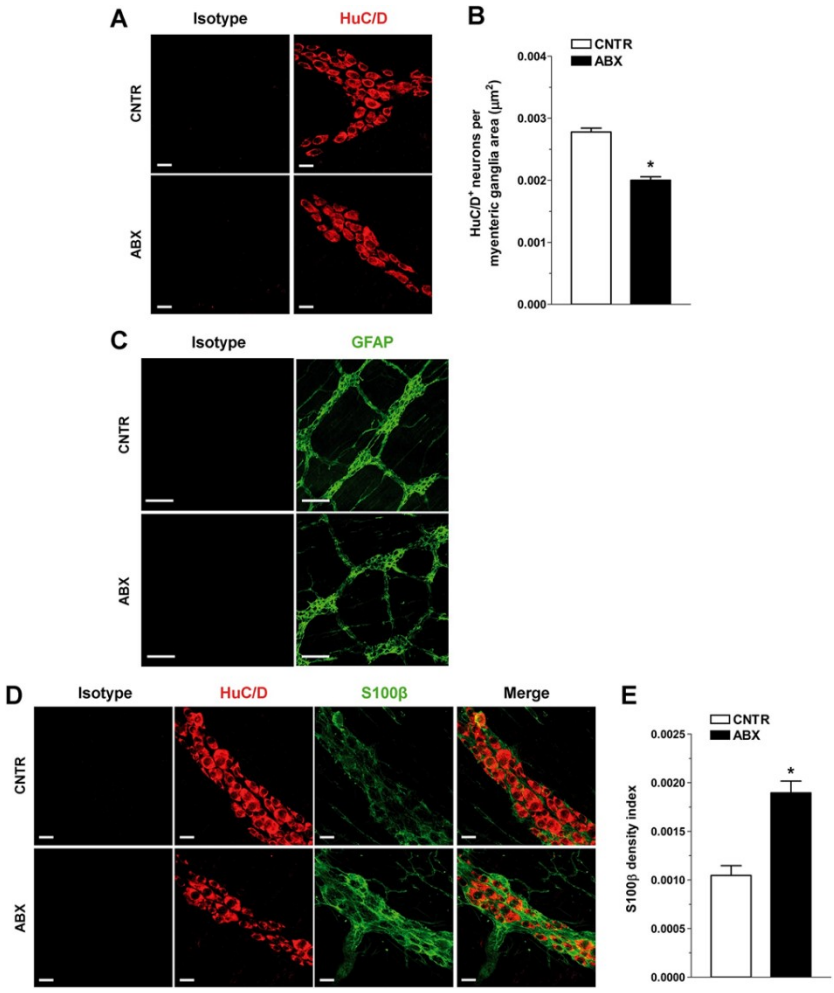


Figure 4.31. Effects of antibiotic treatment on myenteric plexus architecture. (**A**) Representative confocal microphotographs showing the distribution of HuC/D (red, pan-neuronal marker) and (**B**) number of HuC/D$^+$ neurons per myenteric ganglia area in LMMP preparations from CNTR and ABX-treated mice (N = 6 mice per group). (bars = 22 µm). (**C - D**) Representative confocal photomicrographs showing the distribution of GFAP (green), S100β and HuC/D (green and red, respectively) in LMMP preparations from CNTR and ABX-treated mice (N = 6 mice per group). (bars = 100 µm (C) and 22 µm (D)). (**E**) S100β density index in LMMP preparations of CNTR and ABX-treated mice (N = 6 mice per group). Data are reported as mean ± SEM. *P < 0.05 vs CNTR.

In addition, antibiotic treatment induced distortion of EGCs processes within myenteric ganglia, as shown by the altered distribution of the glial fibrillary acidic protein $GFAP^+$ fibers and by a marked increase of S100β density index by 1.4-fold compared with CNTR (**Figures 4.31 C-E**).

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 GI 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 nonpathogenic commensal microbiota, a 'superorganism' consisting of tens of trillions of microbes. The gut microbiota forms a unique relationship within the host and is 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). Intestinal microflora is directly involved in modulating the development and function of the ENS supporting the view that changes in intestinal microbial composition, particularly in early life, induced by infections or antibiotics, perturb ENS integrity favoring the onset of GI 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 (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 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 & Pachnis, 2015).

Among TLRs, TLR4 plays a well-established regulatory role in the innate immune response to infection and in adaptive responses consenting probiotic bacteria colonization. To date, several studies have evaluated the role of TLR4 in gut mucosa, mainly in colon, whereas only few have explored the impact of TLR4 in the ENS of small intestine (Anitha et al., 2012; Kabouridis & Pachnis, 2015). Although polymorphisms in TLR4 gene affecting LPS signaling have been described in patients with chronic IBD, their pathophysiological relevance in neuroimmune cross talk is still unclear (Cario, 2010). In this regard, ENS appears to be directly involved in modulating the inflammatory response, since it expresses several TLRs, including TLR4, in enteric neurons and glial cells (Rumio et al., 2006; Sharkey & Savidge, 2014). In human LMMPs and in rat ENS primary cultures, neural activation with EFS or ATP has been shown to inhibit LPS-induced TNF-α production through enteric neuronal P2X7R (Coquenlorge et al., 2014). However, it is becoming clear that TLR4 overstimulation by periodic intestinal infections, or its under stimulation following excessive use of antibiotics have the potential to affect the balance between ENS-microbial-derived products early in life, setting the basis for developing gastrointestinal functional disease (GFD) in adulthood (Becattini et al., 2016). In the first part of this study, for the first time, we show the role of TLR4 on ENS structural and functional integrity and provide relevant insights into the underlying mechanisms. In particular, we demonstrate that the absence of TLR4 results in: (i) altered distribution of the enteric glial markers GFAP and S100β; (ii) decreased total number of HuC/D^{+} neurons; (iii) an altered $nNOS^{+}$-to-VIP^{+} neuron ratio; (iv) impaired tonic cholinergic excitation; (v) enhanced inhibitory neurotransmission mediated by the coordinated action of both NO (from nNOS and iNOS) and ATP through the interaction with the purinergic P2X7Rs and P2Y1Rs. Previous immunohistochemical analyses have shown the presence of TLR4 in gut neuromuscular layers, as well as in sensory dorsal root ganglia, suggesting that both intrinsic and extrinsic neuronal circuits possess the machinery to respond directly to microbiota-derived stimuli (Barajon et al., 2009). Indeed, our findings showed that TLR4 deficiency does affect the distribution of S100β and GFAP, specific markers for EGCs (Gulbransen & Sharkey, 2012; Neunlist et al., 2014). Recently, the role of EGCs has started to emerge not only as a mechanical support for enteric neurons but as cellular integrative bridge of gut homeostasis involved in

controlling neuroplasticity, mucosal barrier and inflammatory responses by releasing specific gliomediators (e.g., NO, ATP) (Gulbransen & Sharkey, 2012; Neunlist et al., 2014). Considering that the pathophysiological functions of GFAP and S100β in the ENS are still under discussion and that TLR4$^{-/-}$ mice showed no changes in mucosal permeability and inflammatory markers (as shown by us and others) (Peterson et al., 2010; Devaraj et al., 2011), the increases in these regulatory and structural proteins advocate for the presence of an underlying gliopathy in absence of TLR4 signaling. In support of this premise, increases in GFAP expression are associated with EGCs differentiation, inflammation, and injury (Ochoa-Cortes et al., 2016); S100β expression and release by EGCs at μM levels are linked to pathological conditions (Rühl, 2005); and EGCs gliosis, detected by increased GFAP levels and/or S100β immunoreactivity/release, has been reported in ulcerative colitis, microbial infection and neurodegenerative diseases (Ochoa-Cortes et al., 2016). Lipopolysaccharide hyporesponsiveness and delayed GI transit were reported for Tlr4Lps-d mice on a C3H/HeJ background. However, our study was performed at earlier time points (30 min vs. 4 h) following non-absorbable FITC-dextran administration to evaluate the involvement of the small intestine in the delayed transit. Although the reduced pellet frequency and water content may resemble the same colon dysmotility previously described in Tlr4Lps-d mice (Anitha et al., 2012), our mouse model has a spontaneous mutation that results in a complete loss-of-function of TLR4, whereas the Tlr4Lps-d mice have a point mutation causing an amino acid substitution. In our study, no differences in high potassium-induced contraction were revealed, suggesting that TLR4 deficiency does not influence smooth muscle function (Ratz et al., 2005). However, the downward shift of the concentration-response curve to both carbachol and EFS in TLR4$^{-/-}$ preparations may be explained by the different contributing triggers to the onset of the reduced excitatory neuromuscular response, such as impaired cholinergic neurotransmission (e.g. differences in muscarinic and/or nicotinic receptor activity), higher level of inflammatory mediators or enhanced inhibitory non-adrenergic non-cholinergic transmission. By expressing TLR4 and, thus, being sensitive to LPS, enteric neurons appear to be involved in the regulation of the innate tolerance response to microbial-derived products in the intestine, ensuring a balanced immune response with respect to luminal content. Both increased and decreased GI motility have been reported after LPS exposure, depending on the dose, timing between injections and assessments of GI motility, the region of the GI system that is investigated and the type of LPS (Bashashati et al., 2012). Low-grade

inflammation in the gut can alter digestive motility, through changes in the functions of enteric nerves and/or smooth muscle cells, thus highlighting a pathophysiological relationship between bowel inflammation and abnormalities in enteric motor activity (Kabouridis & Pachnis, 2015). However, recent studies have detected no differences in the levels of several inflammatory markers measured in serum and in peritoneal macrophages in TLR4$^{-/-}$ animals compared to C57BL/6J mice (Hritz et al., 2008; Zhang et al., 2008; Devaraj et al., 2011). Alterations in tachykinergic pathways have been shown in functional GI disorders (Margolis & Gershon, 2009; Corsetti et al., 2015). Under NANC conditions, post-stimulus excitatory responses with L-NAME showed no differences between genotypes, consistent with a lack of TLR4 involvement in tachykinergic pathways also confirmed by immunohistochemistry of SP. Since under certain conditions cholinergic nerve activity can be depressed (Anitha et al., 2006), the observed marked NANC-mediated relaxation indicates that impaired cholinergic neurotransmission results from an enhanced inhibitory control on cholinergic and noradrenergic transmission. The main inhibitory neurotransmitter NO can be generated by the three different enzymes, nNOS, eNOS, and iNOS. More than 90% of the total NOS in the small intestine is nNOS, localized in inhibitory neurons. However, iNOS isoform is also constitutively present and accounts for less than 10% of the total enteric NOS activity whereas eNOS isoform is barely detectable (Lu et al., 2006). In case of inflammation the induction of iNOS produces a large amount of NO with consequent intestinal dysmotility (Eskandari et al., 1999). NANC-mediated relaxation was increased in TLR4$^{-/-}$ preparations and was mediated by NO produced by iNOS and nNOS, whereas in WT mice the inhibitory tone was mainly dependent on nNOS-derived NO. Moreover, TLR4$^{-/-}$ myenteric ganglia contained a reduced number of HuC/D^{+} neurons, associated to a proportional reduction of nNOS^{+} neurons in agreement with our functional findings and as previously shown by Anitha et al. (2012) in whole mount preparations. However, we found that this reduction in nNOS^{+} neurons was accompanied with a proportional increase of VIP^{+} neurons. VIP not only acts as a neurotransmitter but also plays a role in neuroprotection and functions as an anti-inflammatory agent (Ekblad & Bauer, 2004). These adaptive changes in the proportion of VIP^{+} and nNOS^{+} neurons with no modifications in SP-containing nerves appear to be phenotypic characteristics of ENS resembling those found in diabetic neuropathy (Voukali et al., 2011). At the functional level, the loss of TLR4 appears to influence the nitrergic pathway engaging other inhibitory transmitters responsible for the increased NANC relaxations. Our findings of

gliosis and enhanced inhibitory tone, sensitive to both L-NAME and suramin (a non-selective P2 receptor antagonist), support the involvement of ATP in the ENS dysfunctions of TLR4$^{-/-}$ mice. ATP is known to play important roles in gut function as well as in inflammation, since its P2 receptors are widely distributed in neurons, glia, smooth muscle, and immune cells (Burnstock, 2014). In mouse ileum, pharmacological studies (Giaroni et al., 2002; Gallego et al., 2014) have shown that in physiological conditions ATP modulates relaxation by acting via P2Y1Rs, the main receptor subtype mediating NANC inhibitory responses, partly by direct action on smooth muscle and partly by activating nNOS^{+} neurons that release ATP and NO. In the presence of inflammation, an overproduction of ATP activates the low affinity P2X7Rs, contributing significantly to activating inhibitory nitrergic neurons (Hu et al., 2001; Antonioli et al., 2014). In this respect, it has been proposed that ATP released by enteric neurons can activate EGCs, which in turn through Ca^{2+} signals and release of gliotransmitters (e.g., ATP, glutamate, among others), communicate with other EGCs and neurons, influencing gut contractility (Ochoa-Cortes et al., 2016). Here, we found higher amplitude of relaxation to ADP (the P2Y1Rs endogenous ligand) and NANC-mediated responses sensitive to P2Y1 and P2X7 inhibition, together with an increase in immunoreactivities of P2Y1Rs, P2X7Rs and iNOS in TLR4$^{-/-}$ myenteric plexus. Our functional results support the notion that TLR4 deficiency influences ATP neurotransmission, activating myenteric P2X7Rs and P2Y1Rs and determining increased smooth muscle relaxation and iNOS-derived NO production potentially by enteric neurons and EGCs. Recent studies so far have shown the involvement of TLR4 in the modulation of enteric neural stem/progenitor cells, and of neural survival (Anitha et al., 2012; Schuster et al., 2014), our work provides the first evidence of a cross-talk between TLR4 and nitrergic/purinergic pathways in enteric neural-glial communication (Rumio et al., 2006; Brown et al., 2015). Specifically, our study advocates for a new scenario in the ENS, where in the absence of TLR4, ATP pathways cooperate with nitrergic neurotransmission through P2Y1Rs and P2X7Rs as recently demonstrated by Brown et al. (2015).

Moreover, TLR4 is highly expressed in the CNS and it has been reported that this receptor has a critical role in neuroinflammation and brain injury (Trotta et al., 2014). Recently, TLR4, in the brain, has been also recognized as a modulator of neuronal survival during sterile injuries (Mollen et al., 2006), playing a role in CNS plasticity (Okun et al., 2009), learning and memory (Okun et al., 2012) and cognitive dysfunction in pathological

settings (Pascual et al., 2011). Evidence for the expression of TLRs in CNS along with their role as key sensors of not only DAMPs but also of physiological factors (e.g., extracellular matrix components such as fibronectin, heparan sulphate, fibrinogen, oligosaccharides of hyaluronan; Yu et al., 2010), strongly suggest that TLRs have a critical role in the nervous system beyond their involvement in controlling host immune responses (Okun et al., 2012). Given the location of TLRs in the nervous system and that their activation by endogenous ligands may underlie their role as key players in regulating neurodevelopment and neuroplasticity (Okun et al., 2012). *Rolls et al*. demonstrated that TLRs signaling play a crucial role in controlling postnatal neuronal plasticity as it has been implicated in differentiation of neural stem/progenitor cells (NPC) into neurons in the hippocampus area (Rolls et al., 2007). Accordingly, we addressed this study by testing the hypothesis that TLR4 signaling is required for the maintenance of neuronal-glial population in the hippocampus. In line with these studies, our data supports the idea that TLR4 sustains neuron homeostasis, since a reduction in the number of HuC/D^{+} neurons in the hippocampus of $TLR4^{-/-}$ mice was found. This is consistent with a study showing that NPC isolated from a mouse model identical to ours showed enhanced neuronal differentiation and proliferation but with lower survival of newly formed neurons (Rolls et al., 2007). In parallel, we examined the impact of TLR4 absence in the glial network of $TLR4^{-/-}$ mice. Astrocytes and microglial cells are the predominant non-neuronal parenchymal cells in the brain and are essential for maintaining CNS homeostasis (Hertz et al., 1990). They are crucial for the induction of innate immune responses within the brain to protect neuronal cells from invading pathogens (Olson & Miller, 2004). Recent studies have demonstrated that both CNS astrocytes and microglial cells express TLRs (Bowman et al., 2003; McKimmie & Fazakerley, 2005). In our model anomalies in astrocyte distribution, as shown by the distribution of GFAP immunoreactivity, were associated with no difference in microglial network. However, it remains to elucidate the mechanisms by which TLR4 signaling influences the formation and plasticity of neurons as well as glial network considering TLRs involvement in the control of immunity and inflammatory responses.

Beside TLR4, another well-established sensor of PAMPs is TLR2. TLR2 is known to strengthen the intestinal epithelial cell barrier and prevent bacterial entry and inflammation by modulating immune response (Rakoff-Nahoum et al., 2004; Round et al., 2011). 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). Furthermore, the expression of TLR2 suggest a role in the neuronal survival in the ENS (Brun et al., 2013), like TLR4 as reported by us and by the group of *Anitha et al*. (Anitha et al., 2012; Caputi et al., 2017a). Beside to the well-known role of TLRs in sensing molecular profiles derived from pathogens or commensal microorganisms, they also recognize endogenous molecular patterns derived from DAMPs, thus helping the repair of host tissue following inflammation. Oxidized phospholipids (OxPLs), a 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 TLRs signaling in immune cells in both in vitro and in vivo. In particular, 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine (OxPAPC) has been demonstrated to inhibit both TLR2- and TLR4-MD2-dependent signaling pathways but not those activated by other TLRs, due to its specific binding to serum accessory proteins such as LBP and CD14 (Bochkov et al., 2002). This interaction competitively inhibits the interaction of bacterial-derived ligands (such as LPS) to both TLR2 and TLR4 (Erridge et al., 2008). Thus, we evaluated the effect of OxPAPC-mediated inhibition of TLR2 and TLR4 on juvenile ENS integrity and intestinal motility. It's well known that changes in physiological functions (i.e. contractility, gastrointestinal transit and mucosal secretion) and in expression levels of neuronal markers occur during ageing. Since TLR4 deficient mice show significant alterations in ENS structure and function compared to age-matched WT mice and very young mice are more prone to age-dependent modifications, we treated mice aged 3 weeks with OxPAPC to better identify the impact of TLR2 and TLR4 signaling on gut ENS integrity during adolescence. The structural evaluation by means of whole mount immunohistochemistry, performed on ileal LMMPs preparations, evidenced anomalies in myenteric ganglia, denoted by lower number of HuC/D^{+} neurons in OxPAPC-treated mice, associated with altered distribution of the glial proteins GFAP and S100β. The correct identification of neurons is critical to investigate and understand morphological and functional changes that may occur during health and disease (Thacker et al., 2011; Desmet et al., 2014). HuC/D protein allows the quantification of individual neurons and is commonly used as a neuronal marker both in central and in the peripheral nervous system. Similar to the CNS astrocytes, EGCs 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). Recently, it has

been demonstrated that aberrant expression and release of the glial functional protein S100β correlate with the gut inflammatory status (Esposito et al., 2007; 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 and GFAP immunoreactivity (Eng et al., 2000). Our findings indicate that the OxPAPC-mediated TLR2 and TLR4 inhibition induces anomalies in both structural and regulatory proteins of neurons or EGCs, affecting the neuronal and glial developmental homeostasis in the ENS. The morphological abnormalities observed in the ENS of OxPAPC-treated mice were associated with impaired gut motor functions. In juvenile mice treated with OxPAPC for 3 days, the receptor-mediated response to carbachol together with the neuromuscular contractions elicited by EFS were significantly increased, however, no changes in ChAT immunoreactivity was found. Since the gut motility is defined by a correct equilibrium between contractions and relaxations and considering that the major excitatory neurotransmission, like the cholinergic system, is affect by the OxPAPC-treatment, another primary neurotransmission involved in regulating motility, in terms of inhibitory control, is the nitrergic one. In agreement with previous reports (Serio et al., 2003), EFS-induced NANC stimulation at 10 Hz was abolished by the NOS inhibitor L-NAME, indicating its nitrergic origin. An impaired NO-mediated relaxation in ileal preparations of OxPAPC-treated mice was accompanied by a loss of nitrergic modulation in intestinal contractility, as shown by a reduced proportional number of $nNOS^{+}$ neurons in myenteric ganglia. Considering that gut−brain disease paradigms can exist (Natale et al., 2011), the structural defects of neurofilaments expressed in the ENS appear to be phenotypic characteristics resembling those found in neurodegenerative disorders of the central and peripheral nervous system (Liem & Messing, 2009). Reduced expression of HuC/D, a RNA-binding protein stabilizing several key transcripts (eg, acetylcholine esterase, neurofilament M, etc) has been described in diabetic neuropathy (Deschênes-Furry et al., 2007). In addition, the expression of S100β and GFAP, specific markers for EGCs (Cirillo et al., 2011a), is significantly affected in gut neuropathies associated with metabolic and inflammatory diseases (Savidge et al., 2007; Liu et al., 2010). Although the pathophysiologic relevance of the observed alterations in these regulatory and structural proteins is still unknown in the ENS, they clearly indicate the presence of an underlying gut neuropathy induced by OxPAPC treatment that appears to have structural and

functional defects mostly similar to those found in TLR2-deficient mice (Brun et al., 2013). Considering that *Latorre* and colleagues, have demonstrated in a model of double knockout (KO) mice for TLR2 and 4 receptors (TLR2/4 DKO) that the deficiency of TLR2 and TLR4 expression modify the pattern of expression of 5-HT receptors as well as the extent of the involvement of these receptors in the enteric motor responses to 5-HT (Forcèn et al., 2015), we evaluated the effect of the acute pharmacological treatment with OxPAPC in juvenile mice on serotonergic pathways. In accordance with previous studies in ileum and colon of TLR2/4 DKO mice (Forcèn et al., 2015 & 2016), we found that the block of TLR2 and TLR4 signaling affected the pattern of myenteric distribution of SERT and both 5-HT_{2A} and 5-HT_3 receptors. An interesting and very recent finding is that both human microbiota and conventional mouse microbiota can overcome the 5-HT_3-mediated response seen in GF animals, suggesting the involvement of a common effect of commensal microbiota regardless of host species (Bhattarai et al., 2017), potentially through TLRs signaling. *Gershon* was the first to reveal the existence of serotonergic neurons and their critical role in regulating constitutive GI motility (Gershon, 2013). The serotonergic system plays a pivotal role in the ENS and 5-HT has been shown to be an essential intestinal physiological neuromodulator of motility, secretion and visceral sensitivity by acting on specific receptors, expressed on enteric neurons (Cirillo et al., 2011b). In the ileum of OxPAPC-treated mice, the 5-HT-evoked contractile effect resulted significantly increased, suggesting the presence of an interactive dialogue between TLR2 and TLR4 receptors and the intestinal serotonergic neurotransmission, since they are both expressed in a variety of intestinal cell populations, including neurons, glial cells, and smooth muscle cells (Barajon et al., 2009; Filippova et al., 2012; Brun et al., 2013). Moreover, it has been recently reported that the activation of TLR3 and TLR4 may modulate the serotonergic system in intestinal epithelial cells (Mendoza et al., 2009; Mendoza et al., 2012). The 5-$HT_{2,3,4,7}$ receptors are involved in the modulation of intestinal motor function (Barbara et al., 2005). In the ileum of OxPAPC-treated mice, 5-HT-evoked a contractile response in presence of the 5-HT_{2A} receptor antagonist comparable to that obtained from control preparations, indicating an impaired 5-HT_{2A} receptor-mediated signaling (Wang et al., 2011). The amino acid TRP together with its metabolite 5-HT is essential not only for gut homeostasis but also for ensuring human body health and any alterations on these factors are linked to a variety of diseases (O'Mahony et al., 2015). OxPAPC treatment determined a significant increase of metabolite 5-HTP associated with a reduction of 5-HT levels in ileal tissue to suggest that

blocking TLR2 and TLR4 signaling affects TRP metabolism. However, the dominant physiological pathway for TRP is actually along the KYN pathway (Kennedy et al., 2017). This pathway is composed by multiple immunoresponsive enzymes tightly regulated by inflammatory mediators (Campbell et al., 2014). The main enzyme that catalyzes TRP conversion into KYN is the IDO, and its activity can be induced by inflammatory cytokines in various immune cells including mast cells (Ciorba, 2013). TRP and KYN metabolites play a role in intestinal immunity and inflammation (Kennedy et al., 2017). Following OxPAPC treatment, a slight but not significant increase of KYN levels in ileal tissue was detected, to underline an involvement of TLRs signaling in 5-HT availability versus KYN metabolism. The downstream metabolites of KYN, quinolinic and kynurenic acid are of particular interest for neurogastroenterology due to their excitotoxic and neuroprotective role in the CNS, respectively. However less is understood regarding their function in the GI tract but both metabolites appear to be involved in immunoregulation (Keszthelyi et al., 2009). Recent studies have focused on the role of TRP metabolism in IBS, with a preferential activation of KYN pathway in TRP metabolism that may represent a key mediator of the altered immunoregulation in IBS (Clarke et al., 2009; Georgin-Lavialle et al., 2016). Overall these data highlight the importance of a correct interaction between both TLR2 and TLR4 and microbiota-derived ligands, which are required for preserving the integrity of the ENS in terms of structure, function and TRP pathways.

In the last part of this thesis, since recently an increasing evidence of microbiota role in a large number of intestinal and extra-intestinal diseases has become steadily apparent (Thursby & Juge, 2017), we evaluated the effect of antibiotic-induced dysbiosis in juvenile mice. In a previous study my group demonstrated a marked reduction of the fecal bacterial load in mice after administration of the same antibiotic cocktail used in this study (Brun et al., 2013), however, according to other studies (Reikvam et al., 2011; Caputi et al., 2017) this in vivo treatment may induce an altered microbial composition. The gut microbiota has co-evolved with the host over thousands of years to confer many benefits to intestinal physiology and form a truly mutualistic relationship with the host (Hooper & Gordon, 2001). However, changes in intestinal microbiota, as a result of an altered microbial composition, known as dysbiosis, may have major consequences for human health. Importantly, childhood and adolescence are the most dynamic periods of change in relation to microbiota and nervous system development (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). 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 also 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 bacterial infections, also perturbs commensal microbial communities and decreases host resistance to antibiotic-resistant microbes (Ubeda & 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). In our study we assessed the effects of gut dysbiosis 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 adolescent mice by oral gavage for 14 days (ABX mice; Reikvam et al., 2011; Brun et al., 2013; Caputi et al., 2017b). 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 neural 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, evidenced 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 LMMPs preparations, suggesting a link between gut-dysbiosis and visceral hypersensitivity mediated by the glutamatergic transmission in the enteric neuronal circuitries. Another key player in pain perception is represented by TRPV1 receptor, also known as the capsaicin receptor or the vanilloid receptor, that it has been reported upregulated in IBS (Holzer, 2008a, b). Abnormalities in TRPV1 mRNA

transcripts were found in mice treated by antibiotics. Hyperalgesia is characterized by altered receptor sensitivity in the gut mucosa, submucous and myenteric plexa compartments and can be triggered by mucosal inflammation, degranulation of mast cells closes to enteric nerves, increased release of neuropeptides and neurotransmitters and possibly by altered microbial environment or infection (Spiller, 2003; Barbara et al., 2004; Dunlop et al., 2005; Verdù et al., 2006). In different dysbiosis models, elicited by antibiotic treatment or induced by either dietary changes or genetic deficiency (i.e. TLRs deficiency), a direct or indirect effect of microbiota in gut motor and sensory functions has been reported (Quigley, 2011; Brun et al., 2013). In parallel, antibiotic treatment increased SP immunoreactivity in ileal neuromuscular compartment and SP-mediated response of small intestine. SP has been described as an enteric neurotransmitter involved in the modulation of the enteric motor and sensory functions, and its intestinal content may be affected by bacteria perturbation or low-grade inflammation resulting from antibiotic treatment (Verdú et al., 2006). Previous studies have reported increases in enteric SP following experimental colitis (Miampamba & Sharkey, 1998) or exposure to inflammatory insult. In our model of dysbiosis, changes in sensory neurotransmitter content may due to increased inflammation, however, the concept of a direct communication between commensals and the enteric nervous system was suggested by *Hooper* et al in which expression of genes encoding enteric neural transmission differed in GF and colonized mice (Hooper et al., 2001). Several studies have shown the ability of the gut microbiota to impact the serotonergic system (O'Mahony et al., 2015); however, a recent study has highlighted the key role of gut microbiota in influencing 5-HT content (Spohn & Mawe, 2017). Specifically, indigenous spore-forming bacteria from mouse and human microbiota have been shown to promote 5-HT biosynthesis from colonic ECs (Yano et al., 2015). Furthermore, considering that in mouse colon antibiotic treatment has been pointed out to alter 5-HT neurotransmission and GI motility (Ge et al., 2017), we next asked whether the gut dysbiosis induced by antibiotic treatment could affect ileal response to 5-HT. In ABX-treated mice 5-HT evoked a higher relaxation response compared to control. All the 5-HT receptor antagonists tested in this study partially blocked the relaxation response to 5-HT in either group of mice, indicating that $5HT_{2A}$ and $5HT_3$ receptors are contributing to the response evoked by 5-HT in small intestine longitudinal preparations. Since the inhibition of $5\text{-}HT_3$ receptor determined 5-HT-dependent response curves, comparable between control and ABX-treated mice, it is feasible an involvement of these receptors in the increased neuromuscular relaxation by

antibiotic-induced dysbiosis. Moreover, in ABX-treated mice, changes in ENS neurotransmitter pathways induced small bowel motor disturbances resulting in altered gut neuromuscular contractility and motility (Caputi et al., 2017b). In parallel, ABX-treated mice showed higher myenteric immunoreactivity of $5HT_{2A}$ and lower immunoreactivity of SERT compared to control mice, highlighting the primary role of microbiota on ensuring a correct serotonergic neurotransmission. A balanced cross talk between the microbiota and the host in TRP utilization and metabolism may also being implicated not only in ensuring gut integrity but also for the homeostasis of human body and any alterations may contribute to develop a variety of diseases (O'Mahony et al., 2015). Emerging data reported the ability of gut microbes to regulate host 5-HT biosynthesis (Yano et al., 2015) since a reduction of the 5-HT serum concentration was associated to a morphological enlargement of intestinal ECs in GF animals (Wikoff et al., 2009; Uribe et al., 1994). Following antibiotic treatment, increased TRP plasma levels were found as previously shown in GF animals which were then normalized by mouse microbial colonization immediately post-weaning (Clarke et al., 2013). Along the TRP metabolic pathway, 5-HTP resulted reduced in ileal tissue after antibiotic treatment, suggesting a reduced availability of this metabolite for 5-HT synthesis. The influence of gut microbes on TRP availability and 5-HT synthesis have been evidenced also by evaluating the activity of the enzyme IDO, responsible for TRP degradation along the KYN pathway. Previous studies in GF mice have shown an implication of KYN pathway with a decrease in the ratio of KYN:TRP (used as an index of IDO activity) which normalizes to levels similar to those found in conventional animals, following introduction of a gut microbiota immediately post-weaning (Clarke et al., 2013.). In our study a lower IDO enzymatic activity was found after antibiotic treatment. In this context, it is also interesting to note that TLRs activation by microbial derived-molecule can lead to alterations in IDO expression, suggesting an involvement of TLRs in the control of KYN pathways (Clarke et al., 2012). Another crucial point reported by several studies, carried out in several animal models, is the bottom-up effects of the enteric microbiota on the development and maturation of the ENS (Hyland & Cryan, 2016). However, enteric neurons and glial cells can sense microbes through TLRs (Anitha et al., 2012; Brun et al., 2013) and other neurochemical receptors as well as through microbial-derived components, microvescicles, neuroactive metabolites or mucosal elements (e.g. enteroendocrine-cell derived mediators) (Hyland & Cryan, 2016). Most of these studies showed that perturbations of microbial communities may affect postnatal ENS

development, inducing adaptive changes in both enteric neurons and glia (Kabouridis et al., 2015). In our study, microbiota dysbiosis after antibiotic treatment during early adulthood affected the assembly of enteric neural circuits, reducing the number of neurons in the myenteric plexus, in analogy with results obtained in GF mice, TLRs knockout mice or autoimmune disorders (i.e. TLRs overstimulation) (Okun et al., 2009; Brun et al., 2013; Collins et al., 2014; Caputi et al., 2017b). In addition, distortion of $GFAP^{+}$ processes and S100β overexpression are highly indicative of myenteric glial network alterations, which may underlie gut gliopathy due to a disrupted host–bacteria crosstalk. Besides exerting a trophic and functional support to enteric neurons, EGCs have been identified as key cellular targets for intestinal microflora. However, in this latter regard, the molecular mechanisms by which luminal microbiota shapes the organization of enteric neuroglial networks and its potential implication in GI disorders remains to be elucidated (Turco et al., 2014; Kabouridis et al., 2015). Due to the emerging role of microbiota on gut activity and organization, and its potential relevance on gut functional disorders, further studies are needed to fully disclose the modulatory role of gut microbiota-derived factors on specific neurotransmission pathways and related effects on motility and pain perception, which may possibly predispose to gut disorders or preserve ENS developmental integrity.

6. CONCLUSIONS

Our study highlights for the first time a novel role for TLR4 in ileum, demonstrating that TLR4 fine-tunes ENS circuitry modulating the inhibitory component of neuromotor activity by NO and ATP co-transmission, essential for maintaining a proper bidirectional neural-glial communication. Our findings provide the basis for a better understanding of the mechanisms underlying GI dysmotility in presence of an anomalous neuroimmune crosstalk, thereby paving the way for the development of suitable pharmacological modulators of TLR4 signaling for the management of functional GI disorders (Caputi et al., 2017a). Moreover, our data support the view that the acute inhibition of TLR2 and TLR4 signaling affects: i) enteric neuronal network, ii) neuromuscular contractility and 5-HT-induced contraction of isolated ileal preparations, iii) 5-HT_3 expression and 5-HT_3-mediated response of myenteric plexus of mouse small intestine, iv) TRP metabolism, determining increased IDO enzymatic activity with consequent increased KYN and lower 5-HT levels in ileal tissue, and possibly neurodegeneration. The components of TRP metabolism may represent a potential marker of the presence of an aberrant TLR2 and TLR4 signaling (e.g. gut dysbiosis, TLR polymorphisms). Thereby targeting the TRP pathway may exert beneficial effect in IBS patients in presence of gut dysbiosis. In conclusion, our findings demonstrated that perturbations of microbiota composition and consequent dysregulated host-bacteria interaction during early adulthood were critical as they significantly altered ENS neurotransmission and potentially established a phenotype susceptible to IBS and/or IBD (Brun et al., 2013; Ungaro et al., 2014; Barbara et al., 2016). In addition, our data also suggested that a 2-week high-dose course of antibiotics in juvenile mice represents a practical model for evaluating the effect of gut commensal bacteria on the maturation of juvenile ENS and visceral sensitivity, without affecting the original postnatal gut microbiota composition, which is inevitable in GF mice.

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