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1. INTRODUCTION

1.1 The enteric nervous system

The enteric nervous system (ENS) is a complex and neuronal network, that extends from the esophagus to the anal sphincter and is composed of ganglia, interconnecting fibers and neuronal fibers impinging on effector tissue. (Furness et al., 2014). Motility patterns, gastric secretion, transport of fluid across the epithelium, blood flow, nutrient handling, interaction with the immune and endocrine systems of the gut are function under the control of the ENS (Furness, 2012; Wood, 2012). Large number of neurons, 200-600 millions in human, the same number of neurons that is found in the human spinal cord, give rise to three major components: the subserous, the myenteric (Auerbach's located between the two smooth muscle layers) and the submucosal (Meissner's located in the submucosal layer) plexuses (Fig. 1.1).

According to their morphology, neurochemical coding, cell physiology, projections to targets and functional roles, approximately 20 distinct types of neurons have been described. Intrinsic primary afferents are sensory neurons which detect mechanical distortion of the mucosa, mechanical forces in the external musculature (tension of the gut wall) or the presence of chemical luminal stimuli and initiate appropriate reflex control of functions including motility, secretion and blood flow (Clerc et al., 2002).

Along the whole gastrointestinal tract, the longitudinal and circular smooth muscle layers and the muscolaris mucosae are innervated by uni-axonal excitatory and inhibitory motor neurons (type I morphology), which receive prominent fast excitatory synaptic potentials (Wood, 2012). The primary neurotransmitters for excitatory motor neurons are acetylcholine (ACh) and tachykinins. Several neurotransmitters have been identified in inhibitory motor neurons, including nitric oxide (NO), vasoactive intestinal peptide (VIP) and ATP-like transmitters, although NO is considered the primary transmitter (Furness et al., 2014). Another important class of enteric neurons is represented by secretomotor and secretomotor/vasodilator neurons regulating the electrolyte and water transport across the intestinal mucosa (Vanner and Macnaughton, 2004).

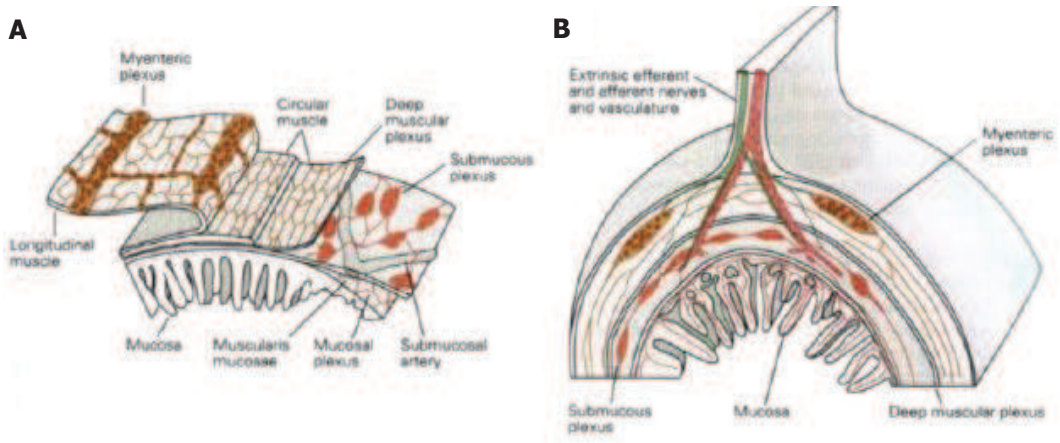

Fig. 1.1: Schematic representation of the different layers of the small bowel (A) and trasversal section of the same (B) (Hansen MB, 2003).

1.2 Plasticity in the enteric nervous system: the enteric micronenvironment

Neuronal plasticity comprises a great variety of changes in neuronal structure and function in response to alterations of input. For the study of neuronal plasticity, enteric nervous system (ENS) show several features that make it a suitable and unique model. Enteric ganglia share morphological (e.g., presence of enteric glial cells, resembling CNS astrocytes (Jessen et al., 1983), and absence of collagen fibers) and functional (e.g., growth factors, neurotransmitters) similarities with the central nervous system (CNS), and their complex organization is unmatched in any other section of the peripheral nervous system (Gershon et al., 1994). Second, enteric ganglia maintain integrated functions in the absence of input from the CNS, which has a modulatory role. The bowel can propel intraluminal contents (peristaltic reflex) or generate the migrating myoelectric complex (MMC, whose progression along the small intestine depends on intrinsic neural activity) independent of extrinsic innervation (Furness and Costa, 1987). Clinical (e.g., recovery of motor function after intestinal transplantation) and experimental (e.g., denervation and pharmacological manipulations) observations suggest that homeostatic control of gut function in a changing environment is achieved through adaptive changes involving enteric ganglia. Enteric neuronal plasticity may also lead to regeneration of neuronal circuitries and allow the recovery of normal intestinal functions after surgical procedures.

Several "players" are involved when considering adaptive changes in the enteric microenvironment: neurons, enteric glial cells, smooth muscle cells, and the interstitial cells of Cajal (which are viewed as the intestinal pacemaker cells) (Sanders KM, 1996). Mast cells may also play an important role in mediation of neuroimmune interactions (Bueno et al, 1997, De Winter, 2012). A possible cross-talk between smooth

muscle cells and dorsal root ganglion cells has also been demonstrated (Ennes et al, 1997), and this finding may affect the understanding of altered visceral sensitivity and reinforce the concept of a brain–gut axis interaction. In the following paragraphs, three of the main non-neuronal cellular components of the enteric microenvironment: glial cells, myocites and the local microbiota will be described.

1.2.1 Enteric Glia

Enteric glial cells represent an important non-neuronal component of the ENS, and it resembles CNS astrocyte. Several studies demonstrated that enteric glial cell do not only contribute to create a protective local microenvirnment, in analogy with the function of astrocytes, but may also have a functional role in enteric information transfer by responding to a variety of neuroligands (Sarosi et al., 1998; Gulbransen and Sharkey, 2012). Moreover , ablation of enteric glial cells in the jejunum and ileum of adult transgenic mice has been found to rapidly lead to a fulminating enteritis with severe inflammation and hemorrhagic necrosis of these organs, associated with degenerative changes in the ENS and this phenomenon is in support with a role in creation of a protective local microenvironment by enteric glia (Bush et al., 1998).

1.2.2 Enteric Smooth Muscle Cells

The enteric myocyte can also display phenotype plasticity similar to that of vascular and bronchial smooth muscle cells, whose plasticity has important pathophysiological implications for atherogenesis and airway remodeling, respectively (Jhonson et al., 1997). Production of cytokines and/or growth factors by myocytes (Van Assche et al., 1999) may alter neuromuscular function and be a basis for neuromotor dysfunction in patients with inflammatory bowel disease (IBD). A study on relaxatory responses mediated by protease-activated receptors in gastrointestinal smooth muscle in an animal model, demonstrated that smooth muscle cells are provided with sensors for inflammatory signals in the gut (Cocks et al., 1999, Giaroni et al., 1999).

1.2.3 The intestinal microbiota

Through the course of evolution, a complex microbial ecosystem has colonized the skin, oral cavity, respiratory, urogenital and GI tract of all mammals, including humans. The community of commensal microorganisms residing in or passing through the GI tract is referred to as the intestinal microbiota (Dethlefsen et al., 2006). The human intestinal microbiota is composed of an astounding number and

species of microorganisms that symbiotically colonize the human gut, comprising approximately 300 to 500 bacterial species, containing nearly 2 million genes (the so-called microbiome). The human intestinal tract is colonized by about ten times more microbial cells than human body cells and thus it contain about 150 times more microbial genes than the human genome (Qin et al., 2010; Wopereis et al., 2014).

Of the 10 bacterial phyla detected in the gut the *Firmicutes*, *Bacteroidetes* and *Actinobacteria* predominate, the *Firmicutes* being the dominant and more assorted phylum (Simren et al., 2013).

The gut microbiota is differently distributed along the GI tract. The microbial density, increases from 101–108 microbial cells in the stomach, and small intestine, to 1010–1012 cells in the colon and faeces (Booijink et al., 2007; Dethlefsen et al., 2006; Gerritsen et al., 2011), indicating that the greater microbial amount of the human microbiota is located in the large intestine. The composition also differs, with predominance of Gram-positive bacteria in the upper GI tract and mainly Gram-negative microorganisms and anaerobes in the colon, where the microbiota composition almost entirely consists of three phyla, *Firmicutes, Bacteroidetes* and *Actinobacteria* (Jones et al., 2009; Martinez et al., 2010). The composition of luminal intestinal microorganisms that populate the colon is quite uniform and faecal material seems to better represent the colonic microbiota composition (Eckburg et al., 2005). The intestinal microbiota coexists in a homoeostatic relationship with the host, playing a role in metabolic, nutritional, physiological and immunological processes in the human body. It exerts important metabolic activities by extracting energy from otherwise indigestible dietary polysaccharides such as resistant starch and dietary fibers (Wopereis et al., 2014). These metabolic activities also lead to the production of important nutrients, such as short-chain fatty acids (SCFA), vitamins (e.g. vitamin K, vitamin B12 and folic acid) and amino acids, all essential nutrients for human beings (Albert et al., 1980; Conly et al., 1994). The gut microbiota has not only a primary role in the development and functionality of the innate and adaptive immune responses, but also in regulating GI sensory and motor functions and intestinal barrier homeostasis (Parkes et al., 2008; Gerritsen et al., 2011).

1.3 Adaptation to a changing environment

Several physiological (development and ageing), clinical (e.g., recovery of motor function after intestinal transplantation) and experimental (e.g., denervation and pharmacological manipulations) observations suggest that homeostatic control of gut function in a changing environment is achieved through adaptive

changes involving enteric ganglia. A survey of enteric ganglia adaptation during development is depicted in the following paragraphs.

1.3.1 Development and ageing

The necessity of adaptation before and after birth suggests that development of new neuronal connections prenatally and neuronal plasticity in adults involve the same cellular mechanisms. The identification of different lineages among enteric neuronal precursors on the basis of their common dependence on specific growth/differentiation factors is a key step in identification of molecules and mechanisms subserving plasticity. Neuronal development is not complete at birth, and the total number of myenteric neurons may increase significantly after birth (Gabella, 1971). During the first month of postnatal life, in rats, neurochemical differentiation is accomplished (Matini et al, 1997). A study carried out in infants and children indicates that the density of ganglion cells in the myenteric plexus decreases significantly with age during the first 3–4 years of life (Wester et al, 1999). This supports the notion that development is an ongoing process in the first years of life, but also need to take patient age into account when interpreting pathological findings (Koletzko et al., 1999).

Several studies exist on the morphological, histochemical, electrophysiological, and functional classification of enteric neurons (Wood, 1994) which are the progeny of neural crest immigrants, together with enteric glial cells. Among the several cell lineages described in the prenatal period, the development of the largest one depends on the glial cell line–derived neurotrophic factor (GDNF) and its receptor tyrosine kinase Ret (Jing et al., 1996). In GDNF or c-ret (the proto-oncogene encoding for Ret) knock out mice, neurons and glia are absent from the bowel caudal to the esophagus and the cardiac stomach. Ret tyrosine kinase can also be activated by neurturin, a neuronal survival factor, which play a crucial role in the maintenance of postmitotic myenteric neurons (Heuckeroth et al., 1999). In neurturin-deficient mice a reduction in the density of nerve fibers, smaller enteric neuronal cell bodies, and abnormal gastrointestinal motility, but not a drastic reduction of enteric neurons, as observed in the absence of GDNF, were observed (Heuckeroth et al., 1999). GDNF may also have a role in the adult gut (Peters et al., 1998). A second lineage depends on the expression of Mash-1 (a transcription factor) and resembles sympathetic neurons because these cells are catecholaminergic. This is a transient feature and may represent an early expression of plasticity in the ENS (Baetge et al., 1989) because these neural precursors develop into neurons that are no longer catecholaminergic (Gershon et al., 1993). No intrinsic adrenergic neurons are detectable in the adult ENS

(Furness and Costa, 1987). Type A neuronal intestinal dysplasia in humans notably includes the absence of sympathetic nerves in the myenteric plexus and mucosa among its features (Ryan et al., 1995).

The study of age-related changes may also be important for the study of neuronal plasticity because one of the underlying mechanisms may be a variation in the trophic support furnished to neurons by the target tissue (Cowen, 1993). Several reports show morphological and possibly functional changes during aging as well as the different roles of neurotrophic factors and extracellular matrix molecules in neuronal plasticity at different stages of life (Cowen, 1998). In a study it was observed that the spatial density of myenteric neurons per serosal surface unit as well as the total number of myenteric neurons was decreased in the small intestine of senescent guinea pigs (Gabella, 1989). The decrease in neuron number might be an aspect of a process of reshaping, not necessarily associated with malfunction, and triggered simply to maintain homeostasis in a different microenvironment. This interpretation may also be taken into account for the differential changes in the distribution of enteric nerves containing different neuroactive substances in three sphincteric regions of aged rats (Belai et al., 1995); an increased density of nerve fibers containing markers for excitatory neurotransmitters (noradrenaline and substance P) was paralleled by a decreased density of nerve fibers containing inhibitory neurotransmitters (calcitonin gene-related peptide (CGRP) or vasoactive intestinal polypeptide (VIP).

1.3.1.1 Influence of gut microbiota on neurodevelopment

Commensal microbes widely contribute to host phenotype and for this reason, mammals have been described as 'superorganisms', and terms such as 'ecological development' have been coined to indicate that development is a process merging both host genetics and microbiota-derived signals (Willing et al., 2011). The influence of the microbiota extends beyond the GI tract, playing a major role in the bidirectional communication between the gut and the CNS (Cryan and Dinan, 2012). Given the importance of gut microbiota in modulating health and neurodevelopment, the brain–gut axis has been extended to the microbiota–gut–brain axis (Rhee et al., 2009; Collins et al., 2012), which represents a complex network of communication between the gut, the intestinal microbiota, and the brain modulating immune, ENS and CNS functions (Mayer, 2011; Collins et al., 2012; Borre et al., 2014).

Prenatal period

Molecular investigation have only recently revealed that microbial exposure start before birth and the fetus appears to receive microorganisms from the mother during gestation (Jimenez et al., 2008; Satokari et al., 2009). The presence of bacterial species in the fetus (such as *Escherichia coli*, *Enterococcus faecium*, and *Staphylococcus epidermidis*) can result from the translocation of the mother's gut bacteria via the bloodstream and placenta (Jimenez et al., 2008). The ENS development take place during pregnancy, when neural-crest derived cells migrate to the developing GI tract, as described in the previous section. In parallel, the brain formation in humans starts at 3–4 weeks of gestation with neurulation. Cortical neurogenesis occurs predominantly during in utero gestation, but can continue up to 2-5 years of age (Herschkowitz et al., 1997; Workman et al., 2013).

Birth and weaning

Postnatally, the microbial gut colonization is dependent on the birth delivery mode. Whereas vaginally born infants are colonized by fecal and vaginal bacteria from the mother, infants born by cesarean delivery are exposed to a different bacterial milieu closely related to that of the human skin and hospital environment (Biasucci et al., 2010; Dominguez-bello et al., 2010). During the first days of life, the infant gut microbiota shows a low diversity and is unstable, then the precise composition of the developing microbiota population is dependent on whether the infant is breast- or formula-fed (Thum et al., 2012). Infants delivered by cesarean delivery are more likely to suffer from allergies, asthma, GI dysfunction, obesity, and diabetes later in life (Jakobsson et al., 2014). Moreover, breastfed infants demonstrate better neurodevelopmental outcomes and higher scores on intelligence tests (Kramer et al., 2008), but it is unclear whether these neurodevelopmental outcomes are a reflection of the microbiota composition. Thus, establishment of pioneer gut microbiota is a crucial stage in neonatal development and seems to represent a critical period not only for ENS, but also for CNS development (Dominguez-Bello et al., 2010).

Childhood and adolescence

The microbiota continue to evolve until adulthood with a gradual increase in *Bacteroides* s pp., a decline in *Lactobacillus* spp. after the age of five, and a decline in *Bifidobacterium* spp. in late teenage (Hopkins et al., 2002; Balamurugan et al., 2008). Significant changes in the composition of the intestinal microbiota come with the introduction of solid food and weaning, since diet plays a crucial role in modulating microbiota composition (Borre et al., 2014; Wopereis et al., 2014). Recent studies demonstrate that

microbiota colonizing the adult human gut is more abundance and displays a higher diversity in bacterial strains to the child gut (Agans et al., 2011; Ringel-Kulka et al., 2013). Several factors, such as the use of antibiotics, stress, harmful environment, diet, and infections, may contribute to instability and immaturity of gut microbiota during childhood and adolescence. Such alterations may result in dysbiosis and potentially have a negative impact on intestinal and mental health, leading to development of gut and brain disorders later in life (Hviid et al., 2011; Kronman et al., 2012; Borre et al., 2014; Desbonnet et al., 2015). The effect of microbiota on the formation of neural circuits in the mammalian gut is highlighted in *germ-free* mice, showing altered spontaneous circular muscle contractions and decreased nerve density in the jejunum and ileum (Collins et al., 2014). In agreement with these observations, diet modifications leading to changes of microbiota composition resulted in significant alterations in gastrointestinal transit time (Kashyap et al., 2013).

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

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

Adulthood

The gut microbiome evolves throughout the lifespan, but microbiota diversity and stability decline with aging (Claesson et al., 2011). Changes also occur in extreme old age when *Bacteroides* spp. decrease while *Enterococcus* spp. and *Escherichia coli* increase (Heuckeroth et al., 1998; Mulligan, 2014). It has recently been shown that the microbial composition of aged individuals is influenced by their residential community, dietary regimen, and their health status (Claesson et al., 2012).

In addition to a range of medications used by the elderly, impaired digestive and motility functions, leading to nutrient malabsorption and a weakened immune system contribute to compromised diversity and

stability of the gut microbiota composition (Biagi et al., 2013). The effects of the microbiota and the complex interactions between enteric ganglion cells, mucosal immune system and intestinal epithelium indicate that other factors may well influence aging of enteric neurons (Saffrey, 2013). Although adulthood does not appear to be a critical or vulnerable phase, it remains a period during which alterations in the microbiota can influence brain and behavior.

1.4 Molecular mechanisms of plasticity in the ENS

Neuroplastic mechanisms involved in physiological, adaptive, or pathological events in the CNS are associated with well-known phenomena such as sensitization/desensitization, short- and long-term potentiation, kindling, long-term depression, and habituation. There is no general consensus on their classification, partly because of the possible overlap in the underlying molecular mechanisms, together with some molecules/mechanisms possibly involved in neuronal plasticity in the ENS. The hypothesis of a dynamic cascade of interacting molecules, which may differ among systems but with similar underlying neuronal modifications, has been proposed (Giaroni et al., 1999). Several pieces of information are now available to document that some molecular mechanisms possibly subserving activity-dependent plasticity in the CNS may also operate in the ENS. Growth-associated protein 43 is expressed in the adult mammalian ENS. Activity-dependent Fos immunoreactivity has been documented in enteric neurons, namely, in intrinsic primary afferent neurons of the submucosal plexus. Behavioral plasticity (ranging from inexcitability to typical electrophysiological behavior) is reported in AH/type 2 neurons (which are considered sensory neurons or, according to recent nomenclature, intrinsic primary afferent neurons). Intrinsic primary afferent neurons are multipolar, may communicate with each other via slow excitatory synaptic potentials in self–reinforcing networks, can develop long-term increases in excitability, and thus are likely candidates for induction of activity-dependent changes. At a neuronal level, neurotransmitter release undoubtedly represents a critical process underlying development plasticity. The release of a given transmitter and its cotransmitters, is strictly dependent on the degree of stimulus, underpinning the so called "Neurotrasmitter plasticity". For instance acetylcholine and tachykinins are located together in excitatory motor neurons and it has been suggested that the release of one or both transmitters may depend on the degree of stimulation (Grider et al., 1989). It was also demonstrated that ATP is co-present and co-transmitted with classical transmitters, such as norepinephrine and acetylcholine (Burnostock, 1990). Different neurotransmitter

pathways in the ENS may undergo adaptive changes in both physiogical and pathological conditions. The possible involvement of enteric glutamatergic, nitrergic and adrenergic pathways in neuroadaptation in the gut is considered in the following paragraphs.

1.4.1 Glutamatergic neurotransmission

As a major excitatory neurotransmitter in the central nervous system (CNS), glutamate plays a fundamental role in the modulation of both physiological (e.g. memory, learning) and pathophysiological (e.g. stroke, epilepsy, neurodegenerative diseases such as Alzheimer's and Parkinson's disease, etc.) conditions (Meldrum, 2000; Genoux and Montgomery, 2007). Increasing evidence suggests that glutamate may also have a role in the regulation of a number of functions in the peripheral nervous system, including the gastrointestinal function (Gill and Pulido, 2001). Immunohistochemical, biomolecular and functional data suggest that the entire glutamatergic neurotransmitter machinery is present in the complex circuitries of the enteric nervous system (ENS) and participates to the local coordination of gut functions, as well as in the brain-gut axis.

The actions of glutamate are mediated by two types of receptors: ionotropic (iGlu) and metabotropic (mGlu) receptors. iGlu receptors are ion channels that flux cations (Na^+ and Ca^{++}) and are classified into three major subtypes according to their sequence homologies, electrophysiological properties and affinity to selective agonists: N-methyl-D-aspartate (NMDA), a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate receptors. iGlu receptors may differently contribute to excitatory post synaptic potentials elicited by glutamate: AMPA and kainate receptors induce a fast depolarizing response followed by a rapid decay, while NMDA receptors induce a more prolonged depolarization phase (Traynelis et al., 2010). Functional NMDA receptors are heterotetrameric proteins usually composed of two obligatory GluN1 subunits and two modulatory GluN2 (denoted A-D) and GluN3 (A-B) subunits, which confer functional variability to the receptor (Traynelis et al., 2010). NMDA receptors are unique among the glutamate receptor family in that the simultaneous binding of glycine to GluN1 and glutamate to GluN2 is required for activation. Another distinctive feature of NMDA receptors is the voltage-dependent block by Mg^{++} that may be overcome by partial depolarization of the resting membrane potential, which may be induced by AMPA or kainate receptor activation (Meldrum, 2000). A further specific feature is the need for glycine as a co-agonist: each receptor unit has two glycine binding sites located on GluN1 subunits and two glutamate

binding sites on GluN2 subunits. AMPA and kainate receptors assemble as homo- or heteromers from four and five subunits, GluA1-4 and GluK1-5, respectively (Traynelis et al., 2010). mGlu receptors exert their effect on intracellular signal transduction cascades by coupling to GTP-binding proteins and are classified into three major groups, Group I, Group II and Group III according to their homology sequence, pharmacological properties and to the related signal transduction pathways (Niswender and Conn, 2010). Group I receptors activate phospholipase C to produce IP_3, leading to Ca^{++} release from intracellular stores, and diacylglycerol to stimulate protein kinase C. Group II and III receptors reduce intracellular cAMP levels, by negatively coupling to adenylate cyclase. mGlu receptors prevalently encompass a regulatory role acting either presynaptically, as auto- or heteroreceptors to modulate glutamate and other neurotransmitter release, or postsynaptically to modulate the effects of glutamate on neurons and glial cells (Niswender and Conn, 2010).

Several studies provided evidence that glutamate does not only play an important modulatory role on the gastrointestinal epithelial function, but may also represent an enteric neurotransmitter (Filpa et al., 2015). This hypothesis has initially been put forward on the basis of functional/pharmacological evidences suggesting that glutamate and different selective agonists and antagonists to glutamate receptors were able to influence gastrointestinal motor and secretory functions (Kirchgessner, 2001). To reinforce this concept, histological and biomolecular data have been provided to demonstrate the presence of the entire glutamatergic neurotransmitter machinery, including vesicular and neuronal transporters, iGlu and mGlu receptors, in the ENS (Kirchgessner, 2001, Carpanese et al., 2014). To further support the hypothesis that glutamate may behave as an enteric neurotransmitter, studies carried out in guinea pig ileum longitunal muscle myenteric plexus (LMMP) preparations, showed that, in analogy with the CNS, glutamate is synthesized from glutamine in myenteric neurons. (Wiley et al, 1991; Kvamme, 1998). Glutamate (which plays a role in long-term potentiation and excitotoxicity in the CNS) has been proposed as an enteric neurotransmitter, and induces depolarizing responses in AH/type 2 neurons as well as in S/type1 neurons, and can cause excitotoxicity in the ENS. Other reports point to a role of metabotropic glutamate receptors for the induction of long-term potentiation in the ENS. A possible existence of a glutamatergic cross-talk between neurons and enteric glial cells, similar to that hypothesized to have a role in synaptic plasticity and neuronal toxicity in the CA1 hippocampal region was demonstrated (Bezzi et al., 1998). Emerging evidence also suggest that glutamate, mainly via NMDA receptor activation, may have an outstanding role

in the modulation of visceral sensitivity, participating to development of visceral pain associated to pathological conditions such as in irritable bowel syndrome (IBS), as described in paragraph 1.5.2 (Filpa et al., 2016). The ability of NMDA receptor antagonists to reduce pelvic and splanchnic afferent stimulation from the colon to mechanical stimuli is highly indicative of an endogenous glutamatergic modulation of mechanosensitive pathways and visceromotor responses to colorectal distension (Kolhekar and Gebhart, 1996; Blackshaw and Gebhart, 2002). In particular, GluN1 NMDA glutamatergic receptor subunit, which is localized to cell bodies of dorsal root ganglion (DRGs) and in peripheral terminals of primary afferent innervating the rat colon, mediates the local release of neuropeptides which play an important role in neurogenic inflammation and hyperalgesia (MacRoberts et al., 2001) . In addition, NMDA receptors in the spinal cord play an important role in the development and maintenance of allodynia and hyperalgesia, by integrating the activity of groups of neurons and amplifying nociceptive signals, thus leading to "wind-up" of central responses to nociceptive stimuli. (Davide and Lodge, 1987). In addition, intoxication with food containing glutamate or glutamate receptor agonists, such as domoic acid (a kainate receptor agonist) (Teitelbaum et al., 1990), suggests a possible role of glutamate excitotoxicity in the ENS.

1.4.2 Nitrergic neurotransmission

Nitric oxide (NO) is a messenger molecule, which has numerous molecular targets. NO controls regulatory functions such as neurotransmission (Schuman et al., 1991) or vascular tone (Rapoport et al,., 1983), regulates gene transcription (Gudi et al., 1999) and mRNA translation (e.g. by binding iron-responsive elements) (Liu et al., 2002) and produces post-translational modifications of proteins (e.g. by ADP-ribosylation) (Brune et al., 1994). An important mode of NO inactivation is represented by its reaction with superoxide anion (O_2^-) to form the potent oxidant peroxynitrite ($ONOO^-$). This compound can cause oxidative damage, nitration and S-nitrosylation of biomolecules including proteins, lipids and DNA (Lee et al., 2003). Nitrosative stress by $ONOO^-$ has been implicated in DNA single strand breakage followed by poly-ADP-ribose polymerase (PARP) activation (Ridnoue et al., 2004).

In mammals NO can be produced by three different isoforms of the NO synthase enzyme. This enzymes are the neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). All NOS isoforms utilize L-arginine as substrate and molecular oxygen and reduced nicotinamide-adenine-dinucleotide phosphate (NADPH) as cosubstrates. Flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) amd (6R-)5,6,7,8-tetrahydro-L-biopterin (BH_4) are cofactors af all isoenzymes. All NOS proteins are homodimer. In

order to synthesize NO, the NOS enzyme goes throught two steps. In the first step NOS hydroxylates L-arginine to N^{ω}-hydroxy-L-arginine; in the second step, NOS oxidizes N^{ω}-hydroxy-L-arginine to L-citrulline and NO (Stuehr et al., 2001). All NOS isoforms bind calmodulin. In the nNOS and eNOS, calmodulin binding is brought by and increase in intracellular Ca^{2+}. In the iNOS isoform calmodulin binds at extremely low intracellular Ca^{2+} concentrations (below 40nM). (Forstermann and Sessa, 2012).

In the ENS, NO, in physiologic conditions, plays a pivotal role in the inhibitory regulation of peristalsis (Furness, 2000). All the NOS isoforms have been localized in myenteric neurons of different species (Vannucchi et al., 2002; Talapka et al., 2011) and are the predominant source for generation of NO that regulates non-adrenergic non-cholinergic inhibition of smooth muscle contraction (Toda et al., 2005). In myenteric neurons, nNOS seems to represent the main source of NO involved in the physiological modulation of non-adrenergic non-cholinergic inhibitory motor responses of the gut. However, expression of iNOS, but not of nNOS and eNOS, prevails during diseases states,such as intestinal inflammation (Miampamba et al., 1999) and I/R injury (Giaroni et al., 2013). In these conditions large amounts of NO can cause damages in different cellular populations, such as neurons, by the formation of peroxynitrite and nitrotyrosine (Rivera et al., 2011). This phenomenon reflect a functional plasticity of myenteric neurons which activate different NOS isoforms depending on either physiological or pathological conditions (Robinson et al., 2011, Giaroni et al., 2013).

1.4.3 Adrenergic neurotransmission

The pharmacological classification of adrenoceptors into two major subtypes (α and β) was based on the order of potency of sympathomimetic amines in several test systems, including the small bowel (Ahlquist and Levy, 1959). Adrenergic receptors were divided into two major classes: β_1- and β_2- subtypes (Lands et al., 1976) and α_1-and α_2- subtypes (Langer, 1977). In general, adrenoreceptor are mainly divided into α_1, α_2 and β receptors, each one divided into several subtypes. Adrenoceptors belong to the G-protein-coupled family of receptors, which mediate intracellular effects by means of a guanine nucleotide-binding regulatory protein: α_1-adrenoceptors induce breakdown of phosphatidylinositol 4,5-bisphosphate by coupling with phospholipase C, probably through a G_q protein, and are also coupled directly to calcium influx (Han et al., 1987). α_2-adrenoceptors either inhibit adenylate cyclase activity or directly modify ion channel activity through an inhibitory G-protein, whereas β-adrenoceptors activate adenylate cyclase through a stimulatory G-protein (Watson and Girdlestone, 1995).

Several key functions of the gut (modulation of vascular tone, inhibition of motility and fluid secretion) are under the control of the adrenergic pathways (Furness and Costa, 1974). The sympathetic nervous system is one of the modulators of gastrointestinal motility, since motor events, such as the peristaltic reflex and the migrating motor complex (MMC), do not require the integrity of adrenergic pathways for activity, but can be profoundly affected by the sympathetic innervation (Crema et al., 1970; Bonaz et al., 1991). Adrenergic fibers in the gut are of extrinsic origin, as demonstrated by the disappearance of noradrenergic terminals after extrinsic denervation (Furness and Costa, 1987). The majority of cell bodies of noradrenergic terminals within the gut wall are located in the prevertebral sympathetic ganglia; some adrenergic fibers originate in the cervical ganglia and reach the stomach and upper small bowel through the vagus nerve (Lundberg et al., 1978). Noradrenaline is well established as the transmitter of postganglionic sympathetic neurons supplying the gastrointetsinal tract (Furness and Costa, 1987). Noradrenrgic axons in the gut are are located in the myenteric and submucous ganglia and around arterioles, but there is also a sparse noradrenergic supply to the circular muscle and to the mucosa (Gabella, 1979). Most adrenergic fibers are located around intramural ganglion cells (Jacobowitz, 1965). It was demonstrated by several studies that, in the guinea pig and in human small intestine, most of the adrenergic varicosities are concentrated near the edges of the myenteric ganglia and adrenergic axons form synapses on nerve processes. The adrenergic innervation of the longitudinal muscle is less abundant than that of the circular layer (Gabella, 1979). Stimulation of the sympathetic supply to sphinteric muscel is excitatory (Furness and Costa, 1974) and is thought to be due to a direct effect of noradrenaline on smooth muscle α-adrenoceptors. Stimulation of sympathetic nerves causes motor inhibition both *in vivo* (Andrews and Lawes, 1984) and *in vitro* (Garry and Gillespie, 1955). It has been demonstrated that adrenoceptors of both α- and β- subtype are localized at different levels of the gastrointestinal tract. α_1-adrenoceptors are located postjunctionally on smooth muscle cells and on intrinsic neurons, while α_2-adrenoceptors may be present both pre- and postsinaptically (De Ponti et al., 1996). α_2-adrenoceptors may act as autoreceptors inhibiting norepinephrine release from adrenergic nerves at a presynaptic level, (Starke et al., 1989). An important postjunctional location of α_2-adrenoceptors are found mainly on smooth muscle cells, but they control water and electrolyte absorption. β_1- and β_2-adrenoceptors are found mainly on smooth muscle cells, but the former may be present on enteric neurons (De Ponti et al., 1996).

Neuronal plasticity in intrinsic enteric neurons is also studied resorting to adrenergic pathways and extrinsic denervation models. Although the ENS is capable of controlling all digestive functions after extrinsic denervation, a period of adaptation is usually required, during which plasticity of intrinsic neurons allows restoration of intestinal function. In the guinea pig, ablation of the inferior mesenteric ganglion was followed by development of supersensitivity to both neuronal and muscular effects of adrenoceptor agonists (inhibition of acetylcholine release by presynaptic/prejunctional α2-adrenoceptors and relaxation of circular muscle by postjunctional β-adrenoceptors). This demonstrate that the extrinsic sympathetic input exerts a physiologically relevant tonic modulation on enteric colonic cholinergic neurons and that adrenoceptors on both neural and smooth muscle cells are acted on by the endogenous adrenergic transmitter (Frigo et al., 1984; Marcoli et al., 1985). However, the fact that functional parameters, such as efficiency of peristalsis or acetylcholine release, are unchanged after long-term suppression of the sympathetic input to the colon suggests the existence of adaptive changes involving increased functional relevance of nonadrenergic inhibitory systems (Frigo et al., 1984; Marcoli et al., 1985). Analogous changes have been observed after chronic blockade of cathecolamine uptake system with desipramine (DMI), a tricyclic antidepressant (Giaroni et al., 2008). Interestingly, in guinea-pig isolated colon the efficiency of in vitro-measured intestinal peristalsis was reduced after acute exposure to DMI and remained unchanged after chronic treatment with the tricyclic antidepressant, suggesting the occurrence of adaptive changes involving different populations of adrenoceptors (Marino et al., 1994).

1.5 Irritable bowel syndrome (IBS)

IBS entails a heterogeneous group of functional lower gastrointestinal tract disorders characterized by abdominal pain or discomfort associated with altered bowel habits and disordered defecation that may be exacerbated by emotional stress. Abnormal defecation can be diarrhea (IBS-D) or constipation (IBS-C), and a subgroup of IBS patients may alternate from one to the other over time (mixed IBS; IBS-M). Urgency to stool often accompanies the diarrheal-state, and patients with the constipation-predominant form of IBS report abdominal tension and the feeling of incomplete evacuation (Wood, 2002). (Longstreth et al., 2006). IBS is reported to occur in 10-20% of the general population in developed countries (Simsek, 2011). IBS is the most common disorder seen by gastroenterologists and can be associated with significant emotional distress, impaired health-related quality of life, disability, and high health care costs (Quigley, 2003). IBS

is reported more frequently by women than men in Western countries, female–male odd ratio being 2:1 and seems to be more common in the ages between 20 and 40. The disorder cannot be explained by specific pathophysiologic mechanisms, since it is not associated with any structural finding or biological marker (Mach, 2004). However, the symptoms of IBS are related to combinations of several known physiological determinants such as abnormal motor reactivity, enhanced visceral hypersensitivity, altered mucosal immune and inflammatory functions (which includes changes in bacterial flora), and altered brain-ENS regulation, which is influenced by psychosocial and socio-cultural factors (Drossman, 2006; Ohman and Simren, 2010; Simren et al., 2013).

Different psychological and biological factors have been described to contribute to the pathophysiology of IBS, including psychosocial disturbances, abnormal motility, visceral hyperalgesia, disturbances of brain-gut interaction, changes in CNS processing, autonomic nervous system disfunction, genetic and environmental factors and postinfectious events, food allergy and dysbiotic changes in the gut microbiota (Daulatzai , 2014; Drossman et al., 2002).

1.5.1 Abnormal GI Motility

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

1.5.2 Visceral Hypersensitivity

Visceral hypersensitivity, modulated by several external and internal factors, is considered the most important factor in the pathophysiology of IBS and it helps to explain the association of pain with GI motility

disorders, which leads to alterations in defecation patterns (diarrhea or constipation) (Katsanos et al., 2012). Visceral hypersensitivity may be generally amplified in a subset of patients with IBS compared with healthy volounteers. (Mertz et al., 1995; Lowen et al., 2015; Moloney et al., 2016). In the gut, extrinsic nociceptors can respond to different kind of stimuli, depending on receptor expression, including stretch, pH, bacterial products, substances released from immune cells, and neurotransmitters released from the ENS or enterochromaffin cells (Sengupta, 2009). Hypersensitivity and sensitization may occur through altered receptor sensitivity at the gut mucosa, at the submucous and myenteric plexus, which may be enabled by mucosal inflammation, degranulation of mast cells close to enteric nerves, or increased serotonin activity, possibly enhanced by alteration of the bacterial environment or infection (Spiller, 2003; Barbara et al., 2004; Dunlop et al., 2005).

The nociceptors have nerve endings throughout the layers of the GI tract (mucosal, submucosal, muscular layers), and their cell bodies are located in the dorsal root ganglion (DRG) of spinal cord. Then, the nociceptive signal is transmitted to the brain throughout the controlateral side of the spinal cord, and reaches cortical areas for localization and limbic areas for the emotional component of the pain response. Although there is no direct evidence for the participation of glutamate to visceral pain transmission, the ability of NMDA receptor antagonist to reduce pelvic and splanchnic afferent stimulation from the colon to mechanical stimuli is highly indicative of an endogenous glutamatergic modulation of mechanosensitive pathways. GluN1 subunit of the glutamatergic NMDA receptor is localized to cell bodies in dorsal root ganglion (DRG) and in peripheral terminals of primary afferent innervating the rat colon and mediates the local release of neuropeptides, such as CGRP and Sunstance P, which play an important role in neurogenic inflammation and hyperalgesia (Filpa et al., 2016, MacRoberts et al., 2001).

1.5.3 Gut microbiota and inflammation in IBS

In physiological conditions, the gut microbiota has a balanced composition that ensures the host health, and disruption of this equilibrium (dysbiosis) confers disease susceptibility (Gibson et al., 2014). Several preclinical (Bercík et al., 2004; Akiho et al., 2005; Verdù et al., 2006; Kanazawa et al., 2014) and clinical studies (Rao et al., 1987) have demonstrated that in the gut, the sensory and motor functions are influenced by both changes in the immune function and by an inflammatory challenge, which represent pathophysiological factors in IBS (Akiho et al., 2005; Verma-Ghandu et al., 2007).

Abnormalities in gut microbiota following enteric infections, associated with an increase in the permeability of epithelial gut barrier, might activate intestinal mast cells and monocytes, thus triggering an immune response (Ohman and Simren, 2010). Mast cells are important elements in the pathogenesis of inflammatory and non-inflammatory bowel disorders, as they can cause stimulation of nerve endings, modulate inflammation and affect intestinal motility (Farhadi et al., 2007). Increased numbers of mast cells in close proximity to nerves in the colonic mucosa is one of the most frequently reported features of immune activity in IBS (Ohman et al., 2015). On the other hand, degranulation of mast cells after enteric infection or allergy may be the prime event that leads to the cascade of gut hypersensitivity through various mediators (Gui, 1998). IBS patients show, in addition, a further immune-related feature, represented by increased levels of circulating proinflammatory cytokines such as IL-6, IL-8, TNF and IL-1β (Scully et al., 2010; Chang et al., 2012), which may have an effect on epithelial barrier function. Thus, a small increase of proinflammatory cytokines level, in the epithelial barrier, can lead to increased intestinal permeability, thereby altering mucosal border homeostasis (Ohman et al., 2015).

1.5.4 IBS and gut-brain axis

IBS is most commonly described bio-psychosocial disorder of the gut-brain axis. The gut–brain axis encompasses a number of fundamental elements, including the CNS, the autonomic nervous system (ANS) (sympathetic and parasympathetic), the ENS, the neuroendocrine hypothalamic–pituitary–adrenal (HPA) axis, and neuroimmune systems, and more recently has expanded to include the gut microbiota, which fulfill key roles in bidirectional communication (Bercik et al., 2011; Cryan and Dinan, 2012; Burokas et al., 2015). The microbiota–gut–brain axis is pivotal in maintaining homeostasis and is involved in the control of diverse physiological functions including motor, sensory, autonomic, and secretory functions of the GI tract to regulate an array of processes from energy metabolism to mood regulation (Burokas et al., 2015; Dinan et al., 2015).

Communication between CNS and ENS implies a bidirectional connection system: the brain influences the function of the ENS, and the gut influences the brain via vagal and sympathetic afferents. The ENS independently controls gut function, the migrating motor complex, and peristalsis, but it is constantly monitored and modified by CNS via both vagal and sympathetic extrinsic nerves. Thus, the IBS symptoms

may be caused by dysfunctions either primarily in the CNS, or in the gut, or by a combination of both (Moloney et al., 2016).

1.5.5 Psychosocial Factors

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

1.5.6 Possible therapies for IBS management

Since IBS is a functional GI disorder, the possible therapies are only symptomatic and may exert only a transient effect with limited benefit. In this regard, it is particularly important to unveil the role of different cellular components of the enteric microenviroment which are involved in IBS development, in order to prevent the occurrence of more obvious symptoms. Since the interplay between the microbiota and the host seem to play a fundamental role in the pathogenesis of IBS, the possibility to influence the gut saprofitic bacteria may represent a potentially useful approach for the management of the disease. For instance, mechanisms by which food may act on the gut may cause symptoms of IBS food suggesting that food may represent an important issue in the management of IBS (Gibson et al., 2015). Thus, since diet is a key element determing microbiota composition therapeutic dietary interventions that modify gut microbiota may be effective for alleviating IBS symptoms (Lee et al, 2017). There is emerging evidence supporting the efficacy of diets that are low in fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (FODMAPs) for IBS patients. Normally, FODMAPs exacerbate IBS symptoms by increasing small intestinal water volume through an osmotic effect, colonic gas production by bacterial fermentation and altering intestinal motility (Staudacher et al., 2014). Indeed, clinical trials reported that a diet low in FODMAPs effectively reduced IBS symptoms. Furthermore,

some probiotic strains (*Lactobacillus and Bifidobacterium)* have been proven to be effective, safe and promising for the treatment of IBS patients, especially those with diarrhea. It was also observed that a combination of specific probiotic and prebiotics (i.e. symbiotics) may be an effective treatment option for IBS patients with constipation (DuPont, 2014). There have also been various attempts to treat intestinal IBS symptoms by using nonsystemic antibiotics (Basseri et al., 2011). Recently, rifaximin has become the first Food and Drug Administration approved antibiotic for the treatment of IBS. Rifaximin is a rifamycin based oral non absorbable antibiotic that has strong antimicrobial effect against aerobic and anaerobic gram positive and gram negative bacteria (Marchese et al., 2000). Clinical trial reported that global symptoms of IBS significantly improved with rifaximin vs placebo, including reduction of flatulence, diarrhea and pain (Meyrat et al., 2012; Sharara et al., 2006). Although not a psychiatric disorder per se, IBS is highly affected by stress and is frequently accompanied by comorbid psychopathology. Psychological comorbidities, such as anxiety and depression, are frequently seen in IBS (Moloney et al., 2016) and become more frequent as disease severity increases. In clinical practice the more severe the clinical presentation is the larger is the relative importance of central mechanisms (Dekel et al., 2013). For these reasons the use of psychotropic drugs (ie tryciclic antidepressants, selective serotonic reuptake inhibitors and atypical antipsychotics) should be reserved for patients with a moderate to severe disorder and seem to benefit IBS patients by reducing anxiety, restoring normal sleep patterns, and possibly through a direct analgesic effect (Calandre et al., 2012; Bryson et al., 1996; Max et al., 1992).

1.6 Catechol-O-methyl-transferase (COMT)

COMT protein is widely distributed in mammalian brain (Lundstrom et al., 1995; Mannisto and Kaakkola, 1999) and its enzyme activity regulates cathecolamine levels (Yavich et al., 2007). In the CNS, genetic variations in human COMT have been associated with physiological functions (Egan et al., 2001; Winterer et al., 2006) and behavioral phenotypes related to prefrontal cortex and hippocampal information processing, including cognition (Blasi et al., 2005; Bertolino et al., 2006), anxiety (Drabant et al., 2006), obsessive-compulsive disorder (OCD) (Pooley et al., 2007), and pain sensitivity (Nackley et al., 2006). Thus, functional COMT genetic variations modulate multiple spheres of mammalian behavior, with remarkable analogy between human and mouse (Papaleo et al., 2008; Mier et al., 2010; Scheggia et al., 2012; Papaleo et al., 2012). A common polymorphism of this gene in human populations is Val158Met (rs4680). This polymorphism is a functional genetic variant determined by a valine to methionine substitution at codon

108 (S-COMT)/158 (MB-COMT) of the COMT gene and dramatically influences COMT enzymatic activity (Lotta et al., 1995; Palmatier et al., 1999). At normal body temperature, the COMT-Val form leads to higher COMT protein levels and enzymatic activity compared with COMT-Met (Chen et al., 2004). Due to the potential importance of COMT in prefrontal neurotransmission, this common functional polymorphism has been related to development of neuropsychiatric disorders that may involve altered catecholamines levels (Papolos et al., 1998; Eisenberg et al., 1999; Jones et al., 2001).

1.6.1 Role of COMT in the pathophysiology of IBS

Recently, an association between gut functional disorders such as IBS and COMT Val158Met polymorphism has been postulated (Camilleri and Katzka, 2012; Karling et al., 2011). IBS is multifactorial, its etiology is complex and multiple causes may concur to its appearance. Many patients also experience comorbid behavioral disorders, such as anxiety or depression, thus IBS can be described as a disorder of the gut–brain axis (Moloney et al., 2016). In the brain, COMT-met variant, associated with low COMT activity, determines higher levels of DA and chronic activation of dopaminergic neurons, which results in lower neuronal content of enkephalin and a decreased activity level of the endogenous pain inhibitory system (Zubieta et al., 2003). Experimental studies in humans (hypertonic saline infusion into the masseter muscle) showed that individuals with the met/met genotype exhibited diminished regional μ-opioid receptors activity, higher sensory and affective ratings of pain in response to painful stimuli compared to the val/val individuals (Zubieta et al., 2003; Karling et al., 2011). Low COMT activity has also been associated to chronic pain conditions such as facial pain (Marbach and Levitt, 1976; Diatchenko et al., 2005), fibromyalgia (Gürsoy et al., 2003) and with non-migraine headache (Hagen et al., 2006), whereas the val/val genotype has been associated to anxiety/ depression (Domschke et al., 2007).

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

2. AIM

Enteric neuronal circuitries display a considerable ability to adapt to a changing microenvironment, which comprises several cellular "players", including neurons, enteric glial cells, smooth muscle cells, interstitial cells of Cajal, immune cells and commensal bacteria (Giaroni et al., 1999). In particular, gut microbiota seems to be directly involved in modulating the development and function of enteric nervous system (ENS), supporting the concept that changes in commensal microbiome composition, induced by infections or antibiotics, can perturb ENS integrity and activity. Neuronal circuitries in the ENS are known to communicate with the Central Nervous System (CNS) via vagal and sympathetic extrinsic pathways: the so called brain-gut axis. Current cutting-edge research suggests that the enteric microbiota, by modifying enteric neuronal circuitries, may communicate with the brain, thus influencing cognitive and behavioural functions. However, early life perturbations of gut microbiota can potentially influence neurodevelopment leading to functional bowel disorders later in life (Ianiro et al., 2016). There is increasing evidence showing that an altered microbiota composition may be related to functional or psychiatric disorders such as irritable bowel syndrome (Bonfrate et al., 2013; Kennedy et al., 2014) and autism (Finegold, 2011; Mayer et al., 2014).

Irritable bowel syndrome (IBS) comprises a heterogeneous group of functional lower gastrointestinal tract disorders characterized by abdominal pain or discomfort associated with altered bowel habits and disordered defecation that may be exacerbated by emotional stress.

This gut disorder cannot be explained by specific pathophysiologic mechanisms, since it is not associated with any structural finding or biological marker (Mach, 2004). However, the symptoms of IBS are related to combinations of several known physiological determinants such as abnormal motor reactivity, enhanced visceral hypersensitivity, altered mucosal immune and inflammatory functions (which includes changes in bacterial flora), and altered brain-ENS regulation, which is influenced by psychosocial and socio-cultural factors (Drossman, 2006; Ohman and Simren, 2010; Simren et al., 2013). Several preclinical (Verdù et al., 2006; Kanazawa et al., 2014), and clinical studies (Rao et al., 1987) have demonstrated that altered immune function and inflammation in the GI tract (as well as GI infections and dysbiosis), which represent key pathophysiological factors in IBS, may affect gut motility and sensitivity (Akiho et al., 2005; Verma-Ghandu et al., 2007). Recently, the association between gut functional disorders, such as IBS, and catechol-O-methyl-transferase (COMT), an enzyme protein which regulates catecholamines levels in mammalian brain (Lundstrom et al., 1995; Mannisto and Kaakkola, 1999) has been proposed. A common polymorphism

of this gene in human populations is Val158Met. In the brain, COMT-met variant, associated with low COMT activity, determines higher catechoalamines levels and chronic activation of catecholaminergic neurons, which results in lower neuronal content of enkephalin and a decreased activity level of the endogenous pain inhibitory system (Zubieta et al., 2003). On the contrary, the val/val genotype has been associated to anxiety/ depression (Domschke et al., 2007). Since chronic pain syndromes (Whitehead et al., 2002) and anxiety/depression (Mayer et al., 2001; Garakani et al., 2003) are associated with low and high COMT activity, respectively, and both are related with IBS and IBS-like symptoms, these two genotypes may be considered as risk factors for the onset of IBS. On reverse, the val/met (intermediate COMT activity) genotype can be considered the low risk, protective genotype (Karling et al., 2011). In this perspective, IBS can be described as a disorder of the gut–brain axis (Moloney et al., 2016).

The aim of this study is to determine the consequences of a genetic-driven defective COMT activity, in the structural and functional integrity of murine ENS. Data obtained with the transgenic mouse model have been compared to those obtained in the gastrointestinal tract of antibiotic treated-mice in order to deplete the microbiota. On the whole the observations will allow to clarify whether COMT participates to development of functional bowel disorders, such as IBS.

3. MATERIAL AND METHODS

3.1 Animal models

In our experimental model a first group of animals consisted of female mice heterozygous for COMT gene (C57BL/6J COMT$^{+/-}$, Charles River Laboratories, Italy) and the respective wild-type (COMT$^{+/+}$, WT) (12±2 weeks). These animals were analyzed in order to evaluate the influence of altered catecholaminergic transmission, due to COMT partial genetic reduction, in the modulation of gastrointestinal motility. COMT$^{+/-}$ animals were provided by Dr. Francesco Papaleo of the Italian Institute of Technology, Genova, Italy. Genotype was confirmed by means of PCR analysis of mouse tail DNA. Heterozygous mice carrying mutations for the enzymatic metabolism systems with a reduced, but not totally deleted function, may represent experimental models more relevant to human clinical situations, where the complete loss of biological functions, caused by genetic mutations, may be rarely observed. Indeed, genetic polymorphisms are associated with a reduced enzymatic activity rather than with a total loss of their activity.

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

All groups of animals were housed in the pathogen-free animal facility of the Department of Pharmaceutical and Pharmacological Sciences of the University of Padova under controlled environmental conditions (temperature 22° ± 2°C; relative humidity 60–70%) with free access to a standard diet and water, and maintained at a regular 12/12-h light/dark cycle. To normalize gut microbiota, mice colonies from both groups were housed in the same room and in the same cages and maintained by the same personnel. All experimental protocols were approved by the Animal Care and Use Ethics Committee of the University of

Padova and by the Italian Ministry of Health and were in compliance with national and European guidelines for the handling and use of experimental animals.

The antibiotic treated animal model was provided by Dr. Cecilia Giron of the Department of Drug Science of the University of Padua.

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

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

3.2 In vitro contractility studies

Experimental procedures on isolated organs were conducted on distal ileal segments prelevated from ABX and $COMT^{+/-}$ and respective control mice. Animals were sacrificed by cervical dislocation. Segments of the ileum were rapidly excised and rinsed with an oxygenated physiological Krebs solution conserved at 37°C. After washing with Krebs solution, distal ileum was excised in 1cm segments. Ileal samples were suspended in 10 ml isolated organ baths, which were previously filled with a Krebs' solution (composition:NaCl 118 mM, KCl 4.7 mM, $CaCl_2 \cdot 2H2O$ 2.5 mM, $MgSO_4 \cdot 7H2O$ 1.2 mM, $K2HPO_4$ 1.2 mM, $NaHCO_3$ 25 mM, $C_6H_{12}O_6$ 11 mM, ph 7.2) continuosly gassed with a mixture of 95% O_2 and 5% CO_2 at 37°C. Ileal segments were vertically positioned in organ baths and connected, by means of a silk ligature, to an isometric transducer. Mechanical activity was recorded with a PowerLab acquisition data system (ADInstruments, UK) and elaborated with a LabChart 6.0 program (ADInstruments, UK). An initial load of 1 g was applied to each intestinal specimen. An equilibration period of 45 min was allowed before starting the experiment. Ileum viability was assessed through the exposition to three pharmacological stimuli with carbachol 1 μM (CCh, cholinergic agonist) separated by three 5 min washouts. This concentration of carbachol is sufficient to evoke a semi-maximal contractile response. In the following phase samples were exposed to carbachol

stimulations at the following concentrations (0.01-100 μM) to obtain a concentration-response curve. To evaluate smooth muscle response, segments were subjected to 60 mM KCl. Ileal preparation were then subjected to Electric Field Stimulation (EFS) using platinum bipolar electrodes attached to a GRASS-S88 (Grass Instrument Co. Quincy, MA) force displacement transducer, which were positioned in parallel to ileal samples in the isolated organ baths. A 40V stimulus, composed of 10 seconds trains of rectangular impulses (1 ms duration) was applied to ileal preparations. The contractile effect was induced by frequencies from 2 to 60 Hz. Relaxation responses were measured at the frequency of 10 Hz after an incubation period of 20 min with atropine 1 μM and guanethidine 1 μM, in order to evaluate the presence of a non adrenergic non cholinergic transmission (NANC). NANC responses were recorded in the presence or absence of L-N^{ω}-Nitroarginine methyl ester chloridrate (L-NAME) 100 μM, an inhibitor of nitric oxide synthase (NOS) in order to unravel the possible involvement of NO. In some experiments 1400W, a selective iNOS inhibitor, was also added in order to evaluate the possible involvement of the different NOS isoforms. All the inhibitors were added 20-30 min before EFS. Concentration-response curves were subjected to a nonlinear regression analysis (fitted to a sigmoidal equation) to calculate maximal tension (Emax) values (Brun et al., 2013). The relaxation response was quantified by calculating the area under the curve, defined as the integrated area under waves (AUC). The isometric contractile response was calculated as the difference between the baseline registered before the stimulus and the response after inducing the stimulus. The value in centimeters obtained from the chart was converted into a measure of tension expressed in g, after the initial calibration. The contractile effects were expressed as percentage of the initial response of the samples to carbachol, whereas the relaxing effects were expressed as percentage variation with respect to contraction levels before the stimulus.

3.3 Gastrointestinal Transit Analysis

Gastrointestinal (GI) transit was measured by evaluating the intestinal distribution of orally gavaged fluorescein isothiocyanate (FITC)-labeled dextran (70 kDa) from the stomach to the colon. $COMT^{+/-}$ transgenic animals, antibiotic treated and the respective control mice were administered FITC-dextran dissolved in 0.9% saline (100 μl of 25mg/ml FITC-dextran solution for each mouse). Thirty minutes after intragastric administration of FITC-dextran, mice were sacrificed and the complete GI tract, from stomach to distal colon, was collected. The stomach and caecum were analyzed separately while the small intestine

was divided into 10 segments of equal length and the colon was divided into 3 segments of equal length. The stomach, caecum and colon were examined separately, whereas the small intestine was divided in 8 equal segments. Luminal contents from each part (both tissue and faecal content) were collected and clarified by centrifugation (12,000 rpm for 10 minutes at 4°C). Supernatant from each sample were placed into separate wells of a 96 well plate and assayed in duplicate with a Krebs solution control and a FITC-dextran control (1:10 part dilution of FITC-dextran and Krebs solution respectively). FITC-dextran fluorescence intensity was measured at 492/521 nm using a fluorimeter (Victor, PerkinElmer). Data were expressed as percentage (%) of fluorescence for each segment and GI transit was calculated as the geometric center (GC) of distribution of the fluorescent marker using the following formula (Wehner et al., 2007):

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

3.3.1 Pellet frequency and fecal water content

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

3.4 RNA isolation and quantitative RT-PCR

Total RNA was extracted from longitudinal muscle/myenteric plexus (LMMP) of ileal preparations of mice undergoing antibiotic treatment and of transgenic $COMT^{+/-}$ and the relative control animals. RNA was extracted with TRIzol (Invitrogen) and preparations were treated with DNase I (DNase Free, Ambion) to remove any traces of contaminating DNA. cDNA was obtained retrotranscribing 2ug of total RNA using the High Capacity cDNA synthesis kit (Applied Biosystems, Life Technologies, Grand Island, NY, USA).

Quantitative RT-PCR was performed on the Abi Prism 7000 real-time thermocyclator (Applied Biosystems) with Power Sybr Green Universal PCR Master Mix (Applied Biosystems) following manufacturer's instructions. Primers were designed using Primer Express software (Applied Biosystems) on the basis of available sequences deposited in public database. Primer sequences were: GluN1, 5'-CAGGAGCGGGTAAACAACAGCAAC-3', 5'-GCAGCCCCACCAGCAGCCACAGT-3'; iNOS, 5'-CAGCTGGGCTGTACAAACCTT-3', 5'-CATTGGAAGTGAAGCGTTTCG-3'; nNOS, 5'-GTGGCCATCGTGTCCTACCATAC-3', 5'-GTTTCGAGGCAGGTGGAAGCTA-3'; S100β 5'-GACTCCAGCAGCAAAGGTGA-3', 5'-ATCTTCGTCCAGCGTCTCCA-3'; β-actin 5-ACCAGAGGCATACAGGGACA-3, 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 ΔΔCt method to compare gene expression (Vigetti et al., 2011). β-actin was used as housekeeping gene. Experiments were performed at least four times for each different preparation. mRNA levels of $COMT^{+/-}$ and antibiotic treated preparations were expressed as the percentage variation vs values obtained in control preparations.

3.5 Western blot analysis

Experiments were conducted in ileal LMMP preparations obtained from antibiotic treated and transgenic $COMT^{+/-}$ and the respective control animals.

LMMP segments 1–2 mm long were homogenized in ice cold isolation buffer [10 mM, Tris-acetate 5 mM, EDTA 1 mM, phenylmethylsulfonyl fluoride (PMSF) 10% protease inhibitor cocktail (Sigma Aldrich, Milan Italy) and 1% phosphatase inhibitor cocktail (Sigma Aldrich, Milan Italy), pH = 7.4]. The crude homogenate was centrifuged at 30 000g for 30 min at 4° C. The supernatant was collected, while the resulting pellet was re-suspended and incubated for 15 min at RT in a second buffer (Tris-HCl 20mM, NaCl 140 mM, pH 7.4, 10% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail).

Quantification of protein concentration in the supernatant and in the pellet was performed by means of the micromethod of Bradford (1976). After protein quantification samples were diluted in Laemli sample buffer (Tris-HCl 300 mM, pH 6.8, glycerole 10%, SDS 2%, β-mercaptoethanole 0,04%). Sample aliquots were then boiled for 5 min at 95°C to mediate protein denaturation.

Protein were then separated on 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted to nitrocellulose transfer membranes (GE Healthcare, Milan, Italy). Membranes were then incubated in a blocking solution consisting of 5% fat-free milk dissolved in PBS-Tween, 2 hours at room temperature in order to saturate aspecific sites. Membranes were then incubated overnight at 4 °C with primary antisera (Table 3.1).

Table 3.1 Primary antibodies used in the study.

Antibody	Dilution	Reference	Host
nNOS	1:200	Santa Cruz Biotechnology, Inc.	Rabbit
iNOS	1:200	Santa Cruz Biotechnology, Inc. (California, USA)	Rabbit
S100β	1:200	Dako	Rabbit
GluN1	1:500	Millipore Co.	Rabbit
α-tubulina	1:1000	Santa Cruz Biotechnology, Inc.	Mouse

After secondary antisera incubation, nitrocellulose membrane were washed 5 times for 5 minutes each time with PSS-Tween, in order to remove the excess of primary antibody. Membranes were then incubated with the proper secondary antibody conjugated to horseradish peroxidase (HRP). Dilution and reactivity of secondary antisera are reported in table 3.2.

Table 3.2 Secondary antibodies used in the study.

Antibody	Dilution	Reference	Host
nNOS	1:10000	Amersham (Milano, Italia)	Donkey anti-rabbit
iNOS	1:10000	Amersham (Milano, Italia)	Donkey anti-rabbit
S100β	1:10000	Amersham (Milano, Italia)	Donkey anti-rabbit

GluN1	1:10000	Amersham (Milano, Italia)	Donkey anti-rabbit
α-tubulina	1:10000	Chemicon	Chicken anti-mouse

The specific protein chemiluminescence was developed using an enhanced chemi-luminescence technique (ECL advance Amersham Pharmacia Biotech, Cologno Monzese, Italy). Signal intensity was quantified by densitometric analysis using the NIH image software 1.61 (downloadable at http://rsb.info.nih.gov/nih-image). In each membrane α-tubulin was monitored and used as protein loading control. Experiments were performed at least four times for each different preparation.

Protein levels were expressed as the percentage variation versus data obtained in control preparations.

3.6 DNA extraction and quantitative RT-PCR from fecal samples

After collection of fecal samples from colon, caecum and ileum of transgenic $COMT^{+/-}$ and wild type mice, fecal contents were promptly frozen and stored in a -80°C freezer for two weeks prior to DNA extraction. DNA from faecal samples of transgenic $COMT^{+/-}$ and wild type mice was extracted by means of the QIAamp DNA Stool Mini Kit (QIAGEN), according to manufacturer's instructions. Briefly, 200 mg of feces were placed in a sterile, round-bottom 2 mL tube containing 1.4 mL ASL lysis buffer and the remainder of the protocol was followed as described by the manufacturer. All samples were finally eluted in 200 µL AE buffer. After DNA extraction, quantitative RT-PCR was performed on the Abi Prism 7000 real-time thermocyclator (Applied Biosystems) with Power Sybr Green Universal PCR Master Mix (Applied Biosystems) following manufacturer's instructions. Primers were designed on the basis of available sequences from literature data (Guo et al., 2008; Yang et al., 2015). Primer sequences were the following: *Bact* 5'-AACGCTAGCTACAGGCTT-3', 5'-CCAATGTGGGGGACCTTC3'; *Firm* 5'-GGAGYATGTGGTTTAATTCGAAGCA-3', 5'-AGCTGACGACAACCATGCAC-3'**.** For quantitative RT-PCR 10ng of fecal DNA were loaded in each well and a final concentration of 500 nmol/L for each primer was used. Primers were designed to have a similar amplicon size and similar amplification efficiency as required for the utilization of the ΔΔCt method to compare gene expression (Vigetti et al., 2011). Experiments were performed at least four times for each different preparation. DNA levels of $COMT^{+/-}$ preparations were expressed as the percentage variation vs values obtained in wild type animals.

3.7 Whole mount staining

Preparation of ileal whole mount samples

Animals were killed by cervical dislocation and the ileum was rapidly excised and gently rinsed with a Krebs solution (NaCl ,118 mM; KCl, 4.7 mM; $CaCl_2 \cdot 2H2O$, 2.5 mM; $MgSO_4 \cdot 7H_2O$, 1.2mM; KH_2PO_4, 1.20 mM; $NaHCO_3$, 24.99mM; Glucose, 11mM). 10cm distal ileal segments were filled with fixative Segments of the mice ileum were fixed for 4 h at room temperature (RT) in 0.2 mol L-1 sodium phosphate-buffer (PBS: 0.14 mol L-1 NaCl, 0.003 mol L-1 KCl, 0.015 mol L-1 Na2HPO4, 0.0015 mol L-1 KH2PO4, pH 7.4, 4% formaldehyde and 0.2% picric acid. Ileal segments were then cleared of fixative and stored at 4°C in PBS containing 0.05% sodium merthiolate (Thimerosal, Sigma-Aldrich).

3.7.1 Immunohistochemistry

LMMP whole mount ileal preparations were prepared according to Giaroni et al, 2011. Briefly, after blocking aspecific sites with PBS, 1% Triton X-100 and 10% normal horse serum (NHS) (Euroclone, Celbio, Milan, Italy) for 2 h, preparations were then incubated with optimally diluted primary antibodies (Table 3.3). Double labelling was performed during consecutive incubation times: firstly, the primary antibody raised against either S100β, iNOS or nNOS was added overnight at 4 °C, then incubation with secondary antibodies followed for 2 h at RT. Whole mounts were successively incubated overnight at 4 °C with a biotinylated antibody to HuC/D (neuronal cell marker).

Table 3.3 Primary antibodies used in the study.

Antibody	Dilution	Reference	Host
nNOS	1:50	Santa Cruz Biotechnology, Inc.	Rabbit
iNOS	1:50	Santa Cruz Biotechnology, Inc. (California, USA)	Rabbit
S100β	1:200	Dako	Rabbit

HUC/D	1:100	Life Technologies	Mouse

Incubation for 2 h with an appropriate secondary antibody was then performed at RT (Table 3.4). Preparations were mounted onto glass slides, using a mounting medium with DAPI (Vectashield; Vector Lab, Burlingame, CA, USA). Negative controls and interference control staining was evaluated by omitting one or both of the primary antibodies, or one of the secondary antibodies. Preparations were analyzed by confocal microscopy on a Leica TCS SP5 confocal laser scanning system (Leica Microsystems GmbH, Wetzlar, Germany) and pictures were processed using Adobe-Photoshop CS2.0 software.

Table 3.4 Secondary antibodies used in the study.

Antibody	**Dilution**	**Reference**	**Host**
nNOS	1:300	Molecular Probes	Donkey anti Rabbit Alexa Fluor 488
iNOS	1:300	Molecular Probes	Donkey anti Rabbit Alexa Fluor 488
S100β	1:400	Molecular Probes	Donkey anti Rabbit Alexa Fluor 488
HUC/D	1:500	Caltag Lab.	Cy3 streptavidin

3.8 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), of at least 4 experiments. Statistical significance was calculated with the unpaired Student's *t* test (when comparing two groups of data) or analysis of variance (ANOVA), when comparing three or more groups of data, followed by Dunnet's or Tukey post-hoc test for multiple variables. Differences were considered statistically significant when *P*

values were <0.05. For statistical analysis the GraphPad Prism software (GraphPad 5.03 Software Inc, La Jolla, USA).

4. RESULTS

4.1 Catechol-O-methyltransferase genetic reduction influences gastrointestinal transit

COMT genetic reduction determined an altered gut distribution of the fluorescent probe, associated with a significant increase of gastrointestinal transit in $COMT^{+/-}$ mice compared to WT, as shown also by the increased value of the geometric centre (Figs. 1 A and B; $GC_{COMT+/-=}$6.5, range: 5.7-7.5 vs GC_{WT} 5.8, range: 4.9-6.4; $p<0.05$). The increase of GI transit in $COMT^{+/-}$ mice was paralleled by a significant increase in stool frequency (+90%±0.5%, n=10; $p<0.05$; Fig. 2), while the value of faecal water content did not change with respect to wild type animals (data not shown).

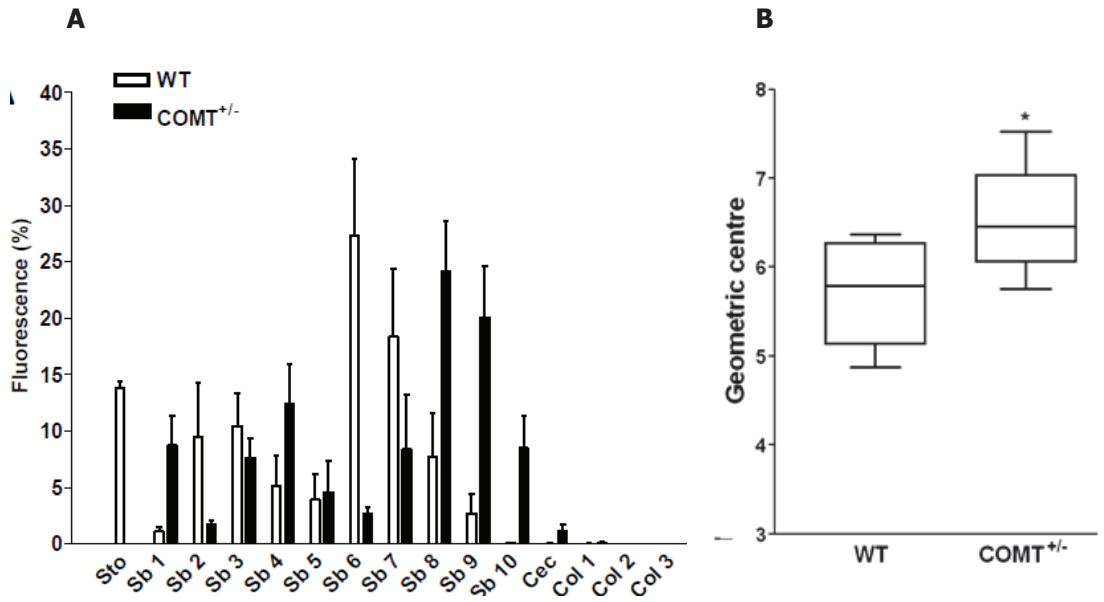

Fig. 1 Gastrointestinal transit in $COMT^{+/-}$ mice. Panel A: Percentage of the nonabsorbable FITC-dextran distribution in 15 gut segments, comprising stomach (Sto), small bowel (Sb 1–10), cecum (Cec), and colon (Col 1–3), 30 minutes after oral administration in WT and $COMT^{+/-}$mice (n=6). Panel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

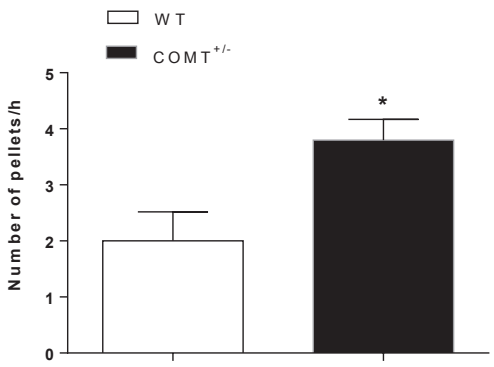

Fig. 2 Pellet frequency in COMT$^{+/-}$ mice Total number of fecal pellets expelled from WT or COMT$^{+/-}$ mice in 1-hour collection period (n=10 mice per group). *p<0.05 vs WT by t-Student test.

4.2 COMT genetic reduction related morphological abnormalities in ENS architecture

In the myenteric plexus of COMT$^{+/-}$ mouse ileum, a marked increase of S100β density was observed with respect to wild type animals (Fig.3).

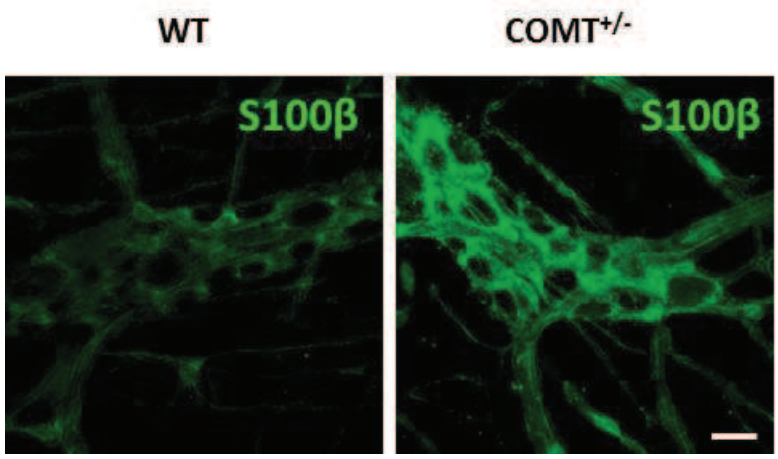

Fig. 3 Effects of antibiotic treatment on ENS architecture after partial genetic deletion of COMT activity. Representative confocal microphotographs showing the distribution of S100β (green) in LMMP preparations from WT and COMT$^{+/-}$ mice. Scale bars= 22 μm.

4.2.1 Biomolecular analysis of S100β mRNA and protein levels after genetic reduction of COMT activity

S100β mRNA and protein level expression in LMMP preparations of $COMT^{+/-}$ mice are shown in Fig. 4. Both parameters significantly increased (mRNA $2^{\wedge\Delta\Delta cT}$: 2.56±0.37, n=15, $p<0.05$ and 126±31.8%, n=6, $p<0.05$ for protein levels) with respect to values obtained in WT mice. (Fig. 4 A- B).

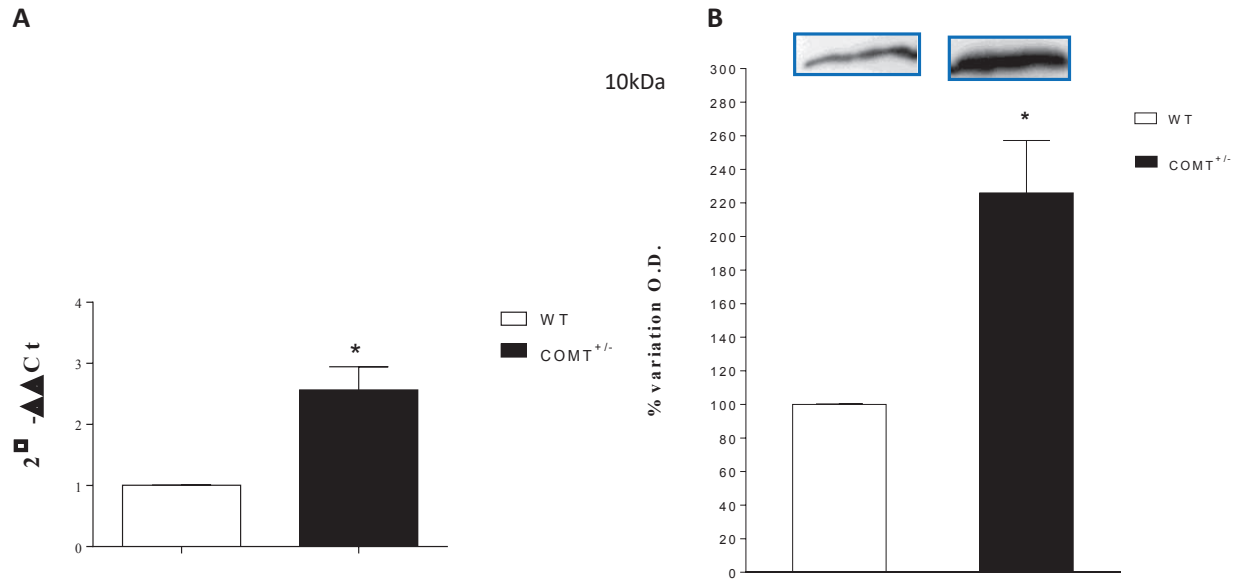

Figure 4. Biomolecular analysis of S100β transcript and protein expression. RT-PCR quantification of S100β transcripts in $COMT^{+/-}$ and in WT LMMP preparations (panel A). Western blot analysis of S100β (panel B) protein expression in $COMT^{+/-}$ and in WT LMMP preparations (panel B). Values of at least four experiments are expressed as mean ± SEM of the variation of the relative gene expression and optical density with respect to relative WT. Vertical bars indicate SEM.*$p<0.05$ by t-Student test.

4.3 COMT genetic reduction effect on excitatory neuromuscular contractility

In order to evaluate COMT genetic reduction effects on excitatory neuromuscular function, concentration-responses curves to the muscarinic receptor agonist, carbachol were performed . The maximal contractile response (Emax) resulted markedly reduced in $COMT^{+/-}$ mice (E_{max}=-41±8%, $p<0.01$, Fig. 5 A) with respect to the value obtained in WT mice. To evaluate the influence of COMT genetic reduction on neuromuscular activity, ileal preparations were subjected either to KCl 60 mM or to electrical field stimulation (EFS). The KCl-induced depolarizing effect was also significantly reduced (-38±5%, $p<0.01$; Figure 5 B) in $COMT^{+/-}$ mice compared with WT. Partial deletion of COMT gene determined changes in EFS-induced neuronal contractile responses, mainly cholinergic in nature, up to 20 Hz (-33±6% at 10 Hz, $p<0.01$; Fig. 5 C).

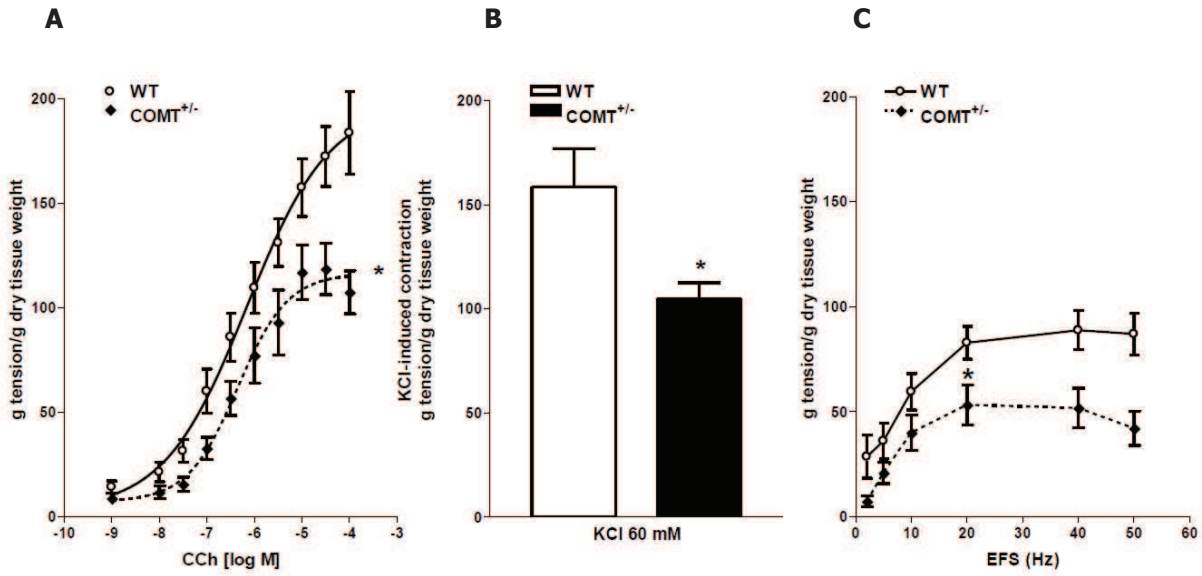

Figure 5 COMT genetic reduction affects ileal neuromuscular contractility. Concentration–response curves to carbachol (CCh, *Panel A*), excitatory response to KCl 60 mM (*Panel B*) and EFS-induced contractions (*Panel C*) in isolated ileal segments from WT and COMT+/- mice. Data are reported as mean ± SEM and are expressed as g tension/g dry tissue weight (n=8). *p<0.01 vs WT by t-Student test.

4.4 Modulation of inhibitory neurotransmission after genetic reduction of COMT activity.

Non-adrenergic, non cholinergic (NANC) inhibitory neurons play an important role in the regulation of GI motility, and NO is the major NANC inhibitory neurotransmitter of the GI tract. To evaluate the influence of COMT genetic reduction on inhibitory neurotransmission, in this set of experiments atropine (1 μM) and guanethidine (1 μM) were added in order to block cholinergic and adrenergic transmission (NANC conditions). EFS at 10 Hz was then performed in presence and absence of the pan-NOS inhibitor L-NAME (100 μM). EFS- induced NANC stimulation at 10 Hz caused a marked relaxation, that resulted significantly greater in ileal preparations obtained from $COMT^{+/-}$ mice compared to WT (+114±10%, $p<0.05$; Fig. 6). Pretreatment of ileal samples with L-NAME significantly blocked EFS (10Hz)-evoked NANC relaxation in WT mice. In $COMT^{+/-}$ mice, relaxation was only partially blocked by the pan-NOS inhibitor (-72±8%, $p<0.05$; Fig. 6).

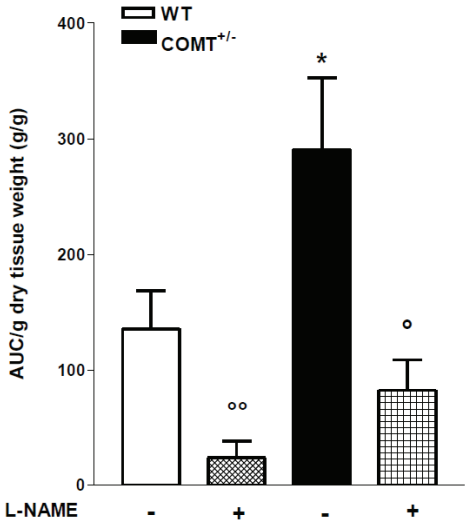

Figure 6 Effect of COMT genetic reduction on inhibitory contractile responses. EFS (10Hz)-evoked NANC relaxation in presence or absence of L-NAME in ileal preparations from COMT$^{+/-}$ and WT mice. Data are expressed as percentage of abolished contraction (AUC)/g dry tissue weight (n=8). *p<0.05 vs WT; °p<0.05, °°p<0.01 vs respective control by one-way ANOVA.

4.4.1 Distribution of nNOS and iNOS neurons in the myenteric plexus after genetic reduction of COMT activity.

To further investigate the impact of COMT genetic reduction on the enteric nitrergic neurotransmission we evaluated the distribution of nNOS and iNOS immunopositive neurons in the myenteric plexus. The number of nNOS^{+} myenteric neurons did not significantly change in LMMP preparations obtained from COMT$^{+/-}$ mice ileum with respect to WT animals. (Fig.7.). In LMMP preparations from the ileum of COMT$^{+/-}$ a marked increase of iNOS^{+} myenteric neurons was observed with respect to WT animals. (Fig. 8).

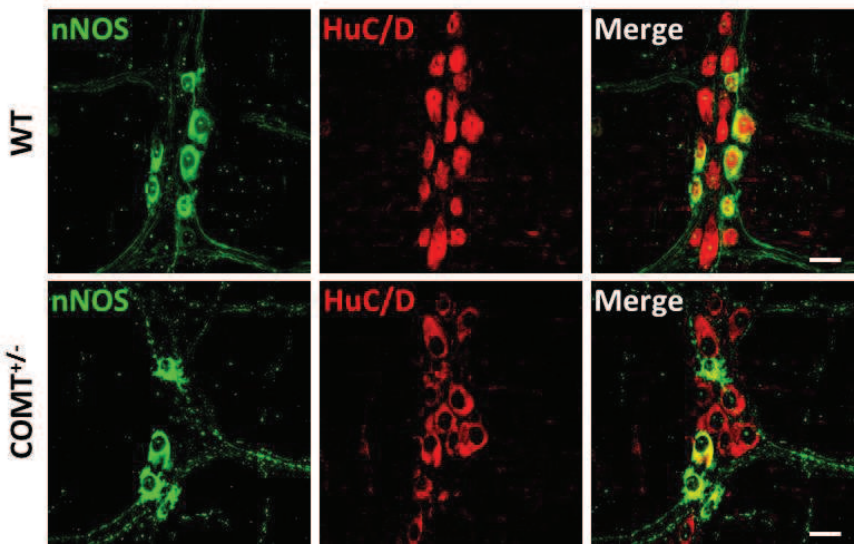

Fig. 7 nNOS immunostaining in COMT$^{+/-}$LMMP preparations. Representative confocal microphotographs showing the distribution of neuronal nitric oxide synthase (nNOS, green; marker for nitrergic neurons) and HuC/D (red, pan-neuronal marker) in ileal LMMP whole mount preparations from WT and COMT$^{+/-}$; scale bar=22 μM).

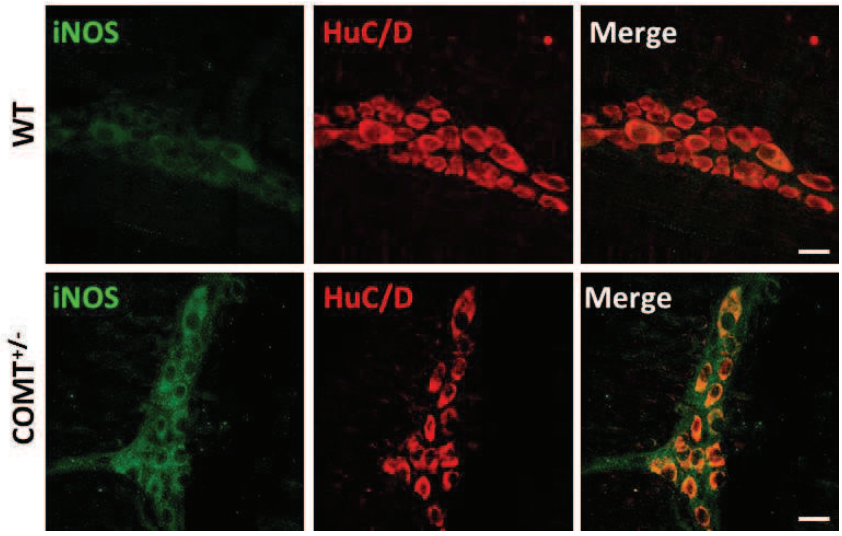

Fig. 8 iNOS immunostaining in COMT$^{+/-}$LMMP preparations. Representative confocal microphotographs showing the distribution of neuronal inducible nitric oxide synthase (iNOS, green; marker for nitrergic neurons) and HuC/D (red, pan-neuronal marker) in ileal LMMP whole mount preparations from WT and COMT$^{+/-}$; scale bar=22 μM).

4.4.2 Biomolecular analysis of nNOS and iNOS mRNA and protein levels after genetic reduction of COMT activity

nNOS mRNA and protein level expression in LMMP preparations of COMT$^{+/-}$ mice are shown in Fig. 9. Both nNOS mRNA and protein levels in COMT$^{+/-}$ mice did not significantly change with respect those obtained in WT mice. (Fig. 9 A- B).

iNOS mRNA and protein level expression in LMMP preparations of COMT$^{+/-}$ mice is shown in Fig. 10. iNOS mRNA and protein levels significantly increased (mRNA $2^{\wedge-\Delta\Delta cT}$: 5.18±0.52, n=12, $p<0.05$ and 149±6, n=4, $p<0.05$, for protein levels) in LMMPs preparations of COMT$^{+/-}$ mice with respect to the relative WT. (Fig. 10 A- B).

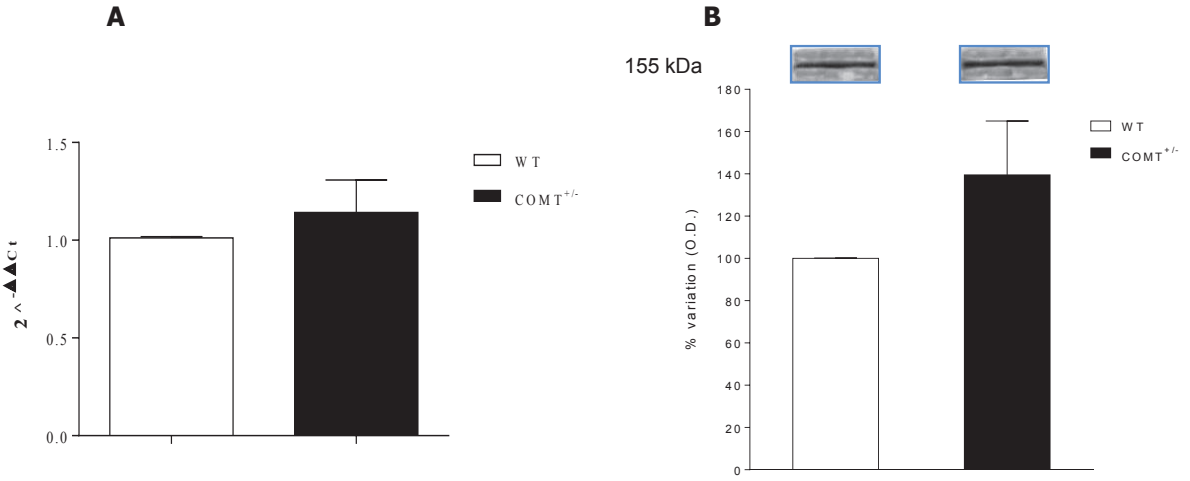

Figure 9 Biomolecular analysis of nNOS transcript and protein expression.RT-PCR quantification of nNOS transcripts in COMT$^{+/-}$ and in WT LMMP preparations (panel A). Western blot analysis of nNOS protein levels in COMT$^{+/-}$ and in WT LMMP preparations (panel B). Values of at least four experiments are expressed as mean ± SEM of the variation of the relative gene expression and optical density with respect to relative controls. Vertical bars indicate SEM.

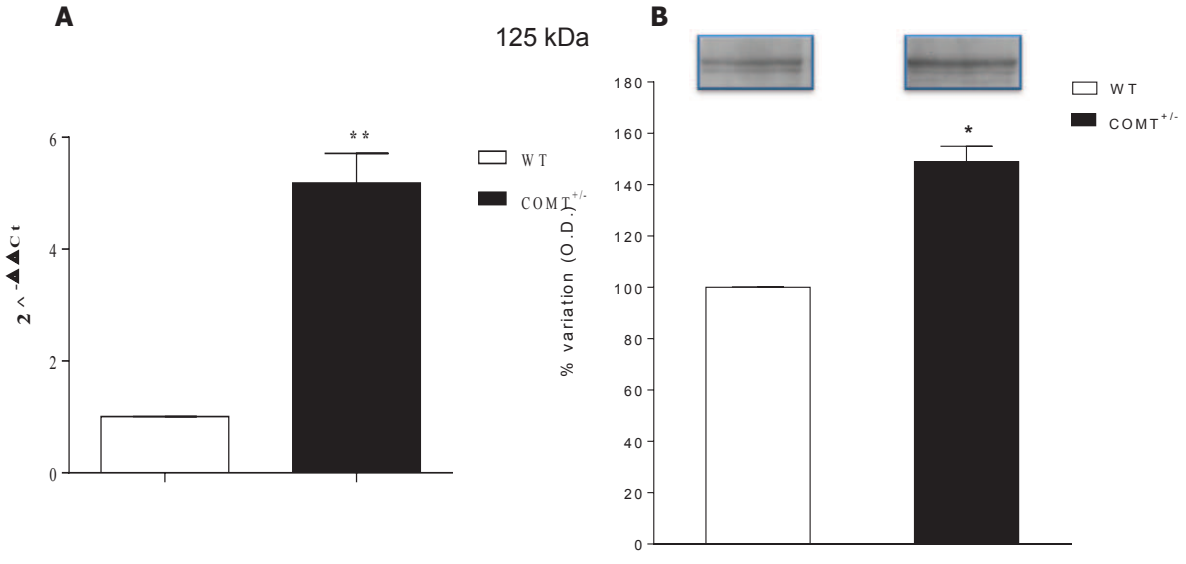

Figure 10 Biomolecular analysis of iNOS transcript and protein expression RT-PCR quantification of iNOS transcripts in $COMT^{+/-}$ and in WT LMMP preparations (panel A). Western blot analysis of iNOS protein levels in $COMT^{+/-}$ and in WT LMMP preparations (panel B). Values of at least four experiments are expressed as mean ± SEM of the variation of the relative gene expression and optical density with respect to relative controls. Vertical bars indicate SEM.*p<0.05; **p<0.01 by t-Student test..

4.5 Modulation of enteric glutamatergic neurotransmission after genetic reduction of COMT activity

mRNA and protein levels of the GluN1 subunit of the NMDA glutamatergic receptor are shown Fig.11. GluN1 mRNA and protein levels significantly increased in LMMPs preparations of $COMT^{+/-}$ mice with respect to the relative WT ($p<0.01$ for both mRNA and protein levels) . (Fig. 11 A- B).

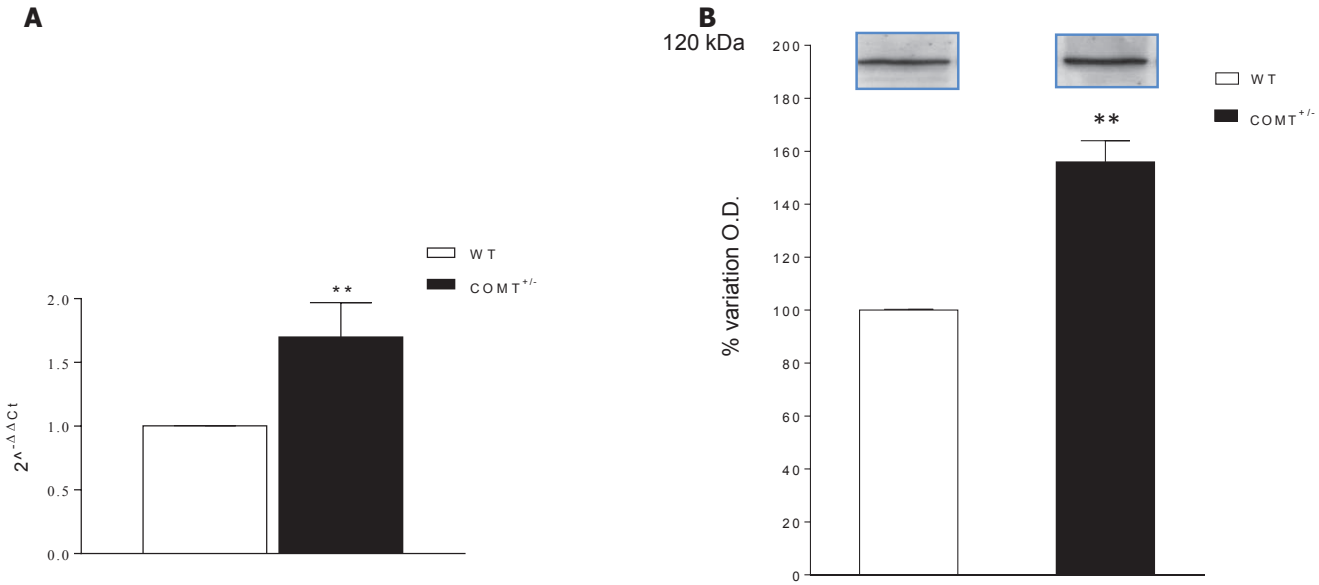

Figure 11 Biomolecular analysis of GluN1 transcript and protein expression RT-PCR quantification of GluN1 transcripts in COMT$^{+/-}$ and in WT LMMP preparations (panel A). Western blot analysis of GluN1 (panel B) protein expression in COMT$^{+/-}$ and in WT LMMP preparations (panel B). Values of at least four experiments are expressed as mean ± SEM of the variation of the relative gene expression and optical density with respect to relative controls. Vertical bars indicate SEM. **p<0.01 by t-Student test.

4.6 COMT genetic reduction effect on Bacterioidetes and Firmicutes mRNA expression in mice fecal samples

Quantitative RT-PCR analysis of *Firmicutes* and *Bacterioidetes* DNA levels in fecal samples from ileum, caecum and colon and of COMT$^{+/-}$ and wild type animals is shown in Fig. 12. *Firmicutes* DNA did not significantly increase in caecum faecal samples, while a trend towards an increase was obtained in colonic samples. In ileal faecal specimens, *Firmicutes* DNA significantly increased ($p<0.05$) (Fig 12 A). *Bacterioidetes* DNA significantly increased in caecum faecal samples ($p<0.05$), and showed a trend towards an increase in the colon. The same parameter significantly decreased in ileal samples ($p<0.001$) (Fig 12 B).

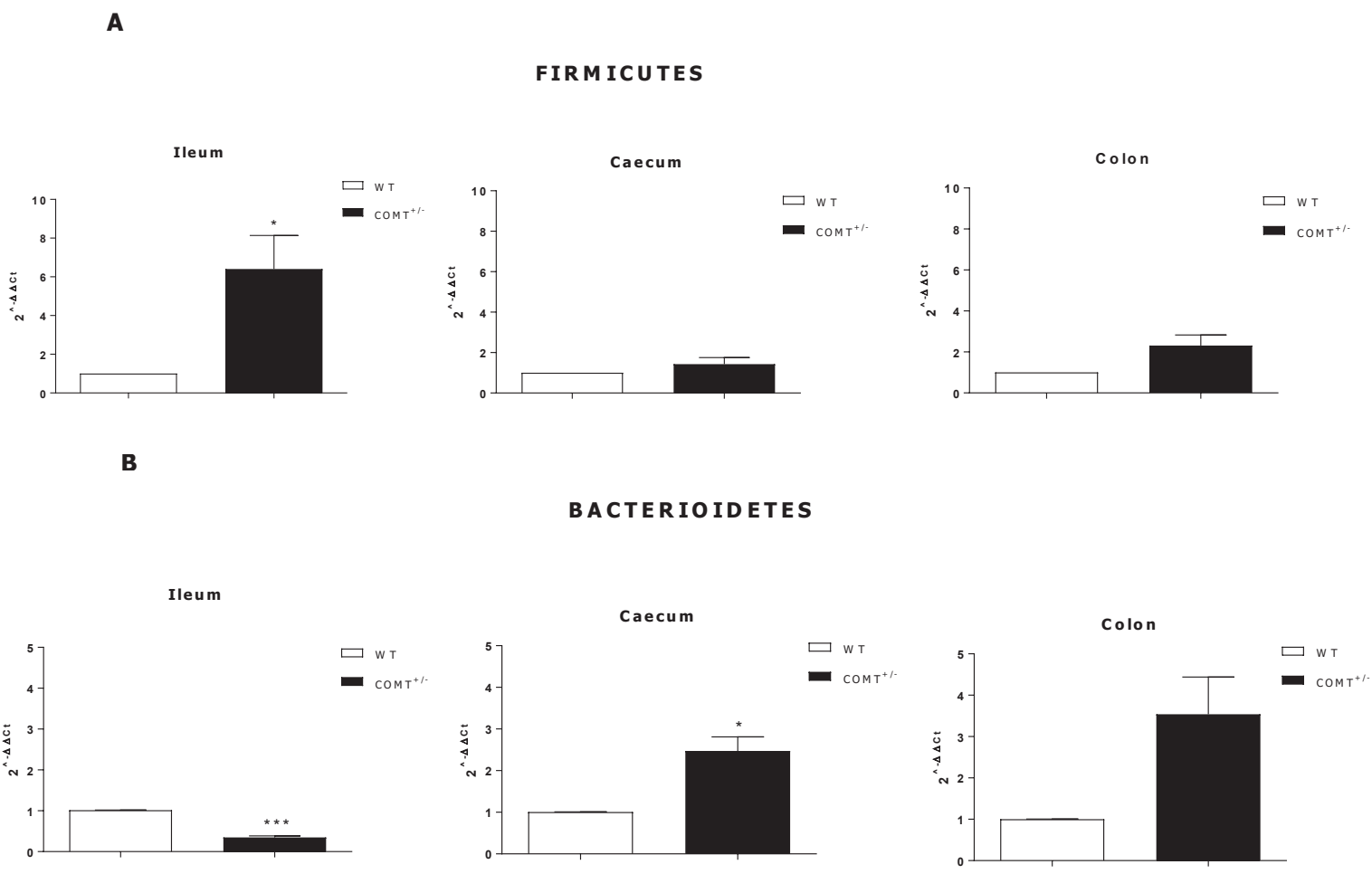

Fig.12 Biomolecular analysis of *Firmicutes* and *Bacterioidetes* transcript RT-PCR quantification of *Firmicutes* (panel A) and *Bacterioidetes* (panel B) DNA in COMT$^{+/-}$ faecal samples and in WT preparations. Values of at least four experiments are expressed as mean ± SEM of the variation of the relative gene expression and optical density with respect to relative WT animals. Vertical bars indicate SEM.*p<0.05, ***p<0.001 by t-Student test.

4.7 Anatomo-pathological alterations of the gastrointestinal tract induced by microbiota depletion

Anatomo-pathological analysis of the gastrointestinal (GI) tract of mice subjected to total microbiota depletion showed caecal swelling (Fig 13, panel A-B) with a significant increase of caecum weight (by 240.2±11.5%, N=17 mice per group; p<0.001) (Fig. 14, panel A) and a significative enlargement of the area of the large intestine (+92±0.1%, p<0.01, Fig. 14, panel B) with respect to control values. These characteristics were previously demonstrated in germ-free mice (Reikvam et al., 2011).

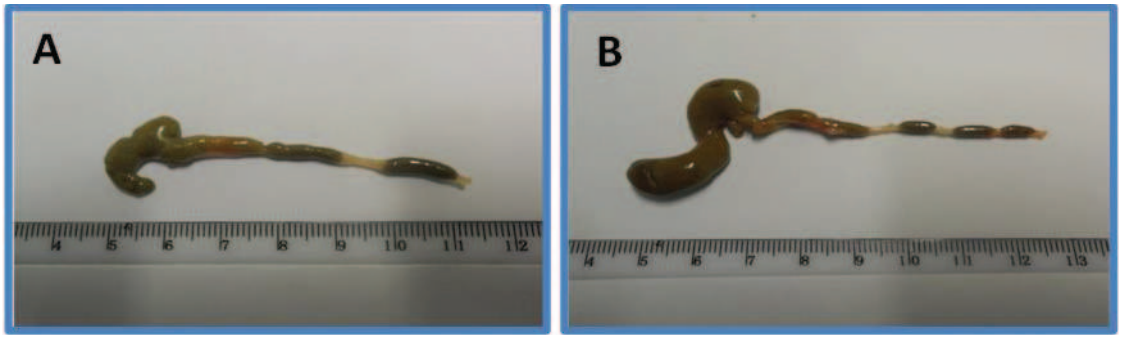

Fig. 13 Portion of the large intestine (caecum, colon and anus) of control mice (panel A) and antibiotic treated mice (panel B)

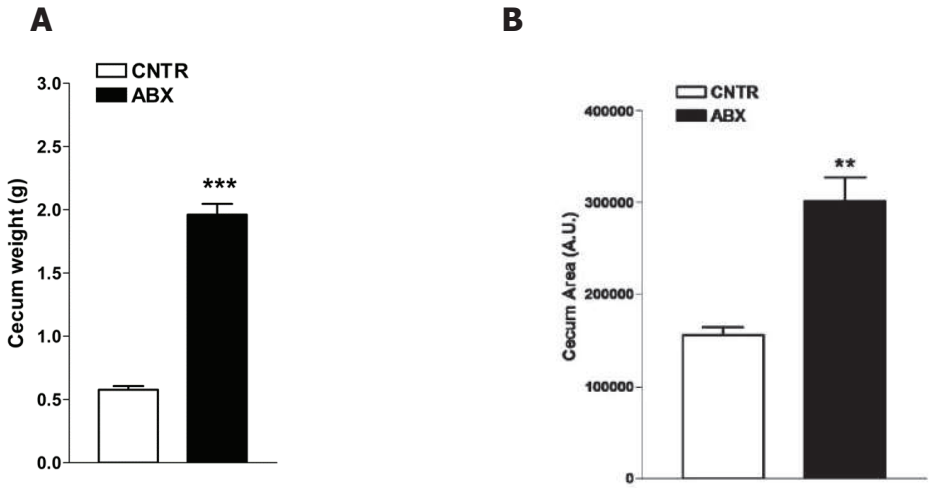

Fig. 14 Effect of the intestinal microbiota depletion on the large intestine weight (panel A) and area (panel B) of control and antibiotic treated mice (ABX), **p<0.01, ***p<0.001 vs control by t-Student test.

4.8 Gastrointestinal transit in antibiotic treated mice

Antibiotic-mediated microbiota depletion determined a significant decrease of GI transit, as evidenced by the altered gut distribution of nonabsorbable FITC-dextran (Fig. 15 A) as well as a reduction of the geometric centre compared to CNTR mice (Fig. 15B, GC_{ABX}=3.5, range: 2.9-4.0 vs GC_{CNTR}=7.3, range: 6.2-7.9; p<0.01). Delayed gastric emptying was also observed in ABX-treated mice, as shown by the 4.5-fold increase of the fluorescent probe gastric content (n=10; P<0.01; Fig. 15 C). The delay of GI transit in ABX-treated mice was paralleled by a significant reduction in stool frequency (-29.5±0.5%, n=15; p<0.01; Fig. 16 A), evaluated during 1 hour collection period, together by an increase of faecal water content (by 35.5±7.2%, p<0.01; Fig. 16 C).

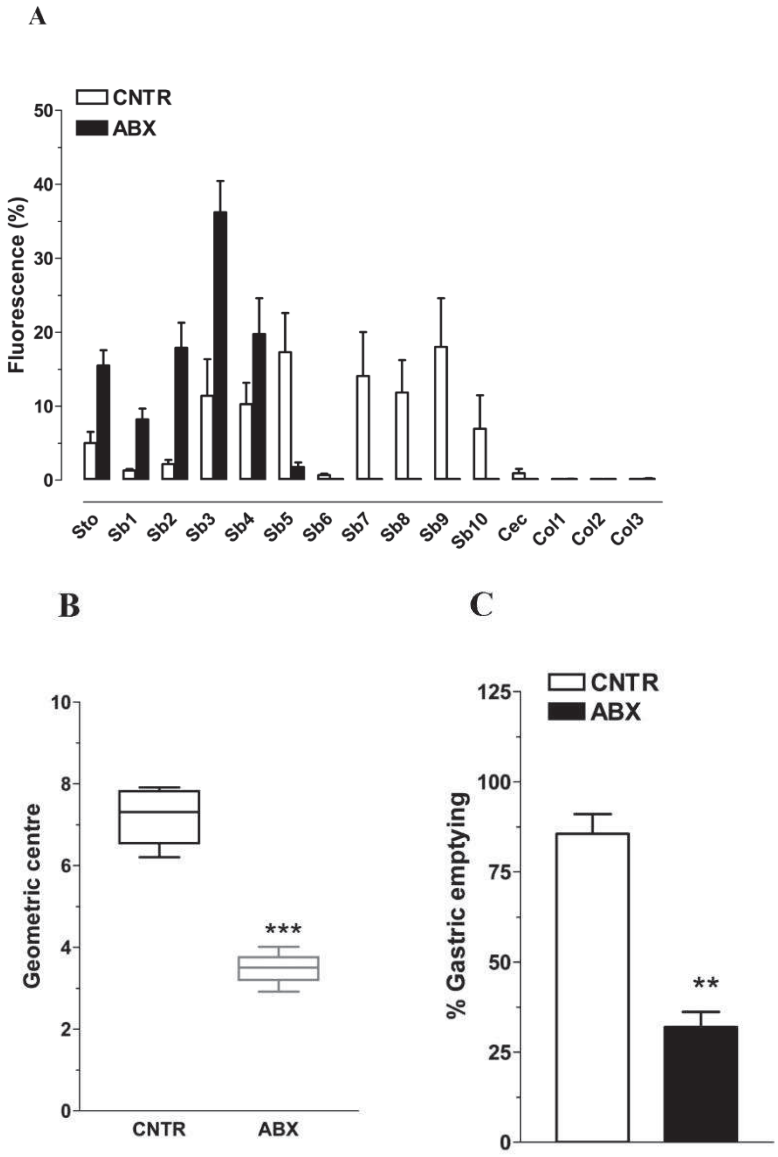

Figure 15 Gastrointestinal transit in ABX mice. *Panel A:* Gastrointestinal (GI) transit was measured as % of nonabsorbable FITC-dextran distribution in 15 gut segments, comprising stomach (Sto), small bowel (Sb 1–10), cecum (Cec), and colon (Col 1–3), 30 minutes after oral administration in CNTR and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

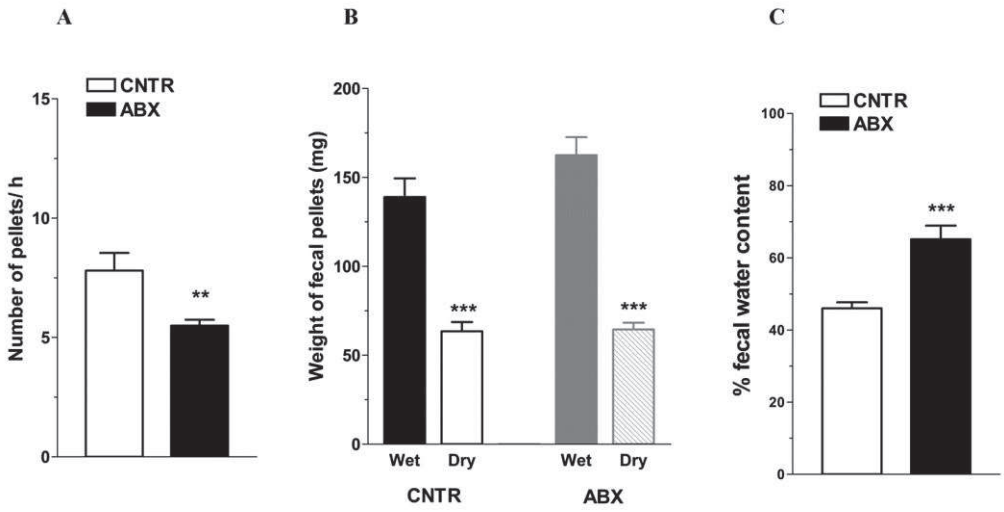

Fig. 16 Antibiotic-induced microbiota depletion pellet frequency and fecal water content. (A) Total number of fecal pellets expelled from CNTR or ABX-treated mice in 1-hour collection period (N=15 mice per group). (B) Wet and dry weight of fecal pellets expelled from CNTR or ABX-treated mice. (C) Percentage of fecal water content of CNTR and ABX-treated mice (N=15 mice per group). **p<0.01, ***p<0.001 vs CNTR by t-Student test.

4.9 Microbiota depletion morphological abnormalities in ENS architecture

Changes in microbiota composition and/or signalling influences the ENS morphology (Brun et al., 2013). In the myenteric plexus of ABX-treated mouse ileum, a marked increase of S100β density was observed with respect to CTRL animals (Fig. 17).

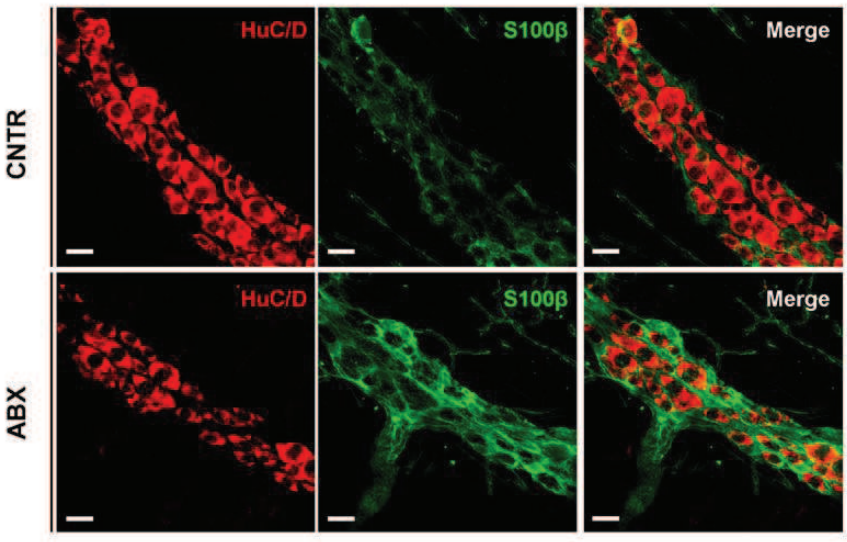

Fig. 17 Effects of antibiotic treatment on ENS architecture. Representative confocal microphotographs showing the distribution of S100β (green) and HuC/D (red) in LMMP preparations from CNTR and ABX-treated mice. Scale bars= 22 μm .

4.9.1 Biomolecular analysis of S100β mRNA levels in antibiotic treated mice

S100β mRNA levels did not significantly change (n=6) with respect to the relative CTRL in LMMPs preparations of antibiotic treated mice (Fig. 18).

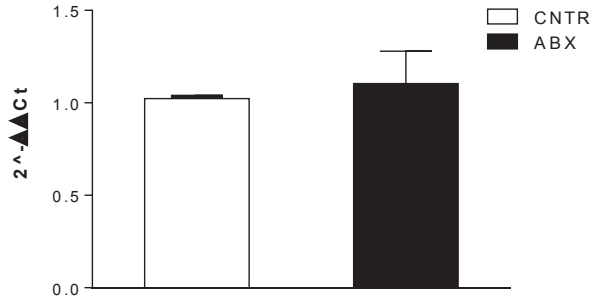

Fig. 18 Biomolecular analysis of S100β transcript.RT-PCR quantification of S100β transcripts in antibiotic treated mice and in control preparations. Values of at least four experiments are expressed as mean ± SEM of the variation of the relative gene expression with respect to relative controls. Vertical bars indicate SEM.

4.10 Antibiotic-induced microbiota depletion effect on excitatory neuromuscular contractility

In order to evaluate COMT genetic reduction effects on excitatory neuromuscular function, concentration-responses curves to the muscarinic receptor agonist carbachol were performed. Ileal segments from ABX-treated mice showed a significant downward shift of the concentration-response curve to CCh and a consequent decrease in maximum response value (Emax = -49.8±6.3%, n=8 mice per group; p<0.05 vs CNTR; Fig. 19 A). Ileal muscular response to the depolarizing agent KCl (60 mM) significantly decreased in ABX-treated mice (by 27.4±8.2%, n=8 mice per group; p<0.01; Fig. 19 B). To clarify whether ileal contraction changes in ABX-treated mice were caused by alterations of the neuromuscular function, we evaluated the effect of electric field stimulation (EFS) at increasing frequencies of stimulation. Alterations in ABX-treated mouse ileum excitatory neurotransmission was confirmed by decreased EFS-elicited contractions (by 59.7±7.1% at 10 Hz, n=8 mice per group; p<0.01; Fig.19 C).

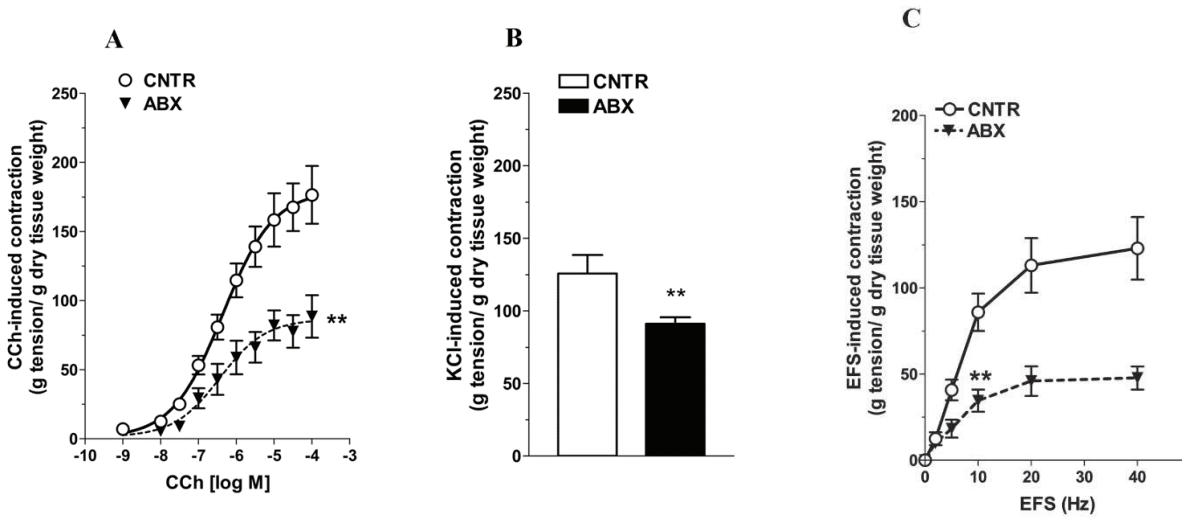

Fig.19 Antibiotic-induced microbiota depletion excitatory contractility. (A) Concentration-response curves to carbachol (CCh, 0.001-100 μM) in isolated ileal preparations from CNTR and ABX-treated mice (N=16 mice per group). (B) KCl 60 mM-elicited excitatory response in isolated ileal preparations from CNTR and ABX-treated mice (N=16 mice per group). (C) Neuromuscular excitatory response induced by EFS (0-40 Hz) in isolated ileal preparations of CNTR and ABX-treated mice (N=8 mice per group). **p<0.01 by t-Student test.

4.11 Antibiotic induced microbiota depletion modulation of inhibitory neurotransmission

In a further set of experiments, the effect of microbiota depletion was evaluated on NO-mediated relaxation responses of mouse ileum to transmural stimulation in the presence of guanethidine and atropine. NANC-evoked relaxations at 10 Hz stimulation frequency markedly increased in ABX-treated mice compared to CNTR (by 70.1±5.2%, n=8; *P*<0.001; Figure 20). In ABX mice, pre-treatment with 1400W, a selective inhibitor of inducible NOS (iNOS), did not affect 10 Hz-EFS induced-relaxations in NANC conditions (Fig. 20). The addition of L-NAME completely abolished the relaxation response induced by EFS in CNTR mice but not in ABX-treated mice (-77.2±6.3%, n=8; *P*<0.001; Fig. 20).

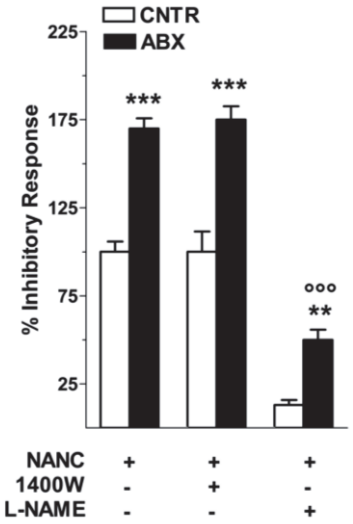

Fig. 20 Antibiotic-induced microbiota depletion inhibitory neurotransmission. 10 Hz EFS-evoked NANC relaxation responses in absence or presence of 1400W or L-NAME (n=8 mice per group).***p<0.001 vs respective control; °°°p<0.001 vs respective control without L-NAME by one-way ANOVA.

4.11.1 Immunostaining of nNOS neurons in antibiotic treated mice.

To further investigate the effect of microbiota depletion on the enteric nitrergic neurotransmission we evaluated the distribution of nNOS immunopositive myenteric neurons. The number of $nNOS^+$ myenteric neurons significantly decreased in LMMP preparations from the ileum of ABX mice with respect to control animals. (Fig. 21).

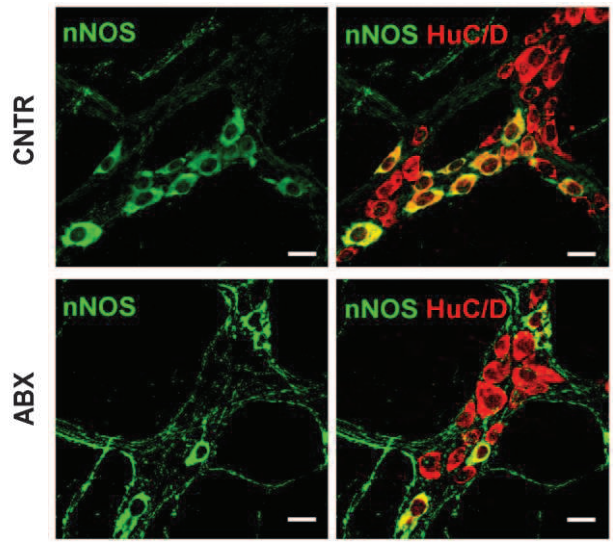

Fig. 21 nNOS immunostaining of antibiotic treated LMMP preparations. Representative confocal microphotographs showing the distribution of neuronal nitric oxide synthase (nNOS, green; marker for nitrergic neurons) and HuC/D (red, pan-neuronal marker) in ileal LMMP whole mount preparations from CNTR and ABX-treated mice; scale bar=22 μM).

4.11.2 Biomolecular analysis of nNOS and iNOS mRNA levels in antibiotic treated mice

In LMMPs preparations obtained from ABX-treated mice, nNOS mRNA levels did not significantly change (n=6) (Fig. 22) while iNOS mRNA levels significantly decreased (mRNA $2^{\wedge\Delta\Delta cT}$: 0.27±0.05, n=6, p<0.001) with respect to the relative controls (Fig. 23)

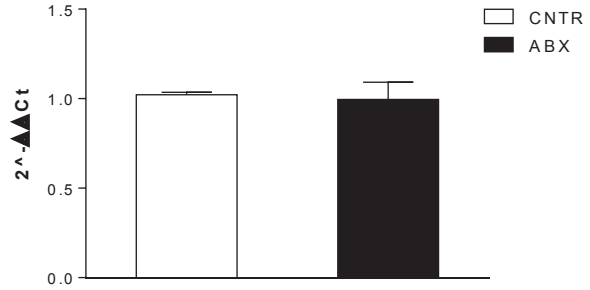

Fig. 22 Biomolecular analysis of nNOS transcript.RT-PCR quantification of nNOS transcripts in antibiotic treated mice and in control preparations. Values of at least four experiments are expressed as mean ± SEM of the variation of the relative gene expression with respect to relative controls. Vertical bars indicate SEM by t-Student test.

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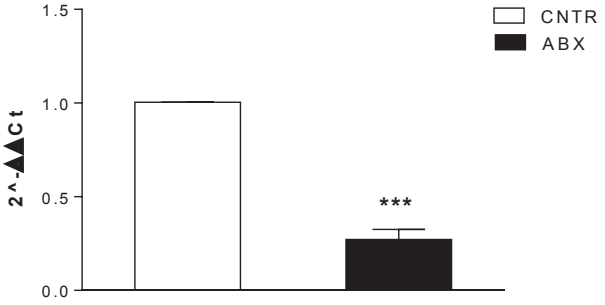

Fig. 23 Biomolecular analysis of iNOS transcript.RT-PCR quantification of iNOS transcripts in antibiotic treated mice and in control preparations. Values of at least four experiments are expressed as mean ± SEM of the variation of the relative gene expression with respect to relative controls. Vertical bars indicate SEM.***p<0.001 by t-Student test..

4.12 Antibiotic induced microbiota depletion modulation of enteric glutamatergic neurotransmission

A significant increase (mRNA $2^{\wedge\Delta\Delta cT}$: 1.3±0.006, n=8, p<0.001) of GluN1 subunit mRNA levels was found in LMMP preparations obtained from ABX-treated mice with respect to control values (Fig. 24).

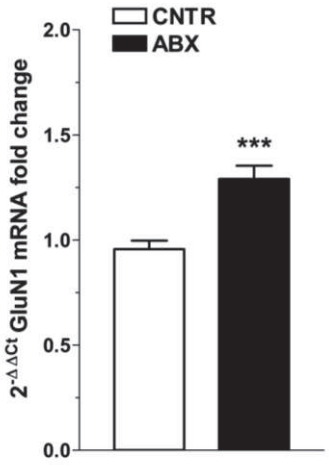

Fig. 24 Antibiotic-induced microbiota depletion effects on enteric glutamatergic trasmission. RT-PCR quantification of GluN1 transcripts in ileal LMMP preparations from CNTR and ABX-treated mice. Values of at least four experiments are expressed as mean ± SEM of the variation of the relative gene expression with respect to relative controls *** P<0.01 vs CNTR.

5. DISCUSSION

The present project aimed to evaluate structural and functional anomalies of the gastrointestinal tract of adult mice characterized by a genetically driven reduction in COMT. In particular, I focused on the possible adaptive changes involving the enteric nervous system (ENS), a complex network of ganglia and interconnecting fibers innervating the gut, that directly coordinates gastrointestinal functions, including motility, secretion, mucosal immunity and visceral perception (Furness 2012). On the whole, data obtained in the present project reflect alterations in motor and sensitive parameters which resemble some features of functional gastrointestinal disorders, such as Irritable Bowel Syndrome (IBS).

Enteric neuronal circuitries display a considerable ability to adapt to a changing microenvironment, which comprises several cellular "players", including neurons, enteric glial cells, smooth muscle cells, interstitial cells of Cajal, immune cells and the microbiota (Giaroni et al., 1999; Furness, 2012). Indeed, the homeostatic control of gut function in a changing environment is achieved through adaptive changes involving enteric ganglia. Neuronal plasticity encompasses a great variety of changes in neuronal structure and function in response to alterations of several inputs and may comprise "neurotrasmitter plasticity", that, at a neuronal level, involves changes in the chemical coding and alterations of neurotransmitter release and functions, both in physiogical and pathological conditions (Giaroni et al., 1999).

The cathecol-O-metiltranferase (COMT) protein is widely distributed in mammalian brain (Lundstrom et al., 1995; Mannisto and Kaakkola, 1999) and its enzymatic activity regulates cathecolamine levels (Yavich et al., 2007). In the CNS, genetic variations in human COMT have been associated with physiological functions (Egan et al., 2001; Winterer et al., 2006) and behavioral phenotypes related to prefrontal cortex and hippocampal information processing, including cognition (Blasi et al., 2005; Bertolino et al., 2006), anxiety (Drabant et al., 2006), obsessive-compulsive disorder (OCD) (Pooley et al., 2007) and pain sensitivity (Nackley et al., 2006). A common human polymorphism of COMT, the val158met, determines low activity of the enzyme with well recognized effects on CNS neurotransmission. In human cortical neurons of the COMT Val158Met genotype adaptive changes underlying plasticity and involving brain-derived neurotrofic factor (BDNF) have been shown to occur (Witte et al., 2012). Accordingly, the Val158Met polymorphism (Val158Met) of the COMT gene, may affect working memory plasticity in adults (Heinzel et al., 2014). Another feature of Val158Met polymorphism is the possible association with visceral pain, one of the main

symptoms associated with Irritable Bowel Syndrome (IBS). IBS entails a heterogeneous group of functional lower gastrointestinal tract disorders characterized by abdominal pain or discomfort associated with altered bowel habits and disordered defecation that may be exacerbated by emotional stress. Additional IBS symptoms include altered mucosal immune and inflammatory functions (which includes changes in bacterial flora) and abnormal motor reactivity (Drossman, 2006; Ohman and Simren, 2010; Simren et al., 2013).

In our model, reduction of the COMT gene, resulted prominent changes in GI motility. On the whole the excitatory cholinergic transmission seems to be reduces as suggested by the the reduction of small intestine neuromuscular responses to both carbachol and EFS in genetically mutated animals. In addition, stimulation with KCl in $COMT^{+/-}$ animals showed a significant reduction in the muscular motor activity which may relate to a defective smooth muscle function. On the other hand enhancement of electrically-induced NANC responses in preparations obtained from $COMT^{+/-}$ animals suggest that the nitrergic inhibitory relaxation may play a relevant role in the regulation of intestinal motility in this model, contributing to enhancement of intestinal transit. Indeed, the modulation of inhibitory inputs to the bowel has a fundamental role in the propagation of relaxation during peristalsis (Furness 2014).

NO is produced by three main nitric oxide synthase isoforms, the neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). Neuronal nitric oxide synthase (nNOS) is constitutively expressed in myenteric neurons and is the predominant source for the generation of NO, mediating non-adrenergic non-cholinergic inhibition of smooth muscle contraction. In myenteric neurons, expression of iNOS, is enhanced during disease states, such as intestinal ischemia and inflammation (Giaroni et al., 2013, Kolios et al., 2004). Inducible NO synthase (iNOS), but not neuronal NOS (nNOS), mRNA, protein levels and immunopositive neurons significantly increased in the LMMP of $COMT^{+/-}$ animals, with respect to wild type animals. This may reflect a plasticity of nNOS and iNOS function in the myenteric plexus in COMT partially deleted animals, with iNOS taking over nNOS role in the modulation of the enteric neuromuscular function. As observed in the CNS, in enteric ganglia, iNOS is also expressed by enteric glial cells. In LMMP preparations of $COMT^{+/-}$ animals, the increase of iNOS expression was associated with the enhancement of both mRNA, protein expression levels and immunostaining of the glial marker, S100β, with respect to wild type mice. Such up-regulation of enteric glial cells usually correlates with a disease states, such as inflammation (Bradley et al. 1997). Indeed, alterations of enteric glia may have detrimental effects on enteric neurons integrity (Thacker et al., 2011).

The ability of L-NAME to partially reverse the inhibitory NANC response is highly suggestive for the involvement of other inhibitory mediators (such as VIP and ATP) in the control of intestinal relaxation (Collins et al., 2014). We cannot also exclude the involvement of adaptive changes occurring at different adrenoceptors, in particular inhibitory alpha2 adrenergic receptors after partial COMT deletion. At this latter regard, adrenergic pathways are indeed involved in the control of several key functions of the gut: they modulate vascular tone and inhibit motility and fluid secretion (DePonti et al., 1996). In particular, adrenergic innervation of the gut, which is mainly of extrinsic origin, exerts a tonic inhibitory control of motor activity. Indeed, in guinea-pig myenteric plexus adaptive changes involving different populations of adrenoceptors have been observed after chronic treatment with the tryciclic antidepressant desipramine (DMI), an inhibitor of cathecolamine uptake (Marino et al., 1994). In analogy with COMT genetic deletion, DMI increases synaptic catecholamine levels. Acute administration of DMI reduced colonic peristalsis (Marino et al., 1994). However, after chronic DMI treatment, the peristaltic function was restored suggesting the possible rearrangements of adrenergic receptor function (Marino et al., 1994; Giaroni et al., 2008). Interestingly, DMI is a medication for IBS (Trinkley et al., 2014). In this scenario, further studies are needed to uncover the neurotransmitter/s and relevant mechanism involved in the enhanced inhibitory response in the $COMT^{+/-}$ mouse intestine.

In the ENS of $COMT^{+/-}$ mice, the levels of GluN1 subunit of the NMDA receptor increased. Along the gastrointestinal tract, the GluN1 subunit is abundantly expressed in myenteric and submucosal ganglia. Adaptive changes involving GluN1 myenteric subunits have already been shown to occur after an I/R damage as well as during inflammation in the gut (Giaroni et al., 2011; Zhou et al., 2011) and may be related to both neuromuscolar dysfunction as well as to altered visceral intestinal sensitivity (MacRoberts et al., 2001; Filpa et al., 2016). Interestingly, visceral hypersensitivity develops in 50-80% of patients with IBS and the origin of this phenomenon is probably based on both peripheral and central nervous system mechanisms (Spiller et al, 2007).

An important component of the enteric microenvironment, which influences the ENS, is represented by the non pathogenic commensal microbiota, which in normal conditions colonizes the intestinal lumen. These microorganisms have a unique relationship with the host and are crucial in guaranteeing intestinal epithelial integrity and barrier function, promoting gut development and maturation of the mucosal immune system (Shroff et al., 1995; Collins et al., 2014). Gut microbiota is directly involved in modulating the development

and function of the ENS supporting the hypothesis that changes in intestinal microbes composition induced by either infections or antibiotics, particularly during early life, can perturb ENS integrity favoring the onset of gastrointestinal disorders (i.e. inflammatory bowel disease, IBD or irritable bowel syndrome, IBS; McVey Neufeld et al., 2013) Recently, the microbiota-gut-brain axis has emerged as a key player in brain neurodevelopment, indicating that microbiota initial colonization and development can influence general and mental health later in life (Diaz Heijtz et al., 2011; Borre et al., 2014). In this view, the microbiota-gut-brain axis has been recognised as an important way that connect emotional and cognitive centers of the brain with peripheral intestinal functions (Cerabotti et al., 2015). 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.

Interestingly, after genetic deletion of COMT activity, some commensal intestinal microbial strains underwent drastic changes. In $COMT^{+/-}$ mice a significant increase of *Firmicutes* DNA in the ileum and colon and a significant decrease of *Bacterioidetes* DNA in the ileum were observed. Analogously, the intestinal microbiota of some IBS patients showed *Firmicutes* enrichment and reduced abundance of *Bacteroides* with respect to control patients (Hong and Rhee, 2014).

In view of the alterations observed in microbiota composition in $COMT^{+/-}$ mice gut, a series of experiments was carried out on mouse gut after microbiota depletion, by means of a massive antibiotic chronic treatment. This treatment has been previously shown to induce a disruptive effect on protective commensal bacteria populations, leading to dysbiosis and potentially causing IBS symptoms (Maxwell et al., 2002; Villarreal et al., 2012).

Macroscopic analysis of antibiotic treated mouse gut suggest that exposure to a broad spectrum cocktail of antibiotics determined a marked enlargement in caecum, a phenotypic characteristic similar to those found in adult germ-free mice (Reikvam et al., 2011), with ensuing dysbiosis. In the ABX model, immunohistochemical data showed that, in accordance to the $COMT^{+/-}$ mice, immunoreactivity to the glial marker S100β, significantly increased in the ileal myenteric plexus, although S100β mRNA levels did not change (Caputi et al., 2015). This may suggest that the perturbation of gut microbiota composition may affect glial proteins, but not the transcript.

Analogously to what was observed in $COMT^{+/-}$ mice, antibiotic-induced microbiota depletion caused disturbances in ileal neuromuscular contractility, with a significant significant reduction to EFS. Additionally,

a downward shift of the concentration-response curve to the non selective cholinergic receptor carbachol was observed. A significant reduction to high potassium-induced membrane depolarization suggest that also the smooth muscle responsiveness may be affected by antibiotic treatment. As observed in the $COMT^{+/-}$ model, in ABX-treated mice EFS-induced NANC stimulation markedly increased compared to controls. Pretreatment with the pan NOS inhibitor L-NAME, only partially abolished NANC responses, suggesting that the depletion of gut microbiota can influence not only the nitrergic-mediated relaxation, but also other inhibitory neurotransmitters (Zizzo et al., 2003). In addition, in antibiotic-treated animals, pre-treatment with the selective inhibitor of iNOS, 1400W, did not change NANC responses, suggesting that iNOS is not involved in the regulation of NANC responses, in this model. In accordance with functional data, a significant reduction in iNOS mRNA was also observed, reinforcing the hypothesis that iNOS may not participate to NANC relaxations. As concerns nNOS, in ABX-treated animals, immunopositive myenteric neurons for this enzyme significantly diminished. Alterations of nitrergic neurotransmission in ABX-treated mice may participate to the significant delay of GI transit. Such delay was also reflected in corresponding changes in stool frequency and water content, indicating a possible state of constipation and alteration of intestinal permeability as a result of antibiotic treatment (Caputi et al., 2015). On the whole, these observations on ABX-treated mouse gut motor functions are only partially superimposed to those observed in the $COMT^{+/-}$ model. Such discrepancies may reflect the different motor symptoms of IBS, characterized either by constipation or by hypersecretion.

In analogy with $COMT^{+/-}$ mice, antibiotic treatment determined a significant increase of GluN1 subunit of NMDA receptor expression in ileal LMMP preparations, suggesting a link between gut-dysbiosis and visceral hypersensitivity mediated by the glutamatergic transmission in the enteric neuronal circuitries.

In conclusion, the partial deletion of COMT determines anomalies in the ENS architecture, affecting excitatory and inhibitory neurotransmission leading impaired gut neuromuscular contractility and visceral hypersensitivity with critical effects on gut function (Caputi et al., 2015). Most of these alterations were observed after microbiota depletion. Interestingly, COMT partial genetic deletion induced changes in gut microbioma composition with respect to control animals suggesting that host may affect gut microbial flora arrangement. These data confirm the importance of studying the interplay between host and microbiota. In addition, both COMT genetic deletion as well as dysbiosis may be critical factors involved in the pathogenesis of functional gut disorders, such as IBS.

In figure 5.1 a schematic representation of the most relevant findings obtained after partial genetic deletion of COMT in mice is showed.

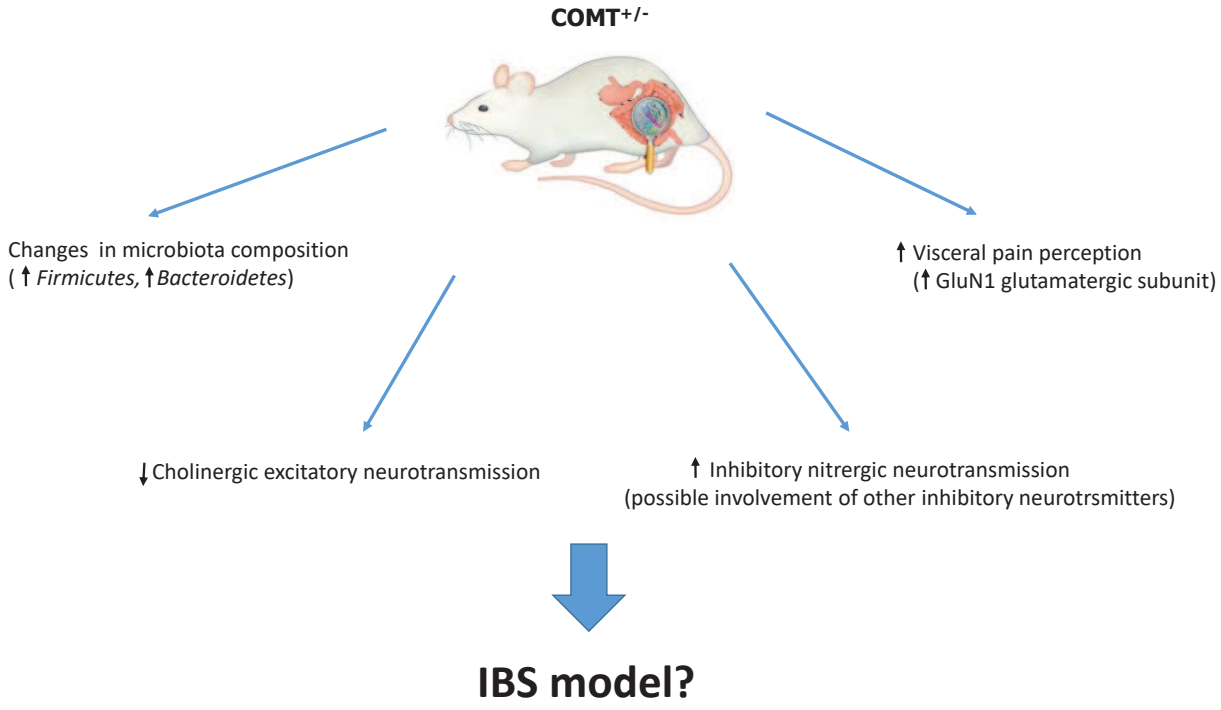

Fig. 5.1 Schematic representation of the relevant data obtained after partial genetic deletion of COMT

Further investigations are needed to clarify the role of COMT in the regulation of molecular pathways involved in IBS pathophysiology. For example, it will be intriguing to evaluate the possible contribute of other inhibitory pathways, such as purinergic, α_2-adrenergic, dopaminergic, in the alterations of GI motility that were observed after partial genetic deletion of the COMT enzyme. Functional experiments on visceral pain perception alterations may shed light on the possible role and involvement of COMT enzyme in IBS pain perception and discomfort.

Owing to the comorbidity of IBS and psychiatric disorders, such as anxiety and depression, it will be interesting to evaluate whether alterations of COMT activity and disbyosis may be related to development of psychiatric and neurodevelopmental disorders. It is known that the microbiota-gut-brain axis is the main way of communication between ENS and CNS, principally via the vagus nerve. For this reason, surgical vagotomy on COMT$^{+/-}$ mice may clarify the possible role of the bidirectional way of communication in the development of psychiatric disorders and its comorbidity with IBS.

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