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Toll-like receptor 4, enteric nervous system and gut neuromuscular function in models of functional and inflammatory bowel disorders

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RIASSUNTO

L'interazione fra costituenti della parete intestinale e microflora commensale costituisce il principale artefice di mantenimento della barriera mucosale, di promozione dello sviluppo dell'apparato digerente e di modulazione delle attività gastrointestinali, quali motilità, secrezione, immunità mucosale e sensibilità viscerale. Un'alterata microflora è stata associata a diversi disordini gastrointestinali (malattie infiammatorie croniche intestinali, MICI o sindrome dell'intestino irritabile, IBS) mentre cambiamenti del microbioma intestinale durante le fasi dell'infanzia e dell'adolescenza, causati da infezioni o antibiotici, predispongono all'insorgenza di queste malattie. Inoltre, disfunzioni del SNE quali anomalie strutturali e/o variazioni nel contenuto di neurotrasmettitori, sono state associate all'insorgenza sia di MICI che di IBS. In questo contesto, giocano un ruolo chiave i recettori Toll-like (Toll-like receptors, TLRs), un sofisticato sistema di proteine che mediano la risposta infiammatoria contro agenti patogeni e attivano segnali benefici assicurando l'integrità tissutale in condizioni fisiologiche e patologiche. Polimorfismi nei geni che codificano i TLRs, inclusi il TLR2 ed il TLR4, sono stati associati a fenotipi diversi di malattia in pazienti affetti da disordini gastrointestinali.

In questa tesi di dottorato si sono valutate le alterazioni strutturali e funzionali del SNE murino in assenza del segnale mediato dal recettore dell'immunità innata TLR4 in modelli di: i) inibizione *ex vivo* dell'attività delle cellule gliali enteriche; ii) obesità, indotta da una dieta ad alto contenuto di grassi; iii) colite sperimentale tramite somministrazione *in vivo* di sodio destrano solfato.

Dapprima è stato approfondito il ruolo del recettore TLR4 nel mantenimento della funzione intestinale effettuando esperimenti di contrattilità in vitro utilizzando preparati di ileo provenienti da topi C57BL/6J WT esposti ad un cocktail di antibiotici per indurre la deplezione del microbiota e da topi $TLR4^{-/-}$ di pari età (9 ± 1 settimane). Gli effetti indotti dalla deplezione indotta dal cocktail di antibiotici sono simili a quelli evidenziati dall'assenza di segnale del recettore TLR4 sulla funzione gastrointestinale, come ad esempio una riduzione significativa della risposta contrattile eccitatoria di tipo colinergico accompagnata da un alterato rapporto di neuroni positivi all'ossido nitrico sintasi neuronale (nNOS) associata ad anomalie nella distribuzione ed espressione della subunità β della proteina S-100, marcatore gliale nucleare e citoplasmatico in grado di legare il calcio. Tali osservazioni sembrano confermare come la mancanza del microbiota intestinale, ed in particolare proprio del recettore TLR4, possa influenzare l'integrità della rete neuronale e gliale enterica. Data l'importanza di un corretto segnale del microbiota commensale nel mantenimento della rete nervosa e del codice neurochimico del SNE è stata valutata la funzione intestinale in vitro mediante esperimenti di contrattilità utilizzando la tecnica dell'organo isolato su segmenti di ileo provenienti da topi WT e $TLR4^{-/-}$. Grazie agli studi funzionali e di immunofluorescenza con microscopia confocale è stato possibile dimostrare che l'assenza del recettore TLR4 non solo determina una diminuzione significativa della riposta eccitatoria ma induce un marcato aumento della neurotrasmissione

inibitoria per l'azione coordinata di ossido nitrico (prodotto sia da nNOS che dalla NOS inducibile (iNOS)) ed ATP, per interazione col recettore purinergico P2Y1. Al fine di caratterizzare l'origine dell'alterato tono inibitorio dei preparati intestinali dei topi $TLR4^{-/-}$, esperimenti ex vivo con la gliotossina fluoroacetato hanno rivelato il ruolo primario dei recettori purinergici P2Y1 espressi nella glia enterica nel sostegno della trasmissione inibitoria. Pertanto, la valutazione dei topi deficienti per il TLR4 ha evidenziato il ruolo primario di questo recettore nella modulazione dell'interazione dei segnali sia neuronali che gliali del SNE per assicurare una corretta attività neuromuscolare intestinale.

Al fine di approfondire il ruolo del recettore TLR4 in condizioni infiammatorie lievi, sono stati valutati gli effetti dell'obesità indotta da una dieta ad alto contenuto di grassi (HFD; 60% kcal di lipidi) sull'integrità funzionale e morfologica del SNE in animali WT e $TLR4^{-/-}$. La dieta HFD determina, da un lato, depressione dell'attività eccitatoria colinergica, dall'altro, aumentato tono inibitorio con conseguente ridotto transito intestinale, associata a gliosi reattiva. Inoltre, è stato evidenziato che l'assenza di TLR4 protegge gli animali dall'incremento di peso e dalle relative anomalie neuromuscolari funzionali e strutturali indotte dalla dieta HFD, ad indicare il ruolo primario di questo recettore nel modulare gli effetti dannosi dell'obesità nel tratto gastrointestinale, come ad esempio la costipazione. Nei topi $TLR4^{-/-}$ la neurotrasmissione serotoninergica, mediata dai recettori 5-HT_3, è incrementata ed insensibile alla ketanserina, antagonista dai recettori 5-HT_{2A}, e non subisce variazioni in seguito alla dieta HFD a sottolineare l'influenza del segnale mediato dal recettore TLR4 nella modulazione della risposta serotoninergica di tipo neuromuscolare. Tali alterazioni sono state associate a livelli tissutali significativamente aumentati di serotonina e dei suoi metaboliti, triptofano e chinurenina, indotti dalla mancanza del recettore TLR4 ed ulteriormente potenziati dalla dieta HFD.

Infine, per valutare l'importanza dell'asse TLR-SNE nel mantenimento della funzione intestinale e nell'attività del SNE in condizioni infiammatorie, è stato valutato l'effetto del destrano sodio solfato, somministrato nell'acqua da bere, così da indurre la colite nei topi WT e $TLR4^{-/-}$. Questo modello di colite sperimentale, riconosciuto per la sua semplicità, riproducibilità e versatilità, offre l'opportunità di mimare i processi infiammatori coinvolti nello sviluppo della colite ulcerosa nell'uomo nonché studiare il coinvolgimento dell'immunità. La colite indotta da DSS ha determinato marcate alterazioni strutturali e del codice neurochimico del SNE, che si traducono in una aumentata risposta neuromuscolare eccitatoria in maniera più severa nei topi WT rispetto a quelli deficienti per il recettore TLR4. È stato evidenziato che nei topi $TLR4^{-/-}$ l'aumentata risposta alla serotonina si riduce in maniera marcata, ed è mediata solo dai recettori 5-HT_3 ma non dai recettori 5-HT_{2A}, nonché livelli tissutali significativamente ridotti di serotonina e chinerunina. Questo risultato evidenzia ulteriormente il ruolo del recettore TLR4 nella modulazione dei processi neuroimmunomediati, come quelli governati dal sistema serotoninergico, che quindi non è solo sotto il controllo del microbiota enterico ma anche dei recettori dell'immunità innata.

In conclusione, i risultati della presente tesi sottolineano il ruolo chiave del recettore TLR4 nella sottile modulazione della glioplasticità, risposta inibitoria neuromuscolare, mediata dalla produzione di NO da parte dell'enzima iNOS, e la motilità gastrointestinale in condizioni patologiche, come nel caso di un basso grado di infiammazione sistemica (ad esempio, obesità) o di un alto grado di infiammazione locale (ad esempio, MICI).

ABSTRACT

The interaction between the constituents of the intestinal wall and commensal microflora is essential for the maintenance of mucosal barrier, the promotion of digestive system development and the modulation of gastrointestinal (GI) activities, such as motility, secretion, mucosal immunity and visceral sensitivity. Alterations in the gut microflora composition have been associated to several GI disorders (e.g. inflammatory bowel disease, IBD, and irritable bowel syndrome, IBS) while changes in intestinal microbiome during childhood and adolescence, caused by infections or antibiotics, predispose at the onset of these diseases. Furthermore, dysfunctions of the enteric nervous system (ENS) such as structural abnormalities and/or changes in the content of neurotransmitters have been associated with the onset of IBD and/or 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 activates beneficial signals to ensure tissue integrity in physiological and pathological conditions. Polymorphisms in the genes encoding TLRs, including TLR2 and TLR4, have been associated with different disease phenotypes in patients with GI disorders. Therefore, in this study, we evaluated the structural and functional alterations of murine ENS in the absence of the signal mediated by TLR4, a receptor of innate immunity, in mouse models of: i) ex vivo inhibition of enteric glial cells activities; ii) obesity induced by a high fat diet (HFD); iii) experimental colitis by in vivo administration of sodium dextran sulfate (DSS).

Firstly, the role of the TLR4 receptor in maintaining intestinal function was investigated by performing in vitro contractility experiments, using ileal preparations from C57BL/6J WT mice exposed to a cocktail of antibiotics to induce microbiota depletion to be compared to mice deficient for TLR4 signaling. The effects provoked by the antibiotic-induced microbiota depletion are similar to those evidenced by the absence of TLR4 signaling on GI function, in particular, a significant reduction of the cholinergic excitatory contractile response accompanied by an altered ratio of neurons positive for the neuronal nitric oxide synthase (nNOS), associated to alterations in the distribution and expression of the glial S100β protein. These observations suggest that the lack of the intestinal microbiota, and, especially, the lack of TLR4 signaling may influence the integrity of the enteric neuronal and glial network. Given the importance of a correct commensal microbiota signal in maintaining the neuroglial network and the ENS neurochemical code, intestinal function was evaluated by in vitro contractility experiments using the isolated organ bath technique on ileal segments from WT and $TLR4^{-/-}$ mice. Thanks to functional and immunofluorescence studies with confocal microscopy, it was possible to demonstrate that the absence of the TLR4 signaling not only determines a significant decrease in the excitatory response but induces a marked increase in inhibitory neurotransmission mediated by both nitric oxide (produced both by nNOS than from the inducible NOS (iNOS)) and ATP, which mediated its actions through the P2Y1 purinergic receptor. In

order to characterize the origin of the altered inhibitory tone in intestinal preparations of TLR4$^{-/-}$ mice, ex vivo experiments with the gliotoxin fluoroacetate were performed revealing the primary role of P2Y1 purinergic receptors, expressed in the enteric glia, in support of inhibitory transmission. Therefore, the evaluation of TLR4$^{-/-}$ mice highlighted the primary role of this receptor in modulating the interaction of both neuronal and glial signals of the ENS to ensure correct intestinal neuromuscular activity.

In order to investigate the role of TLR4 in mild inflammatory conditions, the effects of obesity induced by high-fat diet (HFD; 60% kcal of lipids) on the functional and morphological integrity of the ENS were evaluated in WT and TLR4$^{-/-}$ mice. The HFD determines reduced cholinergic excitatory activity and increased inhibitory tone with consequent slower intestinal transit, associated with reactive gliosis. The absence of TLR4 protects mice from weight gain and the relative functional and structural neuromuscular anomalies induced by HFD, to highlight the primary role of this receptor in modulating the harmful effects of obesity in the GI tract, such as constipation. In TLR4$^{-/-}$ mice the serotonergic neurotransmission, mediated by 5-HT$_3$ receptors, is increased and insensitive to ketanserin, an antagonist of 5-HT$_{2A}$ receptors, and was not subjected to changes following HFD to underline the influence of the signal mediated by the TLR4 in the modulation of the neuromuscular serotonergic response. These alterations were associated to significantly increased tissue levels of serotonin and its metabolites, tryptophan and kynurenine, induced by the absence of the TLR4 signaling and further enhanced by the HFD.

Finally, we evaluated the importance of the TLR-ENS axis in maintaining ENS integrity in inflammatory conditions, obtained by inducing experimental colitis in WT and TLR4$^{-/-}$ mice. This model of experimental colitis, recognized for its simplicity, reproducibility, and versatility, offers the opportunity to mimic the inflammatory processes involved in the development of ulcerative colitis in humans and to study the involvement of immunity. DSS-induced colitis led to marked structural alterations in the neurochemical code of the ENS, which resulted in an increased excitatory neuromuscular response more marked in WT mice than in those deficient for TLR4. In TLR4$^{-/-}$ mice treated with DSS, the increased response to serotonin is markedly reduced and is mediated by 5-HT$_3$ receptors but not by 5-HT$_{2A}$ receptors. These changes were associated to a marked reduction of serotonin and kynurenine tissue levels. These results further highlight the role of the TLR4 receptor in the modulation of neuroimmuno-mediated processes, involving both the serotonergic system and the enteric microbiota.

In conclusion, our findings underline the key role of TLR4 signaling in ensuring gut homeostasis by finely tuning enteric glioplasticity, inhibitory neuromuscular response, mediated by iNOS-produced NO, and gut motility during pathological conditions, such as in case of low-grade systemic inflammation (e.g. obesity) or high-grade acute inflammation (i.e. IBD).

1. INTRODUCTION

1.1 Enteric Nervous System

In 1899, Bayliss and Starling discovered ‘‘the law of the intestine’’ which states: “*Local stimulation of the gut produces excitation above and inhibition below the excited spot. These effects are dependent on the activity of the local nervous mechanism*” (Bayliss & Starling, 1899), highlighting the characteristic neurohormonal features of gastrointestinal (GI) function. In other words, they revealed that the nervous system of the intestine comprises intrinsic neural circuitry necessary and sufficient for generating and coordinating intestinal motility, nowadays known as as peristaltic reflex (Bayliss & Starling, 1899; Hansen, 2003). The extensive intrinsic nervous system is defined as enteric nervous system (ENS), responsible of controlling gut functions independently from the central nervous system (CNS; Furness, 2012). For this reason, the gut is described as “second brain” or “little brain” (Goyal & Hrano, 1996). Moreover, further evaluations of enteric neurons revealed that the ENS is closely similar to the CNS (Furness, 2000). The ENS is the largest division of the peripheral nervous system (PNS; Chalazonitis & Rao, 2018) and it is extended from the esophagus to the anal sphincter within the GI system walls (Furness et al., 2014). The human ENS contains 200–600 million neurons, the same number of neurons composing the human spinal cord (Furness & Costa, 1987; Furness, 2006). The ENS is presumed to be a separate portion of the CNS with which it is in permanent communication through sympathetic and parasympathetic afferent (i.e. vagal, splanchnic and pelvic nerves) and efferent nerves (Harrington et al., 2010). On the other hand, the CNS is connected with the enteric neurons throughout the central autonomic neural network. Through these bidirectional connections the ENS provides neural control of all functions of the GI tract (Goyal and Hirano, 1996). The majority of the enteric neurons are dispersed into small ganglia, which are connected by bundles of nerve processes, forming the two major plexuses, identified as myenteric (or *Auerbach’s*) and submucous (or *Meissner’s*) plexus (**Figure 1.1**; Furness et al., 2014). The myenteric plexus (MP) lies between the longitudinal and circular muscle layers and extends along the entire gut. It primarily provides motor innervation to the two muscle layers and secreto-motor innervation to the mucosa, but there are numerous projections from the MP to the submucosal ganglia and to enteric ganglia of the gallbladder and pancreas. There are also a substantial number of projections from the myenteric neurons to the sympathetic ganglia (Goyal & Hirano, 1996). In large mammals, the submucosal plexus (SMP) consists of more than one layer: an inner network located at the abluminal (serosal) side of the muscularis mucosae and an outer (*Schabadasch’s*) plexus adjacent to the luminal side of the circular muscle coat (Hansen, 2003). Non-ganglionated plexuses also supply through all the layers of the gut (Costa et al., 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**).

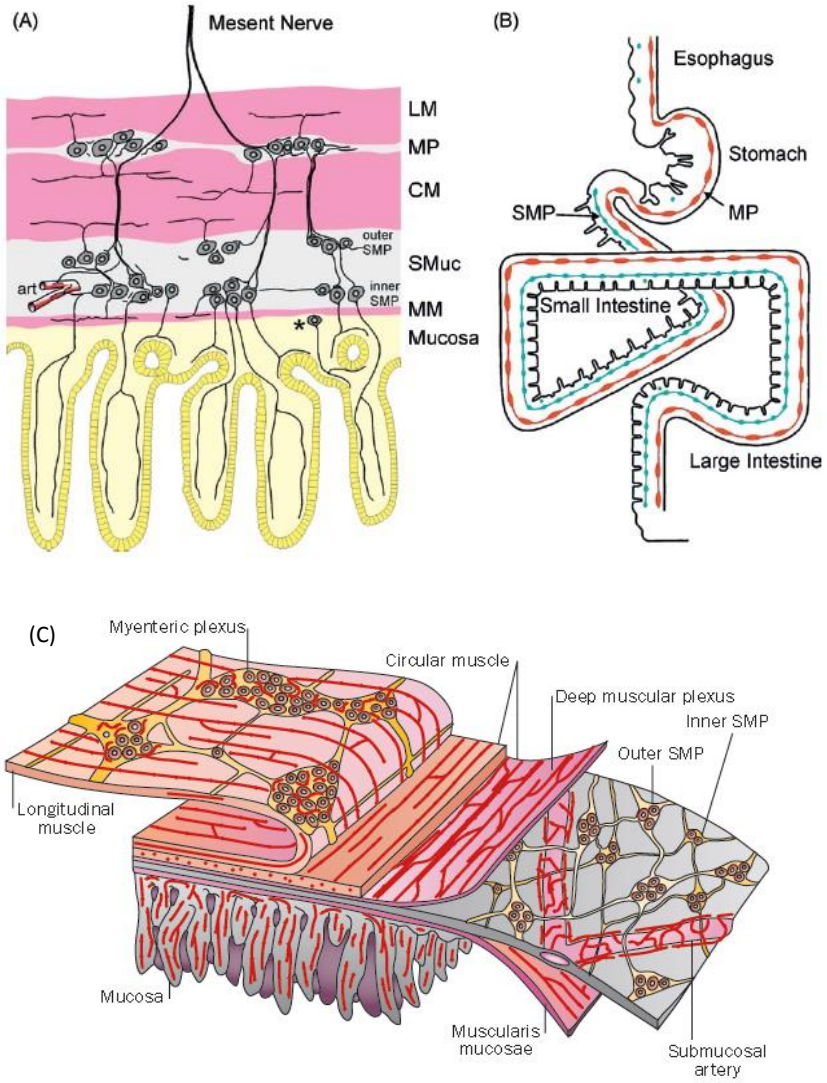


Figure 1.1 Organization of the ENS. **A)** In the small and large intestines, neural cells are contained in ganglia of the myenteric plexus (MP) between the longitudinal (LM) and circular muscle (CM) layers and in ganglia within the submucosa (SM), depicted in transverse section through the gut wall. 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 ENS provides GI homeostasis ensuring restorative, maintenance and adaptive functions. Enteric reflex circuits sense the physiological state of the GI tract, integrating informations and providing outputs to control motility, fluid exchange between the gut and its lumen, epithelial permeability and local blood flow (Gershon, 2005; Furness, 2006). Enteric neurons also interact with the immune and endocrine systems of the gut and all of these functions contribute to the maintenance of the integrity of the intestinal epithelial barrier (Mach, 2004; Furness, 2006; Savidge et al., 2007). In small animals, neurons of MP are mainly responsible for controlling different motility patterns, while SMP's neurons are involved in regulating secretion and absorption.

Alterations of ENS homeostasis heighten the development of enteric neuropathology, highlighting the importance of the ENS. Functional neuropathies have been grouped as sporadic and acquired diseases (e.g. Chagas disease or irritable bowel syndrome, IBS), congenital or developmental disorders (e.g. Hirschsprung disease), neuropathies associated with other disease states (which can be secondary to the other disorder, such as diabetic gastroparesis and other diabetes-related motility disorders), and iatrogenic or drug-induced neuropathies (e.g. postoperative ileus) and those associated with intestinal transplantation, such as neuropathology following ischemia and reperfusion injury (Furness, 2012).

In the ENS, 17 types of neurons have been identified and characterized according to their neurochemical coding, location, shape and morphology, cell physiology, functional roles and projections (Costa et al., 2000; Hansen, 2003). The morphological classification is based on shape and axonal projections created by Dogiel (1899), who was the first to illustrate and define 3 distinct types of enteric neurons: Dogiel types I, II, and III (or filamentous interneuron; Clerc et al., 1998; **Figure 1.2**). Type I neurons are uniaxonal and have short lamellar dendrites. Type II neurons have a large round or oval and smooth cell body, attached to multiaxon with long processes projecting circumferentially. Type III neurons appear as filamentous neurons with long branched processes. This classification is useful for distinguishing larger neurons but is not so clear for smaller neurons (Clerc et al., 1998).

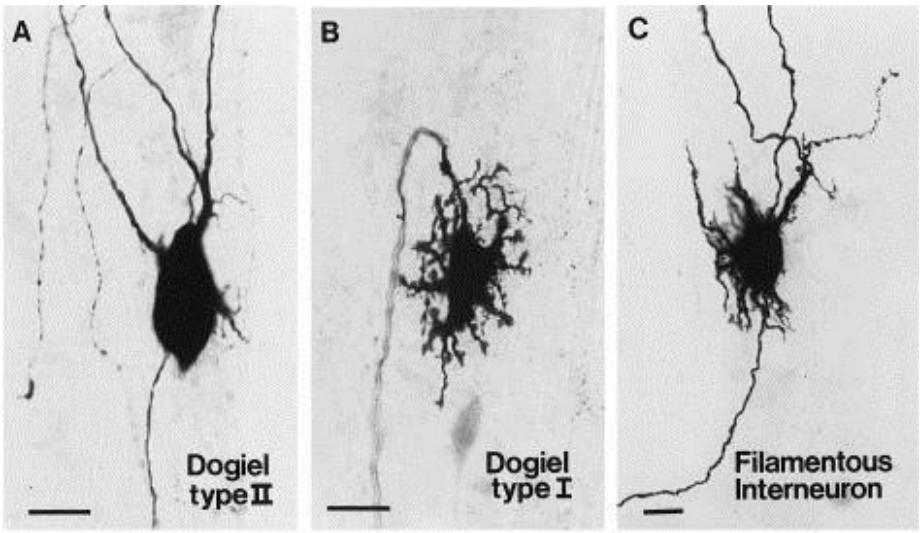


Figure 1.2 Examples of the morphology of a Dogiel type I, Dogiel type II and Filamentous Interneuron (modified from Clerc et al., 1998).

Another type of classification is based on the electrophysiological response. In the 1974, Hirst discovered 2 type of neurons based on differences in membrane potential events. AH (after-hyperpolarizing) neurons are characterized by “slow after-hyperpolarizing potential” (sAHP) whereas S (synaptic) neurons show “fast excitatory postsynaptic potentials” (fESPS). The last classification of enteric neurons depends on their functional proprieties, and they are distinguished in sensory neurons, interneurons and motoneurons. In turn, sensory neurons can be divided in 2 subgroups

intrinsic primary afferent neurons (IPANs) and extrinsic primary afferent neurons (EPANs). IPANs belong to AH type and are described as Dogiel type II. Chemical stimulation activates the chemosensitive IPANs, while the mechanoreceptor myenteric IPANs react to intestinal wall musculature's contraction through mechanosensitive ion channels (Kunze et al., 2000). Sub-mucosal IPANs respond indirectly to mucosal distortions through enterochromaffin cells that release serotonin (5-HT) after mechanical stimuli. EPANs have vagal or spinal afferents, involved in physiological events or stimuli.

Interneurons are both AH- and S-type neurons and belong to Dogiel type II neurons (Costa et al., 2000). They form multisynaptic pathways along the gut tube length controlling the propagation of peristaltic waves. They are interposed between the primary afferent neurons and the motor or secretomotor neurons. The ascending interneurons are mainly cholinergic, whereas the descending ones, that are the majority, have a complex chemical coding including acetylcholine (ACh), nitric oxide (NO), vasoactive intestinal peptide (VIP), 5-HT and somatostatin. Interneurons, which release ACh/NO/VIP/somatostatin, are involved in local motility reflexes, while ACh/5-HT neurons are involved in the local secretomotor reflexes (Costa et al., 2000). Lastly, motoneurons are represented by S-type neurons and Dogiel type I (Hansen, 2003), and distinguished in subclasses: muscular, and secretomotor and vasomotor neurons. The muscular motoneurons innervate the musculature of the entire GI tract (*muscularis mucosae*, longitudinal and circular musculature). They mediate cholinergic and tachykinergic excitatory stimulation but also inhibitory transmission. Secretomotor and vasomotor neurons can control secretion and blood flow and are directly regulated by IPANs through the release of ACh and VIP. The majority of secretomotor and vasomotor neurons have the soma localized in the SMP and some of them project in the MP or *muscularis mucosae* (Hansen, 2003).

1.2 Enteric Neurotransmission

The local control of the ENS depends deeply in the interaction between its different neuronal cell types (Nezami & Srinivasan, 2010). Enteric neurotransmitters can be small molecules (e.g. norepinephrine and serotonin), larger molecules (peptides) or gases (NO and carbon monoxide), they extensively innervate all the structural and functional elements of the gut: smooth muscle, epithelium, immune elements, and vasculature (Sharkey & Savidge, 2014). Enteric neurons, the main components of the ENS, usually express a combination of different neurotransmitters, which defines the neural *chemical coding*. The chemical code depends on the type of neuron, the species and the intestinal segment (Hansen, 2003). More than 30 different neurotransmitters have been identified in the ENS (Galligan, 1998; Furness et al., 2000). 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. Depolarization induces neural excitation, whereas hyperpolarization induces inhibition (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. The gut receives a double extrinsic innervation from the autonomic nervous system (ANS): the cholinergic parasympathetic innervation, responsible of controlling the excitatory activity of intestinal smooth muscle, and the inhibitory noradrenergic sympathetic innervation. Parasympathetic preganglionic fibers generally terminate on cholinergic nerve cells of myenteric and submucosal plexuses. The sympathetic fibers are instead postganglionic and inhibit the release of acetylcholine, secreting norepinephrine that activates the presynaptic α2 receptors (Ganong, 2006).

1.2.1 Cholinergic Neurotransmission

The cholinergic neurotransmission is the major excitatory neurotransmission in the ENS causing excitatory potentials in post-synaptic effectors mediated by ACh (Harrington et al., 2010). A signal transduction from somatic system and ANS is necessary for the activation of cholinergic receptors (Carlson & Kraus, 2018). ACh is a co-transmitter of the greater neural population in the ENS 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; **Figure 1.3;**). 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 within the ENS: nicotinic (nAChRs) and muscarinic receptors (mAChRs; Purves et al., 2001). nAChRs are responsive to the agonist nicotine and are ionotropic ligand-gated ion channels, whereas mAChRs are responsive to muscarine and are G-protein coupled receptors (Carlson & Kraus, 2018). At the cholinergic synapse both classes of receptors are present on nerve terminals (pre-synaptic receptors) or on effector cells (post-synaptic receptors) where they act as autoreceptors, regulating ACh release from nerve terminals (**Figure 1.3**).

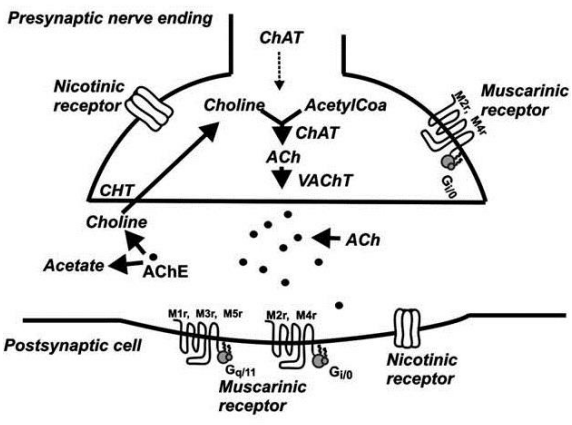


Figure 1.3 Biosynthesis of Acetylcholine (modified from Harrington et al., 2010).

Within the ENS nAChRs are required for the fast synaptic transmission in the ganglia (McGehee, 1999; De Biasi, 2002), in order to propagate reflexes rapidly and to produce fast responses to stimuli (Galligan, 2002). nAChRs activation is the predominant mechanism for the cholinergic neurotransmission in enteric ascending reflex pathways. The mAChRs, once interact with ACh, activate the second messenger cascades and intracellular signaling pathways mediating slow excitatory transmission (Caulfield & Birdsall, 1998; Harrington et al., 2010). These receptors are coupled to numerous G-proteins, therefore eliciting several intracellular responses after their activation. mAChRs activation can either depolarize or hyperpolarize the cell membrane, initiating or inhibiting the action potential, respectively.

1.2.3 Nitrergic Neurotransmission

Non-adrenergic non-cholinergic (NANC) neurons are expressed throughout the GI tract of several animal species (Furness & Costa, 1987; Boeckxstaens et al., 1990). The nitric oxide (NO) is considered the main neuromuscular neurotransmitter of NANC transmission in the parasympathetic system (Boeckxstaens et al., 1991; Toda & Okamura, 2003; Toda & Herman, 2005).

In mammals, NO can be synthesized by three different isoforms of the NO synthase enzyme (NOS): the neuronal NOS (nNOS), the endothelial NOS (eNOS) and the inducible NOS (iNOS). More than half of neural cells in the ENS express nNOS (Kochar et al., 2011). However, all the NOS isoforms are distributed in myenteric neurons of different species (Vannucchi et al., 2002; Talapka et al., 2011) and utilize L-arginine as substrate in presence of molecular oxygen and reduced nicotinamide-adenine-dinucleotide phosphate (NADPH) as co-substrates. In order 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).

In physiologic conditions, NO plays a crucial role in the inhibitory regulation of peristalsis (Furness, 2000). The intracellular mechanisms of relaxation induced by NO are mediated via activation of guanylyl cyclase (GC), expressed in several GI cell types, including smooth muscle cells (SMC), interstitial cells of Cajal (ICC), and fibroblast-like cells. The expression of iNOS, but not of nNOS and eNOS, is induced during diseases states, such as intestinal inflammation (Miampamba & Sharkey, 1999) and ischemia/reperfusion (I/R) injury (Giaroni et al., 2013). In these conditions, large amounts of NO can harm different cellular populations, such as neurons, due to the formation of peroxynitrite and nitrotyrosine (Rivera et al., 2011). This phenomenon reflects a functional plasticity of myenteric neurons that, by sensing the surrounding conditions (e.g. physiological versus pathological status) activate different NOS isoforms (Robinson et al., 2011; Giaroni et al., 2013). However, it is still not clear whether changes in the levels of NO exert pro- and anti-inflammatory actions (MacEachern et al., 2015).

1.2.4 Serotonergic Neurotransmission

Serotonin (5-HT), once of the most extensively studied neurotransmitter of the CNS, is predominantly secreted in the gut. As a matter of fact, the 95% of 5-HT is stored in GI system whereas about 5% is found in the brain (Banskota et al., 2019). 5-HT was discovered in 1937 by Vittorio Erspamer who firstly denominated this neurotransmiter 'enteramine', being extracted from rabbit enterochromaffin cells (ECs) and gut epithelial cell (Erspamer & Arsero, 1952; Banskota et al., 2019). Later, in 1948, Page and Rapport discovered a vasoactive compound from bovine serum and named it serotonin; but only in 1952 it became clear that enteramine and serotonin were the same molecule (Terry & Margolis, 2017; Banskota et al., 2019).

Among the 95% of gut 5-HT content, EC cells and enteric neurons stored about 90% and 10%, respectively (Kim & Camilleri, 2000). In the ENS, 5-HT neurons constitute about 2% of all myenteric neurons (Kim & Camilleri, 2000). 5-HT acts as both hormone and neurotransmitter; in the CNS it regulates mood, anxiety, memory and depression while in the gut 5-HT regulates sensory, motor and secretory functions through interactions with intrinsic and extrinsic nervous pathways (Stasi et al., 2014). Indeed, it can modulate ENS development and neuroplasticity, motility, inflammation and mucosal regenerative processes (Terry & Margolis, 2017). Moreover, 5-HT cannot cross the blood-brain barrier, indeed the brain has to synthesize its own 5-HT (Molderings, 2012). 5-HT mediates many functions through activation of 14 different 5-HT receptors (5-HTRs) that are separated into seven different subclasses (Jonnakuty & Gragnoli, 2008; Molderings, 2012; Terry & Margolis, 2017). They are distributed on enteric neurons, ECs, smooth muscle and immune cells (Gebauer et al., 1993). 5-HTRs are classified according to their structure, transduction signal, and pharmacology, until now seven types of families and multiple subtypes of 5-HTRs have been identified: the 5-HT_3 receptor is coupled to an ion channel, whereas $5\text{-HT}_{1,2,4,5,6,7}$ receptors are coupled to G proteins (Jonnakuty & Gragnoli, 2008; Stasi et al., 2014). In the intestine five receptor classes are expressed: $5\text{-HT}_{1,2,3,4,5,7}$ receptors. In particular, 5-HT_4R and 5-HT_3R are the most studied and are localized presynaptically or on the sensory and myenteric neurons, respectively (Hoyer et al., 2002; Gershon, 2004).

Once released, 5-HT is transported into surrounding epithelial cells by the serotonin reuptake transporter (SERT) and degraded to 5-hydroxyindoleacetic acid. SERT is expressed in enteric neurons and mucosal enterocytes (Terry & Margolis, 2017). 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). All of the enterocytes in the lining of the intestines show to express SERT for the selective removal of 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). Furthermore, SERT is also expressed by platelets that

are deputed to store peripheral 5-HT (Terry & Margolis; Banskota et al., 2019). In the colonic myenteric plexus, it has been discovered that, single serotonergic neurons innervate EGCs, submucosal neurons, interstitial cells of Cajal, and blood vessels, suggesting that they represent neurons involved in the coordination of motility, secretion, and blood flow (Ochoa-Cortes et al., 2016).

1.2.5 Purinergic Neurotransmission

The neurotransmitter adenosine 5'-triphosphate (ATP) is released by non-adrenergic non-cholinergic (NANC) nerves and triggers inhibitory junction potentials (Burnstock, 2018), playing a central role in enteric mechano-sensory transduction, synaptic and neuromuscular transmission modulating both motility and secretion (Gallego et al., 2008). Purines are released from intrinsic innervation, sympathetic or sensory motor neurotransmission during axon reflexes, to act directly on smooth muscle purinoceptors mediating relaxation or contraction or on prejunctional nerve terminals for modulating transmitter release from motor and inhibitory neural control pathways (Burnstock, 2014). ATP and the other purines interact with the P2 purinoreceptors, the ionotropic (P2XRs) and metabotropic (P2YRs) subtypes, expressed in both myenteric and submucous enteric plexus. They participate in sympathetic transmission and neuromodulation involved in enteric reflex activities, as well as influencing gastric and intestinal epithelial secretion and vascular activities (Burnstock, 2014). P2XRs are ligand gate cation channels; once they are activated, they mediate fast synaptic transmission. Moreover, it has been found that they contribute to pain transmission and they can be involved in several functional GI disorders including IBS (Wu et al., 2017; Burnstock, 2018). P2YRs are coupled to G proteins; once activated, they predominately mediate slow synaptic transmission (Gallego et al., 2008; Bornstein, 2008; King, 2015; Burnstock, 2018). P2Y1 is one of purinergic receptors that mediates purinergic inhibitory neuromuscular transmission in the guinea-pig colon and mouse jejunum and mediates the fast-inhibitory junction potential (IJP) and the non-nitrergic relaxation of the human colon (Gallego et al., 2008). Of note, we recently discovered that P2Y1 is expressed both in neurons and in EGCs, andinfluences the NANC-mediated relaxation in isolated ileal segments of $TLR4^{-/-}$ mice (Caputi et al., 2017a).

Together, these receptors affect many enteric reflexes and motor patterns (Galligan, 2008). Changes in purinergic signaling in the ENS may contribute to some pathological mechanisms.

1.3 Enteric Glial Cells

The ENS is composed of enteric glial cells and neurons (EGCs; Jessen, 2004) in a ratio of 4:1 (Rühl, 2005). EGCs were observed, for the first time, by Dogiel in 1899, but only after anatomical analysis by Gabella in the 1970s the enteric glia was recognized to be a distinctive class of peripheral astrocytes (Gulbransen and Sharkey, 2012). The word "glia" derives from a Greek word that means glue and for

many years EGCs were believed to provide mainly structural support (Ochoa-Cortes et al., 2016) as suggested by their distribution around the enteric neurons (Gulbransen and Sharkey, 2012; Neunlist et al., 2013; Sharkey & Savidge, 2014). EGCs possess the same characteristics of brain astrocytes and are found in both the myenteric and submucosal plexuses, but also outside of the ENS, such as intramuscularly (Gulbransen and Sharkey, 2012; Sharkey & Savidge, 2014; Ochoa-Cortes et al., 2016). They possess many neurotrophic, neuroprotective and neuro-immunomodulatory properties (Neunlist et al., 2014; Capoccia et al., 2015). Moreover, they have a pivotal role in the maintenance of ENS homeostasis (Gulbransen & Sharkey, 2012; Yu & Li, 2014; Boesmans et al., 2015; Ochoa-Cortes et al., 2016) and in the severity of gut diseases (Neunlist et al., 2014). Even if they derived from neural crest progenitor cells, EGCs differ from the Schwann cells, the major glial cells in the PNS (Gershon & Rothman, 1991; Rühl, 2005). They are described as small cells extremely irregular and stellate-shaped associated with neuron cell bodies in enteric ganglia and nerve fiber clusters in interganglionic connectives (Gershon & Rothman, 1991; Gulbransen and Sharkey, 2012).

In 1994, *Hanani and Reichenach* proposed a classification scheme for EGCs, based only on cell morphology and location within the plexus (Hanani & Reichenach, 1994). Later on, *Boesmans and colleagues* (2015) re-examinated EGC subtype classification considering not only morphological features and distribution within the gut wall but also molecular and physiological attributes, classifying 4 types of EGCs. The type I or protoplasmic EGCs exhibit a star-shaped, irregularly branched processes, resembling protoplasmic astrocytes of the CNS and closely embrace neuronal cell bodies and fibers within myenteric and submucosal ganglia (intraganglionic EGCs). The type II of EGCs are more elongated, for interganglionic EGCs. The type III or mucosal enteric glia consists of several long branches that reach the mucosal epithelial cells. The type IV or intramuscular EGCs are distributed between smooth muscle cells, running with neuronal fibers in the musculature (Boesmans et al., 2015; **Figure 1.4**).

EGCs are usually identified by the expression of three biomarkers: glial fibrillary acidic protein (GFAP), S100β protein and the SRY box–containing gene 8/9/10 (SOX 8/9/10; Boesmans et al., 2015; Capoccia et al., 2015).

The transcription factor SOX10 is expressed in the multipotent neural crest (NC) progenitors and EGCs. Deletion of SOX10 has been found in patients with Waardenburg–Hirschsprung disease (Bondurand & Sham, 2013), underling the important role of SOX10 in ENS development and homeostasis. The EGCs, like all the glial cells, are highly active in both healthy CNS and ENS, but in response to injury and inflammation they undergo in a state defined as reactive gliosis that is characterized by an overexpression of GFAP or S100β (Sofroniew, 2009; Burda et al., 2013; Boesmans et al., 2015; Capoccia et al., 2015). Reactive gliosis is not a single uniform process but is a progressive change that lead to ENS alterations and is detected in inflammatory bowel diseases (IBD) and functional GI disorders (Ochoa-Cortes et al., 2016). EGCs communicate through Ca^{2+} signals that

permit to integrate information transmitted by neurons, glial cells, immune cells, or other cells in the gut microenvironment (Ochoa-Cortes et al., 2016). Moreover, EGCs express neurotransmitter receptors, suggesting that, like astrocytes, they are active participants in neuronal communication, and they can influence synaptic transmission (Gulbransen & Sharkey, 2009). We have recently shown that the absence of Toll-like receptor 4 (TLR4) signaling determines an enteric reactive gliosis associated with an increase in relaxation, mediated by the inhibitory NANC neurotransmission through a complex neuronal-glia signaling constituted by P2X7 and P2Y1 receptors, and NO produced by nNOS and iNOS. In the gut, TLR4 is expressed in EGCs and neurons and its signaling mediates enteric neuronal survival and host-immune responses (Turco et al., 2014; Caputi et al., 2017a).

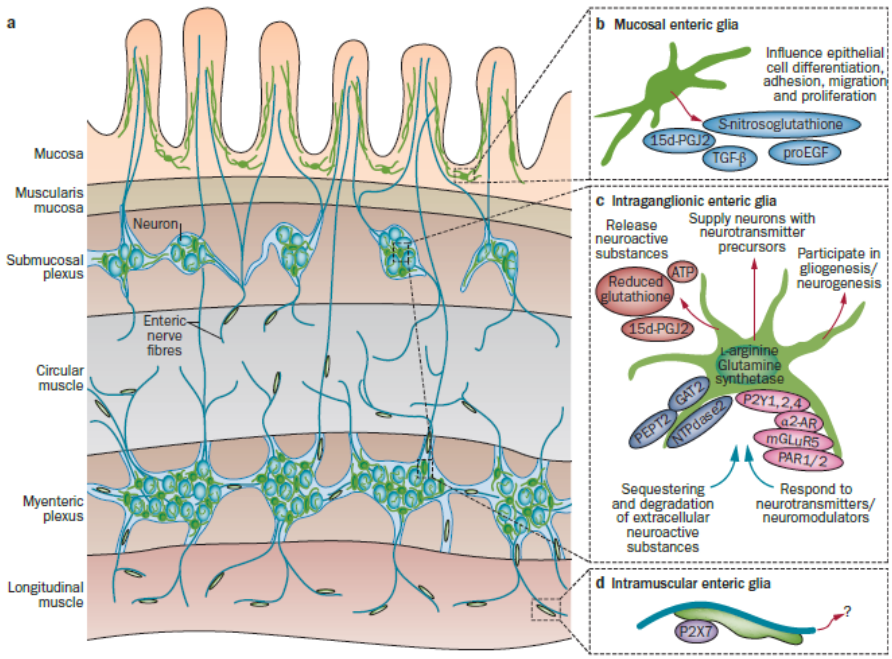


Figure 1.4. Subpopulations of enteric glia. (**a**) Subpopulations of enteric glia are located within the gut wall with different physiological functions and signaling mechanisms. (**b**) Mucosal enteric glia lies in the mucosa directly beneath the epithelial cells. (**c**) Intraganglionic glia surrounds 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 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).

Other studies revealed the importance of EGCs in protecting and regulating enteric neurons and the consequences related to their dysfunction. Neuron-glial communication occurs in both rodent (Gulbransen & Sharkey, 2009) and human ENS (Liñán-Rico et al., 2015). *In vitro* models using cultured EGCs demonstrated the capacity of EGCs to respond to neurotransmitters, especially ATP (Gulbransen & Sharkey, 2009). Moreover, EGCs can receive cholinergic and serotonergic signaling from the neurons (Ochoa-Cortes et al., 2016). In the EGCs it has been found also the expression of iNOS protein with consequent NO-production (Cirillo et al., 2011b).

The role of glial cells in the nervous system has been studied using transgenic mice for EGCs proteins or the substance fluorocitrate (FC) and its precursor fluoroacetate (FA) (Fonnum et al., 1997), that selectively inhibit glial cells metabolism (Ochoa-Cortes et al., 2016).

FA and FC are toxins that specifically block the aconitase enzyme of Krebs cycle of glial cells both in CNS and in ENS (Fonnum et al., 1997; Nasser et al., 2007; MacEachern et al., 2015). Recently, it has been shown that *in vivo* administration of FC determined alterations of GI motility and gut contractility (Nasser et al., 2007). Then, *Aubé et al.* clearly demonstrated that loss of enteric glia, by genetic manipulation, was associated with reduced motility, increased intestinal barrier permeability and disrupted cholinergic and nitrergic myenteric neurotransmission (Aubé et al., 2006). *Bush et al.* found that the ablation of EGCs led to significantly compromise epithelial and vascular integrity in the GI tract, resulting in inflammation (Bush et al., 1998). Moreover *Cornet et al.* found that autoimmune depletion of enteric glia can cause severe enterocolitis (Cornet et al., 2001). In rodents, a significant fraction of enteric neurons and EGCs develops during the early postnatal period, and changes associated with feeding or luminal microflora composition appears to affect ENS development and the maturation of the mucosal immune system after birth (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 emphasized by the reduced number of enteric neurons and the associated deficits in gut motility observed in germ-free (GF) mice (Anitha et al., 2012). Moreover, 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., 2015b). These outcomes reveal the complex and intricate connection between the microbiota and EGCs as regulators of neuroimmune control of host defense in the intestinal mucosa (Sharkey et al., 2018) and of maturation of intestinal neural-glial circuits. Fascinatingly, reconstitution of GF mice with conventional microbiota normalized the density of EGCs network and gut physiology (Kashyap et al., 2013; Kabouridis et al., 2015b), underlining ENS plasticity and microbiota influence on ENS homeostasis. Furthermore, the potential role of the microbiota and the mucosal immune system in the activation of glial progenitors is currently not known, but it is established that glial cells are capable to direct influence immune responses (Turco et al., 2014). Indeed, upon bacterial stimulation, EGCs upregulate expression of MHC class II, actively responding to the colonization of the gut lumen by microbiota and participating in antigen presentation to the adaptive immune system (Turco et al., 2014). Taken together, these observations highlight the dynamic host–microbe interactions which is essential for EGCs development, suggesting that an improved understanding of this crosstalk will provide important insights into the pathophysiology of GI diseases.

1.4 Toll-Like Receptors

Environmental microorganisms are potential enemies for all living organisms which can respond triggering an immune response mediated by the innate and/or acquired immunity. Until few years ago the innate immune system activated by TLRs was thought to be only a non-specific response accomplished by neutrophils, dendritic cells and macrophages. Nowadays it is known to be that the activation of dendritic cells by TLR ligands is also necessary for their maturation and consequent ability to initiate adaptive immune responses, including opsonization, activation of complement, coagulation cascades, phagocytosis, activation of proinflammatory signaling pathways and apoptosis (Janssens & Beyaert, 2003; Akira et al., 2006; Javaid et al., 2019). On the other hand, acquired immune responses arise in the late phase of infection as slower processes mediated by T and B cells, expressing highly diverse antigen receptors, being thus capable of responding to a wide range of potential harmful microbes with consequent generation of immunological memory (Akira et al., 2006). Moreover, the innate immune system has a central function in the activation and shaping of the adaptive immune response through the induction and the release of cytokines and co-stimulatory molecules (Medzhitov, 2007; **Figure 1.5**).

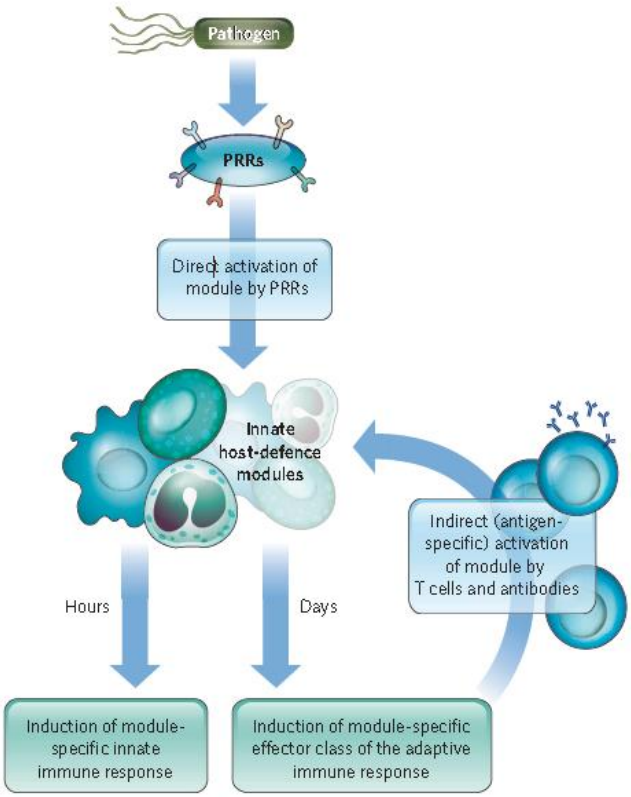


Figure 1.5. Activation of host-defense mechanisms (modified from Medzhitov, 2007).

The innate immune system uses nonclonal sets of recognition proteins, called pattern recognition receptors (PRRs; Janssens & Beyaert, 2003). Toll like receptors (TLRs) are one of the most important

family of the PRRs. The discovery of the TLRs began when the receptor 'Toll' has been identified in *Drosophila melanogaster* as a key controller of embryonic development (Akira & Takeda, 2004; Okun et al., 2011). Subsequent genetic studies have then discovered other critical genes in the dorsal-ventral patterning of the embryo, beside Toll, such as tube, pelle, cactus, the NF-κB homolog dorsal, and other seven genes upstream of Toll (Belvin & Anderson, 1996). In the 1995 for the first time, *Hultmark et al.* identified Toll-1 as an activator of the immune response in a *Drosophila* cell line. About the same period, a human homolog of Toll was identified and mapped to chromosome 4p14 (Taguchi et al., 1996). Afterward, Toll signaling was shown to be also involved in the antifungal response of *Drosophila* (Lemaitre et al., 1996). In the 1997, *Medzhitov et al.* described the first TLR in mammalian. In 1998 *Rock et al.* characterized five human TLRs that are involved in controlling immune responses with no role in the development, whereas the Drosophila Toll-pathway is implicated in both immunity and developmental processes (Valanne et al., 2011). TLRs are type I transmembrane proteins responsible in the recognition of pathogen microbes referred to as pathogen-associated molecular patterns (PAMPs). PAMPs are appropriate for innate immune recognition because they are: i) constant among microorganisms of a given class; ii) products of pathways that are unique to microorganisms, allowing discrimination between self and non-self-molecules; iii) essential 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 part of the cell wall structure, such as lipopolysaccharide (LPS), peptidoglycan (PG), lipoteichoic acids (LTA) and other lipoproteins (Patten & Collett, 2013). Other important PAMPs are beta-glucan, a cell wall component of fungi, and viral nucleic acids, all structures recognized by TLRs. An important aspect of pattern recognition is that TLRs themselves do not distinguish between pathogenic microorganisms and symbiotic (non-pathogenic) microorganisms, since the receptor ligands are shared between pathogens and commensals (Medzhitov, 2007). Until now, 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 TLR12 and TLR13 have been lost from the human genome (Kawai & Akira, 2010; Patten & Collett, 2013; **Table 1.1**). Studies in mice deficient for each single type of TLRs have demonstrated that every TLR has a distinct function in relation to PAMPs recognition and activation of immune responses (Akira et al., 2006).

TLR1, 2, 4 and 6 recognize lipid-based structures whereas TLR4 interacts with LPS of Gram-negative bacteria, causing 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; Patten & Collett, 2013). TLR3, 7, 8 and 9, being localized intracellularly, detect nucleic acids derived from viruses and bacteria.

TLR3 recognizes double stranded RNA (dsRNA) generally produced by several viruses during replication. TLR7 recognizes synthetic imidazoquinoline-like molecules, guanosine analogs such as loxoribine, viral single stranded RNA (ssRNA) and small interfering RNA (Akira et al., 2006; **Table 1.1**). TLRs are expressed on a variety of cells, including immune cells, such as macrophages, DCs, B cells, specific types of T cells, and also fibroblasts, epithelial cells and neurons. Expression of TLRs is rapidly altered following the appearance of pathogens, the increased levels of cytokines or the onset of environmental stressors (Akira et al., 2006). Furthermore, TLRs can be localized extracellularly or intracellularly. Some 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 the 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; Patten & Collett, 2013).

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

Table 1.1. TLRs location and characteristics (modified from Kumar et al., 2009).

The engagement of TLRs by microbial components triggers the activation of signaling cascades, leading to the induction of genes involved in antimicrobial host defense such as those encoding

proinflammatory cytokines and chemokines (Patten & Collett, 2013). 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, 2010). 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-beta (TRIF)/TIR-domain-containing molecule 1 (TICAM1) and TRIF-related adaptor molecule (TRAM; Yamamoto et al., 2002; Oshiumi et al., 2003; **Figure 1.6**).

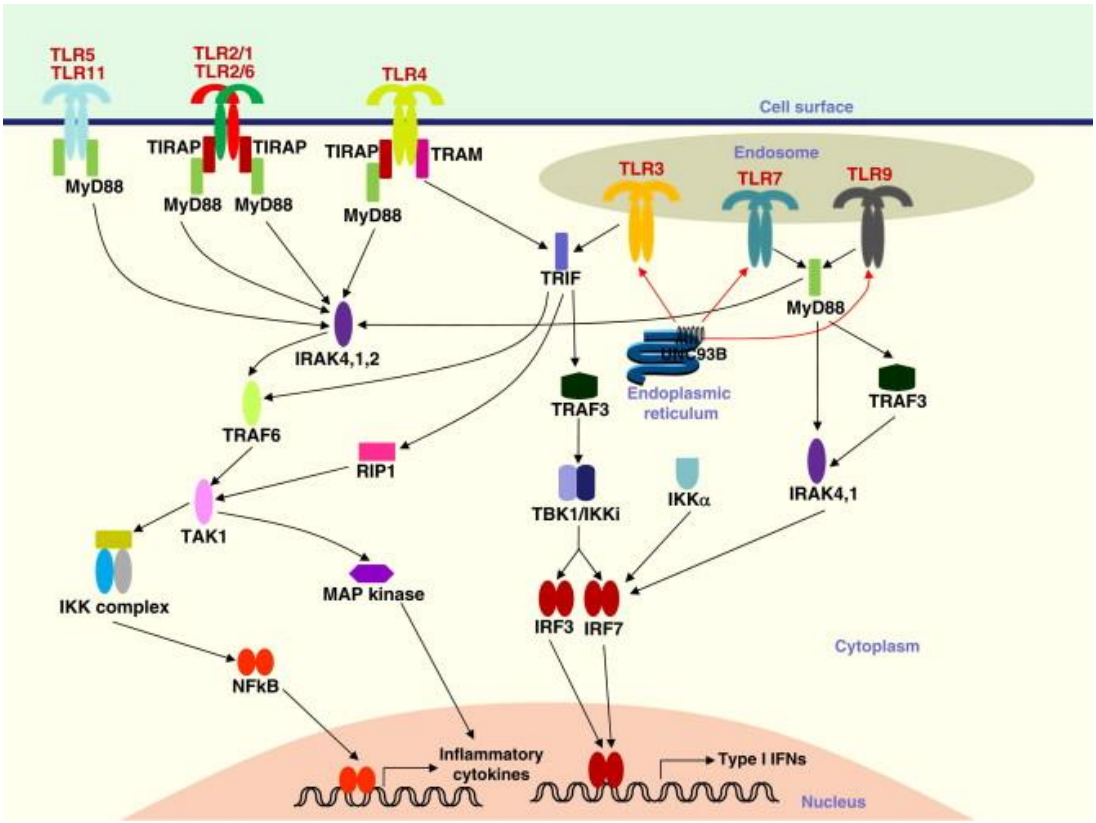


Figure 1.6. TLR signaling in conventional dendritic cells, macrophages and dendritic cells (modified from Kumar et al., 2009).

The differential responses mediated by distinct TLRs ligands can be explained in part by the selective interaction with 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. Instead, 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, 2010). 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).

1.4.1 Toll-Like Receptors 4 & Enteric 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). An inappropriate activation of the immune system by commensal bacteria or pathogens may lead to the pathogenesis of intestinal disease (Podolsky, 2002). TLRs play a role in the crosstalk between the intestinal microbiota and the host, as they specifically recognize conserved microbial molecular structures, called MAMPs and protect against pathogenic microorganisms (Martin et al., 2010). Intriguingly, GF animal studies demonstrate that the microbiota is necessary for the development of gut mucosal immunity (Macpherson & Harris, 2004).

The expression of TLRs in both CNS and ENS (Barajon et al., 2009) indicates that TLRs are involved not only in the regulation of the host immune responses but have a role in the 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 which have been shown that are expressed by enteric neurons and glia, suggesting that ENS lineages can directly sense microbial microbiota. TLR2 and TLR4 signaling seems to be also 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).

Anitha et al. (2012) showed that GF and antibiotic-treated mice exhibited reduced motility and fewer $nNOS^{+}$ neurons. These effects were 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 $MyD88^{-/-}$, suggesting that TLR4 signaling is critical for 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 PRR and 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 both nitrergic and purinergic neurotransmission and in modulating the

distribution of EGCs in the ileum (Caputi et al., 2017a). Therefore, the crosstalk between TLR4 and nitrergic/purinergic pathways in neural-glial communication is likely to be a prerequisite for ensuring normal gut physiology and the pathology. Polymorphisms in TLRs genes or, in general, a defective TLR4-mediated 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 or DAMPs, mainly derived from tissue injured by oxidative stress. For instance, 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). Moreover, it has been discovered that, in the premature gut, the activation of TLR4 leads to apoptotic processes together with a reduction of cell proliferation impairing epithelial cells regeneration (Mihi & Good, 2019). A higher activation of TLR4 it has been found also in necrotizing enterocolitis, that is a GI disease associated with a marked microbial colonization in the immature gut that triggers the activation of TLR4 causing impairment in the epithelial barrier (Mihi & Good, 2019) suggesting that TLR4 can be a target to treat some GI disorders.

1.5 Gut Microbiota

The intestinal microbiota is the community of commensal microorganisms, such as bacteria, archaea and eukarya, living in the GI tract (Dethlefsen et al., 2006), a mammal organ with the largest interfaces (250–400 m^2) between the host, environmental factors and antigens. The gut microbiota is described as a "superorganism", composed of more than 90% microbial cells and 10 million microbial genes (the so-called microbiome), forming a mutualistic relationship with the host, fundamental for intestinal homeostasis (Hooper & Gordon, 2001; Nicholson et al., 2005).

The gut microbiota is differently distributed along the GI tract with the highest content in the large intestine. There are different bacterial *phyla*, such as *Firmicutes* (e.g. *Lactobacillus, Clostridium, Enterococcus*), *Bacteroidetes* (e.g. *Bacteroides*; Dethlefsen et al., 2006), *Actinobacteria (e.g. Bifidobacteria), Proteobacteria (e.g. Escherichia coli), Fusobacteria, Verrucomicrobia* and *Cyanobacteria* (Eckburg et al., 2005; Qin et al., 2010). It has several functions such as pathogen protection, nutrition and immune modulation but also metabolic activities (Guinane & Cotter, 2013). Indeed, it can extract energy from indigestible dietary polysaccharides, resistant starch and dietary fibres (Wopereis et al., 2014), with consequent production of short-chain fatty acids (SCFA), bile acids, vitamins (e.g. vitamin K, vitamin B12 and folic acid) and amino acids, all essential nutrients for human beings (Agus et al., 2018).

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). Different groups have recently revealed that microbial exposure start before birth when the fetus receives microorganisms from the mother during gestation (Jiménez et al., 2008; Satokari et al., 2009). The presence of bacterial species in the fetus (such as *Escherichia coli, Enterococcus faecium*, and *Staphylococcus epidermidis*) results from the translocation of the mother's gut bacteria via the bloodstream and placenta (Jimenez et al., 2008). Postnatally, the microbial gut colonization is dependent on the birth delivery mode. For instance, vaginally born infants are colonized by fecal and vaginal bacteria from the mother, whereas infants born by cesarean delivery are exposed to bacteria associated with 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, and then the precise composition of the developing microbiota population is dependent on whether the infant is breast- or formula-fed (Thum et al., 2012). Furthermore, the microbiota composition is unique for each individual and begin stable following the first year of life (Murphy et al., 2015). Alterations in the gut microbiota signature early in life can predispose to immune disorders (Penders et al., 2007). Many studies have been focused on exploring the effects of microbiota on gut physiology, by using GF models and antibiotics treatment regimens (**Figure 1.7**).

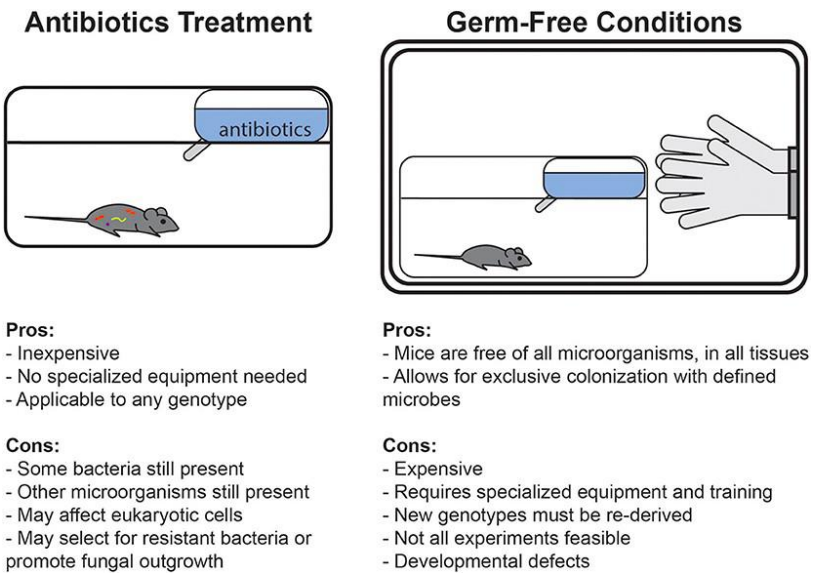


Figure 1.7. Comparison of the advantages and disadvantages of antibiotics-treated and germ-free mouse model systems (Kennedy et al., 2018).

GF mice are bred in isolators, which fully block exposure to microorganisms and keep them free of detectable bacteria, viruses, and eukaryotic microbes. Using GF mice is possible to understand the effect of complete absence of microbes or allow 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 alternative method to avoid some of these complications has been the use of antibiotic treatment. Treatment with broad-spectrum antibiotics is commonly used to deplete the gut microbiota and can be readily applied to any mouse genotype or disease condition.

Antibiotics can deplete bacterial populations in mice, which are normally colonized since birth whereas in GF conditions the mice are completely sterile since they are born. Antibiotics can selectively deplete different members of the microbiota because they have differences in mechanism of action. Indeed, 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., 2015). On the other hand, a cocktail of different classes of antibiotics can be used to broadly deplete the gut microbiota (Caputi et al., 2017b). Mice treated with 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 (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). 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 in potential negative impact on intestinal health, leading to development of gut disorders later in life (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). 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., 2011). 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 of different disorders associated with aging. Indeed, in presence of a variety of diseases such as inflammatory bowel disease (IBD), neurological

disorders, cancer, and obesity, there is an imbalance of microbial populations defined as dysbiosis, leading to bacteria tissue infiltration and enhanced inflammatory response (Singer-Englar et al., 2019). This response is mediated by TLRs, which play a key role in host recognition of microbes; specifically, TLR4 has been implicated in the recognition of bacterial LPS, which are elements of the cell walls of Gram-negative bacteria, and TLR5 is involved in the recognition of bacterial flagellin triggering the expression of proinflammatory cytokines and chemokines (Singer-Englar et al., 2019).

1.6 Obesity Pandemic

The World Health Organization (WHO) defined obesity as one of the greatest public health challenges of the 21st century, assuming worldwide epidemic proportions with increasingly rates (Karalis et al., 2009; Gonzalez-Muniesa et al., 2017). In 2014, in the United States, 68.5% of adult population was considered obese or overweight (Murphy et al., 2015). In 2018, WHO estimated that worldwide obesity was tripled since 1975 with a marked increase of obese/overweight children. In 2016 NCD Risk Factor Collaboration (NCD-RisC), subsequently to a population-based studies of 186 countries covering the 99% of the world's population, found that the prevalence of obesity in men from 1975 to 2014 was marked increase from 3.2% to 10.8% (**Figure 1.8A**), in women from 6.4% to 14.9% (**Figure 1.8B**).

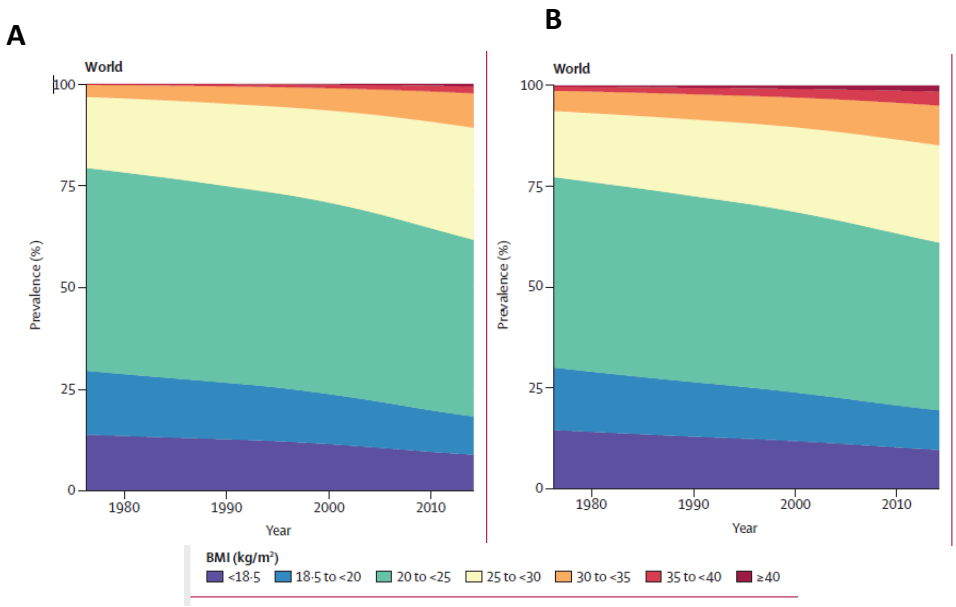


Figure 1.8. Trends in age-standardised prevalence of BMI categories in men (**A**) and in women (**B**) modified by NCD Risk Factor Collaboration).

Obesity results from a dysregulation between caloric intake and energy expenditure associated with a low physical activity resulting in an excess of body fat accumulation (Heber, 2010; Gonzalez-Muniesa et al., 2017). Interestingly, obesity is defined as a hall marker of metabolic syndrome, which is known to increase the individual's risk of developing type 2 diabetes, and cardiovascular disease (Vijay-Kumar et al., 2010). In fact, obesity is considered a causal factor of numerous metabolic and

endocrine disorders including heart disease, diabetes, bone and joint disorders and some forms of cancer (Sellayah et al., 2014).

There are different methods to determine an obese grade, including densitometry, imaging-based methods, and dual-energy X-ray absorptiometry that are mostly used for research purposes, whereas body mass index (BMI), defined as the body mass divided by the square of the body height, and universally expressed in units of kg/m^2, is the most widely used parameter to diagnose obesity for its low cost and simplicity (Nguyen & El-Serag, 2010; Gonzalez-Muniesa et al., 2017; Müller & Geisler, 2017). WHO has defined overweight and obesity values of BMI between 25.0 and 29.9 kg/m^2 and greater than 30.0 kg/m^2, respectively (Nguyen & El-Serag, 2010).

Moreover, BMI is used in epidemiologic studies as a tracker of worldwide obesity incidence. The ethnic differences in body fat distribution have already destabilized the generalizability of BMI, for example in Southeast Asia, requiring new BMI limits for the definition of obesity (Heber, 2010).

Furthermore, ethnic differences have, also, been found in the susceptibility to metabolic syndrome which is genetically increased in Asians, Latinos, and Native Americans (Heber, 2010). As shown in **Figure 1.9**, from 1975 to 2014 the men BMI was increased from 21.7 kg/m^2 to 24.2 kg/m^2 (**Figure 1.9A**), and in women from 22.1 kg/m^2 to 24.4 kg/m^2 (**Figure 1.9B**). Looking at the specific regional mean BMI, in 2014 men from Central Africa and South Asia showed lower BMI range (21.4 kg/m^2) compared to those from with higher BMI range of about 29.2 kg/m^2. Women BMI ranged from 21.8 in kg/m^2 in South Asia to 32.2 kg/m^2 in Polynesia and Micronesia (NCD-RisC, 2016).

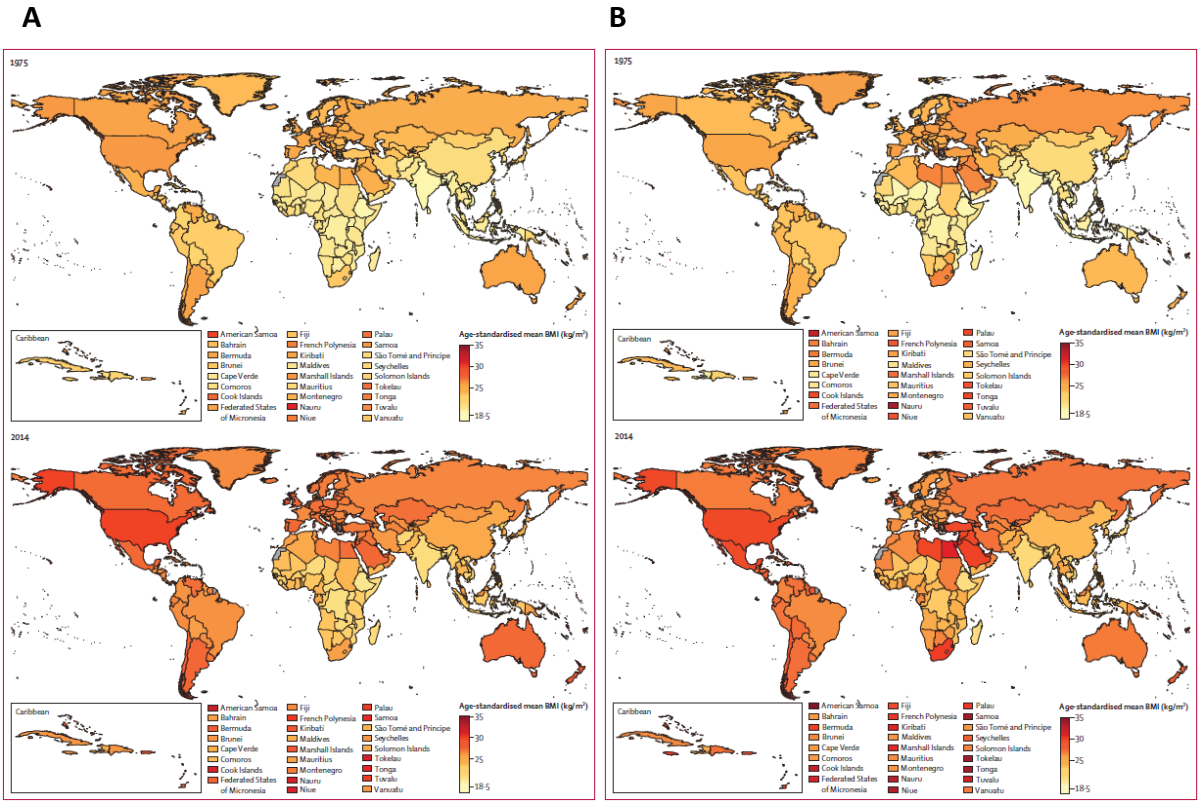


Figure 1.9. Age-standardized mean BMI in men (**A**) and in women (**B**) by country in 1975 and 2014 (modified by NCD Risk Factor Collaboration 2016).

The intensification in obesity rates reflects changes in lifestyle, including nutritional and environmental challenges and higher susceptible genetic background (Karalis et al., 2009). Nowadays, BMI is increasing in most countries in parallel with the intensification of people living in cities. Previously, BMI was lower in rural areas than in cities because of a higher energy expenditure in the daily work and domestic activities. Over the years, in high-income and industrialized countries, service and office jobs have become more common, and cars are used for transport with more spending on food and increased consumptions of processed carbohydrates. All these changes, defined as 'urbanization of rural life', contributed to the increase of BMI (NCD-RisC, 2019).

1.7 Obesity and Gut Microbiota

The etiology of obesity is not fully understood; gene-environment interactions but also human evolution, alterations in the immune system and in gut microbiota can lead to a state of over-nutrition (Karalis et al., 2009; Sellayah et a., 2014; Murphy et al., 2015; Muscogiuri et al., 2019). Genetic susceptibility currently explains only a small part of the overall susceptibility to obesity but cannot explain the rise in incidence of this disease (Tilg, 2010).

Several studies have been made to identify host-environmental factors that can affect energy balance. Studies in GF mice have shown that their total body fat was lower compared to that found in conventionally raised mice (Tilg, 2010) and the administration of high-fat diet (HFD) to GF mice did not cause weight gain or adiposity, or other metabolic effects, seen in conventionally raised mice, highlighting the key role of gut microbiota in host metabolism (de La Serre et al., 2010). Microbiota mainly contains organisms from *Bacteroides* and *Firmicutes* phyla. The first group of bacteria has a strong capacity of digest dietary polysaccharides, in fact it is more abundant in healthy and lean people compared to obese people. Indeed, in presence of an obese phenotype, microbiota is less rich in bacterial components with an increase of *Firmicutes*/*Bacteroides* ratio causing a dysbiosis compared to the healthy subjects. Interestingly, new studies characterized the microbiota populations in presence of anorexia and, conversely to the obese phenotype, it has been found an increase of *Bacteroides* with a reduction of *Firmicutes* phyla (Muscogiuri et al., 2019). *Turnbaugh et al*. (2008) demonstrated that the consumption of a HFD alters the mouse gut microbiota in a single day; moreover colonization of adult GF mice animals with a distal gut microbiota harvested from the ceca of conventionally-raised donors fed a low-fat polysaccharide-rich diet produces a marked increase in body fat content within 10–14 days (Turnbaugh et al., 2006). Moreover, *Turnbaugh et al*. (2006) showed that when adult GF mice fed with standard diet (SD) were colonized with a microbiota harvested from an genetically obese mice or lean donors, adiposity in beneficiaries of the obese microbiota increased greater compared to who received the lean microbiota, to indicate that the obese gut microbiota has an increased and transmissible capacity to promote fat deposition (Turnbaugh et al., 2006). Different mechanisms have been proposed to explain the role of gut

microbiota in the development of obesity. Firstly, the ability of microbiota to ferment dietary polysaccharides not digested by humans to obtain short chain fatty acids (SCFA). SCFA, once are adsorbed, can induced lipogenesis and increase triglyceride content (Chakraborti, 2015). The second mechanism is related to the capacity of microbiota of suppressing the adenosine monophosphate kinase resulting in a decreased liver fatty acid oxidation causing an increase of fat accumulation. The third hypothesis is related to the onset of a low-grade of inflammation due to an innate response to LPS that can bind TLR4 causing the activation of proinflammatory pathways (Chakraborti, 2015). HFD, both in animals and humans, alters gut microbiota composition (more in favor of Gram-negative phylum), which in turn increases the production and intestinal permeability of LPS, resulting in its high plasma concentration and development of "metabolic endotoxemia" (Bakker et al., 2015; Chakraborti, 2015). Moreover, *de La Serre et al.* (2010) demonstrated that HFD causes an increase of LPS levels with a marked intestinal TLR4 activation. Interestingly, *Shi et al.* (2006) found that $TLR4^{-/-}$ mice are resistant to diet-induced obesity and insulin resistant; furthermore, also mice lacking the TLR4 adapter protein CD14 do not develop diet-induced obesity (Cani et al., 2007). However, LPS, responsible of gut inflammation and adiposity resulting in obesity, is known to be a Gram-negative bacterial product. Recent observations have shown that microbiota in obese people are rich in *Prevotellaceae* (a subgroup of *Bacteroidetes*), which is a good source of LPS (Graham et al., 2015). *Anitha et al.* (2016) have recently discovered that intestinal dysbiosis in HFD-fed mice contributes to the delayed intestinal motility via TLR4-dependent neuronal loss (Anitha et al., 2016). The innate immune system is involved in the development and severity of metabolic syndrome and obesity, acting not only through TLR4 signaling, but also via other TLRs as shown in mice deficient for toll-like receptor 5 (TLR5) which are prone to develop obesity and insulin resistance (Tilg, 2010).

Animals models are essential for studying the pathogenesis and therapy of metabolic disorders (Buettner et al., 2007). Based on the epidemiological studies demonstrating a positive relationship between dietary fat intake and obesity, HFD-induced obesity is the most common model used in research (Hariri & Thibault, 2010). Usually, the experimental used HFD contains between 30 to 78% of total energy intake (Buettner et al., 2007) compared to the normal rodent diet with 10% of fat. The most applied HFD contains 45 or 60% of fat, however, the letter has the advantage of the rapid onset of obesity, reducing time and saving cost. Moreover, the European or American diet contains about 40% of fat as energy intake (Speakman, 2019).

A mouse strain particularly susceptible to the effects of dietary fat is the C57BL/6J (B6) mouse. This animal develops severe obesity, hyperglycemia, and hyperinsulinemia when weaned on a high-fat diet (Parekh et al., 1998; Buettner et al., 2007).

Diet-induce obesity is increasing, and is characterized by a low grade of inflammation, that is also associated to changes in 5-HT availability (Bertrand et al., 2011). Moreover, serotonergic system, together with the dopaminergic system, is involved in feeding behavior, motivation to eat, (food-

related) reward learning, and energy expenditure (van Galen et al., 2018). Changes in 5-HT-mediated response is also considered one of the "neurotransmitter hypotheses" that justify an increase of food intake in obese individuals due to a decreased serotonin-mediated homeostatic feedback in response to food intake together with a decreased dopamine-mediated reward (van Galen et al., 2018). 95% of 5-HT in the body is released by ECs and it can act on different serotonergic receptors (Spiller, 2008). *Bertrand et al.* (2012) showed that in rats fed with HFD, ileal ECs release more 5-HT compared to the healthy control associated with a reduced capacity of SERT to reuptake 5-HT and an increase of 5-HT mucosal levels. Increased peripheral 5-HT levels and TPH1 polymorphisms are associated with obesity (Young et al., 2015). Indeed *Crane et al.* (2015) demonstrated that mice $TPH1^{-/-}$ fed with HFD have significantly reduced weight gain, lower body fat and lower fasting, highlighting the role of 5-HT in the onset of obesity.

1.8 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) comprises a group of idiopathic, chronic, and relapsing inflammatory disorders of the GI tract (Becker et al., 2015). Ulcerative colitis (UC) and Crohn's disease (CD) represent the two main forms of IBD and are described as autoimmune diseases characterized by inflammation of the small or/and large intestine, in which elements of the digestive system are attacked by the body's own immune system (Fakhoury et al., 2014). The etiology of these diseases is still not well understood (Tripathi & Feuerstein, 2019); there are several contributing factors such as commensal microbiota, hereditary factors and environmental triggers (Forbes, 2002; Baumgart, 2008). Indeed, the main hypothesis of IBD pathogenesis suggest the presence of a disturbed interaction of the host immune system with the commensal microflora and/or other luminal antigens (Baumgart, 2008). This unusual response might be due to the damaged integrity of both intestinal epithelium and mucosal immune system (Podolsky, 2002). Both diseases are chronic and relapsing inflammatory gut disorders, distinguished by the location of the inflammation in the GI tract and by the pattern of histological alterations in the bowel wall (Podolsky, 2002; Becker et al., 2015). Moreover, IBD patients exhibit diarrhea, abdominal pain, bloody stools, and vomiting (Fakhoury et al., 2014). CD and UC share similar characteristics but differ in the location and nature of the inflammatory changes. In CD, inflammation can affect any part of the GI tract, while UC is characterized by an inflammatory response localized in the mucosal layer of the colon and/or the rectum (Fakhoury et al., 2014; Sammut et al., 2015).

UC is a pathology that can be developed at any age, but typically arises between age 15 and 35, affecting equally both sexes (Ghosh et al., 2000; Sammut et al., 2015). Surprisingly, in these last years, IBD has been diagnosed also in children (Fakhoury et al., 2014). First-degree family members of an affected patient show a risk of IBD that is up to 20 times higher compared to the rest of the population (Podolsky, 2002). Many studies have been performed to identify specific candidate genes,

including major histocompatibility-complex loci that are responsible of the pathology onset. It has been found a link between chromosome 16, in particular the gene NOD2, and development of CD, without any clear association with UC (Podolsky, 2002). NOD2 is a cytoplasmic protein expressed in macrophages and involved in activating the PRRs for bacterial LPS, namely TLR4, and modulating NF-κB activation and macrophage apoptosis with an increase of proinflammatory cytokines (Fakhoury et al., 2014).

It has been shown that patient with CD from Europe and North America are more likely to have variants of NOD2 compared to persons without the disease. Persons who are homozygous for the variant of NOD2 may have a 20-fold or more increase in susceptibility to CD, with a particular tendency for ileal disease.

Beside the genetic factors, microbiota plays also a pivotal role in the pathogenesis of IBD. For instance, in IBD animal models from both experimental and spontaneous intestinal inflammation, the absence of bacteria ameliorated the disease, suggesting that the bacteria can control intestinal inflammation (Gkouskou et al. 2014).

Commensal bacteria can induce an immune response if they enter directly in contact with mucosal immune cells. To avoid this interaction, the bowel wall is covered by a single layer of epithelial cells, responsible of protecting the bowel wall from bacteria attack (Goto & Kiyono, 2012). If these protective mechanisms fail, bacteria can penetrate in the bowel wall, causing inflammatory immune reactions such as those that occur in patients with IBD (Cario, 2012).

In healthy subject the microbiota consists of 1000 different species, including more than 10^{12} cells, conferring healthy benefit to the host such as nutrition, protection against pathogens, immune system development and metabolic processes (Becker et al., 2015). Moreover, in the healthy gut, the flora is dominated by the phyla *Firmicutes* and *Bacteroidetes*, followed by species belonging to the *Actinobacteria* and *Proteobacteria* (Qin et al. 2010). By contrast, in IBD patients, commensal bacteria have fewer complex profiles: both *Firmicutes* and *Bacteroidetes* are decreased, whereas *Actinobacteria* and *Proteobacteria* are considerably increased (Frank et al. 2007; Chassaing & Darfeuille-Michaued, 2011; Morgan et al., 2012), causing a condition defined as dysbiosis. It is still uncertain whether the dysbiosis is the primary cause of inflammation or simply a secondary phenomenon of IBD (Eichele & Kharbanda, 2017). Furthermore, IBD patients, compared to healthy controls, have fewer bacteria with anti-inflammatory properties leading to gut inflammation. In the clinic, antibiotics are used to treat IBD-related conditions, highlighting the role of microbiota in the development of IBD (Becker et a., 2015).

IBD is increasing in the populations and, in order to explore the mechanism underlying IBD and to find potential treatment strategies, several animal studies have been used. Indeed, 66 different kinds of animal models have been established to understand IBD pathophysiology, that can be classified into chemically induced, cell-transfer, congenital mutant, and genetically engineered models

(Mizoguchi, 2012). In 1993, the appearance of spontaneous colitis was discovered in mice knockout for interleukin (IL)-2, IL-10, or T-cell receptor (TCR) α. Since now, more than 40 different types of genetically engineered knockout (KO) and congenital gene mutant mouse strains have been found to spontaneously develop colitis and/or ileitis (**Figure 1.10**).

All these models suggest that IBD is mediated by highly complicated molecular mechanisms (Mizoguchi, 2012). As reported in **Figure 1.10**, IBD models are classified into five major model groups: congenital (spontaneous gene mutation), chemically induced, genetically engineered, cell-transfer and spontaneous. The genetically engineered model can be divided in other 6 groups based on the lack or overexpression of a specific gene, the use of cell-specific promoter to overexpress the gene, or innate models that are immune-deficient mouse-based system. The principal model is the genetically engineered IL-10 KO mouse that spontaneously develops colitis after 3 months of age. C3H and Balb/c mouse backgrounds are more susceptible to this type of colitis, compared to the C57BL/6 strain, and enteric microbiota plays a major role in this type of colitis (Mizoguchi, 2012).

Beside the use of genetically engineered mice, there are also other models of colitis such as the chemical-induced model.

There are three different chemical models for experimental IBD, such as trinitrobenzene sulfonic acid (TNBS, or dinitrobenzene sulfonic acid (DNBS)), oxazolone and dextran sulfate sodium (DSS). DNBS-induced colitis is a model of CD which involves NOD2-dependent mechanisms (Mizoguchi, 2012). DSS colitis is a popular experimental protocol, being an easy, time and cost-saving model. DSS, a polymer of sulfated polysaccharide, is a colitogenic chemical with anticoagulant properties that induces epithelial damage (Eichele & Kharbanda, 2017).

It is administrated in drinking water to induce intestinal injury characterized by bloody diarrhea, ulcerations, intestinal inflammation, body weight loss, and shortening of colon length (Mizoguchi, 2012). The effectiveness of the DSS-induced colitis depends on several factors, such as DSS concentration (usually between 1%-5%), duration and frequency of administration (acute or chronic), molecular weight of the manufactured DSS, animal strain (C3H/HeJ, C57BL/6 and BALB/c mice strains are more susceptible), and microbial environment in the animal facility (GF versus specific pathogen-free). Depending on these factors, the animals can develop acute colitis, chronic colitis or even colitis-induced dysplastic lesions (Eichele & Kharbanda, 2017; **Table 1.2**).

Acute colitis is obtained by DSS continuous administration to understand the induction phase of intestinal injury. Another possibility is to treat mice with DSS for a few days followed by the recovery phase changing DSS to normal drinking water and then examine after termination of recovery days (Mizoguchi, 2012). During DSS administration, mice can exhibit marked weight loss (about 5%-10% of initial body weight), altered stool consistency leading to diarrhea and hematochezia (Eichele & Kharbanda, 2017). The administration of DSS induced a change in the gut microbiota, with an increase of Gram-negative anaerobes such as *Bacteroidaceae* (Okayasu et al., 1990).

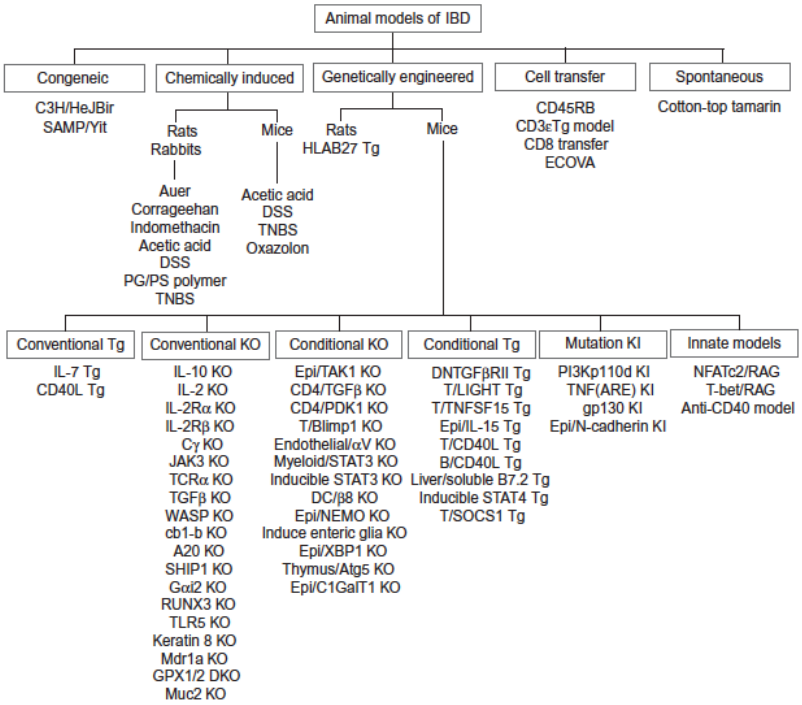


Figure 1.10. Classification of animal models of experimental IBD (modified from Mizoguchi, 2012).

Compared to the other IBD models, in DSS colitis enteric bacteria contribute to the suppression of the acute colitis. As shown by *Rakoff-Nahoum et al.* (2004), wide spectrum of antibiotics can cause lethal colitis with intestinal bleeding after DSS administration, with higher mortality rate in mice deficient for MyD88 or TLR4$^{-/-}$ mice (Rakoff-Nahoum et al., 2004). Furthermore, mice kept under GF environment develop lethal colitis with massive intestinal bleeding in response to DSS (Mizoguchi, 2012). In contrast to the majority of IBD models, DSS-induced colitis recovers spontaneously after termination of DSS treatment, allowing to study the mechanism of recovery processes from intestinal inflammation such as epithelial healing and development of regulatory immune cells with ability to enhance the wound healing (Mizoguchi, 2012).

FACTORS	VARIABLES	DESCRIPTION
DSS	Molecular weight	40-50 kDa for tissue penetration
	Dosage concetration	From 1.5% to3% are the most used
	Duration of therapy	Acute: 5-10 days administration Chronic: 4-5 repeating cycles of DSS and sterile water
	Manufacturer/batch	Various manufactures with differing potency
HOST	Genetically susceptible strain of animal	Susceptible strains: C3H/HeJ, C57BL/6, BALB/C
ENVIRONMENT	Housing conditions	Groups vs individual unit, frequency of cage changes alters coprophagy by host
	Microbial state	GF vs specific pathogen free vs wild type

Table 1.2. Factors influencing the effectiveness of dextran sodium sulfate (DSS)-mediated colitis (modified by Eichele & Kharbanda, 2017).

Experimental colitis animal models have provided meaningful and indispensable insights into the histopathological and morphological changes in the intestinal tract related to the pathogenesis of human IBD, but none can fully reflect human IBD and each model has some demerits, but all together they provide important interventions for developing new therapeutic measures such as cell-specific therapy and personalized therapy to save the lives of patients with IBD more safely and more effectively (Mizoguchi, 2012).

New evidence demonstrated that the catabolism of tryptophan (TRP), followed by 5-HT production, may also have repercussion in ENS neurotransmission and can modulate the severity of colitis. The 90% of 5-HT in the body is produced by EC cells and, once it is released, has a pivotal role in controlling gut motility and secretary functions, promoting growth and turnover of mucosal epithelium, and maintaining intestinal homeostasis (Chen et al., 2016; Stavely et al., 2018). 5-HT is a neurotransmitter in both CNS and ENS (Mawe & Hoffman, 2013), indeed, 2% of ENS' neurons are serotonergic (Costa et al., 1996). The development of intestinal inflammation involves the action of neuromodulators and cytokines that transmit signals in a bidirectional way, between enteric neurons and immune cells; 5-HT is one of the most essential paracrine/neurocrine messengers that participate in this cross talk (Terry & Margolis, 2017).

Animal models, but also data from humans, support a proinflammatory role for mucosal 5-HT in intestinal inflammatory disease. It has been discovered that the EC cells, the major producers of enteric 5-HT, are increased in intestinal biopsy specimens taken from individuals with CD and UC (Belai et al. 1997; El-Salhy et al. 1997; Coates et al. 2004). Moreover, in mice KO for tryptophan hydroxylase 1 (TPH1) the severity of colitis is significantly diminished (Terry & Margolis, 2017).

1.9 Serotonergic Pathways and Tryptophan Metabolism

As mentioned above, 5-HT is a critical signaling molecule in in intestinal inflammatory disease and is involved in a wide range of physiological functions (Gershon & Tack, 2007; Terry & Margolis, 2017). 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 (l-AAD) producing 5-HT (Terry & Margolis, 2017). The first step of 5-HT synthesis can be mediated by 2 different tryptophan hydroxylases, TPH1 and TPH2, which are found, respectively, in EC cells and neurons of SNC and SNE (Gershon & Tack, 2007). The 90% of 5-HT production in the gut occurs in EC cells as shown in **Figure 1.11**.

TPH1 deletion (TPH1KO) does not alter the content of 5-HT in the brain, whereas deletion of TPH2 (TPH2KO) virtually eliminates brain 5-HT, but hardly alters the total 5-HT content of the gut at all to indicate that this is because the EC cell pool of 5-HT dwarfs that in enteric neurons (Gershon, 2012). However, it has been discovered that in TPH1KO mice bowel motility was not affected whereas in

TPH2KO mice gut motility was abnormal (Gershon, 2012). 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) suggesting that 5-HT in ECs cells serves purposes that are not only related to GI motility, such as peristalsis, secretion, vasodilation and perception of pain or nausea (Mawe & Hoffman, 2013).

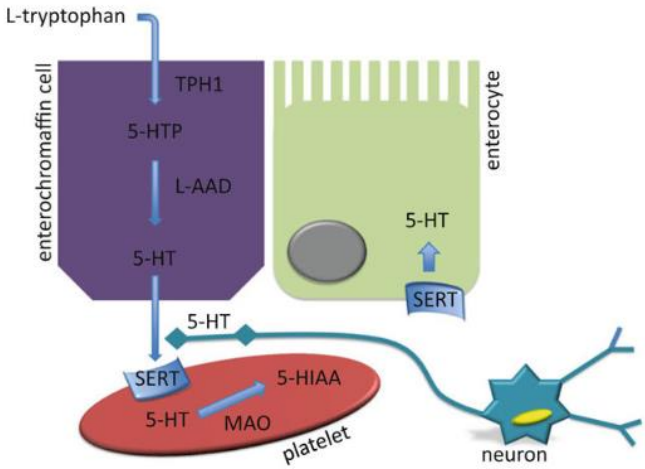


Figure 1.11. 5-HT biosynthesis. L-tryptophan is taken up by enterochromaffin (EC) cells (purple) where it is converted by tryptophan hydroxylase 1 (TPH1) to 5-hydroxytryptophan (5-HTP). The enzyme L-amino acid decarboxylase (L-AAD) then produces 5-hydroxytryptophan (5-HT) which is released into the extracellular space and can either act locally in the intestine, via its receptors in the intestinal mucosa or intercalated dendrites of the submucosal and myenteric plexuses, or be taken up by platelets (red) via the serotonin transporter (SERT). Locally acting 5-HT will be taken up by the enterocytes, via SERT, where it is broken down by monoamine oxidase (MAO) and metabolized to 5-hydroxyindoleacetic acid (5-HIAA). The 5-HT taken up by platelets is either released at a distal site, where it can execute hormonal actions, or metabolized, as in the enterocytes, by MAO within the platelet, and later excreted by the kidneys (modified from Terry & Margolis, 2017).

Emerging evidence also suggests that the serotonergic system can feel the influence of gut microbiota, especially in the periods before the developments 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 showing its key role 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.* (2015), 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. Changes in microbiota composition have been found also in several gut inflammation diseases, indeed 5-HT is implicated in UC, CD and celiac disease (Terry & Margolis, 2017). Moreover increase 5-HT mucosal levels appears to be proinflammatory and becoming important in the onset of the gut pathology.

TRP metabolism depends on three major pathways in the GI tract (**Figure 1.12**): (i) the direct transformation of TRP by the gut microbiota into an array of molecules, including ligands of the aryl hydrocarbon receptor (AhR); (ii) the kynurenine pathway (KYN) in both immune and epithelial cells via indoleamine 2,3-dioxygenase (IDO) 1; and (iii) 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-α. 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).

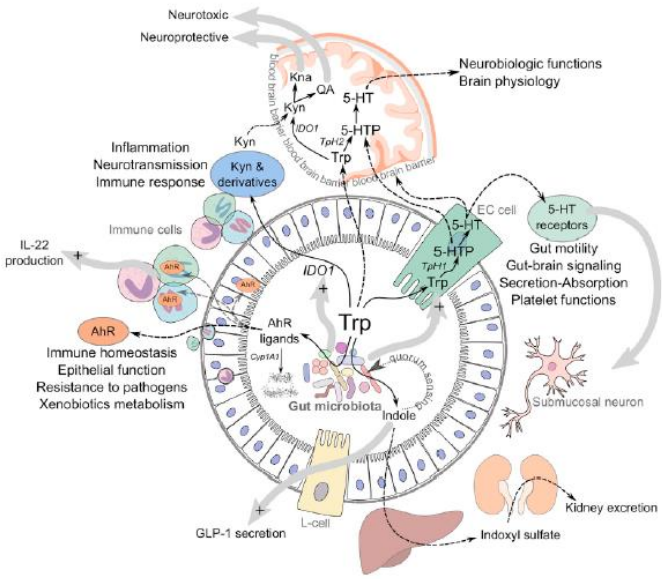


Figure 1.12. Integrated trp metabolism under the control of the gut microbiota in host physiology. Dietary TRP can be directly converted by gut microbiota into AhR ligands that are able to tune local and distant host functions, including immune homeostasis and barrier physiology. Gut microbiota also 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 studies have encouraged 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 increased 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 is considered as neuro-protective whereas quinolinic acid has been considered excitotoxic (Stone et al., 2012). Less is known about 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 and the development of GI functional disorders or/and IBD.

2. AIM

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. Furthermore, TLRs are involved in the low-grade inflammation, such as obesity, and in immunological alterations found in patients with irritable bowel syndrome (IBS) or IBD. IBD, generally, is confined to the colon with occasional extension into terminal ileum; however, several studies have shown similar functional abnormalities in the small intestine of patients suffering from UC as well as in experimental animal models of colitis. Therefore, the present research was focused to evaluate the effects of experimental colitis in small intestine of WT and $TLR4^{-/-}$ mice. Nowadays, 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 primary aim of the present PhD final thesis was to characterize the role of intestinal microbiota in the control of gut homeostasis by assessing the role of TLR4 in the neuromuscular function in presence of low- or high-grade inflammation. In particular, this research was focused on evaluating:

- ✓ TLR4 deficiency and ABX treatment in tuning structural and functional integrity of ENS;
- ✓ TLR4 signaling on enteric glia phenotype and related consequences on GI functions;
- ✓ TLR4 deficiency on the severity of HFD-induced obesity or DSS-induced colitis and, accordingly, on ENS homeostasis and ileal serotonergic pathway, to evidence potential consequences in the microbiota-gut axis.

3. MATERIALS and METHODS

3.1 Mice

For characterizing the role of TLR4 in tuning structural and functional integrity of ENS, 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. Juvenile C57BL/6J mice (CNTR, 3 ± 1 weeks old) were used for investigating the effect of 2-week antibiotic-induced gut dysbiosis on ENS architecture (Caputi et al., 2017b). All 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 all the 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 of the University of Padova, and by the Italian Ministry of Health, and were in compliance with national and European guidelines for the handling and use of experimental animals.

3.2 *In vivo* treatments

Animals were randomized to the following treatment groups:

i. To induce gut dysbiosis, we used a previously established pharmacological strategy, 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). WT mice, aged 3 ± 1 weeks, 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 antibiotic cocktail was 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; **Figure 3.1**). WT mice, treated with vehicle (tap water), were used as control mice (CNTR group).

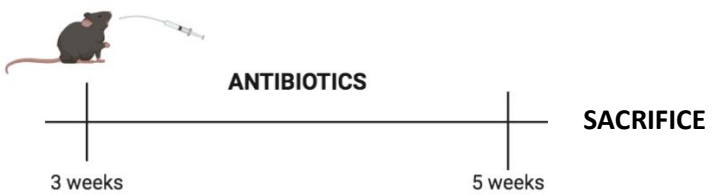


Figure 3.1. Antibiotics treatment for 2 weeks.

ii. To reproduce an obese status, we used a commercial standard diet (SD; 12% kcal from fat; altromin international) and a fat-enriched diet (HFD; 60% kcal from fat; mucedola srl) that were administered to WT and TLR4$^{-/-}$ mice, aged 9 ± 1 weeks, for 8 weeks (**Figure 3.2**; Antonioli et al., 2019).

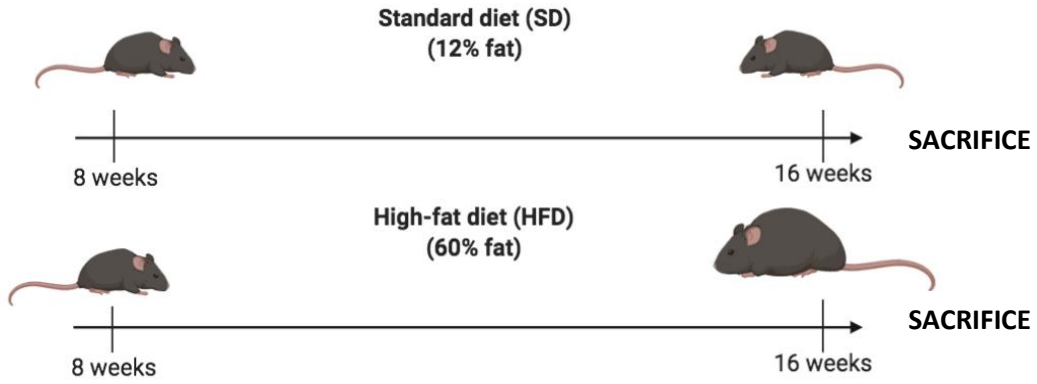


Figure 3.2. Standard and High-fat diet for 8 weeks.

iii. To induce experimental colitis, WT and TLR4$^{-/-}$ mice were treated for 5 days with 2% dextran sodium sulfate (DSS) dissolved in drinking water and then switched to regular drinking water for 3 days (**Figure 3.3**; Prescott et al., 2013; Wirtz et al., 2017). The mice were monitored every day, evaluating the disease activity index (DAI) (**Table 3.1**; Yang et al., 2016). The experimental endpoint was reached when a DAI value ≥ 2 was measured.

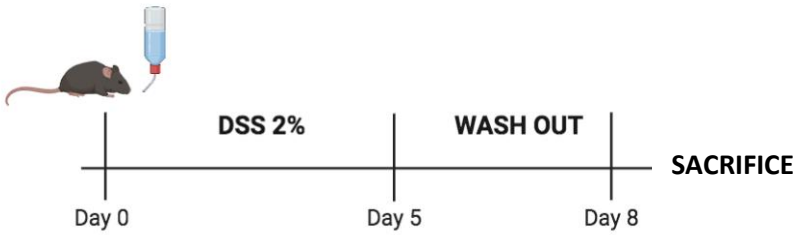


Figure 3.3. DSS treatment for 5 days.

DAI score	Weight loss (%)	Stool consistency	Occult/gross bleeding
0	None	Normal	Normal
1	1–5		
2	5–10	Loose stools	Hemoccult positive
3	10–15		
4	>15	Diarrhea	Gross bleeding

Table 3.1. Calculation of the Disease activity index (DAI). DAI was determined by the sum of the scores obtained by the evaluation of the percentage of body weight loss, status of stool consistency and presence of gross bleeding.

At the end of the *in vivo* treatments, animals were killed by cervical dislocation. All the following experimental procedures were conducted blindly. After the collection of the whole gastrointestinal

(GI) tract, the macroscopic damage in the small and large intestines was analyzed by eye-inspection as reported in **Table 3.2**.

Macroscopic tissue damage	Score
No damage	0
Hyperemia without ulcers	1
Hyperemia and wall thickening without ulcers	2
One ulceration site without wall thickening	3
Two or more ulceration sites	4
0.5 cm extension of inflammation or major damage	5
1 cm extension of inflammation or severe damage	6–10

Table 3.2 Calculation of macroscopic tissue damage. Macroscopic tissue damage was graded by visual inspection of a blinded observer according to the extent and severity of inflammatory changes in mucosal architecture of small and large intestines.

The third evaluation was the histological analysis following the criteria shown in **Table 3.3**.

Ileal mucosal damage	Score
Inflammatory cells	
Presence of occasional inflammatory cells in the lamina propria	0
Increased numbers of inflammatory cells in the lamina propria	1
Confluence of inflammatory cells, extending into the submucosa	2
Transmural extension of the infiltrate	3
Tissue damage	
No mucosal damage	0
Discrete lymphoepithelial lesions	1
Surface mucosal erosion or focal ulceration	2
Extensive mucosal damage and extension into deeper structures of the bowel wall	3

Table 3.3. Calculation of ileal mucosal damage index. Histological scores were blindly determined on H&E stained ileal tissue sections collected on day 8 post-DSS administration.

3.3 *In vitro* treatments

To assess the impact of enteric glia on ENS function, ileal segments from WT and TLR4$^{-/-}$ mice were incubated for 2 hour with 10 μM fluoroacetate (FA), an inhibitor of glia function at 37° C with 95% O_2 and 5% CO_2, before contractility analysis and IHC experiments (McClain & Gulbransen, 2017).

3.4 Histology Analysis

Ileal specimens, fixed in 10% buffer formalin, embedded in paraffin and cut into 4 µm-sections were stained with haematoxylin and eosin (H&E). Ten slides for each genotype were analyzed blindly (Caputi et al., 2017a).

3.5 Measurement of Metabolic Parameters

Blood samples were taken from the tail vein of mice exposed to SD or HFD, after 1-hour starvation, and triglycerides, cholesterol and glucose levels were measured using Multicare Insensor (BSI Srl, Arezzo, Italy), according to the manufacturer's instructions.

3.6 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. 100 µl of 25 mg/ml FITC-dextran, dissolved in 0.9% saline, were administered by gavage to the 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 and the colon were divided into 10 and 3 segments of equal length, respectively. Tissue luminal and fecal contents were centrifuged at 12,000 rpm for 10 minutes at 4°C, and fluorescence intensity of the supernatants were measured using a fluorimeter (Victor, PerkinElmer) at excitation 485 nm and emission at 525 nm. Gastrointestinal transit was calculated as the geometric center (GC) of distribution of the fluorescent marker using the following formula (Wehner et al., 2007):

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

3.7 Stool Frequency and Colonic Emptying

Fecal pellet output and water content was assessed in sham and treated non-fasted WT and $TLR4^{-/-}$ mice (N=6). Fecal water content provides an indication of constipation, diarrhea or malabsorption. Each animal was placed in a clear clean cage and were examined throughout a 60-minute period. The number of stool pellets extruded per hour (stool frequency) was used as an index of colonic emptying. Pellets were collected immediately after expulsion and placed in sealed tubes weighed beforehand. These were weighed to obtain wet weights and then dried overnight at 65°C to obtain constant dry weights. Water content was calculated from the difference in wet and dry weights expressed as a percentage. (Li et al., 2006; Anitha et al., 2012; Caputi et al., 2017a).

3.8 *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 and 2017b). Experiments were performed on full-thickness distal ileum segments isolated from sham and treated WT and $TLR4^{-/-}$ mice. After performing midline laparotomy, the ileum was collected, quickly transferred into a dissecting dish filled with Krebs solution, and its contents were gently flushed out. Distal ileal 1-cm segments were suspended between the organ holder and the isometric force transducer (Grass transducer; Grass Instruments, West Warwick, RI, USA) by a silk thread. The strips were allowed to equilibrate for 1 h in 10-mL organ baths containing oxygenated (95% O_2 and 5% CO_2) 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) maintained at 37°C. Tension of the segments was set to 1 g and changes in ileal 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 increasing concentrations of CCh (0.01-100 µM), added cumulatively, to obtain a concentration-response curve. 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 a S88 stimulator (Grass Instrument, Quincy, MA, USA) in basal or non-adrenergic non-cholinergic (NANC) conditions, obtained by adding 1 µM guanethidine and 1 µM atropine to the organ bath. The neuronal basis of the responses was verified after pretreatment with tetradotoxin (TTX, 1 µM) for 30 minutes. To evaluate nitrergic-mediated neurotransmission, ileal preparations were pre-incubated for 20 minutes with 100 µM Nω-nitro-L-arginine methyl ester (L-NAME), a pan-NOS inhibitor, or 10 µM 1400W, a iNOS inhibitor, in NANC conditions and follow by 10 Hz-EFS-mediated NANC responses to induce relaxation. In order to assess the purinergic origin, the relaxation response was also studied after addition of 1 µM MRS2500, a P2Y1 receptor antagonist. Concentration-response curves to 5-HT (0.3–30 µM) were obtained in a non-cumulative manner (Forcèn et al., 2015). In order to evaluate the involved 5-HT receptors type, a submaximal dose of 5-HT (30 µM) was tested in the presence of 1 µM ketanserin, a $5\text{-}HT_{2A}R$ antagonist, or 0.1 µM ondansetron, $5\text{-}HT_3R$ antagonist, left in contact to the tissue for at least 30 min. Concentrations of the drugs 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, except for 30 µM 5-HT responses that were expressed as gram tension/% 1 µM CCh response. 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 and 2017b).

3.9 Immunohistochemistry on Ileal Whole Mount Preparations

Fresh isolated distal ileum 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 attached myenteric plexus (LMMPs) were prepared as previously described (Caputi et al., 2017b). 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 the 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 0.3% Triton X-100) for 45 minutes with gentle shaking. After blocking nonspecific sites with PBT containing 5% BSA for 1.5 hour at room temperature, LMMPs were incubated with primary antibodies (**Table 3.4**) diluted in PBT/BSA 5% 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 3.5**). 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-WM
GFAP (chicken)	polyclonal	Merk Millipore	1:100	IHC-WM
S100β (rabbit)	EP1576Y	Merk Millipore	1:100	IHC-WM
IBA1 (rabbit)	polyclonal	Wako Corporation	1:800	IHC-WM
SOX10 (rabbit)	monoclonal	abcam	1:50	IHC-WM
nNOS (rabbit)	polyclonal	Life Technologies	1:100	IHC-WM
iNOS (rabbit)	polyclonal	Santa Cruz Biotechnology	1:50	IHC-WM
P2Y1 receptor (rabbit)	polyclonal	Alomone Labs	1:100	IHC-WM
connexin-43 (rabbit)	polyclonal	Alomone Labs	1:100	IHC-WM
Choline acetyltransferase (ChAT; goat)	Polyclonal	Merk Millipore	1:50	IHC-WM

Table 3.4. Characteristics of the primary antibodies used for immunofluorescence analyses. IHC-WM = immunohistochemistry in whole mount preparations.

SECONDARY ANTIBODY	SOURCE	DILUTION	APPLICATION
goat anti-rabbit IgG Alexa Fuor 488 conjugate	Life Technologies	1:1000	IHC-WM
goat anti-chicken IgG Alexa Fuor 488 conjugate	Life Technologies	1:1000	IHC-WM
Streptavidin Alexa Fuor 555 conjugate	Life Technologies	1:1000	IHC-WM
Streptavidin Alexa Fuor 488 conjugate	Life Technologies	1:1000	IHC-WM
dylight 650 goat anti-rabbit IgG	Life Technologies	1:1000	IHC-WM
donkey anti-goat IgG Dy Light 549 conjugate	Jackson ImmunoResearch	1:500	IHC-WM

Table 3.5. Characteristics of the secondary antibodies used for immunofluorescence analyses. IHC-WM = immunohistochemistry in whole mount preparations.

3.10 Confocal Image Acquisition and Analysis

Images were acquired with a Zeiss LSM 800 confocal imaging system (Oberkoken, Germany) equipped with a 40× objective (NA 0.95) and an oil-immersion 63× objective (NA 1.4). Z-series images, composed of 10 to 15 plane forming Z-stack of 512 × 512 pixels, were captured and processed as maximum intensity projections. Z-stacks of 8- or 10-µm depth were obtained for LMMPs of about 4-6 animals per group. All microscope settings were set to collect images below saturation and were kept constant for all images. In LMMPs total neuron population of myenteric ganglia was analyzed by counting the number of HuC/D^+ cells in 10 randomly-chosen images per mouse (N=8 mice/group), and normalized to the area of the ganglia. To evaluate the distribution of nitrergic neurons in ileal myenteric plexus, the number of $nNOS^+$ enteric neurons was blindly counted and normalized as described before (Caputi et al., 2017a). Protein immunoreactivity was determined

in LMMPs whole mount preparation by measuring the fluorescent intensity for each antigen in 10 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 units (A.U.).

3.11 Tryptophan Metabolites Analysis

Plasma and ileal samples were stored at -80°C for subsequent 5-HT metabolites analyses. Plasma samples were diluted in milliQ H_2O and centrifuged at 13,000 g for 2 minutes before high-performance liquid chromatography (HPLC) analysis. 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 sonicated with Elmasonic S30 sonicator (Elma, Singer, Germany; four 15-second bursts, 60W) and centrifuged at 13,000 rpm 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 rpm for 10 minutes at 4°C, as previously described (Giron et al., 2008; Comai et al., 2016), for protein determination with the Lowry assay (Lowry et al., 1951). The ileal 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. 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 (Comai et al., 2016).

3.12 Real Time Quantitative RT-PCR

Total RNA was extracted from mucosa-deprived whole wall (WW) samples with TRIzol (Invitrogen) and treated with DNase I (DNase Free, Ambion) to remove any traces of contaminating DNA. cDNA was obtained retrotranscribing 0,5 µg of total RNA using the High Capacity cDNA synthesis kit (Applied Biosystems, Milan, Italy). Quantitative RT-PCR was performed on the Abi Prism 7000 real-time thermocyclator (Applied Biosystems, Milan, Italy) with Power Sybr Green Universal PCR Master Mix (Applied Biosystems, Milan, Italy) following manufacturer's instructions. Primers were designed using Primer Express software (Applied Biosystems, Milan, Italy) on the basis of available sequences deposited in public database. Primer sequences were: iNOS fw 5'- CAGCTGGGCTGTACAAACCTT -3', rv

5’- CATTGGAAGTGAAGCGTTTCG -3’; β-actin fw 5’-ACCAGAGGCATACAGGGACA-3’, rv 5’-CTAAGGCCAACCGTGAAAAG-3’. For quantitative RT-PCR a final concentration of 500 nmol/L for each primer was used. Primers were designed to have a similar amplicon size and similar amplification efficiency as required for the utilization of the 2-ΔΔCt method to compare gene expression, as previously described (Bistoletti et al., 2019). β-actin was used as housekeeping gene.

3.13 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 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.

3.14 Materials and Reagents

SUBSTANCE	SOURCE
Ampicillin	Sigma-Aldrich, Italy
Atropine sulfate	Sigma-Aldrich, Italy
Bovine serum albumin	Sigma-Aldrich, Italy
$CaCl_2 \cdot 2H_2O$	Merck, Italy
Carbachol	Sigma-Aldrich, Italy
Dextran sodium sulfate	Sigma-Aldrich, Italy
D-glucose	Sigma-Aldrich, Italy
Fluorescein isothiocyanate-dextran 70 KDa	Sigma-Aldrich, Italy
Fluoracetic acid	Sigma-Aldrich, Italy
Guanethidine	Sigma-Aldrich, Italy
KCl	Carlo Erba, Italy
Ketanserin	Tocris Bioscience
KH_2PO_4	Carlo Erba, Italy
Metronidazole	Sigma-Aldrich, Italy
$MgSO_4 \cdot 7H_2O$	Sigma-Aldrich, Italy
CITIFLOUR AF-1	SIC, Italy
MRS2500	Tocris Bioscience
Na_2PO_4	Merck, Italy
NaCl	Carlo Erba, Italy
Neomycin	Sigma-Aldrich, Italy
Nω-Nitro-L-arginine methyl ester hydrochloride	Sigma-Aldrich, Italy
Ondansetron	Tocris Bioscience
Paraformaldehyde 8% aqueous solution	Electron Microscopy Science, Italy
Serotonin	Sigma-Aldrich, Italy
Sucrose	Sigma-Aldrich, Italy
Tetradotoxin	Sigma-Aldrich, Italy
Vancomycin	Sigma-Aldrich, Italy
1400W	Sigma-Aldrich, Italy

4. RESULTS

4.1 Microbiota and immune system in ENS homeostasis

A highly dynamic connection between microbiota and gut homeostasis it has been recently established. For instance, in the *Drosophila melanogaster* the microbiota improves intestinal stem cell proliferation and regeneration of the gut epithelium whereas, under GF conditions, larvae exhibit reduced development, underlining the importance of commensal microbiota in ensuring host metabolism and energy balance (Kabouridis & Pachnis, 2015). It is known that the bidirectional communication between microbiota and its host is beneficial and regulates host homeostasis (Caputi et al., 2017a and 2017b). Indeed, in the host, the presence of microorganisms is sensed by pattern recognition receptors (PRRs). The family of TLRs belongs to the most important subclass of PRRs that plays a key role in the maintenance of symbiosis between gut microbiota and its host (Rakoff-Nahoum et al., 2004; Kabouridis & Pachnis, 2015). TLR4, which recognizes lipopolysaccharide (LPS), a major component of outer membrane of Gram-negative bacteria, is expressed by immune cells as well as enteric neurons and glia, suggesting that most of ENS cell population have the capacity to respond directly to stimuli derived from the microbial flora (Barajon et al., 2009; Kabouridis & Pachnis, 2015). Furthermore, microbiota perturbations can affect postnatal ENS development, causing adaptive changes in both enteric neurons and glia (Kabouridis et al., 2015a).

4.1.1 Antibiotic-induced microbiota dysbiosis and TLR4 deficiency alter enteric glial phenotype

Development of the intestinal microbiota is a key dynamic process in the first years of life but is also a critical time for ENS development and maturation (Brun et al., 2013; Borre et al., 2014). Considering that changes in microbiota composition and alterations on TLR4 signaling are known to influence ENS morphology (Brun et al., 2013; Hyland & Cryan, 2016; Caputi et al., 2017a and 2017b), we have thus evaluated ENS integrity by immunohistochemistry.

In ileal myenteric plexus of both $TLR4^{-/-}$ and ABX-treated mice, the numbers of HuC/D^{+} and $nNOS^{+}$ neurons were significantly lower than in control mice (-13±2% and -30±1%, respectively, in $TLR4^{-/-}$ mice; -26±2% and -29±2%, respectively, in ABX mice; **Figure 4.1**).

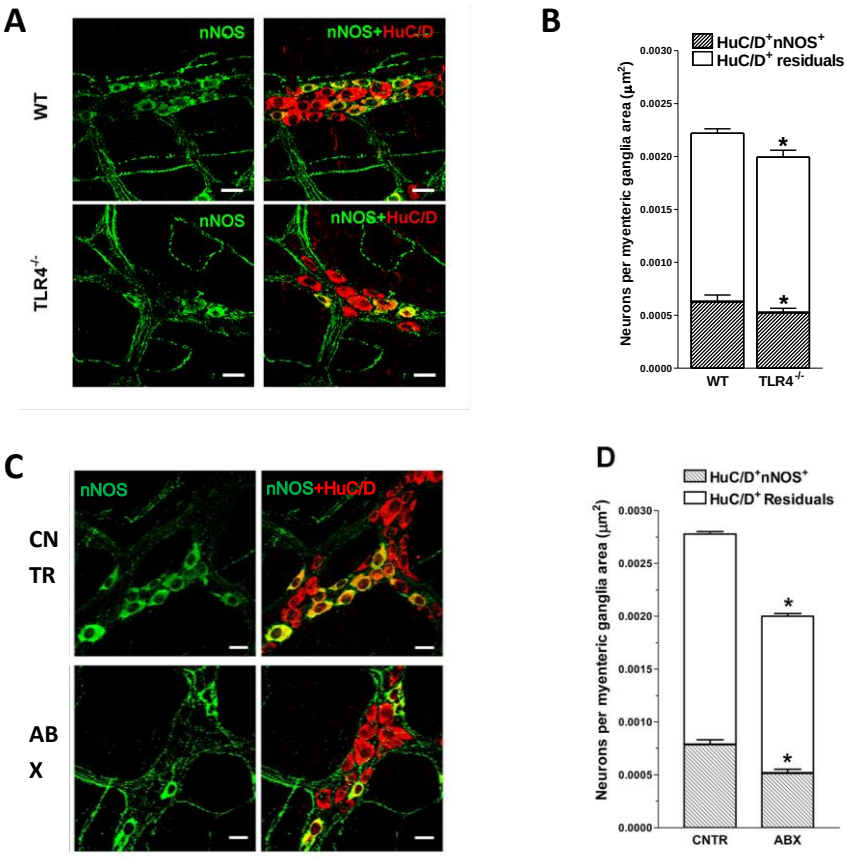


Figure 4.1. TLR4 signaling and antibiotic-induced microbiota dysbiosis affect neuronal phenotype. **A-C**) Representative confocal microphotographs showing the distribution of nNOS (green) and HuC/D (red) in WT and TLR4$^{-/-}$ LMMPs preparations (**A**) and in WT LMMPs preparations from CNTR and ABX-treated mice (**C**) (bars = 22 µm). **B, D**) Total number of HuC/D^{+}nNOS^{+} neurons and residual HuC/D^{+} neurons in WT and TLR4$^{-/-}$ LMMPs preparations (**B, C**) and in WT LMMPs preparations from CNTR and ABX-treated mice. *P<0.05 vs respective control. N=8 mice/group.

In TLR4$^{-/-}$ and antibiotic-treated mice an alteration of the EGCs markers, S100β and GFAP, was found (**Figure 4.2**). LMMPs preparations from TLR4$^{-/-}$ and ABX mice showed a significant increase in S100β immunoreactivity compared to control mice (+20±4%, +100±2%, respectively **Figure 4.2**). These findings were associated to a 3-fold increase in the process length of GFAP^{+} gliofilaments in TLR4$^{-/-}$ LMMPs and a distortion of GFAP^{+} gliofilaments in ABX LMMPs (data not shown; Caputi et al., 2017a and 2017b).

Increased immunofluorescence of both glial markers is a sign of higher expression of these proteins, related to the presence of a inflammatory process, which usually determines a nonspecific defensive reaction of glial cells in response to harmful stimuli (Ochoa-Cortes et al., 2016). Usually the 'reactive gliosis phenotype' is produced in the pathogenesis of IBD, but also in response to bacterial and viral infections, and in presence of functional gastrointestinal disorders (FGIDs; e.g. irritable bowel syndrome (IBS); Ochoa-Cortes et al., 2016).

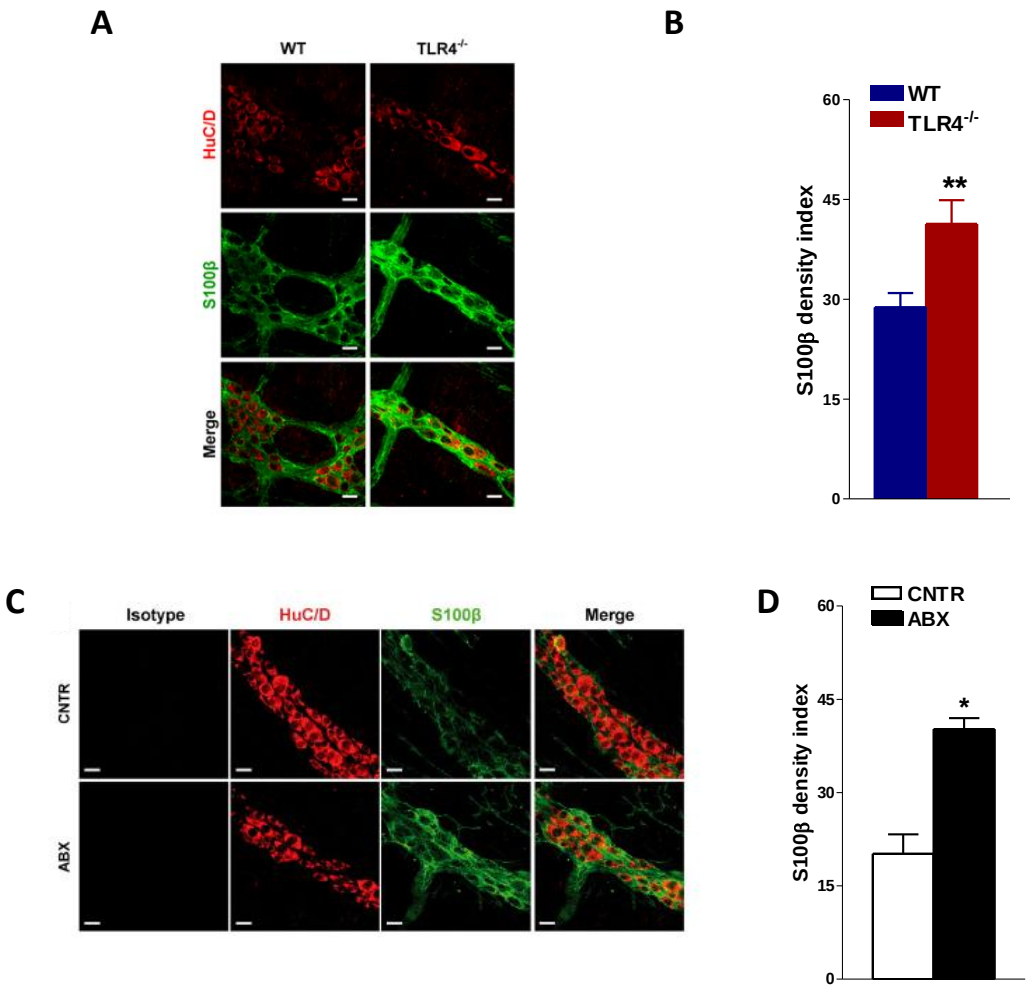


Figure 4.2. TLR4 signaling and antibiotic-induced microbiota dysbiosis affect glial phenotype. **A-C**) Representative confocal microphotographs showing the distribution of S100β (green) and HuC/D (red) in WT and TLR4$^{-/-}$ LMMPs preparations (**A**), and in WT LMMPs preparations of CNTR and ABX-treated mice (**C**) (bars = 22 µm). **B-D**) Analysis of S100β density index in ileal LMMPs whole-mount preparations of WT and TLR4$^{-/-}$ (**B**), and in WT LMMPs preparations of CNTR and ABX-treated mice (**D**). *P<0.05 vs CNTR; **P<0.01 vs WT. N=8 mice/group.

4.1.2 Antibiotic-induced microbiota dysbiosis and TLR4 deficiency affects excitatory neuromuscular contractility

Considering the neuron-glia plasticity mediated by an altered microbiota, we decided to evaluate the receptor and nonreceptor-mediated contractile response of ileal segments. To better understand ileal excitatory responses, cumulative concentration-response curves to the non-selective cholinergic receptor agonist CCh were performed. Ileal segments from both TLR4$^{-/-}$ and ABX-treated mice showed a downward shift of the concentration-response curve to CCh associated with a significant decrease in the maximum response compared to control mice (Emax=-27±8% and-49±6%, respectively; **Figure 4.3**).

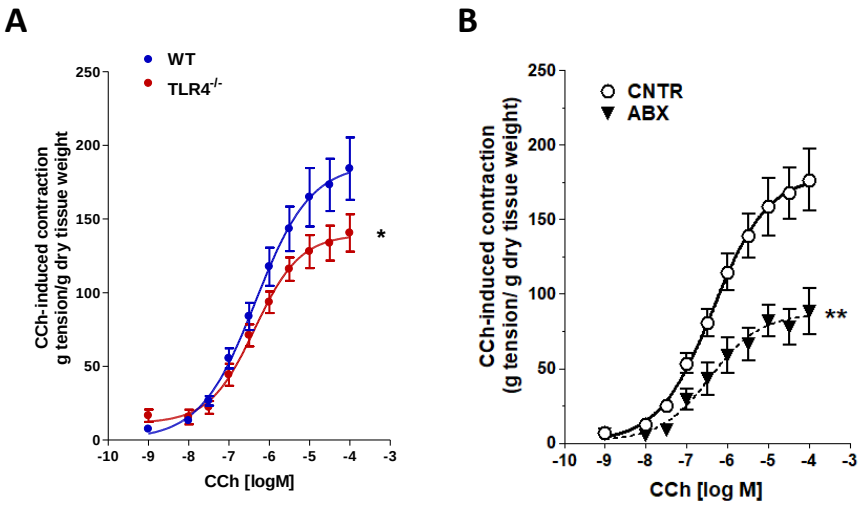


Figure 4.3. TLR4 deficiency and antibiotic treatment impair the contractile responses to CCh. Concentration–response curves to CCh of TLR4$^{-/-}$ (**A**) and ABX (**B**) ileal preparations. *P<0.05 vs WT; **P<0.01 vs CNTR. N=8 mice/group.

To confirm that ileal contraction changes were caused by alterations in neuromuscular function, we evaluated the effect of Electric Field Stimulation (EFS) at increasing frequency of stimulation on ileal preparations. In LMMPs preparations from TLR4$^{-/-}$ and ABX-treated mice (**Figure 4.4**), we obtained a significant reduction of EFS-elicited contractions compared to control value at 10 Hz, by 30% and 60%, respectively. The EFS-induced contractions up to 10 Hz induced a cholinergic response of neuronal origin, as previously shown by *Brun et al.* (2013).

TLR4$^{-/-}$ and ABX-treated mice obtained comparable alterations in the excitatory neuromuscular response and enteric reactive gliosis. Considering that all together these data indicate that the absence of both TLR4 signaling and commensal bacteria alters structural and functional integrity of ENS in a similar manner, we focused our research on the role of TLR4 in ENS homeostasis during normal and pathological conditions.

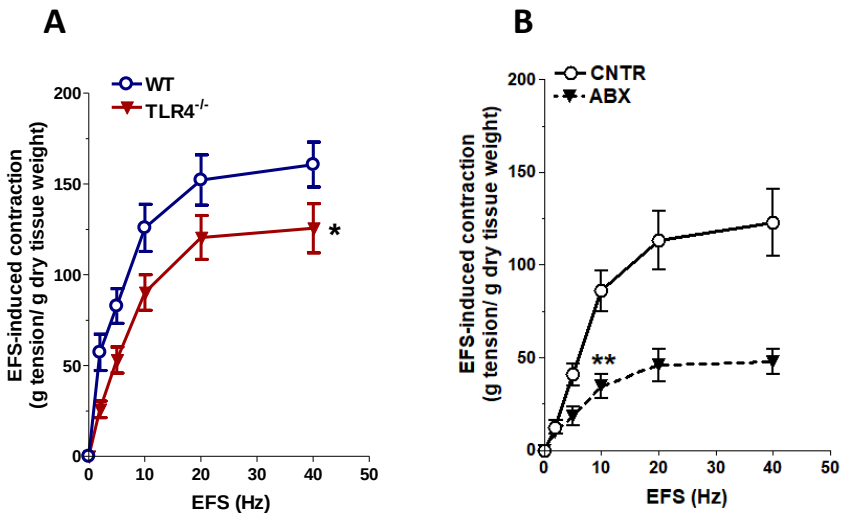


Figure 4.4. TLR4 deficiency and antibiotic treatment alters ileal neuronal response. EFS-induced excitatory responses in TLR4$^{-/-}$ (**A**) and ABX-treated (**B**) ileal preparations. *P<0.05 vs WT; **P<0.01 vs CNTR. N=8 mice/group.

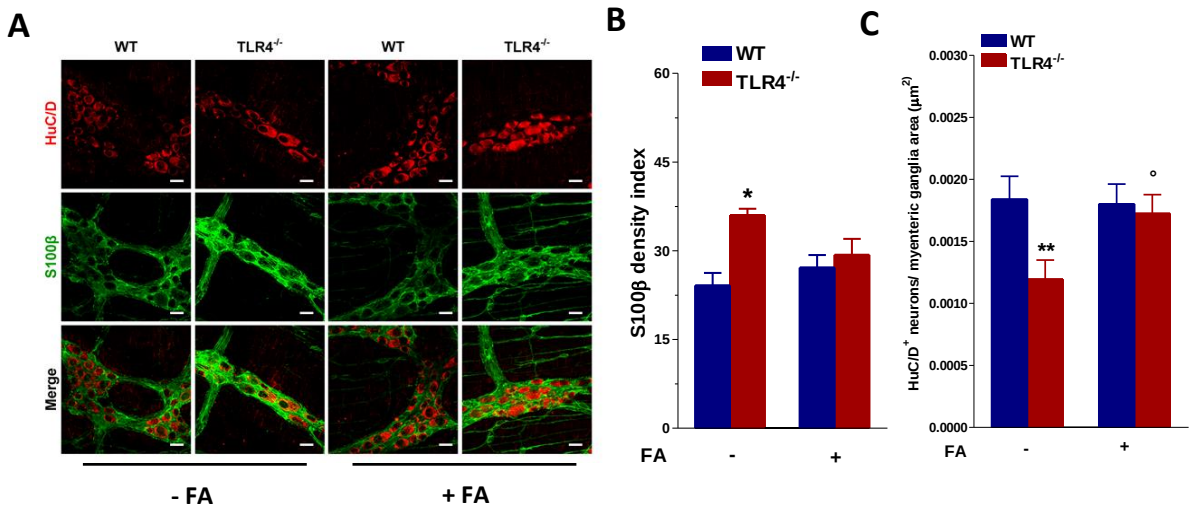
4.2 TLR4 and enteric glial cells

The absence of TLR4 signaling causes alterations in ileal neuromuscular function and glial phenotype leading to ileal dysmotility and reactive gliosis (Caputi et al., 2017a). To better understand the role of enteric glia in gastrointestinal (GI) modifications of TLR4$^{-/-}$ mice, we treated isolated ileal segments with the gliotoxin fluoroacetate (FA). Fluorocitrate (FC) and its precursor FA are commonly used as experimental tools to disrupt glial functions in both central nervous system (CNS) and enteric nervous system (ENS; Fonnum et al., 1997; Nasser et al., 2006; McClain & Gulbransen, 2017). Our goal was to determine whether the disruption of ECGs by FA could restore the function and the structure of ileal ENS in TLR4$^{-/-}$ mice.

4.2.1 *In vitro* fluoroacetate treatment reduces enteric reactive gliosis in TLR4$^{-/-}$ mice

TLR4 is expressed in the ENS (Rumio et al., 2006; Barajon et al., 2009) and is required for the regular growth of small intestine (Riehl et al., 2015). Firstly, we evaluated the impact of TLR4 absence on glial phenotype by immunohistochemistry. In ileal tissue from TLR4$^{-/-}$ mice the immunofluorescence of the glial markers S100β, SOX10 and GFAP was significantly higher compared to that found in WT mice (+49±1%; +25±2%; +15±4%, respectively; **Figure 4.5A,B,D,E,F**). The total number of HuC/D^{+} neurons was found significantly lower in the myenteric plexus of TLR4$^{-/-}$ mice compared to WT mice (-20±4%; **Figure 4.5A, C**).

After treatment with FA, in the LMMPs of TLR4$^{-/-}$ mice we found a slight but not significant increase of SOX10 immunofluorescence associated to an increase of the total number of HuC/D^{+} neurons suggesting a mechanism of neuroplasticity (Boesmans et al., 2015; **Figure 4.5A,B,C**). Treatment with FA determined a myenteric glial phenotype comparable between genotypes without any change in SOX10 immunofluorescence in TLR4$^{-/-}$ mice compared to WT mice (**Figure 4.5**).



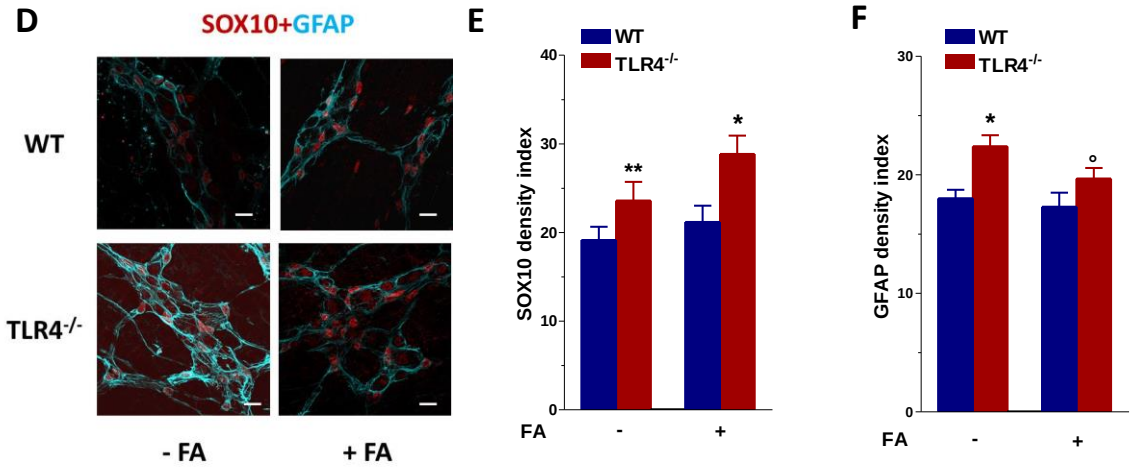
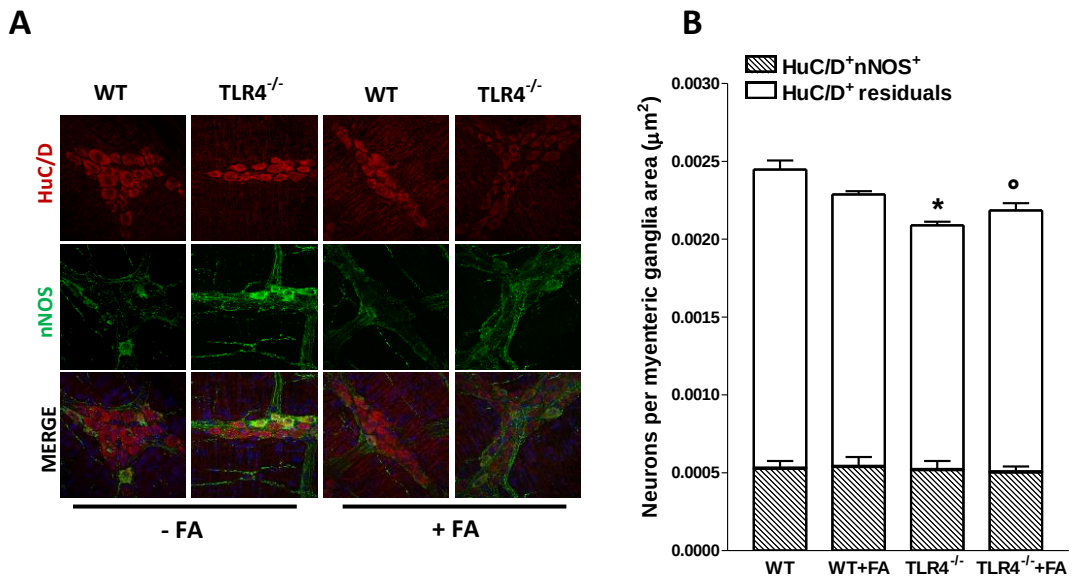


Figure 4.5. Effect of fluoroacetate (FA) treatment on enteric glial phenotype. **A-D**) Representative confocal microphotographs showing the distribution of S100β (green), HuC/D (red) (**A**) and SOX10 (red) and GFAP (cyan) (**D**) in LMMPs preparations of WT and TLR4$^{-/-}$ mice (bars = 22 µm). (**B, E, F**) Analysis of S100β, SOX10 and GFAP density index in ileal LMMPs preparations of WT and TLR4$^{-/-}$ mice. (**C**) Total number of HuC/D^{+} neurons in ileal LMMPs preparations of WT and TLR4$^{-/-}$ mice. *P<0.05, **P<0.01 vs WT; °P<0.05 vs respective control without FA. N=6 mice/group.

4.2.2 Fluoroacetate *in vitro* treatment alters nitrergic neurotransmission of small intestine in TLR4$^{-/-}$ mice

We have recently demonstrated that in NANC conditions, EFS at 10 Hz causes a 1.32-fold increase in relaxation in TLR4$^{-/-}$ mice preparations together with a proportional increase of nNOS^{+} neurons related to a partial loss of HuC/D^{+} neurons (-20±4%; Caputi et al., 2017a; **Figure 4.6**). In TLR4$^{-/-}$ mice the disruption of EGCs' activity, following the *in vitro* FA incubation, re-established an inhibitory response comparable to WT mice (**Figure 4.6C**) with a proportional reduction of nNOS^{+} positive neurons (**Figure 4.6A,B**).



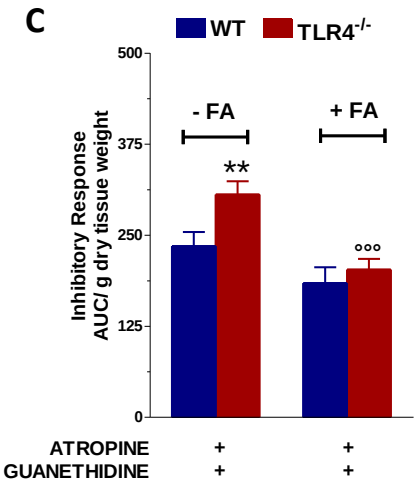


Figure 4.6. In vitro treatment with fluoroacetate (FA) alters nitrergic neurotransmission of small intestine in TLR4$^{-/-}$ mice. **A**) Representative confocal microphotographs showing the distribution of nNOS^{+} (green) and HuC/D^{+} (red) neurons in LMMPs preparations of WT and TLR4$^{-/-}$ mice (bars = 22 µm). **B**) Number of HuC/D^{+}nNOS^{+} and residual HuC/D^{+} neurons in ileal LMMP preparations WT and TLR4$^{-/-}$ mice. **C**) 10 Hz-EFS-evoked NANC relaxation responses in WT and TLR4$^{-/-}$ mice. *P<0.05, **P<0.01, ***P<0.001 vs WT; °P<0.05, °°°P<0.005 vs respective control without FA. N=6 mice/group.

Considering these data and the involvement of iNOS in NO-mediated relaxation and the higher immunoreactivity of iNOS in EGCs (Caputi et al., 2017a), we assessed whether the disruption of the ECGs' activity impairs the iNOS-mediated inhibitory response. Following *in vitro* incubation with the iNOS inhibitor 1400W, we revealed an inhibitory response comparable to that obtained after *in vitro* treatment with FA (MacEachern et al., 2015; **Figure 4.7A**), suggesting that NO release is mediated by iNOS expressed in EGCs, as shown by the higher immunoreactivity in TLR4$^{-/-}$ LMMPs (**Figure 4.7B,C**). Indeed, disruption of enteric glial cells activity with FA determined a marked reduction of iNOS immunoreactivity in both genotypes (**Figure 4.7B,C**).

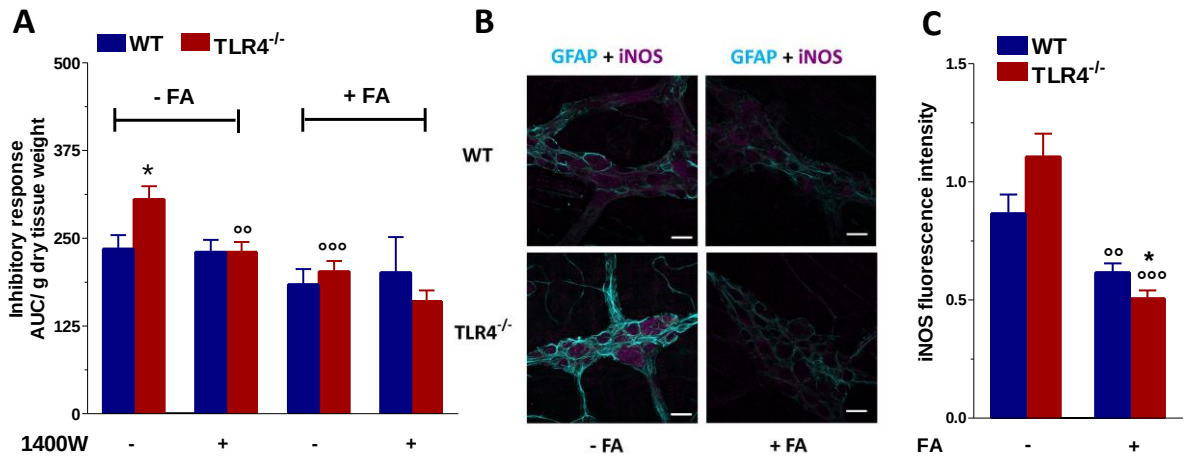
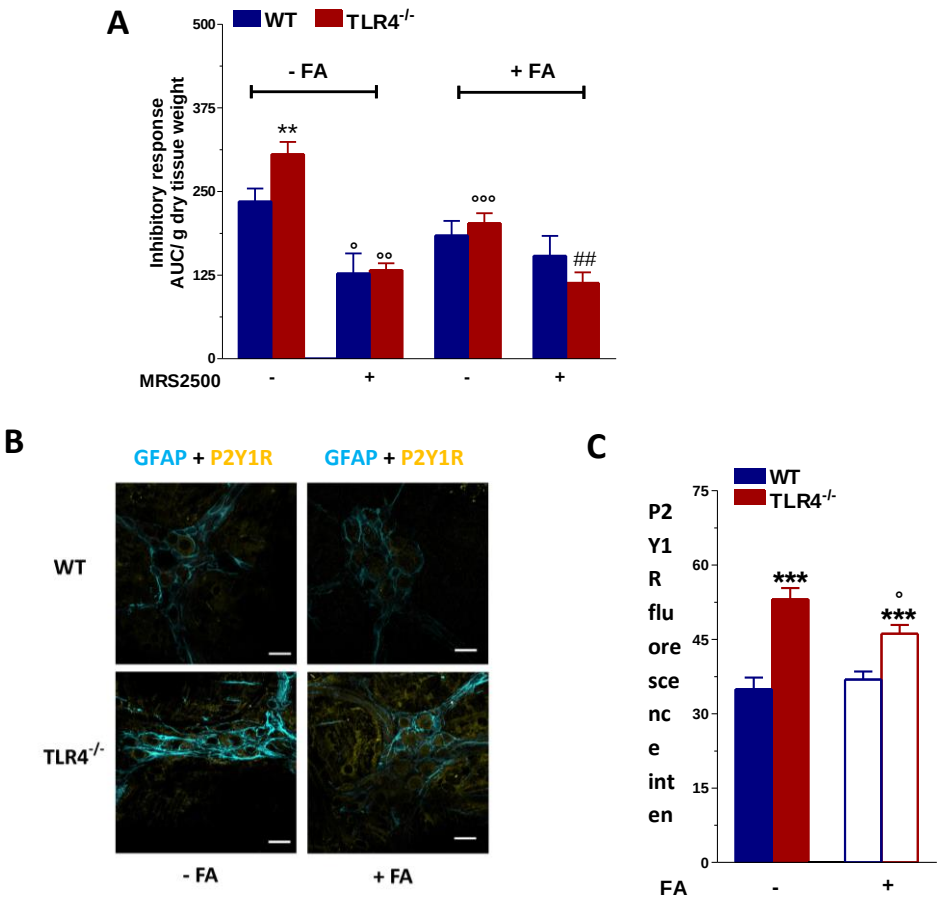


Figure 4.7. TLR4 signaling and EGCs modulates NO receptor-mediated relaxation. **A**) 10 Hz EFS-evoked relaxation in NANC conditions with or without 10 µM FA or 10 µM 1400W (iNOS inhibitor) in WT and TLR4$^{-/-}$ mice. **B**) Representative confocal microphotographs showing the distribution of GFAP (cyan) and iNOS (magenta) in LMMPs preparations of WT and TLR4$^{-/-}$ mice (bars = 22 µm). **C**) Analysis of iNOS fluorescence intensity in LMMPs whole mount preparations of WT and TLR4$^{-/-}$ mice. *P<0.05 vs WT; °°P<0.01, °°°P<0.001 vs respective control without FA. N=6 mice/group.

4.2.3 Fluoroacetate *in vitro* treatment influences purinergic-mediated inhibitory response of TLR4$^{-/-}$ ileal segments

We have previously shown that TLR4$^{-/-}$ mice has a higher ADP-mediated relaxation due to a marked P2Y1R immunoreactivity and the inhibition of P2Y1R activity with MRS2500 in presence of L-NAME markedly reduced the amplitudes of NANC-mediated relaxation (by 45±3%; Caputi et al., 2017a). Therefore, we decided to evaluate the role of EGCs activity in purinergic-mediated relaxation in NANC conditions in presence or absence of FA. The addition of MRS2500 in NANC conditions determined a reduction in the inhibitory response of TLR4$^{-/-}$ ileal preparations (-55±3%; **Figure 4.8A**), restoring an inhibitory response comparable to WT mice. The combination of FA and MRS2500 determined a reduction of NANC-mediated relaxation comparable to that obtained only with MRS2500 in both WT and TLR4$^{-/-}$ mice (**Figure 4.8A**). In ileal myenteric plexus of TLR4$^{-/-}$ mice was found an increase of immunoreactivity of P2Y1R compared to WT mice (+41±3%) but no changes were found following FA treatment (**Figure 4.8B,C**). P2R are necessary during the development of glial cells (Scemes et al., 2004), and in the CNS their activation is associated to astrogliosis whereas in the ENS they control connexin-43 hemichannels, causing glial-driven neuron death (Brown et al., 2016). Accordingly, we evaluated the immunofluorescence of connexin-43 in LMMPs of TLR4$^{-/-}$ mice that was comparable to WT and no changes were found following FA treatment (**Figure 4.8D,E**).



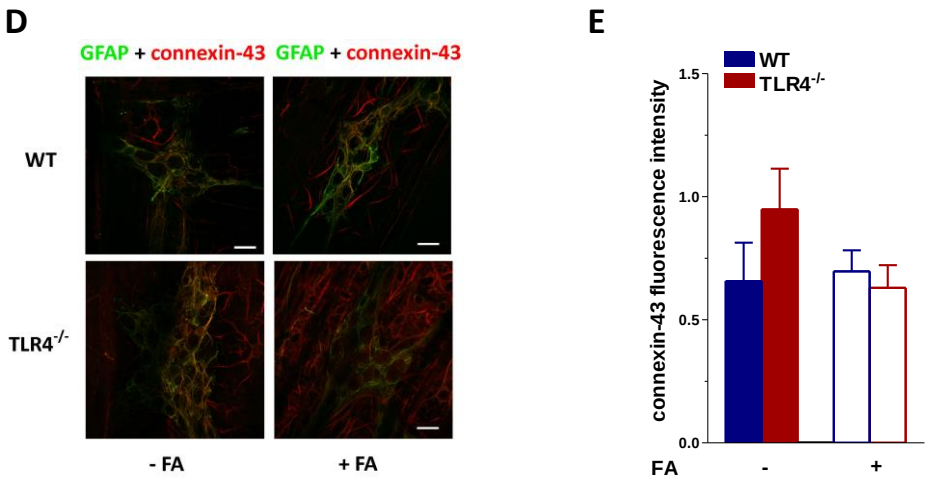


Figure 4.8. TLR4 signaling modulates P2Y1 receptor-mediated relaxation. **A**) 10 Hz EFS-evoked relaxation in NANC conditions with or without 10 µM FA or 10 µM MRS2500 (P2Y1 inhibitor) in WT and TLR4$^{-/-}$ mice. **B**) Representative confocal microphotographs showing the distribution of GFAP (cyan) and P2Y1R (yellow) in LMMPs preparations of WT and TLR4$^{-/-}$ mice (bars = 22 µm). **C**) Analysis of P2Y1R fluorescence intensity in LMMPs whole mount preparations of WT and TLR4$^{-/-}$ mice. **D**) Representative confocal microphotographs showing the distribution of GFAP (green) and connexin-43 (red) in LMMPs preparations of WT and TLR4$^{-/-}$ mice (bars = 22 µm). **C**) Analysis of connexin-43 fluorescence intensity in LMMPs whole mount preparations of WT and TLR4$^{-/-}$ mice. **P<0.01, ***P<0.001 vs WT; °°P<0.01, °°°P<0.001 vs respective control without FA; ##P<0.01 vs respective control with FA. N=6 mice/group.

Finally, we evaluated the inhibitory response in presence of the pan-NOS inhibitor L-NAME and MRS2500. As shown in **Figure 4.9,** in both genotypes the pre-treatment with these inhibitors completely blocked EFS-evoked NANC relaxation. Conversely, after FA treatment, this response was only partially abolished meaning that the disruption of EGCs activity could influence other inhibitory neurotransmissions, such as serotonergic or dopaminergic.

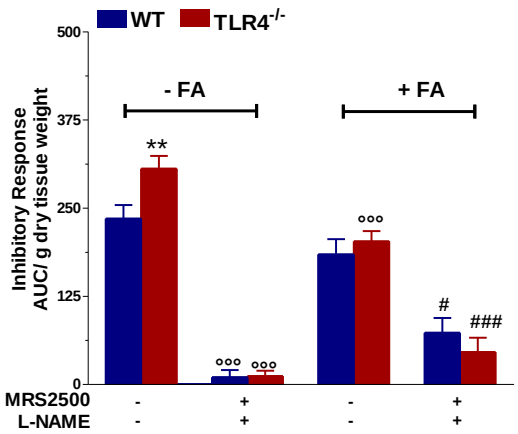


Figure 4.9. TLR4 signaling modulates nitrergic and purinergic relaxation. **A**) 10 Hz EFS-evoked relaxation in NANC conditions with or without 10 µM FA or 10 µM MRS2500 (P2Y1R inhibitor) or 1 µM L-NAME (pan-NOS inhibitor) in WT and TLR4$^{-/-}$ mice. **P<0.01 vs WT; °°°P<0.001 vs respective control without FA; #P<0.05, ###P<0.001 vs respective control with FA. N=6 mice/group.

4.3 Influence of TLR4 signaling on high-fat diet-induced dysmotility in mouse small intestine

Excessive consumption of high-fat diet (HFD) has contributed to the obesity epidemic, however this relationship is more complex than the simple concept of energy balance. It is known that HFD induces alterations in gut microbiota (Murphy et al., 2015). Studies in GF mice have shown that their total body fat was lower compared to that found in conventionally raised mice (Tilg, 2010), suggesting a key role of gut microbiota in host metabolism. HFD and enteric dysbiosis can lead to a chronic low-grade inflammation (Bakker et al., 2015), associated to the development of obesity. In the last decade, the innate immune system has been shown to influence the development and severity of metabolic syndrome and obesity, as revealed in mice deficient for the Toll-like receptor 5 (TLR5) which are prone to obesity and predisposed to insulin resistance (Tilg, 2010). *Anitha et al.* (2016) have discovered that intestinal dysbiosis in HFD-fed mice contributes to the delayed intestinal transit via TLR4-dependent neuronal loss (Anitha et al., 2016). A mouse strain particularly susceptible to the effects of dietary fat is the C57BL/6J (B6) mouse which develops severe obesity, hyperglycemia, and hyperinsulinemia when weaned on a HFD (Parekh et al., 1998).

4.3.1 8 week-HFD determines an obese phenotype

To confirm that HFD induces obesity not only in WT mice but also in $TLR4^{-/-}$ mice, we evaluated the body weights and the metabolic parameters on WT and $TLR4^{-/-}$ mice fed for 8 weeks with a SD or HFD. The body weights of WT and $TLR4^{-/-}$ mice were significantly increased after HFD compared to the mice fed with SD (+33±1% and +21±2%, respectively; **Figure 4.10A,B**), reaching after 8 weeks a value of body weight comparable between genotypes (Kim et al., 2007), however, higher than 26 g, considering the study of *Hohos et al.* (2018). Moreover, after HFD the plasma levels of cholesterol and triglycerides were significantly increased in WT mice (+42±3% and +22±1%, respectively; Nezami et al., 2014) and in $TLR4^{-/-}$ mice (+22±2% and +52±3%, respectively; **Figure 4.10D,E**). After SD, in $TLR4^{-/-}$ mice glucose plasma levels were higher compared to WT mice (+17±1%), after HFD only WT mice showed a significant increase of glucose levels compared to WT mice fed with SD (+20±1%; **Figure 4.10C**; Nezami et al., 2014).

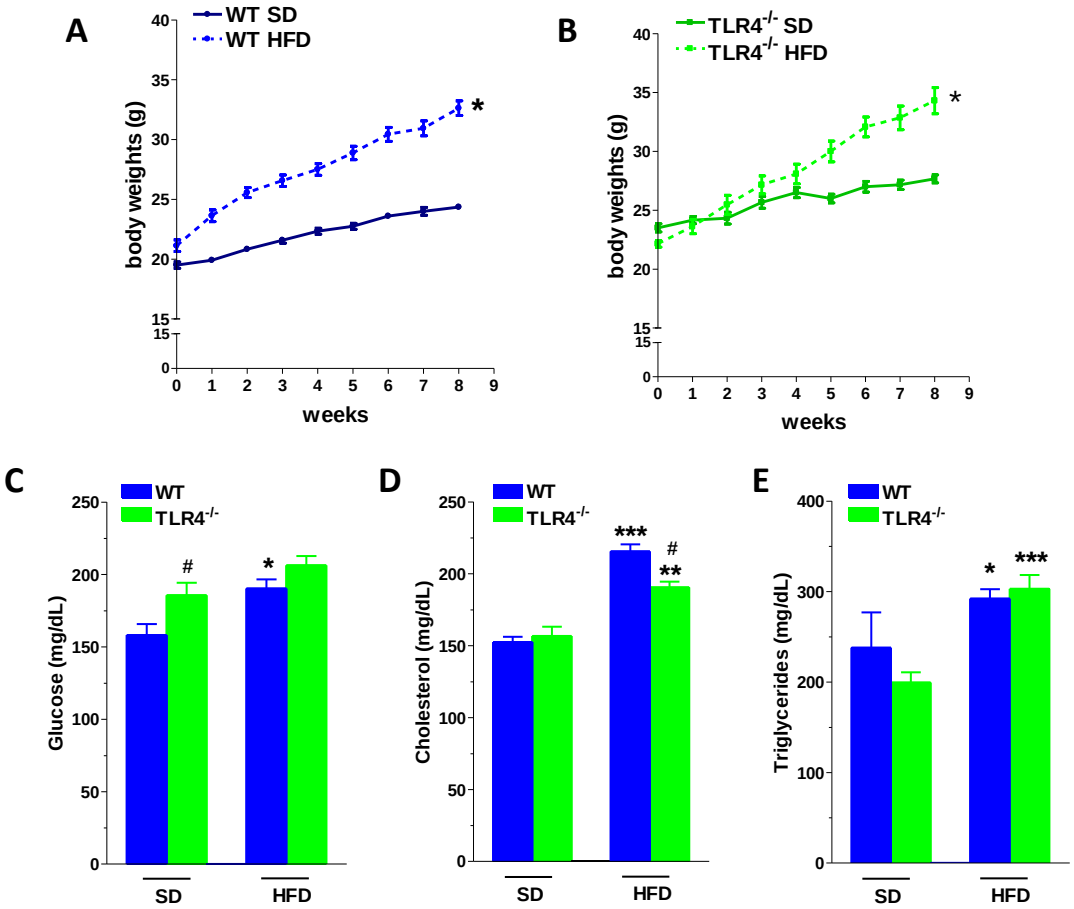


Figure 4.10. HFD induces obesity in both genotypes. **A-B**) Increase of body weights after 8 weeks of HFD in WT (**A**) and TLR4$^{-/-}$ (**B**) mice. **C-D-E**) Glucose (**C**), cholesterol (**D**), triglycerides (**E**) plasma levels in WT and TLR4$^{-/-}$ mice fed with SD or HFD for 8 weeks. *P<0.05, **P<0.01, ***P<0.001 vs respective control in SD; #P<0.05 vs WT. N=8 mice/group.

4.3.2 HFD impairs gastrointestinal motility

Mice fed with HFD it has been shown to have GI dysmotility and delayed transit (Anitha et al., 2016). Previously we have discovered that, in SD, TLR4$^{-/-}$ mice were subjected to a significant reduction of the geometric center compared to WT (GC_{WT}=6.9±0.5 and $GC_{TLR4-/-}$=6.3±1, respectively; **Figure 4.11A**) together with a fecal pellets/hour and stool water content significantly lower in TLR4$^{-/-}$ mice compared to WT mice (**Figure 4.11B,C**; Caputi et al., 2017a). However, HFD did impaired GI transit and fecal pellets/hour only in WT mice compared to WT mice in SD (GC_{WT}=5.5±0.5 and $GC_{TLR4-/-}$=6.5±1; **Figure 4.11A**; Nezami et al., 2014) suggesting that the absence of TLR4 partially protects against the HFD detrimental effects. Reduced stool water content was obtained in both WT and TLR4$^{-/-}$ mice fed with HFD compared the SD mice, as previously shown by *Nezami et al.* (2014). These data are also in agreement with the findings of *Kim et al.* (2011) in mice deficient for TLR4 that were protected from developing obesity-mediated low grade of inflammation.

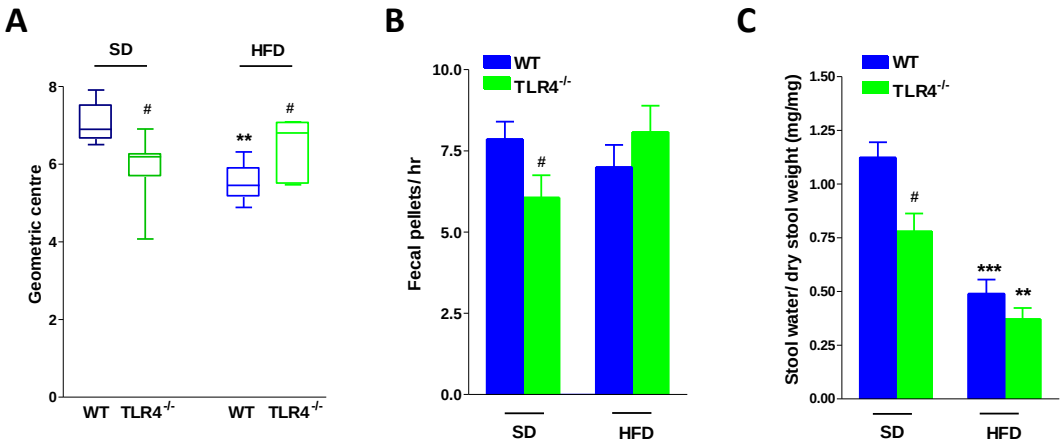


Figure 4.11. HFD alters GI transit. **A**) Geometric center calculated following the analysis of the GI transit obtained by measuring the distribution (%) of non-absorbable fluorescein isothiocyanate labeled dextran (FITC-dextran, 70 KDa) in gut segments, 30 minutes after oral administration in WT and TLR4$^{-/-}$ mice fed with SD or HFD. **B**) Pellet frequency per hour in WT and TLR4$^{-/-}$ mice fed with SD or HFD. **C**) Stool water content in WT and TLR4$^{-/-}$ mice fed with SD or HFD. **P<0.01, ***P<0.001 vs respective control in SD; #P<0.05 vs WT. N=5 mice/group.

4.3.3 HFD affects excitatory neurotransmission of the small intestine

As previously shown by *Caputi et al.* (2017a) the neuromuscular function of isolated ileal preparations from TLR4$^{-/-}$ mice, determined by performing frequency-response curves to EFS, was significantly reduced compared to WT mice in SD (by 18±1.5% at 20 Hz, **Figure 4.12**).

After 8 weeks of HFD, both genotypes showed a significantly reduction of EFS-induced contraction compared to WT and TLR4$^{-/-}$ mice in SD (by 20±2% and 23±2% at 20 Hz, respectively; **Figure 4.13**) reaching a value comparable between the two groups of animal at 40 Hz.

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 (Caputi et al., 2017b). 10 Hz-EFS-mediated responses determined a significant reduction of contraction (-23±3%, **Figure 4.13A**) only in ileal segments of WT HFD mice compared to WT SD mice suggesting that in WT mice HFD alters the cholinergic neurotransmission. Indeed, *Nezami et al.* (2014) found a reduction in ChAT^{+} cholinergic neurons.

We then tested whether the reduced excitatory contraction could be the result of an increase of the inhibitory component, especially in the nitrergic neurotransmission, considering that is the primary inhibitory pathway in the gut (Zizzo et al., 2004; Lomax et al., 2010; Kabouridis & Pachnis, 2015).

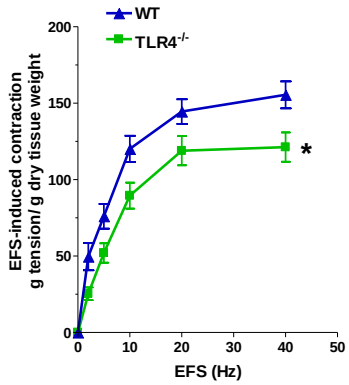


Figure 4.12. TLR4 deficiency alters ileal excitatory contractility in SD-fed mice. EFS-(0-40 Hz)-induced contraction in isolated ileal preparations of SD-fed WT and TLR4$^{-/-}$ mice. *P<0.05 vs WT. N=5 mice/group.

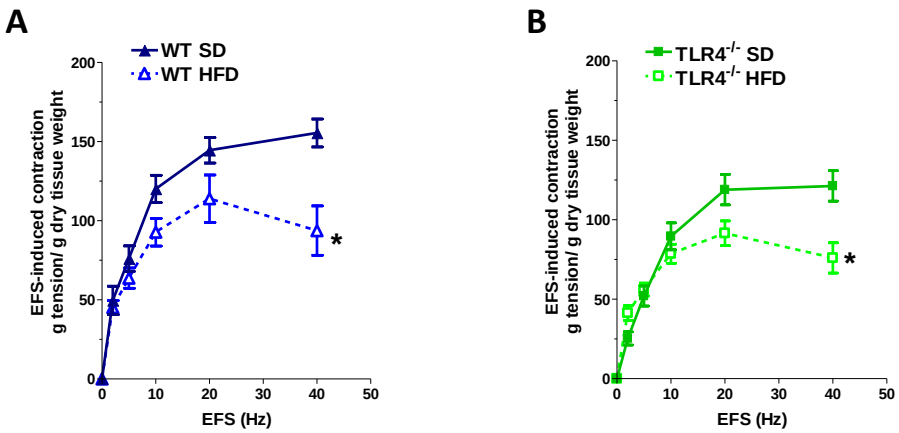


Figure 4.13. HFD impairs ileal excitatory contraction. (**A-B**) EFS-(0-40 Hz)-induced contraction in isolated ileal preparations from WT (**A**) and in TLR4$^{-/-}$ (**B**) mice fed with SD or HFD. *P<0.05 vs respective control in SD. N=5 mice/group.

4.3.4 HFD modulates inhibitory neurotransmission of the small intestine

As previously shown by *Caputi et al.* (2017a), in NANC conditions EFS at 10 Hz caused a 1.32-fold increase in relaxation in TLR4$^{-/-}$ mice preparations (**Figure 4.14**). Pre-treatment with the pan-NOS inhibitor L-NAME almost completely blocked EFS-evoked NANC relaxation in WT segments. Conversely, in TLR4$^{-/-}$ mice, this response was partially abolished by L-NAME suggesting a marked inhibitory neurotransmission in transgenic mice (**Figure 4.14**).

Pre-treatment with 1400W, a selective inhibitor of iNOS, significantly reduced the NANC-mediated relaxation in TLR4$^{-/-}$ mice (by 23±2%), whereas no changes were recorded in WT mice (**Figure 4.15**). These data suggest that most of NO-induced relaxation is mediated by iNOS in TLR4$^{-/-}$ mice. 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). During inflammation, there is an increase of NO release due to the induction of iNOS with consequent intestinal dysmotility (Eskandari et al., 1999).

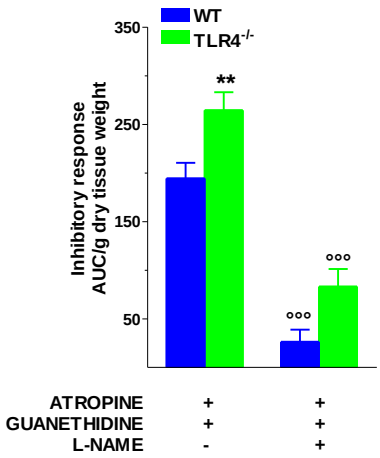


Figure 4.14. TLR4 deficiency increases ileal NO-mediated inhibitory response in SD-fed mice. 10 Hz-EFS-evoked NANC relaxation with or without L-NAME in WT and TLR4$^{-/-}$ preparations from SD-fed mice. **P<0.01 vs WT; °°°P<0.001 vs control without L-NAME. N=5 mice/group.

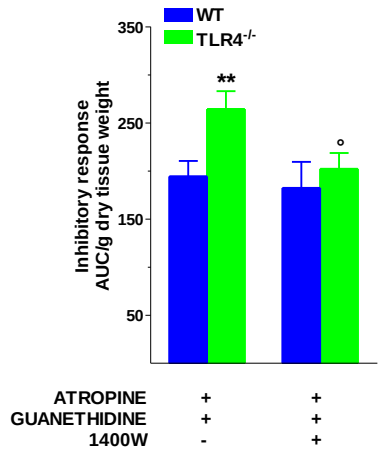


Figure 4.15. TLR4 deficiency determines higher inhibitory response mediated by NO released from iNOS in SD-fed mice. 10 Hz-EFS-evoked NANC relaxation with or without 1400W in WT and TLR4$^{-/-}$ preparations. **P<0.01 vs WT; °P<0.05 vs control without 1400W. N=5 mice/group.

After HFD, WT mice obtained a significantly reduction in the inhibitory response compared to WT mice in SD (by 26±1%, **Figure 4.16**). The pre-treatment with 1400W determined a reduction of the NANC-mediated relaxation, suggesting the presence of a low-grade inflammation after HFD (by 15±1%; **Figure 4.16**), as previously shown by *Kim et al.* (2011) that revealed in WT HFD mice increased LPS levels and TLR4 engagement with consequent activation of NF-kB and higher expression of iNOS. Pre-treatment with L-NAME almost completely blocked EFS-evoked NANC relaxation in WT mice fed with SD or HFD (**Figure 4.16**).

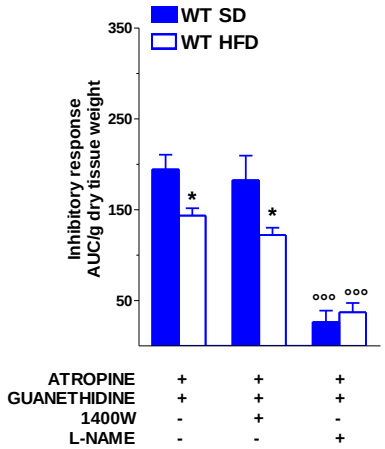


Figure 4.16. HFD affects nitrergic-inhibitory response of ileal segments from WT mice. 10 Hz EFS-evoked relaxation in NANC conditions with or without 10 µM 1400W (iNOS inhibitor) and 100 µM L-NAME (nNOS inhibitor) in WT mice. *P<0.05 vs respective control in SD; **°°°**P<0.001 vs respective control in presence of atropine and guanethidine. N=5 mice/group.

In transgenic mice, 8 weeks-treatment with HFD determined a significantly reduction of the inhibitory response compared to TLR4$^{-/-}$ mice in SD (by 24±2%, **Figure 4.17**). The addition of 1400W did not change the NANC-mediated relaxation obtained in TLR4$^{-/-}$ mice fed with HFD (**Figure 4.17**) suggesting that the absence of TLR4 protects against the development of a low-grade inflammatory state caused by HFD, as previously shown by *Kim et al.* (2007) in TLR4$^{-/-}$ mice in which HFD had no effect on NF-kB activity and cytokine release. Pre-treatment with L-NAME almost completely blocked EFS-evoked NANC relaxation compared to the inhibitory response found in TLR4$^{-/-}$ fed with SD (**Figure 4.17**), reaching an inhibitory response comparable to the found in WT mice.

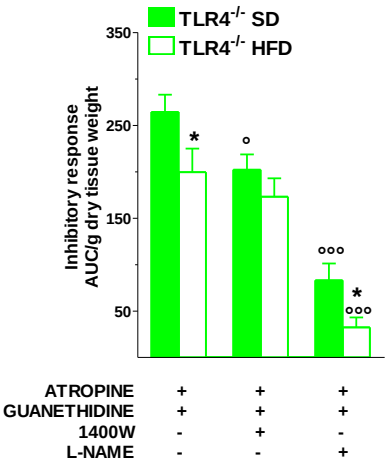


Figure 4.17. HFD affects nitrergic-inhibitory response of ileal segments of TLR4$^{-/-}$ mice. 10 Hz EFS-evoked relaxation in NANC conditions with or without 10 µM 1400W (iNOS inhibitor) and 100 µM L-NAME (nNOS inhibitor) in TLR4$^{-/-}$ mice. *P<0.05 vs respective control in SD; °P<0.05, **°°°**P<0.001 vs respective control in presence of atropine and guanethidine. N=5 mice/group.

4.3.4 HFD influences neuroglia phenotype in small intestine myenteric plexus

TLR4$^{-/-}$ myenteric ganglia showed a significant reduction of nNOS^{+} neurons (by 26±1%) compared to WT mice fed with SD. After HFD intake, the number of nNOS^{+} neurons decreased by 23% in myenteric ganglia of WT mice, with slight but not significant changes in LMMPs of TLR4$^{-/-}$ mice. However, a significant increase of the total number of HuC/D^{+} neurons was observed and was associated to a proportional reduction of nNOS^{+} neurons in both genotypes fed with HFD (**Figure 4.18**) confirming the results obtained by others in the large intestine (Anitha et al., 2016) and in the small intestine (Soares et al., 2015). Moreover, in WT mice fed with HFD the area of myenteric ganglia was increased compared to WT mice fed with SD suggesting a role of diet in the neuroplastic changes of ENS.

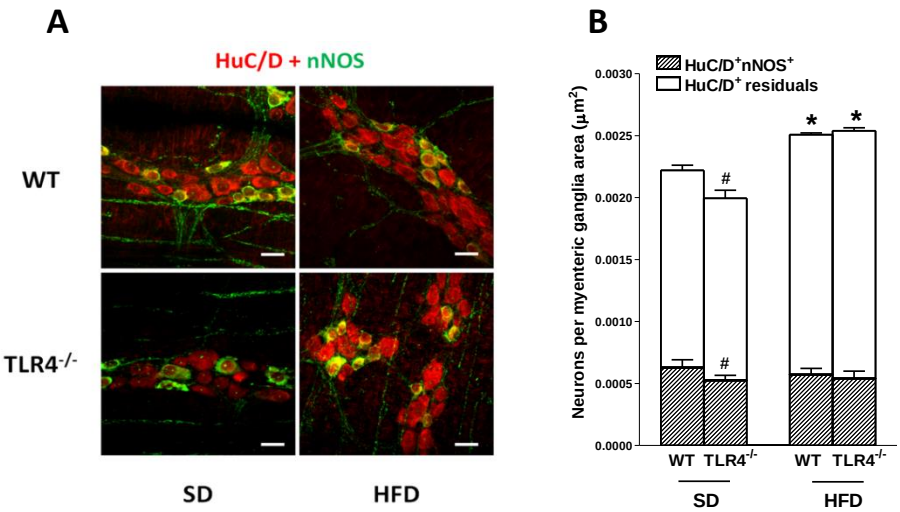


Figure 4.18. TLR4 signaling modulates neuron phenotype. **A**) Representative confocal microphotographs showing the distribution of nNOS^{+} (green) and HuC/D^{+} (red) neurons in WT and TLR4$^{-/-}$ LMMPs preparations of mice fed with SD or HFD (bars = 22 µm). **B**) Total number of HuC/D^{+} and nNOS^{+} neurons in WT and TLR4$^{-/-}$ LMMPs preparations of mice fed with SD or HFD. #P<0.05 vs WT; *P<0.05 vs respective control in SD. N=5 mice/group.

For assessing the effect of diet on ENS structure we evaluated the immunofluorescence of the glial marker S100β (**Figure 4.19A**), GFAP and SOX10 (**Figure 4.19C**). TLR4$^{-/-}$ myenteric ganglia showed a significant reduction of the total number of HuC/D^{+} neurons together with a significant increase of S100β immunofluorescence compared to WT mice in SD (+79±2%). Higher expression of the glial markers such as GFAP, S100β and SOX10 are indicative of ENS neuroplasticity and reactive gliosis (Sofroniew, 2009; Burda et al., 2013; Capoccia et al., 2015). As shown by **Figure 4.19**, the absence of TLR4 signaling caused a significant increase of S100β, GFAP and SOX10 density index, indicative of inflammation in EGCs compared to that found in WT mice fed with SD (+79±2%, +15±1%, +24±1%, respectively).

HFD administration determined reactive gliosis in WT mice, causing 2.4-fold increase of S100β immunoreactivity together with 1.2-fold and 1.6-fold increase of GFAP and SOX10 immunoreactivity,

respectively. In TLR4$^{-/-}$ mice, HFD appears to normalize enteric gliosis reducing S100β and GFAP immunoreactivity (-25±2%, -33±2%, respectively; **Figure 4.19**). SOX10 density index was found significantly increase after HFD in TLR4$^{-/-}$ mice (+22±1%; **Figure 4.19C,E**), reaching an immunofluorescence intensity comparable to WT HFD mice. All together these data suggest an involvement of TLR4 signaling in the gliopathy development induced by HFD (Caputi et al., 2017a).

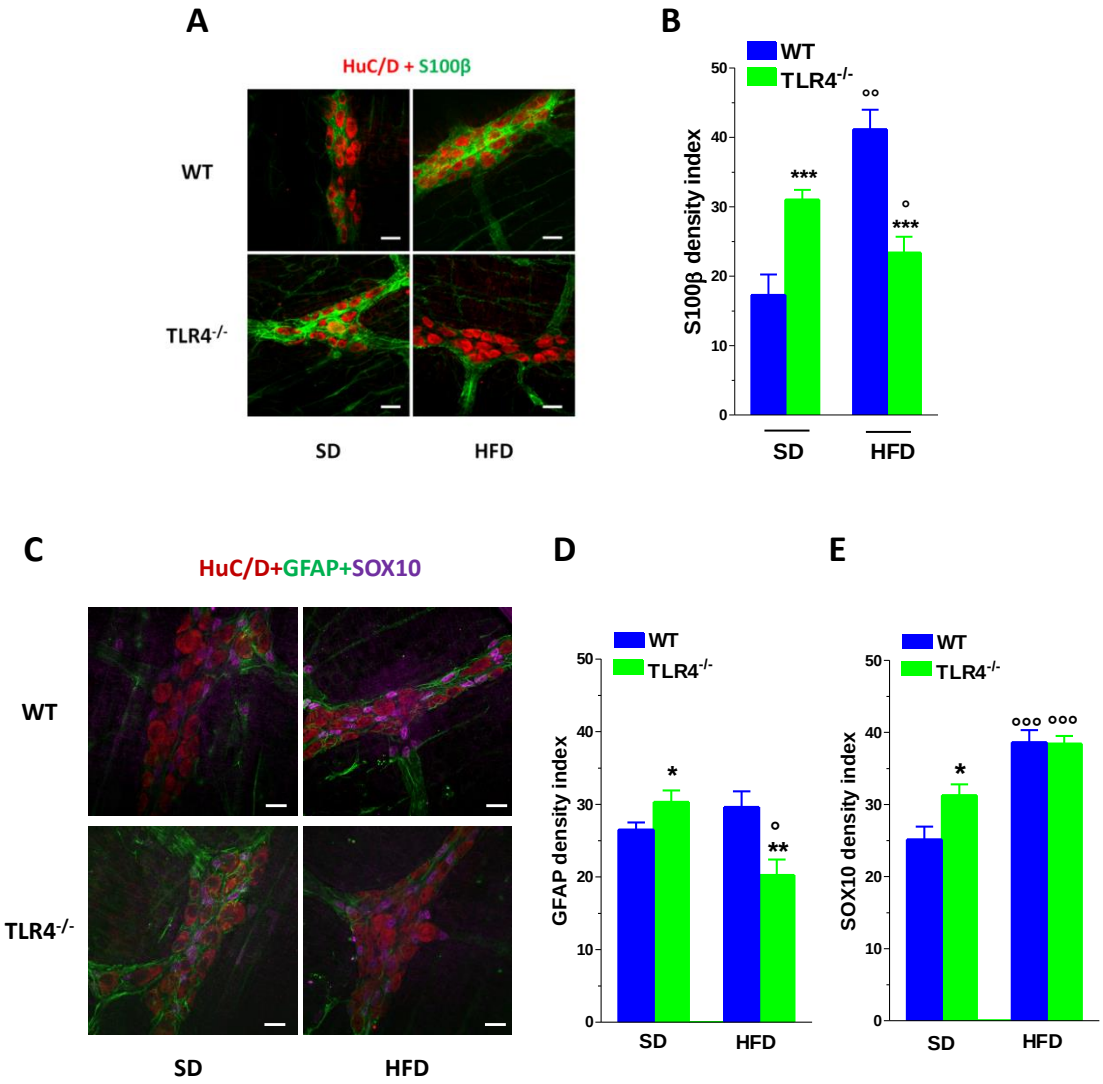


Figure 4.19. HFD affects glial phenotype in WT and TLR4$^{-/-}$ mice. **A**) Representative confocal microphotographs showing the distribution of HuC/D^{+} (red) and S100β^{+} (green) neurons in WT an TLR4$^{-/-}$ LMMPs preparations of mice fed with SD or HFD (bars = 22 µm). **B**) Changes in S100β density index in WT and TLR4$^{-/-}$ LMMPs preparations of mice fed with SD or HFD. **C**) Representative confocal microphotographs showing the distribution of HuC/D (red), and GFAP (green) and SOX10 (magenta) in WT and TLR4$^{-/-}$ LMMPs preparations of mice fed with SD or HFD (bars = 22 µm). **D-E**) Changes in GFAP (**D**) and SOX10 (**E**) density index in WT and TLR4$^{-/-}$ LMMPs preparations of mice fed with SD or HFD. *P<0.05, **P<0.01, ***P<0.001 vs WT; °P<0.05, °°P<0.01, °°°P<0.001 vs respective control in SD. N=5 mice/group.

4.3.5 HFD modifies serotonergic neurotransmission

Serotonin (5-HT), a monoamine neurotransmitter, affects feeding behavior and obesity in the central nervous system (CNS) and regulates glucose and lipid metabolism (Watanabe et al., 2016). Studies in

mice and rats have shown that after HFD the level of 5-HT is increased probably due to an inhibitory effect on food intake and body weight gain (Bertrand et al., 2011; Kim et al., 2011). Therefore, we decided to evaluate the role of 5-HT in ileal contractility in both groups of animals after 8 weeks of SD or HFD. It is known that 5-HT activates both intrinsic excitatory and inhibitory enteric motor neurons (Michel et al., 1997). Isolated ileal preparations from WT and TLR4$^{-/-}$ mice fed with SD were incubated with increasing concentrations of 5-HT to obtain a non-cumulative concentration-response curve. As shown in **Figure 4.20**, in transgenic mice 5-HT-response is significantly higher compared to WT mice in SD (Emax=+59±3%).

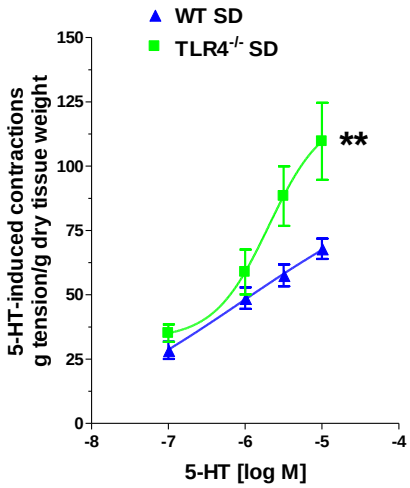


Figure 4.20. TLR4 signaling affects 5-HT-mediated ileal contraction. Non-cumulative concentration-response curves to 5-HT (0.3–30 μM) of isolated ileal preparations from WT and TLR4$^{-/-}$ mice fed with SD.**P<0.01 vs WT. N=5 mice/group.

HFD administration determined a 1.2-fold increase of the contraction mediated by 5-HT as shown by Emax values (Emax=+20±1%) in WT mice compared to WT SD mice (**Figure 4.21A**), whereas in TLR4$^{-/-}$ mice HFD determined a significantly reduction of the 5-HT-mediated response (Emax=-42±4%) compared to TLR4$^{-/-}$ SD mice (**Figure 4.21B**).

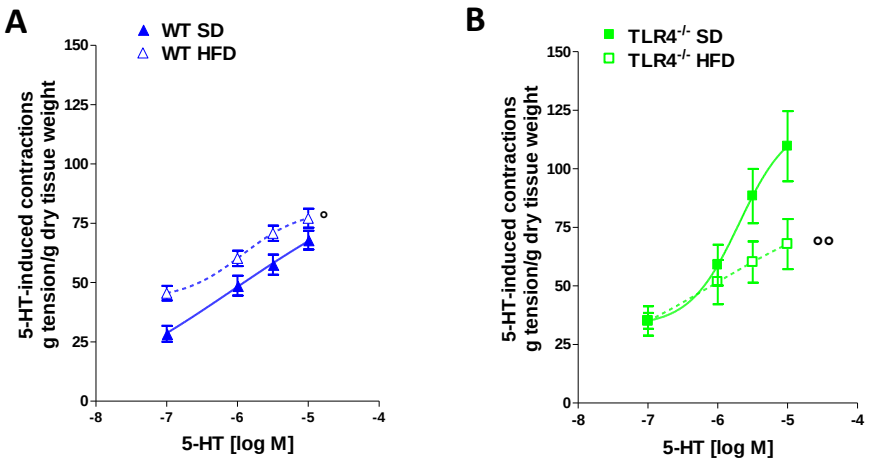


Figure 4.21. HFD affects 5-HT-mediated ileal contraction. **A-B**) Non-cumulative concentration-response curves to 5-HT (0.3–30 μM) of isolated ileal preparations from WT SD and WT HFD (**A**) and from TLR4$^{-/-}$ SD and TLR4$^{-/-}$ HFD (**B**).°P<0.05, °°P<0.01 vs respective control in SD. N=5 mice/group.

Considering that 5-HT can act trough different receptors such as 5-HT_{2A}, 5-HT_3, 5-HT_4 and 5-HT_7 that are all expressed in the gut, we evaluated the role of the 5-HT_3 receptor, known to be involved in GI transit, gut motility and intestinal secretion (Crowell, 2004) and of 5-HT_{2A} receptor, involved in regulating intestinal contractility (Briejer et al., 1997).

Since 5-HT response was found to be altered in $TLR4^{-/-}$ mice fed with SD, we sought to test muscular function mediated by 30 µM 5-HT after the development of obesity and in absence or presence of 0.1 µM ondansetron, a 5-HT_3R antagonist, or 1 µM ketanserin, a 5-HT_{2A}R antagonist. As shown by **Figure 4.22A**, HFD for 8 weeks in WT mice determined a significant increase in the 5-HT mediated contraction (+50±2%) that was completely abolished in the presence of ondansetron. Pre-treatment with ketanserin determined 2.8-fold increase in 5-HT-mediated contraction in WT mice that was not affected following HFD treatment.

In $TLR4^{-/-}$ mice, HFD caused a significantly reduction in 5-HT-mediated contraction (by 42±4%; **Figure 4.22B**) that was still completely sensitive to ondansetron but not to ketanserin (**Figure 4.22B**), suggesting that TLR4 signaling influences 5-HT_{2A}R-mediated response.

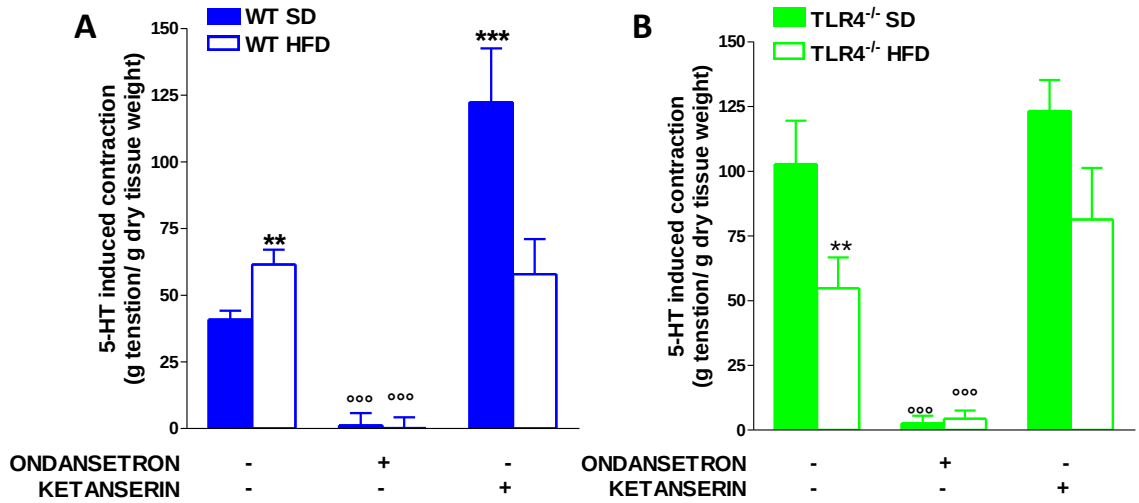


Figure 4.22. HFD affects 5-HT-mediated ileal contraction. **A-B**) Contraction induced by 30 µM 5-HT in absence or presence of 0.1 µM ondansetron or 1 µM ketanserin by ileal preparations from WT (**A**) and $TLR4^{-/-}$ (**B**) mice fed with SD or HFD. **P<0.01, ***P<0.001 vs respective control; °°°P<0.001 vs respective control without 5-HTR antagonist. N=5 mice/group.

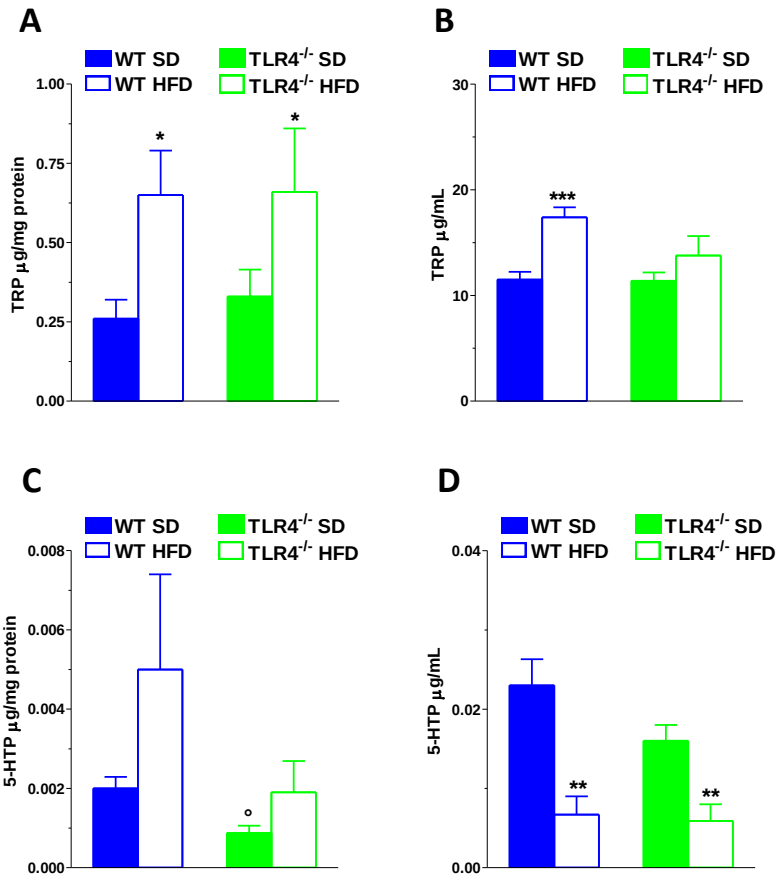
4.3.5 HFD alters tryptophan metabolism

Considering the alterations in the 5-HT mediated response, we tested if obesity affects TRP metabolism, which is involved in controlling small intestine homeostasis and is catabolized by gut microbiota (O'Mahony et al., 2015; Yano et al., 2015). In both ileal tissue and plasma TRP levels were comparable in both genotypes (**Figure 4.23A,B**). In $TLR4^{-/-}$ mice, a significant reduction of 5-HTP levels in both ileal tissue (-56±1%; **Figure 4.23C**) and plasma (-31±1%; **Figure 4.23D**) was found, together with a 1.5-fold increase in 5-HT tissue levels (**Figure 4.23E**) compared to WT mice with a slight but not significant reduction of 5-HT plasma levels (**Figure 4.23F**).

The dominant metabolic pathway for TRP is along the KYN pathway (Le Floc'h N et al., 2011), catalyzed by the ubiquitous IDO (Badawy, 2017). In TLR4$^{-/-}$ mice, we found a significant increase of KYN levels in ileal tissues (+66±1%, **Figure 4.23G**) together with a significant reduction of its levels in the plasma (-25±1%, **Figure 4.23H**).

In WT mice HFD caused a marked increase of TRP, 5-HT and KYN levels in ileal tissues compared to WT fed with SD (+152±5%, +148±3%, +300±4%, respectively; **Figure 4.23A,E,G**). After the plasma analysis of TRP metabolism, we found 1.5-fold increase in TRP levels (**Figure 4.23B**), a significant reduction in 5-HTP and KYN levels (-70%±1%, -23%±1%, respectively; **Figure 4.23D,H**).

TLR4$^{-/-}$ mice fed with HFD, obtained a 2-fold increase of both TRP and KYN levels in ileal tissue compared to TLR4$^{-/-}$ SD mice (**Figure 4.23A,G**) with no changes in 5-HTP and 5-HT levels (**Figure 4.23C,E**). Moreover, HFD determined in TLR4$^{-/-}$ mice a significant increase of TRP and 5-HT plasma levels (+21±1%, +176±5%, respectively; **Figure 4.23B,F**), together with a significant reduction of 5-HTP levels compared to TLR4$^{-/-}$ mice fed with SD (-63±1%, **Figure 4.23D**).



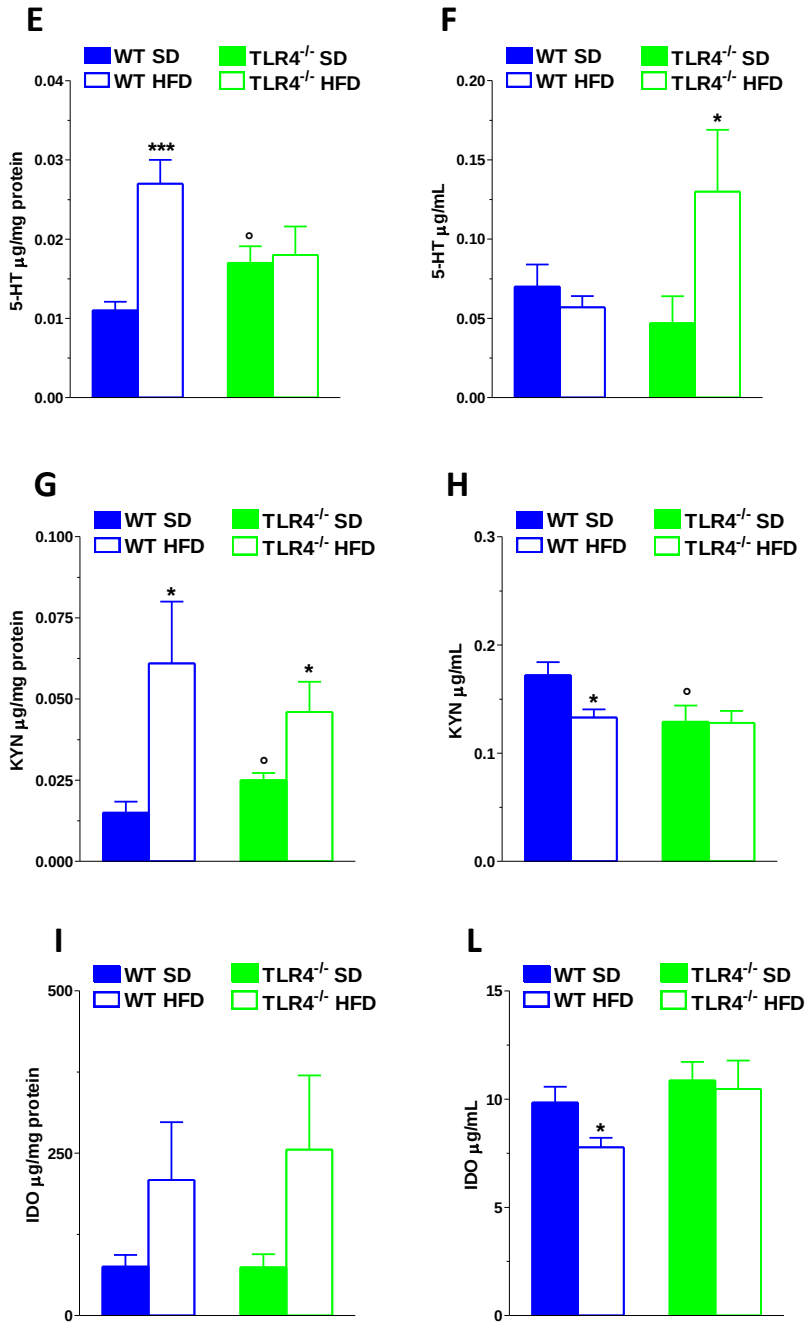


Figure 4.23. HFD-induced obesity alters TRP metabolism. Levels of TRP (**A,B**), 5-HTP (**C,D**) 5-HT (**E,F**), KYN (**G,H**) and IDO (**I,L**), measured by HPLC analysis, in ileal tissue or plasma of WT and TLR4$^{-/-}$ mice fed with SD or HFD. *P 0.05, **P<0.01, ***P<0.001 vs respective control in SD; °P<0.05 vs WT SD. N=5 mice/group.

4.4 Innate immunity and DSS-induced colitis in mouse small intestine

IBD appears to arise from an inappropriate response of mucosal immune system to the microbiota and other noxious antigens. In intestinal ecology, indigenous commensal microorganisms maintain homeostasis along with the elements of the host immune system. However, the occurrence of an innate immune defect, epithelial barrier leakage and/or mucosal injury can harm the otherwise beneficial host–microbe equilibrium, determining changes in intestinal microbiota composition and activity with consequent detrimental effect disease severity (Lu et al., 2018). The crosstalk between the intestinal microbiota and host immunity are mediated, at least in part, through TLRs. TLR-mediated signalling pathways appear to be involved in the progression of IBD, performing beneficial or harmful functions (Fakhoury et al., 2014; Lu et al., 2018). In patients with UC, TLR4 gene expression is upregulated in the intestinal epithelia, suggesting that TLR4 might participate in UC development (Lu et al., 2018). Animal models are indispensable for understanding the pathogenesis of IBD (Wirtz & Neurath, 2007), and there are evidences that in absence of bacteria the intestinal inflammation is ameliorated (Gkouskou et al., 2014). An established chemical model of UC is the one based on the administration of a noxious chemical, such as dextran sodium sulfate (DSS), in drinking water to induce colitis (Wirtz & Neurath, 2007; Gkouskou et al., 2014). Studies on $TLR4^{-/-}$ and $MyD88^{-/-}$ mice suggest that TLR signalling is required to limit bacterial translocation after DSS induced intestinal epithelial injury suggesting TLR involvement in the maintenance of the epithelial barrier (Fukata et al., 2005). Moreover, the disruption of the mucosal barrier, induced by injury, allows the exposure of TLR ligands produced by commensal microbiota, resulting in enhanced inflammatory response and severity of intestinal inflammation (Rakoff-Nahoum et al., 2004). Furthermore, new evidence demonstrated that the catabolism of TRP, followed by 5-HT production, could be implicated in ENS neurotransmission and colitis severity.

4.4.1 DSS-induced colitis severity

DSS-induced colitis determines intestinal injury, causing disruption of mucosal barrier leading to a higher exposure of pathogens and increasing TLR-mediated immune response (Rakoff-Nahoum et al., 2004). We hypothesized that the absence of signaling mediated by TLR4 should partially protect against the DSS-detrimental effects (Rakoff-Nahoum et al., 2004; Fukata et al., 2005).

The body weights of both WT and $TLR4^{-/-}$ mice were significantly reduced after DSS-induced colitis compared to those of respective sham mice (-17±2% and -13±2%, respectively; **Figure 4.24**), reaching a value comparable between genotypes. These changes in body weights are a typical sign of DSS-induced colitis (Meers et al., 2018).

The mice were monitored daily, evaluating the disease activity index (DAI) as shown by the **Table 3.1** (Yang et al., 2016). The experimental endpoint was obtained when the mice reached a DAI value

higher ≥2, based on the reduction of both body weight and number of fecal pellets/hours together with an increase of stool water content (**Figure 4.26B,C**).

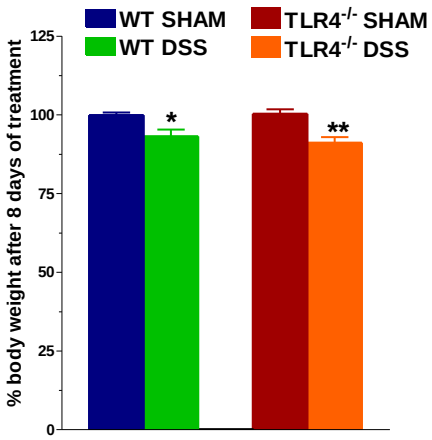


Figure 4.24. DSS-induced colitis causes a comparable reduction of body weight in both genotypes. Percentage of body weight after 8 days of treatment with 2% DSS in WT and TLR4$^{-/-}$ mice. *P<0.05, **P<0.01 vs respective control in sham conditions. N=8 mice/group.

Ah shown in **Table 4.1**, both genotypes obtained a DAI ≥2 compared to sham conditions, suggesting the onset of colitis. In DSS-treated mice we found stool consistency reduction and the presence of occult bleeding.

MICE	DAI
WT SHAM	0
TLR4$^{-/-}$ SHAM	0
WT DSS	6
TLR4$^{-/-}$ DSS	5

Table 4.1. DAI scores of mice treated with 2 % DSS or tap water (sham).

Histological analysis confirmed the presence of colitis in WT mice characterized by a marked increase of Peyer's patches, an intense granulocyte infiltrate extending throughout the mucosa and submucosa, sometimes involving the muscular layer. As shown in **Figure 4.25**, TLR4$^{-/-}$ ileal morphology was comparable to that found in WT mice, except for villi height, which was significantly diminished (Caputi et al., 2017a), whereas the treatment with DSS causes an inflammatory response that was less severe compared to WT specimens (**Figure 4.25**).

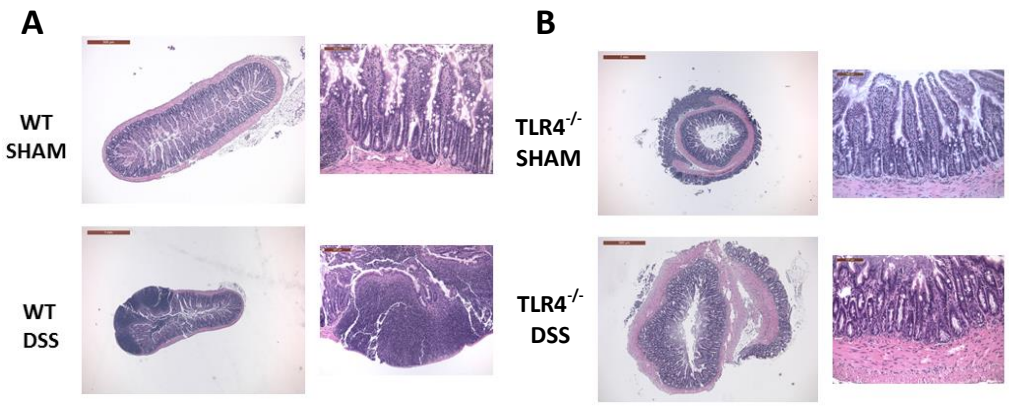


Figure 4.25. Representative hematoxylin-eosin stained sections of distal ileum at day 8 (20×,10×,5×,2.5×) in WT (**A**) and TLR4$^{-/-}$ (**B**) mice in absence or presence of DSS treatment (WT SHAM bars = 500 µm, 100 µm; WT DSS bars = 1 mm, 200 µm; TLR4$^{-/-}$ SHAM bars = 500 µm, 100 µm; TLR4$^{-/-}$ DSS bars = 1 mm, 100 µm).

4.4.2 DSS-induced colitis impairs gastrointestinal motility

One of the main symptoms of UC patients is the constipation, different studies showed prolonged small and large intestine transit time (Fischer et al., 2017; James et al., 2018) together with reduced stool frequency (Bassotti et al., 2014; James et al., 2018).

Recently, we have demonstrated that, in sham condition, TLR4$^{-/-}$ mice show a significant reduction of the geometric center ($GC_{TLR4\text{-}/\text{-}\ sham}$=6.2±2 vs. $GC_{WT\ sham}$=6.9±0.5; **Figure 4.26A**), together with a fecal pellets/hour and stool water content significantly lower in TLR4$^{-/-}$ mice compared to WT mice (Caputi et al., 2017a).

In WT mice, DSS-induced colitis determined a marked reduction of geometric center ($GC_{WT\ DSS}$= 4.8±0.5; **Figure 4.26A**) together with a significant decrease of fecal pellets per hour and increased stool water content (**Figure 4.26B,C**), as previously shown by *Ghia et al.* (2009). In TLR4$^{-/-}$ mice, DSS-induced colitis determined no changes in the transit time ($GC_{TLR4\text{-}/\text{-}\ DSS}$=6.5±1; **Figure 4.26A**) and increased stool water content (**Figure 4.26C**). These data suggest that the absence of TLR4 signaling partially protects against the constipation induced by colitis (Fischer et al., 2017; James et al., 2018).

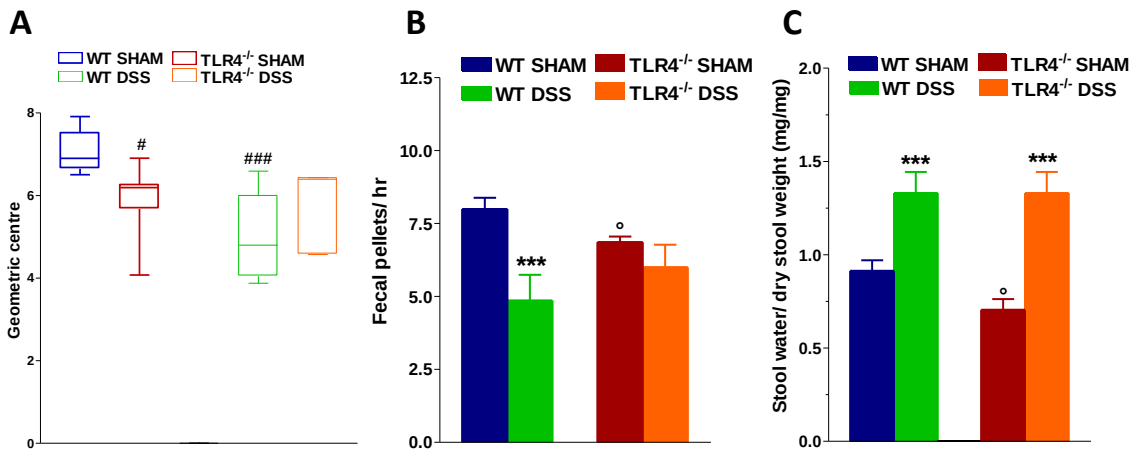


Figure 4.26. DSS-induced colitis alters GI transit. **A**) Geometric center obtained by the analysis of the GI transit in WT and TLR4$^{-/-}$ mice in presence or absence of DSS treatment. **B**) Pellet frequency per hour in WT and TLR4$^{-/-}$ mice in absence or presence of DSS treatment. **C**) Fecal water content in WT and TLR4$^{-/-}$ mice in absence or presence of DSS treatment. #P<0.05, ###P<0.001 vs WT SHAM; ***P<0.001 vs respective control in sham condition; °P<0.05 vs WT SHAM. N=6 mice/group.

4.4.3 DSS-induced colitis affects excitatory neuromuscular response

Considering the changes in the GI transit time following DSS treatment, we evaluated the excitatory cholinergic response on freshly isolated ileal preparations by analyzing cumulative concentration-response curves to the non-selective cholinergic receptor agonist CCh. As previously shown by *Caputi et al.* (2017a), isolated ileal preparations from TLR4$^{-/-}$ mice showed a significant downward shift of the concentration-response curve to CCh with a related decrease in the maximum response compared to WT mice (Emax=-26±8%, **Figure 4.27A**). DSS-induced colitis determined in WT and TLR4$^{-/-}$ mice a significant increase of the contraction mediated by CCh, as shown by the Emax values (+37±4% and +69±5%, respectively; **Figure 4.27B,C**), which resulted to be comparable between the two genotypes.

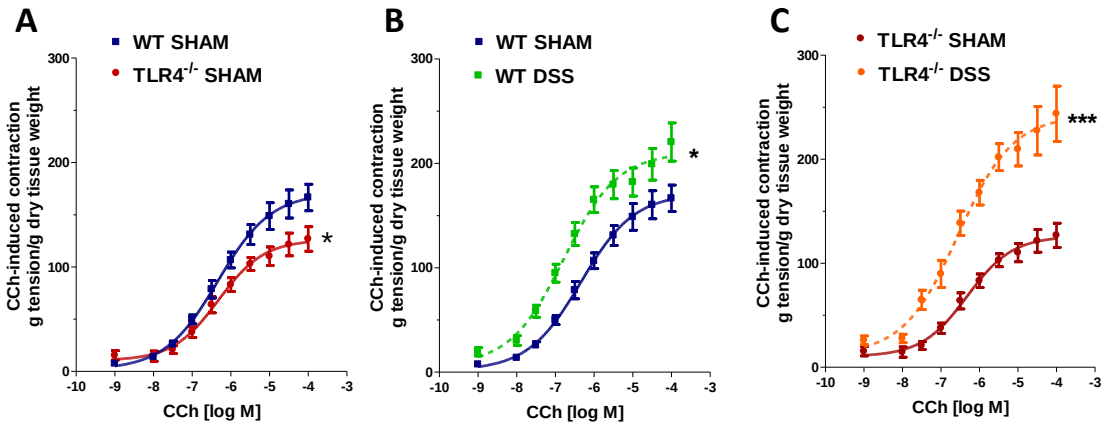


Figure 4.27. DSS-induced colitis affects cholinergic neuromuscular response. Concentration-response curves to CCh (0.001-100 µM) in isolated ileal preparations of WT and TLR4$^{-/-}$ mice (**A**), in absence or presence of DSS treatment (**B** and **C**). *P<0.05 vs WT sham; ***P<0.001 vs TLR4$^{-/-}$ sham. N=6 mice/group.

For verifying whether the changes in contraction were determined by alterations in ENS-mediated transmission we performed frequency-response curves to EFS. In TLR4$^{-/-}$ ileal segments we found an altered neurotransmission reflected by reduced EFS-elicited contractions (by 22±2% at 40 Hz; **Figure 4.28A**; Caputi et al., 2017a). DSS-induced colitis determined in WT and TLR4$^{-/-}$ mice a significant increase of the EFS-induced contraction, as shown by the increase of the maximum response at 40 Hz (+48±3%, +49±5%, respectively; **Figure 4.28B,C**), determining Emax values comparable between genotypes.

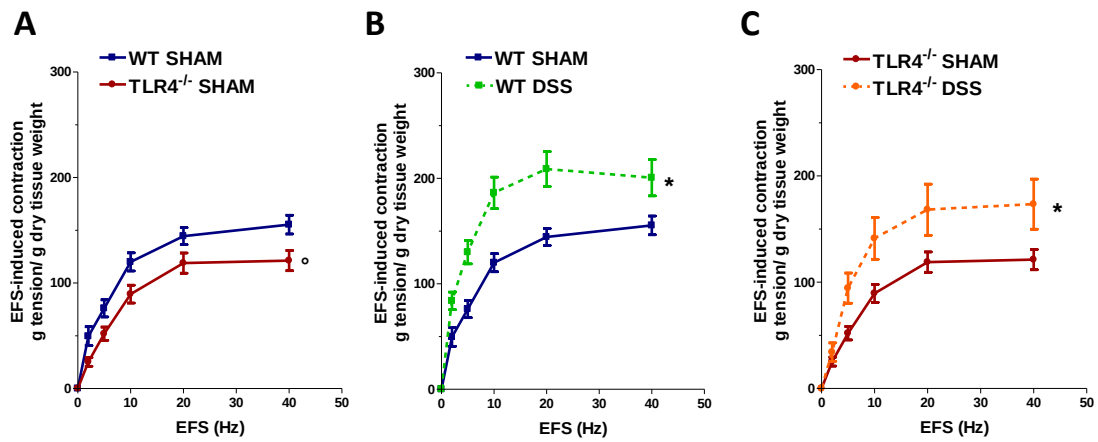


Figure 4.28. DSS-induced colitis affects ileal excitatory response. EFS-(0-40 Hz)-induced contraction in isolated ileal preparations from WT and TLR4$^{-/-}$ mice (**A**) in absence or presence of DSS treatment (**B** and **C**). °P<0.05 vs WT sham; *P<0.05 vs respective control in sham condition. N=6 mice/group.

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 (Caputi et al., 2017b). 10 Hz-EFS-mediated responses determined a significant reduction of contraction (-28±3%; **Figure 4.28A**) in ileal segments of TLR4$^{-/-}$ mice compared to WT mice; the presence of colitis maintained comparable alterations in the response induced by 10 Hz-EFS, suggesting an altered cholinergic neurotransmission in TLR4$^{-/-}$ mice.

We, thus, examined the immunofluorescence distribution of choline acetyltransferase (ChAT) in myenteric ganglia of WT and TLR4$^{-/-}$ mice. ChAT, the enzyme responsible for ACh synthesis, is typically used for assessing the status of the myenteric cholinergic network (Johnson et al., 2018).

DSS-induced colitis in WT mice determined a significant increase of ChAT immunoreactivity compared to WT sham mice (+16±1%; **Figure 4.29A,B**) as previously shown by *Winston et al.* in a rat model of DSS-induced colitis (2013). In TLR4$^{-/-}$ mice a higher ChAT immunoreactivity was found (+77±2%) that was not affected following DSS-mediated colitis.

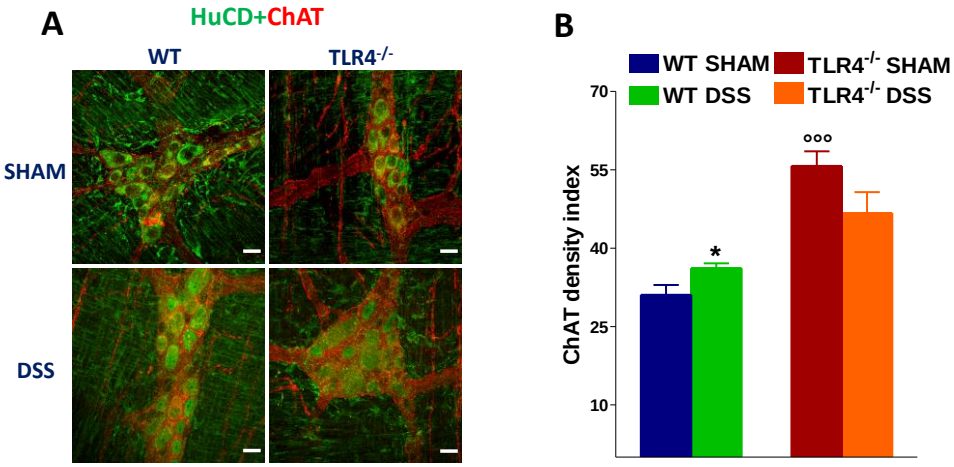
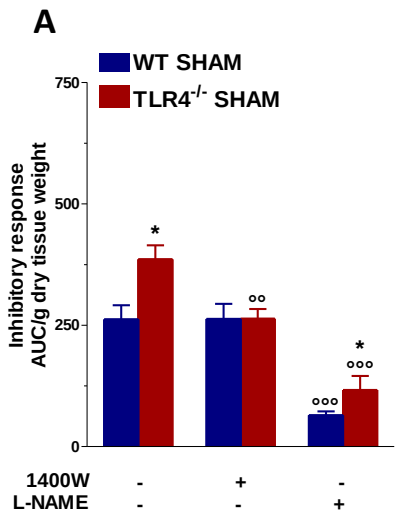


Figure 4.29. DSS-induced colitis influences myenteric cholinergic network. **A)** Representative confocal microphotographs showing the distribution of ChAT (red) and HuC/D (green) in LMMPs preparations of WT and TLR4$^{-/-}$ mice in absence or presence of DSS treatment (bars = 22 µm). **B**) Changes in ChAT density index in ileal LMMPs preparations of WT and TLR4$^{-/-}$ mice in absence or presence of DSS treatment. *P<0.05 vs respective control in sham condition; °°°P<0.05 vs WT SHAM. N=6 mice/group.

4.4.4 DSS-induced colitis increases inhibitory neurotransmission

As previously shown by us (Caputi et al., 2017a), EFS at 10 Hz in NANC conditions caused a 1.32-fold increase in ileal relaxation in TLR4$^{-/-}$ mice preparations (**Figure 4.30A**). Pretreatment with 1400W, a selective inhibitor of iNOS, significantly reduced the NANC-mediated relaxation in TLR4$^{-/-}$ mice (by 23±2%), whereas no changes were recorded in WT mice (**Figure 4.30A**). Pretreatment with the pan-NOS inhibitor L-NAME almost completely blocked EFS-evoked NANC relaxation in WT segments. Conversely, in TLR4$^{-/-}$ mice, this response was partially abolished by L-NAME, indicating the involvement of NO produced by both nNOS and iNOS together with other inhibitory neurotransmitters in transgenic mice (**Figure 4.30A**).



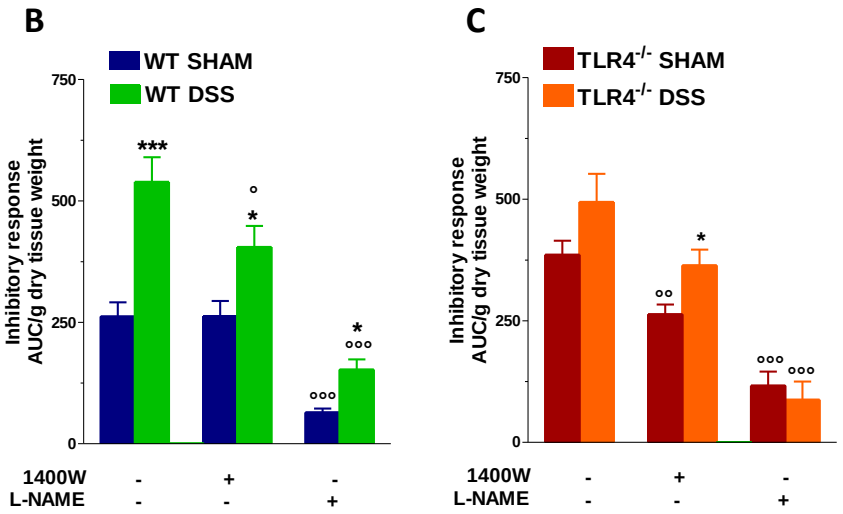


Figure 4.30. DSS-induced colitis increases ileal NO-mediated inhibitory response. 10 Hz-EFS-evoked NANC relaxation with or without 1 µM 1400W, iNOS inhibitor or 100 µM L-NAME, pan-NOS inhibitor, in WT and TLR4$^{-/-}$ mice (**A**), in absence or presence of DSS treatment (**B** and **C**). *P<0.05, ***P<0.001 vs respective control in sham condition; °P<0.05, °°P<0.01, °°°<P 0.001 vs respective control in NANC condition. N=6 mice/group.

In WT mice, DSS-induced colitis determined a 2-fold increase in NANC inhibitory response (**Figure 4.30B**). Pre-treatment with 1400W or L-NAME significantly reduced the inhibitory response (-25±2% and -72±1%, respectively), without, however, reaching values comparable to those obtained in sham conditions (**Figure 4.30B**), to indicate an enhanced iNOS derived NO-production, potentially by an up-regulation of the glial marker S100β during colitis (Kubes & McCafferty, 2000; Cirillo et al., 2009).

In TLR4$^{-/-}$ mice, DSS-induced colitis determined a slight but not significant increase of NANC-inhibitory response (**Figure 4.30C**). Pre-treatment with 1400W reduced partially the inhibitory response without reaching values comparable to those evidenced in TLR4$^{-/-}$ sham mice, whereas incubation with L-NAME determined an inhibitory response equivalent to the response obtained in sham conditions (**Figure 4.30C**), to suggest an enhanced nNOS derived NO-production during colitis in TLR4$^{-/-}$ mice.

To better characterize the effect of colitis in the nitrergic neurotransmission, we analyzed the distribution of nitrergic neurons in the myenteric plexus by confocal immunohistochemistry (**Figure 4.31A**). The development of colitis in WT mice determined a significant reduction of the total number of HuC/D^{+} neurons (-8±1%), associated to a proportional increase in nNOS^{+} neurons (**Figure 4.31A,B**). TLR4$^{-/-}$ mice showed a significant reduction of HuC/D^{+} neurons (-16±1%) together with a proportional increase of nNOS^{+} neurons compared to WT mice (**Figure 4.31A,B**). However, DSS-induced colitis did not change either the total number of HuC/D^{+} and nNOS^{+} neurons, to indicate that TLR4 signaling is involved in the detrimental effects of colitis on nitrergic neurochemical coding and inhibitory neurotransmission.

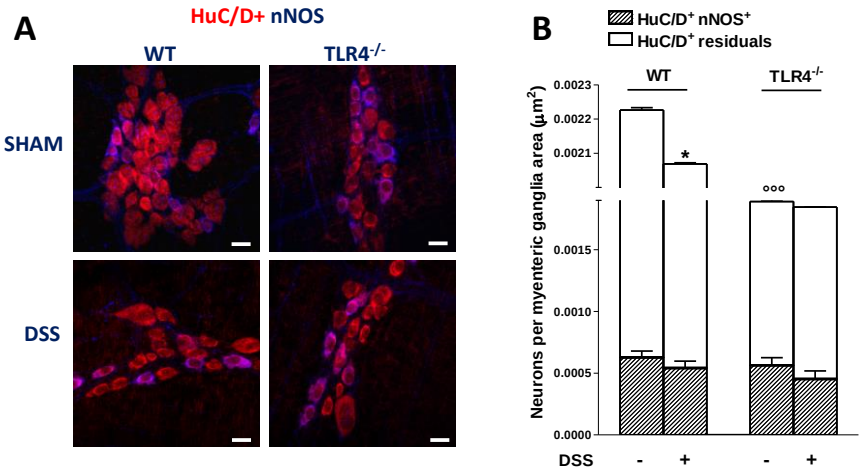


Figure 4.31. TLR4 signaling and DSS-induced colitis affects neurochemical coding. **A**) Representative confocal microphotographs showing the distribution of $nNOS^+$ (blu) and HuC/D^+ (red) neurons in WT an $TLR4^{-/-}$ LMMPs preparations of mice in absence or presence of DSS treatment (bars = 22 µm). **B**) Total number of HuC/D^+ or/and $nNOS^+$ neurons in WT and $TLR4^{-/-}$ LMMPs preparations of mice in absence or presence of DSS treatment. *P<0.05 vs WT SHAM; °°°P<0.001 vs WT SHAM. N=6 mice/group.

We then evaluated the expression of iNOS, enzyme dominantly expressed during inflammatory reactions and found increase in human biopsies of patients with colitis (Gochman et al., 2012). Indeed, in $TLR4^{-/-}$ ileal segments we found a slight but no significant increase in iNOS mRNA levels. DSS treatment caused a 4-fold increase of iNOS mRNA levels in WT mice with no changes in $TLR4^{-/-}$ DSS mice (**Figure 4.32**), confirming our functional data on inhibitory neuromuscular response (**Figure 4.30**).

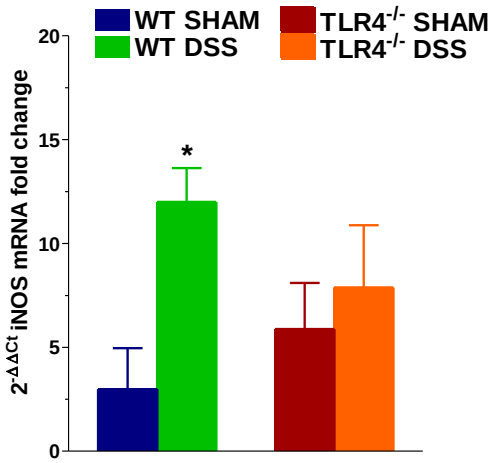


Figure 4.32. TLR4 signaling and DSS-induced colitis affects iNOS expression. RT-PCR quantification of iNOS transcript performed in ileal LMMP+SM preparations of WT and $TLR4^{-/-}$ mice in absence or presence of DSS treatment *P<0.05 vs WT SHAM. N=3 mice/group.

4.4.5 DSS-induced colitis impacts glial phenotype

Considering the structural and functional changes induced by colitis, we evaluated EGCs integrity by immunohistochemistry analysis of the glial markers, SOX10, GFAP and S100β. In WT mice, DSS-induced colitis determined about a 1.3-fold increase in both SOX10 and S100β density index, together with a slight but not significant increment of GFAP (**Figure 4.33**). TLR4$^{-/-}$ mice, in absence of colitis, showed higher SOX10, GFAP and S100β immunoreactivity (+21±2%, +20±1%, and +68±1%, respectively), such increase is considered a sign of reactive gliosis; however in presence of colitis, a reduction in SOX10 immunofluorescence was found with no changes in GFAP and S100β immunostaining (**Figure 4.33**), to suggest an involvement of TLR4 signaling in EGCs phenotype during inflammation (Cerantola et al., 2017).

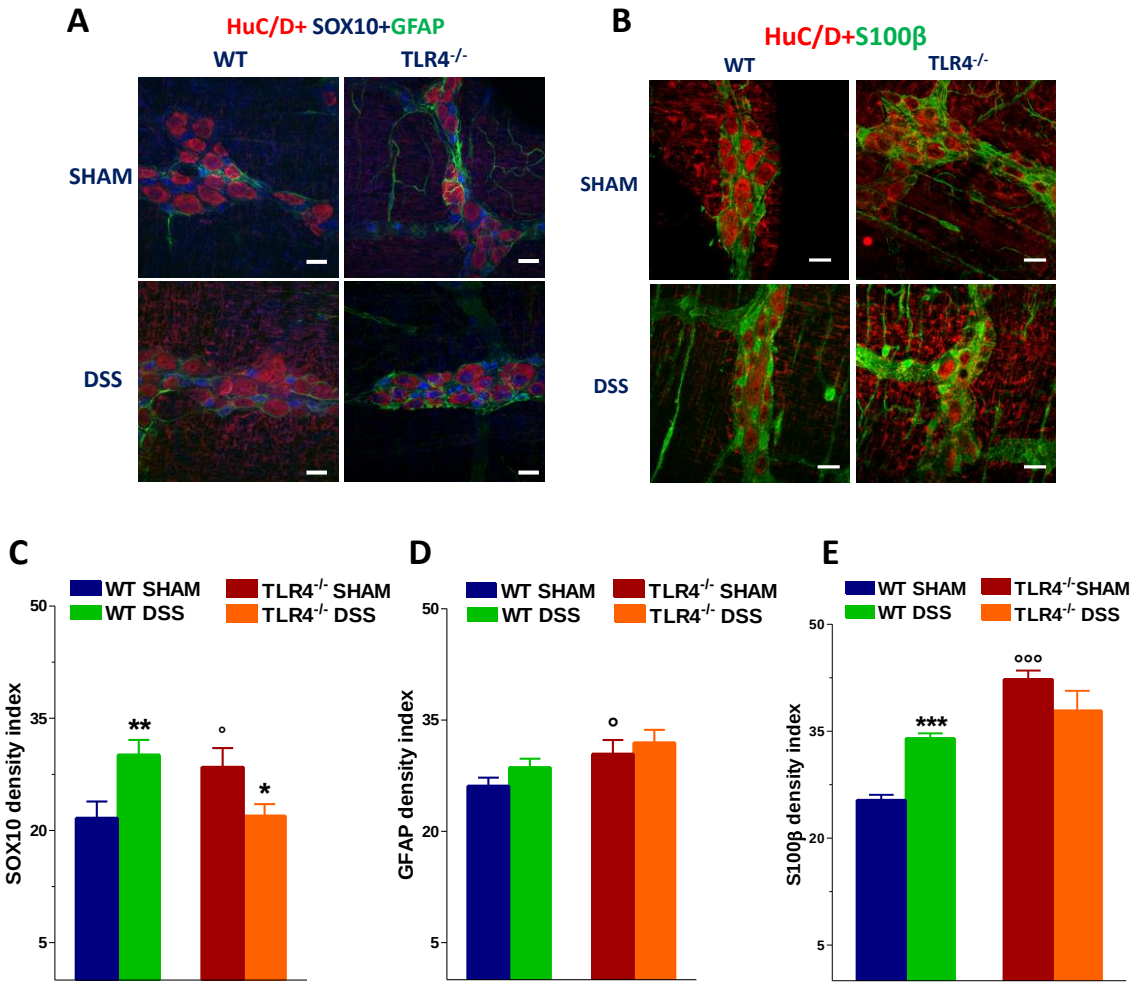


Figure 4.33. DSS-induced colitis influences enteric glial phenotype. **A,B**) Representative confocal microphotographs showing the distribution of (**A**) GFAP^{+} (green) and SOX10^{+} (blu) glial cells and HuC/D^{+} (red) neurons, and (**B**) HuC/D^{+} neurons (red) and S100β^{+} (green) glial cells in LMMPs preparations of WT and TLR4$^{-/-}$ mice in absence or presence of DSS treatment (bars = 22 µm). **C,D,E**) Analysis of SOX10, GFAP and S100β density index in ileal LMMPs preparations of WT and TLR4$^{-/-}$ mice in absence or presence of DSS treatment. *P<0.05, **P<0.01, ***P<0.01 vs respective control in SHAM condition; °P<0.05, °°°P<0.01 vs WT SHAM. N=6 mice/group.

Furthermore, we evaluated the distribution of macrophages through the analysis of immunofluorescence distribution of IBA1, a macrophage marker. As shown by **Figure 4.34**, DSS-induced colitis determined a 2-fold increase in IBA1 immunoreactivity in WT mice, indicative of the presence of an immune-mediated process in the ileal LMMP. In TLR4$^{-/-}$ mice, a significant increase in IBA1 immunofluorescence (+47±2%) was found compared to WT preparations, that was reduced to levels comparable to those found in WT preparations in absence of colitis (**Figure 4.34A,B**), to suggest an involvement of TLR4 signaling in macrophage recruitment in the neuromuscular layers during colitis associated with a marked expression of inducible iNOS (**Figure 4.30**; Tamoutounour et al., 2012).

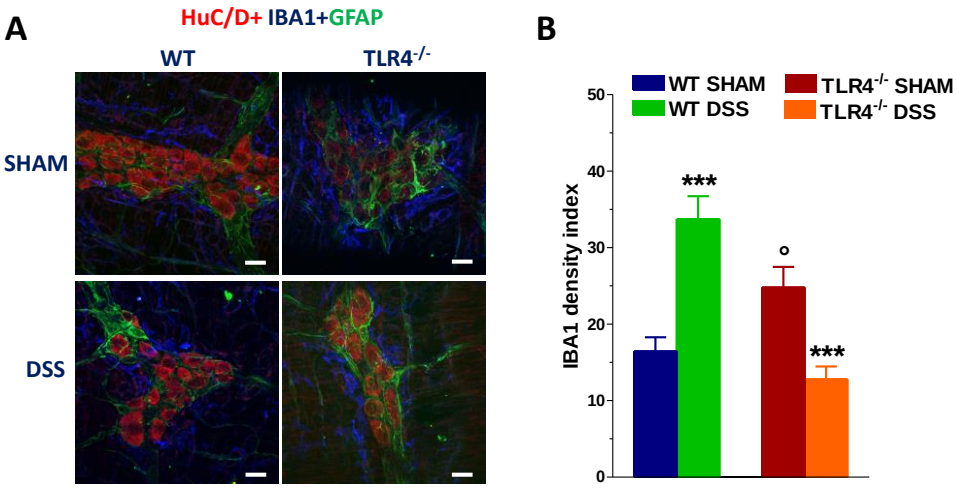


Figure 4.34. DSS-induced colitis increases TLR4 signaling and DSS-induced colitis alters macrophage phenotype. **A**) Representative confocal microphotographs showing the distribution of GFAP (green), HuC/D (red) and IBA1 (blu) in LMMPs preparations of WT and TLR4$^{-/-}$ mice SHAM or DSS (bars = 22 µm). **B**) Analysis of IBA1 density index in ileal LMMPs preparations of WT and TLR4$^{-/-}$ mice in absence or presence of DSS treatment. ***P<0.01, vs respective control in SHAM condition; °P<0.05 vs WT SHAM. N=6 mice/group.

4.4.6 DSS-induced colitis modify serotonergic neurotransmission

5-HT is recognized to be a neurotransmitter essential for ensuring enteric neurotransmission and intestinal homeostasis (Ghia et al., 2009), modulating the neuroimmune responses during intestinal inflammation (Margolis & Pothoulakis, 2009). For instance, it has been demonstrated that, in animal models of colitis, the administration of 5-HT or its precursor 5-HTP enhances the severity of inflammation (Ghia et al., 2009; Chen et al., 2016), highlighting its role as a paracrine factor involved in controlling peristalsis and secretion as well as neuroendocrine modulator of inflammatory responses (Margolis & Pothoulakis, 2009).

We first evaluated the ileal contractile response evoked by the non-cumulative addition of exogenous 5-HT and found in transgenic mice a significantly higher curve (Emax=+59±3%, **Figure 4.35**) compared to that obtained from WT preparations.

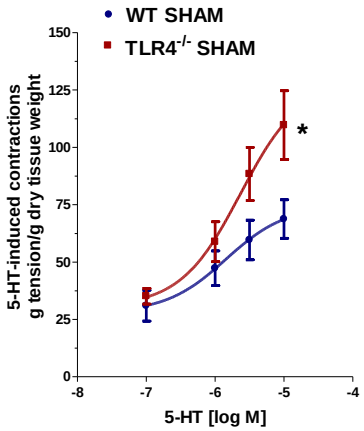


Figure 4.35. TLR4 signaling affects 5-HT-mediated ileal contraction. Non-cumulative concentration-response curves to 5-HT (0.3–30 µM) of isolated ileal preparations from WT and TLR4$^{-/-}$ mice SHAM. *P<0.05 vs WT SHAM. N=6 mice/group.

Since 5-HT response was found to be altered in TLR4$^{-/-}$ mice, we tested neuromuscular response induced by 30 µM 5-HT after DSS treatment and in absence or presence of 0.1 µM ondansetron, a 5-HT$_3$R antagonist, or 1 µM ketanserin, a 5-HT$_{2A}$R antagonist.

As shown by **Figure 4.36A**, in WT mice DSS treatment determined a 1.25-fold increase in 5-HT-mediated contraction that was completely abolished by pretreatment with ondansetron. 5-HT-induced contraction, in presence of ketanserin, determined a 2.75-fold increase in contraction in WT mice that was not affected following DSS treatment.

In TLR4$^{-/-}$ mice, DSS-induced colitis determined a significant reduction of 5-HT-mediated contraction (-51±2%), which was still completely sensitive to ondansetron but not to ketanserin (**Figure 4.36B**), suggesting that TLR4 signaling influences 5-HT$_{2A}$R-mediated response.

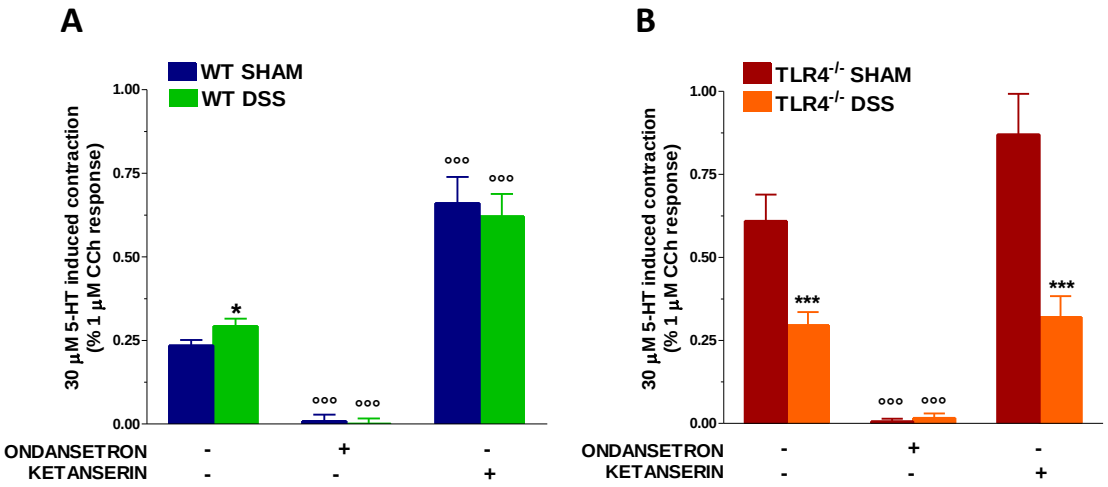


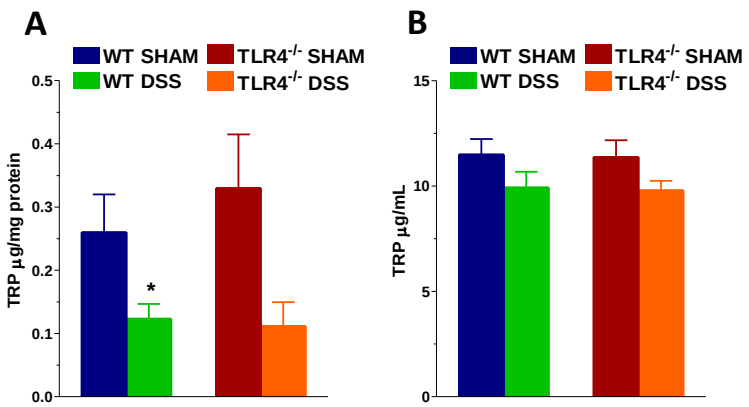
Figure 4.36. DSS-induced colitis affects 5-HT-mediated ileal contraction. **A-B**) Contraction induced by 30 µM 5-HT in absence or presence of 0.1 µM ondansetron or 1 µM ketanserin by ileal preparations from WT (**A**) and TLR4$^{-/-}$ (**B**) mice following treatment with DSS or tap water. *P<0.05, ***P<0.001 vs respective control; °°°P<0.001 vs respective control without 5-HTR antagonist. N=6 mice/group.

4.4.7 DSS-induced colitis impairs tryptophan metabolism

Interestingly, it has been shown that DSS treatment determines higher levels of 5-HT, associated to an increased number of EC cells and low expression of SERT mRNA whereas 5-HT, in turn, regulates DSS-induced colitis severity (Bertrand et al., 2011; Chen et al., 2016). However, *Coates et al.* (2004) found reduced levels of mucosal 5-HT in patients with UC. These data show that it is still not clear the role of 5-HT in colitis; therefore, in view of the changes in the ileal motor response to 5-HT, we tested whether the absence of TLR4 signaling and the presence of colitis may affect TRP metabolism.

TLR4$^{-/-}$ mice exhibit a tendency to higher TRP levels in ileal tissue compared to WT mice (+27±2%; **Figure 4.37A**), with no differences in plasma levels (**Figure 4.37B**). A significant reduction of 5-HTP levels in both ileal tissue (-56±1%; **Figure 4.37C**) and plasma (-31±2; **Figure 4.37D**) was found, suggesting a reduced catabolism of TRP or a faster metabolism to 5-HT. Indeed, TLR4$^{-/-}$ mice showed a 1.5-fold increase in 5-HT tissue levels (**Figure 4.37E**) compared to WT mice with a slight but not significant reduction of 5-HT plasma levels (**Figure 4.37F**). In TLR4$^{-/-}$ mice we found a marked increase of KYN ileal tissue levels (+66±0.9%, **Figure 4.37G**) together with a significant reduction in the plasma levels compared to WT mice (-25±0.9%, **Figure 4. 37H**). In WT mice, DSS-induced colitis caused a clear reduction of TRP, 5-HTP and 5-HT in ileal tissues levels compared to sham mice (-52±2%, -75±3%, -36±1%, respectively; **Figure 4.37A,C,E**) whereas in plasma only 5-HT concentration resulted markedly increased (+200±4%; **Figure 4.37F**), together with a significant reduction of KYN (-26%, **Figure 4.37H**). TLR4$^{-/-}$ mice in presence of colitis showed a significant reduction of 5-HTP, 5-HT and KYN levels in ileal tissue (-81±2%, -76±3%, -96±1%, respectively; **Figure 4.37C,E,G**) that was associated to a significant reduction of 5-HTP concentration (-75±1%; **Figure 4.37D**) and a 2.7-fold increase in 5-HT level in plasma samples (**Figure 4.37F**).

Our data support the hypothesis that colitis determines marked changes in microbiota composition with consequent effect on TRP catabolism and gut-brain axis (Terry & Margolis, 2017).



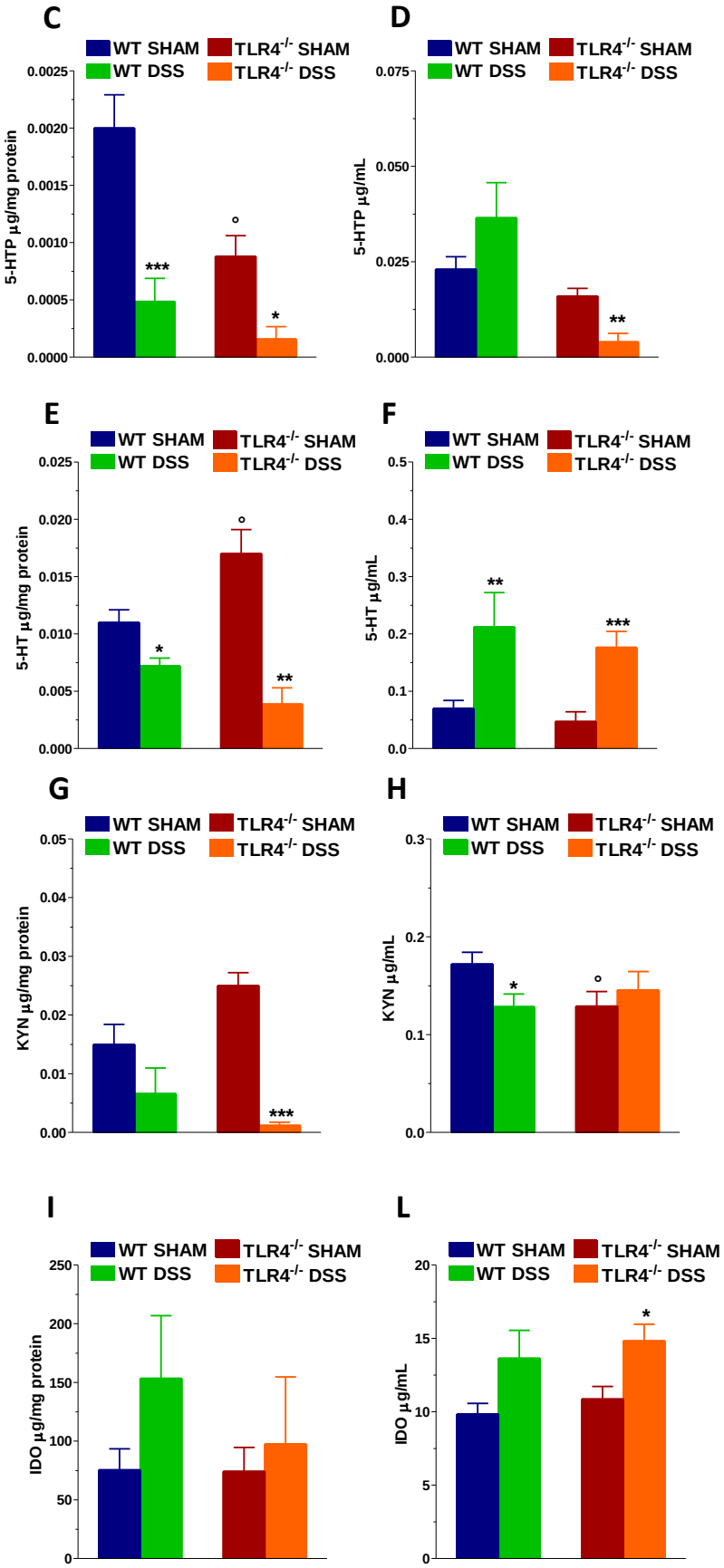


Figure 4.37. DSS-induced colitis alters TRP metabolism. TRP levels (**A,B**), 5-HTP levels (**C,D**) 5-HT levels (**E,F**), KYN levels (**G,H**) and IDO (**I,L**) in ileal tissue or plasma measured by HPLC analysis in WT and TLR4$^{-/-}$ mice following DSS treatment. Data are reported as mean ± SEM. *P 0.05, **P<0.01, ***P<0.001 vs respective control in SD; °P<0.05, °°P<0.01 vs WT SD. N=6 mice per group.

5. DISCUSSION

Homeostasis (ομοιος [similar] and στάσις [status]) 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. Specifically, gut 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 (Caputi et al., 2017a and 2017b). Enteric neuronal circuitries display a considerable ability to adapt in consequences of changes in the microenvironment, which comprises several cellular "players", including neurons, EGCs, smooth muscle cells, interstitial cells of Cajal, and immune cells (Giaroni et al., 1999; Furness, 2012; Yoo & Mazmanian, 2017). This ‘second brain’ of the organism is indirectly exposed to the external luminal environment, which is mainly populated by the (non-pathogenic) commensal microbiota, a ‘superorganism’ consisting of microorganisms that form a unique relationship with its host and then are crucial in ensuring intestinal epithelial integrity and barrier function, promoting gut development and maturation of the mucosal immune system (Shroff et al., 1995; Collins et al., 2014; Obata & Pachnis, 2016). Gut microbiota 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. IBD or IBS; McVey Neufeld et al., 2013; Obata & Pachnis, 2016; Hyland & Cryan, 2016). Recently, the microbiota-gut-brain axis emerged as a key player also in the neurodevelopmental phases of the brain, indicating that events during initial colonization and microbiota development can impact general and mental health in later life (Diaz Heijtz et al., 2011; Borre et al., 2014; Martin et al., 2018). 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 detect the presence of microorganisms throughout TLRs, an important subgroup of PRRs that are involved in the recognition and response to microbial components, derived from pathogens (PAMPs) or endogenous ligands derived from host cells damaged (DAMPs), and in detecting molecular component derived from commensal microorganisms. Thus, TLRs play a crucial role in the innate immunity protection from infection, in the control of tissue integrity and in the symbiosis between gut microbiota and the host (Kabouridis and Pachnis, 2015; Caputi & Giron, 2018). Among TLRs, TLR4 plays a well-established regulatory role in the innate immune response to infection and in adaptive responses consenting probiotic bacteria colonization. Currently, 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; Hug et al., 2018). 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; Brun et al., 2015). In human LMMPs and in rat ENS primary cultures, neuronal 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 under stimulation following excessive use of antibiotics can affect the balance between ENS-microbial-derived products early in life, setting the basis for developing GI functional disease in adulthood (Becattini et al., 2016).

Effect of enteric dysbiosis or TLR4 deficiency on small intestine neuromuscular function

In the first part of this study, we compared the effects of gut microbiota depletion and the absence of TLR4 signaling on the structure and function of ENS. Gut microbiota depletion has been achieved by using a previously published protocol for manipulation of intestinal microbiota based on the administration of a cocktail of broad spectrum antibiotics, given to mice by oral gavage for 14 days (ABX mice; Reikvam et al., 2011; Brun et al., 2013, Caputi et al., 2017b). Ampicillin, vancomycin, neomycin and metronidazole display activity against the full spectrum of bacteria; especially, vancomycin and neomycin show activity against Gram positive and Gram negative, respectively, whereas ampicillin is active against both gram negative and positive aerobic and facultative strains, which is potentially important for preventing antibiotic resistance (Kollef, 2005). Metronidazole is a bactericidal agent widely used for its activity against many anaerobic and certain protozoan and parasitic infections. The depletion of gut microbiota through oral administration of broad-spectrum antibiotics is a sustainable alternative to the use of mice bred in sterile environment from the birth. GF animals are used for such comparative studies, but they need expensive GF facilities as well as specific expertise for animal manipulation and infrastructure management (Reikvam et al., 2011). Compared with animals living in a conventional microbiological environment, GF animals display several phenotypic characteristics, such as an immature and underdeveloped lymphoid system (Smith et al., 2007).

Our data shows that antibiotic-induced microbiota depletion or the absence of TLR4 signaling causes similar disturbances in ileal neuromuscular contractility, with a significant downward shift of the concentration-response curve to the non-selective cholinergic receptor carbachol, along with a significant reduction to EFS at increasing frequencies (0-40 Hz). We then evaluated the structure of ileal myenteric ganglia in ABX and $TLR4^{-/-}$ mice. The confocal microscopy analysis performed on LMMPs revealed several anomalies in the expression and distribution of typical neuronal and glial proteins in the ENS. In the myenteric plexus of ABX or $TLR4^{-/-}$ mice we found a comparable pattern of reduction of both HuC/D^{+} and $nNOS^{+}$ neurons together with an increase of the glial markers S100β and GFAP (Caputi et al., 2017a and 2017b; **Figure 5.1**). Enteric glial damages have detrimental

consequences for enteric neurons (Thacker et al., 2011). Indeed, the perturbation of gut microbiota composition compromises ENS architecture with effects on neuronal and glial survival and these anomalies surprisingly mostly appear to be due to the absence of TLR4 signaling. Moreover, most of the anomalies appear to result from a crosstalk between TLR4 and EGCs (Caputi et al., 2017a).

Changes in glial markers expression during glial cell differentiation, inflammation and injury, highlight that the levels of these proteins are associated to the functional state of ECGs (Cirillo et al., 2011a). Moreover, 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). 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; Grubišić et al., 2018). We have recently shown (Caputi et al., 2017a) that $TLR4^{-/-}$ mice has reactive gliosis as confirmed by the increase of S100β, GFAP and SOX10 immunoreactivity compared to control mice (Boesmans et al., 2015; Ochoa-Cortes et al., 2016). 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 evidence, increases in GFAP expression are associated with EGCs differentiation, inflammation, and injury (Ochoa-Cortes et al., 2016), whereas S100β expression and its release by EGCs at μM levels are linked to pathological conditions (Rühl, 2005). Moreover, EGCs gliosis, detected by increased GFAP levels and/or S100β immunoreactivity/release, are considered a defensive response of glia to protect neural networks from damage during disease or injury (Burda & Sofroniew, 2014).

Impaired enteric glial activity and TLR4 deficiency on small intestine neuromuscular function

We then tested the effect of fluoroacetate (FA), a gliotoxin, to better understand the role of EGCs in the GI modifications of $TLR4^{-/-}$ mice. FA and its metabolite FC are metabolic poisons commonly used to study glial cells functions; they cause the disruption of the tricarboxylic acid cycle enzyme aconitase (McClain & Gulbransen, 2017) and they are preferentially taken up by glial cells (Fonnum et al., 1997, McClain & Gulbransen, 2017). Other glial cells ablation models are available based on transgenic mice, expressing herpes simplex virus thymidine kinase from the mouse glial fibrillary acidic protein (GFAP) promoter, or on mice having glial disruption induced by injection of activated haemagglutinin specific $CD8^{+}$ T cells (Bush et al., 1998; Aubé et al., 2006). Treatment with FA did not change the glial phenotypes and the number of total neurons in WT mice as shown by *Nasser et al.* after treatment with FC (2006). FA treatment in $TLR4^{-/-}$ mice, determined a glial phenotype

comparable between genotypes with no changes in SOX10 immunofluorescence and an increase of total number of HuC/D^{+} neurons compared to WT mice, suggesting the onset of a mechanism of neuroplasticity (Boesmans et al., 2015). SOX10, one of the three glial markers analyzed, is one of the earliest neural crest cell markers of ENS progenitors. It has been found that the deletion of SOX10 causes a failure in the development of enteric ganglia, highlighting its role in the homeostasis of ENS (Laranjeira et al., 2011). During development the expression of the transcription factor SOX10 reveals the distribution of enteric neural precursor cells (ENPCs) which will generate neurons, whereas in adult mice, SOX10 is expressed in enteric glia; indeed SOX10^{+} cells have neurogenic activity during embryogenesis that is missing in the adult stages (Kulkarni et al., 2018). However, *Laranjeira et al.* (2011) demonstrated that under treatment with 4-hydroxytamoxifen mature EGCs are capable of generating multilineage ENS progenitors and activating neurogenic programs. Therefore, neurogenesis is undetectable in the ENS of adult animals, but in response to injury mature glial cells might generate enteric neurons (Laranjeira et al., 2011). Our results in TLR4$^{-/-}$ might be explained by the finding of *Laranjeira et al.* (2011), indeed following FA treatment, we maintained higher levels of SOX10 associated to an increase of total number of neurons to indicate that the disruption of glial cells caused an increase of SOX10 expression, determining neurogenic activity with a higher number of HuC/D^{+} neurons. In adult animals, a substantial phenotypic plasticity among EGCs subtypes has been shown by the observations of *Boesmans et al.* (2015). All subtypes of EGCs originate from a common progenitor which will generate diverse glial subtypes depending on its final destination and physiological context. Potentially, the aptitude of glial cells to gain different properties is not restricted to embryonic or early postnatal stages but is maintained throughout adult life pointing to a previously unacknowledged dynamic phenotypic plasticity of the ENS, capable of adjusting its molecular characteristics in response to diverse challenges associated with nutrition, microbiota, mechanical factors, or disease (Boesmans et al., 2015).

In the ENS, the major inhibitory non-adrenergic, non-cholinergic (NANC) neurotransmitter is endogenous NO that 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 (Bódi et al., 2019). 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; Bódi et al., 2019). In case of inflammation the induction of iNOS produces a large amount of NO with consequent intestinal dysmotility (Eskandari et al., 1999; Bódi et al., 2019) together with an upregulation of iNOS in epithelial and immune cells, in neurons and EGCs of the ENS, determining altered epithelial function and water and ion transport dysregulation (Seago et al., 1995; Green et al., 2004; MacEachern et al., 2015). Indeed, we recently shown that, in NANC conditions, isolated ileal segments from TLR4$^{-/-}$ mice have an increase of EFS-mediated relaxation associated with a proportional increase of nNOS^{+} neurons and an increase of both iNOS activity and immunoreactivity,

whereas in WT mice the inhibitory tone was mainly dependent on nNOS-mediated NO production (Caputi et al., 2017a). To better understand the role of EGCs in the neuromuscular function, we evaluated the effect of FA in the inhibitory response following 10 Hz-EFS-stimulation. After FA treatment, in WT mice we found a slight but not significant reduction of inhibitory response to indicate that EGCs do not participate in the ileal relaxation in a healthy control and confirming the data found by *Aubé et al.* (2006) in jejunal segments from a transgenic model of glial ablation. In $TLR4^{-/-}$ mice, the ablation of EGCs, re-established an inhibitory response comparable to WT mice supporting a role for enteric glia in intestinal physiology. Moreover, iNOS activity in $TLR4^{-/-}$ mice was abolished following *in vitro* FA treatment confirming that the absence of TLR4 signaling is involved in controlling EGCs activity and iNOS expression. These data confirmed the findings of *MacEachern et al.* (2015) showing that FA prevented NO release specifically from iNOS suggesting that FA directly targets enteric glial metabolism. Smooth muscle relaxation in the colon, as well as in the small intestine, is mediated by the release from enteric inhibitory motor neurons of not only NO but also ATP (Mañé et al., 2014). Among all the purinergic receptors, P2Y1Rs is one the receptor subtype involved in mediating inhibitory neurotransmission in the GI tract (Mañé et al., 2014). Moreover, we have recently shown that in $TLR4^{-/-}$ mice P2Y1Rs staining is increased in both neurons and EGCs of myenteric ganglia of small intestine together with a higher inhibitory response (Caputi et al., 2017a). Furthermore, another study showed that, in the CNS, stimulation of P2Y1Rs enhances reactive astrogliosis (Franke et al., 2001). Therefore, we evaluated the activity of P2Y1Rs following disruption of glial cells. In both the genotypes, the combination of FA and MRS2500 determined a reduction of inhibitory response comparable to the response obtained only in presence of MRS2500. P2Y1Rs immunoreactivity did not change in both the genotypes after FA treatment. These data indicate that the relaxation mediated by P2Y1Rs is mostly due to the activation of neural and not glial P2Y1Rs. *McClain et al.* (2014) showed that following the activation of P2Y1Rs, Ca^{2+} responses are generated leading to the opening of connexin-43 (Cx43) hemichannels. Cx43 hemichannels are necessary for the propagation of Ca^{2+} responses among glia, highlighting the role of EGCs in modulating GI motility through Cx43 activity (McClain et al., 2014; Brown et al., 2016). In the CNS, astroglial Cx43 hemichannels are involved in the release of neuroinflammatory mediators, contributing to the development of neuropathic pain and neurodegeneration (Brown et al., 2016). *Brown et al.* (2016) demonstrated that, in isolated preparations of mouse ENS, the direct activation of P2Y1Rs together with the opening of Cx43 hemichannels in glial cells affects neuronal survival leading to loss of neuronal density. However, in our ENS samples, we found no changes in Cx43 density index in absence or in presence of FA treatment in both genotypes. *Brown et al.* (2016) found that EGCs influence the release of ATP through Cx43 hemicannels following the stimulation mediated by the activation of P2Y1Rs and the presence of NO. Thus, we evaluated the inhibitory response in presence of MRS2500 and L-NAME, a pan NOS inhibitor. In absence of FA treatment in both the genotypes 10

Hz-EFS abolished completely the relaxation to confirm that purinergic and nitrergic neurotransmissions are the principal inhibitory pathways in the gut (Mañé et al., 2014). However, following FA treatment, in both the genotypes the inhibitory response was only partially abolished, to suggest that the disruption of EGCs might cause the arise of other inhibitory neurotransmissions, such as serotonergic or dopaminergic one.

TLR4 deficiency on small intestine neuromuscular function in HFD-induced obesity or inflammatory bowel disorders

To better characterize the role of TLR4 signaling in the gut neuromuscular functions in pathological conditions, we exposed mice to a low-grade (i.e. HFD-induced obesity) and high-grade inflammation (i.e. DSS-induced experimental colitis). HFD is known to determine a chronic systemic mild inflammation causing changes in gut microbiota alterations (Duan et al., 2018). Indeed, the presence of low-grade systemic inflammation associated with obesity usually involves a complex network of signals interconnecting several organs, including CNS, however the causal pathways between obesity, inflammation, neurodegeneration and metabolic disease remain incompletely understood (Sanmiguel et al., 2015). DSS-induced colitis is an acute inflammation, affecting primarily the colon and moderately the small intestine, by triggering the disruption of intestinal microbiota and epithelial barrier integrity (Li et al., 2017; Mourad et al., 2017).

TLR4 activity is subject to regulation by dietary components; indeed saturated fatty acids can trigger its signalling mediating inflammatory processes, whereas unsaturated fats can act conversely (Vellosso et al., 2015). Moreover, the diet plays a pivotal role in the IBD onset and in the development of obesity (Rizzello et al., 2019; Sugihara et al., 2019). Dietary nutrients can directly regulate the gut microbiota composition but also the immuno-modulatory function (Sugihara et al., 2019).

Obesity is considered a worldwide epidemic and is one of the public health challenges of the 21st century (James, 2004). HFD contributes to the development of obesity that is associated with enteric neuronal dysfunction and GI motility disorders. Obesity can be defined as the excess accumulation of adipose tissue in the human body that impairs both physical and psychosocial health and well-being (James, 2004). Moreover, HFD induces gut microbiota dysbiosis and it has been shown that GF mice have lower body compared to conventionally raised mice (Tilg, 2010; Reichardt et al., 2017). Several studies on HFD-induced obesity showed that HFD determines enteric neurodegeneration via TLR4 in mice (Reichardt et al., 2017) and impaired GI transit associated with a neuronal loss mediated by TLR4 signaling and increased plasma LPS concentrations (Anitha *et al.,* 2016), with consequent release of proinflammatory cytokines and myenteric neurons apoptosis (Arciszewski et al*.,* 2005; Coquenlorge et al*.,* 2014; Voss & Ekbald, 2014).

UC and CD represent the two main forms of IBD. Since the recognition of CD and UC, back in the 1600 and 1800, the etiology and the genetic predisposition of IBD have been partially outlined

(Baumgart, 2008) but the exact pathogenesis of the disease is still unclear (Tripathi & Feuerstein, 2019). However, the most accredited hypothesis of IBD onset is an anomalous interaction between genetic susceptibility and exposure to environmental risk factors, determining an altered host immune response against commensal microflora and other luminal antigens (Baumgart, 2008). UC patients are characterized by recurrent inflammation in the mucosal layer of the rectum and colon, associated to diarrhea with blood and mucus, and abdominal pain (Sammut et al., 2015). Moreover, *Fakhoury et al.* (2014) showed that in patients with IBD there is an upregulation of NOD2, leading to an increase of TLR4 activity with marked release of proinflammatory cytokines. The presence of colitis determines disruption of mucosal barrier and higher exposure of TLR ligands produced by commensal microbiota (Rakoff-Nahoum et al., 2004).

UC is generally confined to the colon with occasional extension into terminal ileum and with the rare occurrence of systemic toxicity. However, many studies have shown that small intestine is affected by UC showing structural, biochemical and neural alterations, determining dysmotility, higher intestinal permeability and neuronal excitability in UC patients as well as in experimental animal models of colitis (Mourad et al., 2017).

In the HFD-induced obesity model, we evaluated the body weight, and glucose, cholesterol and triglycerides plasma levels. In both the genotypes we obtained a significant increase of body weight reaching values >26 g (Hohos et al., 2018), however, in $TLR4^{-/-}$ mice only after 5 weeks of HFD reached a body weight significantly different from that one of mice fed with SD. Daily administration of HFD induces the development of obesity together with hyperglycemia and hyperinsulinemia (Parekh et al., 1998); indeed in WT mice we found a significantly increase in glucose plasma levels after 8 weeks of HFD as shown by *Anitha et al.* (2016) with no changes in $TLR4^{-/-}$ mice (Nezami et al., 2014). Recently, TLR4 has emerged as an interesting candidate for a cellular link between inflammation and insulin resistance. In addition to lipopolysaccharides from Gram-negative bacteria (Shi et al., 2006; Jackson et al., 2015), TLR4 can be activated by saturated free fatty acids during hyperlipidemic state associated with obesity and secondary to long-term ingestion of a HFD (Schaeffler et al., 2009; Holland et al., 2011). Humans with TLR4 mutations have been shown to tend to be protected against developing diabetes (Manolakis et al., 2011). In agreement with previous studies, the administration of a HFD results in peripheral insulin resistance, as evident by obesity, hyperglycemia, compared to mice fed a normal diet (Jackson et al., 2015). Additionally, TLR4-mutant mice were partially protected against these metabolic disorders, suggesting that activation of TLR4 contributes to HFD-induced peripheral insulin resistance (Jackson et al., 2015; Razolli et al., 2015). In addition, food intake was comparable in the TLR4 mutant compared to WT mice fed a long-term HFD, suggesting that the observed changes in body weight and glucose dysregulation are linked to the loss of TLR4 function rather than a change in energy intake. The absence of TLR4 partially protects against the detrimental metabolic effect of obesity. Indeed, several studies (Shi et al., 2006;

Kim et al., 2007; Manolakis et al., 2011; Jackson et al., 2015; Tsukumo et al., 2015) found the activation of inflammatory pathways in WT mice fed with HFD whereas the absence of TLR4 signaling blocked the inflammatory effects induced by fat diet.

In DSS-induced colitis, both the genotypes showed similar clinical symptoms including diarrhea, occult blood and sometimes rectal bleeding together with a reduction of body weights, as previously shown by Yang et al. (2016). *Rakoff-Nahoum et al.* (2004) evaluated the effect of DSS in mice MyD88$^{-/-}$, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice. They found a severe mortality in MyD88$^{-/-}$ mice after DSS treatment, whereas in TLR2$^{-/-}$ and TLR4$^{-/-}$ mice they obtained an increased susceptibility to DSS. These data suggest that the MyD88-dependent signaling is fundamental for the protection against DSS-induced mortality and morbidity (Rakoff-Nahoum et al., 2004). TLR4$^{-/-}$ mice had epithelial damage similar to that found in control mice after DSS treatment but obtained less acute inflammatory cell infiltrate with fewer neutrophils in the lamina propria and submucosa compared to control mice as previously shown by *Fukata et al.* (2005).

As shown by several studies, gut inflammation begins when TLR4 stimulation, following the binding of its own ligands such as LPS or other PAMPs, determines the activation of NF-κB causing the production of different cytokines including TNFα and IL-1β (Eichele & Kharbanda, 2017). The absence of TLR4 could potentially reduce the detrimental effects induced by DSS in gut morphology.

Recent studies have demonstrated that inflammation leads to several changes in the neuron's circuitry, causing alterations in the hyperexcitability of neurons, peristaltic reflex, synaptic facilitation, and attenuated inhibitory neuromuscular transmission (Mawe, 2015). All together these neuromodulated effects can cause constipation that is one of the main features of obesity and IBD (Bertand et al., 2012; Mawe, 2015). However obese patients can have shorter transit times suggesting that fatty diets modulate GI motility differently (Bertand et al., 2012). Several reports indicate that diarrhea, another feature of UC, should be associated with increased motility but this is not supported by clinical investigation. Indeed, diarrhea in IBD seems to be linked to decreased contractile activity. *Rao et al.* found decreased small intestinal transit rates and they reported that rectosigmoidal transit was increased and stool frequencies were elevated in active colitis relative to quiescent disease (Mawe, 2015). In our models, HFD and DSS treatment caused a marked decrease in transit time in WT mice, with no changes in TLR4$^{-/-}$ mice suggesting that the absence of TLR4 signaling partially protects against the HFD- and DSS-induced constipation. However, in HFD models we found a reduction in stool water content with no changes in the number of fecal pellets in both the genotypes due to changes in gut microbiota with consequent increase of LPS levels and enteric neuroplasticity (Anitha et al., 2012). In DSS-induced colitis we found an increase of stool water content in both genotypes with a strong reduction in the number of fecal pellets only in WT mice, a typical sign of the disease (Ungaro et al., 2017; Maruta et al., 2018; Xu et al., 2019).

Based on our data of GI dysmotility, we tested the neuromuscular excitatory cholinergic response. Both genotypes responded with a significant reduction in cholinergic neurotransmission following HFD or a significant increase in the contraction after DSS treatment. These differences in cholinergic neuromuscular response can be explained by changes in cholinergic neural phenotype. Indeed *Nezami et al.* (2014) found a significant reduction in $ChAT^+$ neurons in proximal colon of mice fed with HFD, whereas in DSS-induced colitis model ChAT immunofluorescence has been found increased as shown by our results in WT mice (Green et al., 2004; Winston et al., 2013) to indicate a primary role of TLR4 signaling in neuronal plasticity. *Green et al.* (2004) reported an increase of ChAT immunoreactivity in a colitis model, suggesting that the DSS treatment caused a shift in the equilibrium of cholinergic control from muscarinic to nicotinic neurotransmission with an altered ion transport and EGCs activity. The activation of nicotinic receptors, expressed on myenteric plexus, elicits an enhanced production of iNOS-mediated NO in EGCs and neurons (Green et al., 2004). Indeed, during intestinal inflammation it has been reported an increase of NO production mediated by an upregulation of iNOS in gut epithelial and immune cells as well as in enteric neurons and glia, determining altered epithelial function and water and ion transport dysregulation (Seago et al., 1995; Green et al., 2004; MacEachern et al., 2015). The increase of iNOS has been evaluated in several model of gut inflammation such as TNBS- or DSS-mediated colitis and ileitis induced by adjuvant (Seago et al., 1995; Green et al., 2004; MacEachern et al., 2015). On the other hand, *Kim et al.* (2007) demonstrated that HFD causing an increase of LPS levels determines TLR4 engagement, NF-kB activation and higher expression of iNOS.

To characterize the effects of these cholinergic anomalies, we evaluated the NANC-mediated relaxation in isolated ileal segments from WT and $TLR4^{-/-}$ mice after HFD and DSS treatment. In absence of inflammation WT mice showed an inhibitory response mediated by only nNOS activity, whereas $TLR4^{-/-}$ mice had an inhibitory response mediated by both iNOS and nNOS (Caputi et al., 2017a). After development of obesity or in presence of colitis, we found in WT mice an altered and opposite inhibitory tone, however, in both cases associated to an higher response mediated by iNOS-produced NO, suggesting the presence of a low-grade inflammation (Kim et al., 2007). On the other hand, $TLR4^{-/-}$ mice fed with HFD showed a reduction of inhibitory response mainly due to the lesser release of iNOS-produced NO, effect that was not found following DSS treatment (**Figure 5.1**). A reduction of iNOS expression, even if not significative, has been previously shown in the colon of $TLR4^{-/-}$ mice fed with HFD (Kim et al., 2007), to further suggest that changes in the gut microbiota determined by HFD may play a pivotal role in the induction of LPS-induced inflammatory status in the intestine and may contribute to the phenotype observed in HFD mice.

Soares et al. (2015) found that, in a model of HFD, in the ileum the $nNOS^+$ neuronal density is increased together with a reduction of number of cells positive for nNOS. Indeed, in our model the reduced NO-mediated relaxation in ileal preparations of WT HFD mice was accompanied by a

reduction of nNOS$^+$ neurons with an increase of HuC/D$^+$ neurons as shown by *Anitha et al.* (2016). On the other hand, WT DSS mice exhibited a marked NO-mediated relaxation in ileal preparations accompanied by a loss of myenteric HuC/D$^+$ neurons associated to a proportional increase in nNOS$^+$ neurons. A reduction of the total number of neurons has been shown in other models of colitis (Sanovic et al., 1999; Boyer et al., 2005; Linden et al., 2005). TLR4 signaling appears to be markedly involved in the myenteric neuroplastic changes observed during experimental colitis but not in myenteric neural network anomalies revealed after 8 weeks of HFD.

In response to injury, stress, or inflammation, astrocytes of the CNS acquire new properties referred to reactive glia or astrogliosis. Given the similarities between CNS astrocytes and EGCs, EGCs also become reactive when exposed to an inflammatory environment and are involved in intestinal epithelial barrier dysfunction (Pochard et al., 2018). The majority of EGCs, located in the myenteric ganglia, co-expressed S100β, GFAP, and SOX10 whereas EGCs outside the plexi are more heterogeneous and expressed either one, two, or three markers. An upregulation of these glial markers is the sign of the onset of reactive gliosis (Cabarrocas et al., 2003). The differential percentage of EGCs expressing combinations of GFAP, S100β, and SOX10 within the glial subtypes I–III of the myenteric plexus indicates that, at the cell population level, glia expression of specific genes shows a dynamic plasticity that is highly influenced by temporally and spatially controlled factors (Boesmans et al., 2015). Several works showed that the levels of EGCs markers are regulated by proinflammatory cytokines and by microbial components, such as LPS. Indeed, EGCs, cultured with TNF-α, IL-1β, LPS, or with a cocktail of LPS and IFNγ, show an increase of GFAP, whereas S100β is upregulated by both LPS and IFNγ but also by the enteroinvasive *Escherichia coli* (Pochard et al., 2018). Several studies demonstrated that, in the hypothalamus, cellular responses following HFD results in the onset of reactive astrogliosis (Thaler et al., 2012; Buckman et al., 2013; Dorfman & Thaler, 2015). Moreover, IBD patients showed an increase in the expression of both GFAP and S100β in the mucosal inflamed areas compared with non-inflamed areas (von Boyen et al., 2011; Pochard et al., 2018). In agreement with previous findings (Cabarrocas et al., 2003; von Boyen et al., 2011; Thaler et al., 2012; Buckman et al., 2013; Dorfman & Thaler, 2015; Pochard et al., 2018), we revealed an increase of SOX10, S100β and GFAP immunofluorescence in WT mice after HFD or DSS treatment (**Figure 5.1A,B**). In TLR4$^{-/-}$ myenteric ganglia HFD determined an increase of SOX10 immunostaining whereas DSS treatment induced a reduction of SOX10 immunoreactivity with no changes in GFAP and S100β (**Figure 5.1C,D**). SOX10 is a nuclear localized transcription factor, expressed by multipotential ENS progenitors (Boesmans et al., 2015) and is maintained in immature and mature EGC. These data indicate an involvement of TLR4 pathway not only to control glial commitment but also to regulate neuronal progenitors, as previously shown in normal and pathological conditions (Anitha et al., 2012; Anitha et al., 2016).

Macrophages are pivotal in coordinating intestinal immune homeostasis and inflammation (Zhu et al., 2014; Jones et al., 2018). *Zhu et al.* (2014) characterized the macrophage populations in a mouse model of DSS-induced colitis. After 7 days of treatment they found an increase of M1 macrophages population with a reduction of M2 macrophages populations, suggesting a disequilibrium in the macrophage phenotype during colitis (Zhu et al., 2014). In WT LMMPs we obtained an increase of the macrophage marker IBA1 during colitis but not in $TLR4^{-/-}$ myenteric plexus to underline the primary role of TLR4 signaling in macrophage recruitment and phenotype during inflammation.

Gut inflammation is also associated to an alteration of 5-HT production mediated by enterochromaffin (EC) cells. EC cells are the best-characterized subgroup of gut endocrine cells, which are dispersed throughout the gut mucosa and are the main source of 5-HT in the gut (Ghia et al., 2009). 5-HT modulates numerous GI functions, such as peristalsis, secretion, vasodilation, pain, nausea, including ENS development and neurogenesis (Gershon, 2013; Mawe & Hoffman, 2013; Terry & Margolis, 2017). Moreover, mucosal 5-HT mediates immune responses and may, potentially, influence intestinal inflammation (Margolis & Pothoulakis, 2009). Once 5-HT is released from EC cells, it can activate different 5-HT receptors to initiate gut motility. The most studied 5-HT receptors are $5\text{-}HT_3R$ and $5\text{-}HT_4R$, both expressed on ENS neurons of both myenteric and submucosal plexi (Mawe & Hoffman, 2013; Terry & Margolis, 2017). Once $5\text{-}HT_3R$ is activated EPSPs are induced together with activation of intrinsic and extrinsic afferent nerves determining gut contraction (Terry & Margolis, 2017).

In the ileum of $TLR4^{-/-}$ mice, 5-HT-evoked contractile response resulted significantly increase compared to WT mice, suggesting the presence of an interactive dialogue between the intestinal serotonergic neurotransmission and TLR4, being expressed in a variety of intestinal cell populations, including neurons, glial cells, and smooth muscle cells (Barajon et al., 2009). The role of 5-HT in GI disorders is still controversial and several studies have suggested a relationship between the serotonergic system, obesity and IBD (Bertrand et al., 2012; Mawe & Hoffman, 2013). Neural 5-HT has a suppressive effect on food intake and tends to decrease body weight gain (Watanabe et al., 2016); moreover studies in mice and rats showed that 5-HT's levels increased after HFD associated with a reduced capacity of SERT to reuptake 5-HT (Bertrand et al., 2011; Kim et al., 2011). Furthermore, the administration of 5-HT or its precursor 5-HTP enhances the severity of inflammation (Ghia et al., 2009; Chen et al., 2016). The activation of 5-HTRs can have beneficial effects against the detrimental consequences of colitis. 5-HT can exert its activity binding different 5-HT receptors (5-HTRs); indeed 5-HT induces contraction or relaxation of smooth muscle depending on the receptor activated. $5\text{-}HT_1$, $5\text{-}HT_2$, $5\text{-}HT_3$, $5\text{-}HT_4$, and $5\text{-}HT_7$ are the receptor subtypes implicated in the regulation of gut motility (Zhao et al., 2006). Furthermore, $5\text{-}HT_3R$ is involved in the regulation of major digestive functions, such as gastric emptying and pancreatic secretion, as well as in the control of food intake under several feeding paradigms (Weber et al., 2009). *Weber et al.* (2009)

demonstrated that oral treatment with tropisetron, 5-HT_3R antagonist, reduced intestinal motility and almost completely blocked weight gain associated with glucose feeding. *Utsumi et al*. (2016) demonstrated that, daily administration of ondansetron (5-HT_3R antagonist) reduced the severity of DSS-induced colitis and the up-regulation of inflammatory mediator expression. These two reports underline the importance of 5-HT_3R in the development of inflammation independently from the causes such as HFD or DSS.

Zhao et al. (2006) showed that following an infection the expression of several 5-HT receptors was upregulated, but only the 5-HT_{2A} was involved in infection-induced functional changes together with an hypercontractility to 5-HT. Indeed, *Nonogaki et al.* (2006) showed that in a model of obesity the hypothalamic 5-HT_{2A}R mRNA levels were increased, underlining that 5-HT can be an important therapeutic target for a number of brain-gut pathologies (Zhao et al., 2006). Systemic administration of sarpogrelate, a 5-HT_{2A}R antagonist, suppressed overfeeding, body weight gain, and hyperglycemia in obese mice (Nonogaki et al., 2006).

Colonic biopsies from IBD patients have shown higher expression level of 5-HT_{2A}R (Xiao et al., 2016). Treatment with ketanserin, a 5-HT_{2A}R antagonist, reduces the severity of colitis induced by DSS (Xiao et al., 2016). In this regard, we evaluated the effect of ondansetron and ketanserin on 5-HT-mediated neuromuscular contraction. HFD as well as DSS treatment determined in isolated ileal segments from WT mice an increase of 5-HT-mediated contraction that was completely abolished in presence of 5-HT_3R antagonist to suggest a higher activity or expression of 5-HT_3R in ileal segments (Utsumi et al., 2016).

In WT ileal LMMPs the inhibition of the 5-HT_{2A}R augmented 5-HT-mediated contraction which was further increase during colitis but not after HFD. Surprisingly, in $TLR4^{-/-}$ ileal preparations HFD as well as DSS-induced colitis determined a significantly reduction of 5-HT-mediated response which resulted insensitive to pretreatment with ketanserin to highlight an involvement of TLR4 pathways on 5-HT_{2A}R-mediated response in pathological conditions.

Recent studies have suggested that changes in 5-HT transporter reuptake and modifications in 5-HTRs activity could be effective for controlling IBD activity and associated symptoms (Layunta et al., 2018). It has been discovered that 5-HT treatment ameliorates the obesity and diabetes of mice fed with HFD diet, without any change of food intake but increasing the energy expenditure (Watanabe et al., 2016). Peripheral 5-HT it has been thought to play a key role in the relief of obesity accelerating energy consumption in skeletal muscle (Watanabe et al., 2016). Human data and animal models of IBD are still controversial about the role of 5-HT. Indeed, in different animal model of colitis, EC cells number, 5-HT levels and release were found to be increased whereas SERT mRNA expression was shown to be reduced (Bertrand et al., 2010; Mawe & Hoffman, 2013; Chen et al., 2016). Unexpectedly, a reduced level of mucosal 5-HT was revealed in human biopsies of UC patients (Coates et al., 2004). Moreover, it has been shown that changes in 5-HT levels in the human gut is

implicated is several GI disorders, such as IBS celiac disease but also diverticular disease, IBD and colorectal cancer (Manocha & Khan, 2012), demonstrating its role in the homeostasis of immune system.

Tryptophan (TRP) is metabolized to 5-hydroxytryptophan (5-HTP) which is further metabolized to 5-HT (Agus et al., 2018). The amino acid TRP and its metabolite 5-HT are essential not only for gut homeostasis but also for ensuring health, and any alterations on these factors are linked to a variety of disease (O'Mahony et al., 2015). Gut microbiota has a pivotal role in 5-HT production; indeed, GF mice have impaired 5-HT production in the large intestine and low 5-HT levels in the blood (Agus et al., 2018).

In TLR4$^{-/-}$ ileum we found higher TRP tissue levels together with a reduction of both plasma and tissue 5-HTP concentration compared to WT mice, to indicate a faster metabolism to 5-HT, possibly triggered by an impaired luminal microbiota activity. 5-HT ileal tissue levels resulted to be higher compared to those found in WT mice, suggesting the presence of a low-grade gut inflammation (Ghia et al., 2009; Kwon et al., 2019).

However, the dominant physiological pathway for TRP is along the KYN pathway (Kennedy et al., 2017). IDO is the key enzyme that metabolizes TRP into KYN, and its activity can be modulated by several inflammatory cytokines, expressed in various immune cells (Ciorba, 2013). TRP together with KYN metabolites play an important role in intestinal immunity and inflammation (Kennedy et al., 2017). In TLR4$^{-/-}$ mice a slight but not significant increase of KYN levels was found in ileal tissues, to underline an involvement of TLR4 signaling in TRP metabolism.

In WT ileum, HFD determined a significantly increase of TRP, 5-HTP and 5-HT tissue levels compared to sham mice whereas DSS-induced colitis caused a marked reduction of TRP metabolites. A reduction of KYN plasma levels was detected in both WT HFD and WT DSS mice compared to sham mice. In TLR4$^{-/-}$ mice, HFD determined only a significant reduction of 5-HTP plasma levels and an increase of KYN tissue level compared to TLR4$^{-/-}$ SD mice. Whereas, DSS-induced colitis caused a reduction of 5-HT ileal tissue levels with an increase of 5-HT plasma levels in a similar manner as revealed in WT DSS specimens to suggest a possible increase of plasma platelet content in 5-HT (Terry & Margolis, 2017).

Overall these data highlight the importance of a correct interaction between TLR4 and microbiota-derived ligands that is required for preserving the integrity of ENS in terms of structure, function, and TRP pathways.

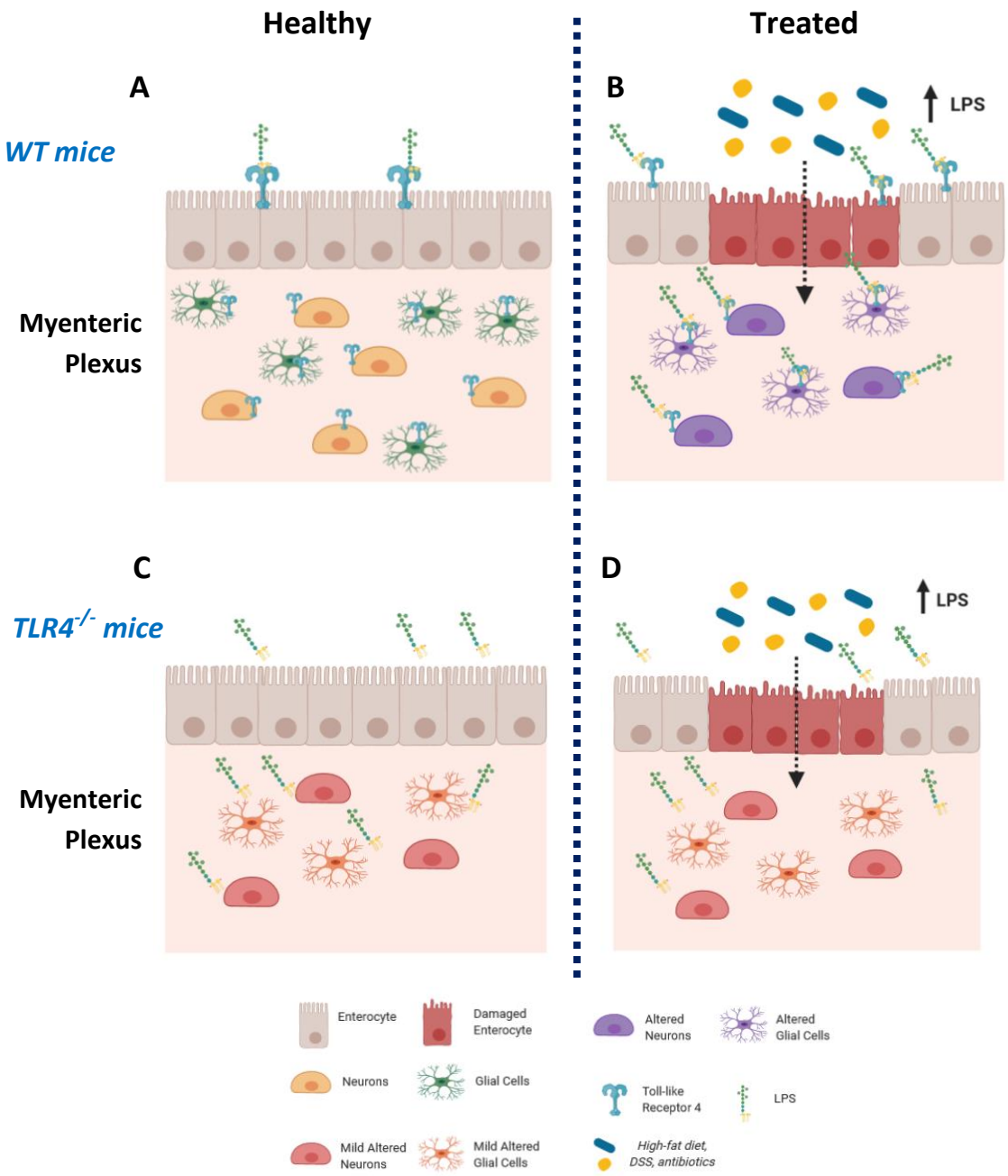


Figure 5.1. Schematic representation depicting the similar effects determined by different in vivo treatments (i.e. antibiotics-induced microbiota depletion, HFD-induced obesity and DSS-induced colitis) on small intestine myenteric plexus of WT mice (**A, B**) and TLR4$^{-/-}$ mice (**C, D**).

6. CONCLUSIONS

Gut microbiota and ENS are able to communicate with each other to maintain a homeostatic relationship. Intestinal microbiota controls host development and function, however we are still far from understanding how gut bacteria influence these systems throughout life. Indeed, 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 but also obesity. Increasing evidence points to a crucial role for the gut microbiota in modulating signaling pathways and neurotransmitters content in ENS.

TLR4 signaling appears to be highly involved in the detrimental effects induced by obesity and colitis underlining its pivotal role in ENS homeostasis. Of note, among all alterations determined in the structural and functional integrity of ENS, antibiotic-induced enteric dysbiosis determines anomalies of glial network, GI transit time and iNOS-produced NO neuromuscular relaxation, similar to those found in TLR4$^{-/-}$ mice (**Table 6.1**). Intriguingly, our findings clearly point out the ability of TLR4 to finely tune some important functions of the GI tract, such as glial plasticity (evidenced by changes in S100β^{+} glial cells density), gut motility (e.g. GI transit time) and inhibitory response mediated by iNOS-produced NO, as summarized on **Table 6.1** where it is manifest that the absence of TLR4 signaling prevents these inflammatory adaptive response to noxious conditions. Therefore, TLR4 can be considered an interesting target for exploitation in immunotherapy and it may be capable of decreasing the severity of disease pathophysiology.

GENOTYPE	TREATMENT	S100β^{+} GLIAL CELLS	GI TRANSIT TIME	iNOS-PRODUCED NO INHIBITORY RESPONSE
WT	ABX	↑	↑	↑
TLR4$^{-/-}$	SHAM	↑	↑	↑
WT	HFD	↑	↑	↑
TLR4$^{-/-}$	HFD	↓	=	↓
WT	DSS	↑	↑	↑
TLR4$^{-/-}$	DSS	=	=	=

Table 6.1. Biomarkers of murine ENS structural and functional integrity that appear to be finely modulated by TLR4 signaling during different conditions, such as antibiotic treatment (ABX), deficiency of TLR4 signaling (SHAM), high-fat diet-induced obesity (HFD), experimental colitis (DSS).

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