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ANALISI DEL RUOLO DI MEDIATORI ENTERICI NELLA FISIOPATOLOGIA DELLE MALATTIE INFIAMMATORIE CRONICHE INTESTINALI

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TESI DI:

DR. MICHELANGELO AUTERI

TUTOR:

PROF.SSA ROSA SERIO

COORDINATORE DEL DOTTORATO:

PROF. GIUSEPPE FERRARO

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SUMMARY

Inflammatory Bowel Diseases (IBD) are severe gastrointestinal (GI) disorders, including Crohn's Disease (CD) and Ulcerative Colitis (UC), characterized by a chronic intestinal inflammatory reaction progressively causing tissue damage and a series of related major symptoms including defective GI motor activity, diarrhea, rectal bleeding, malabsorption, weight loss, fever. A plethora of factors contributes to the physiopathology of IBD, including genetic susceptibility, environmental factors, increased intestinal permeability and, above all, the establishment of an aberrant immune reaction of the Gut-Associated Lymphoid Tissue (GALT) to harmless antigens derived from commensal microbiota. Due to the complex interplay of these diverse factors, the exact cause of IBD is still unknown, and the appropriate treatment of IBD is still a clinical issue, since current therapeutic strategies are restricted to classical anti-inflammatory drugs, not leading to the complete resolution and often associated with different side effects. Hence, research on novel factors and pathways involved in the physiopathology of IBD are absolutely needed to improve currently available therapies. Recently, several researches have provided evidence for the potential effect in IBD of mediators of the enteric nervous system (ENS), demonstrating that modification in the contribution and role of some enteric mediators could lead to the pathological changes of GI motor patterns, as well as to the modulation of the local inflammatory event, controlling immune cell activity within the GALT.

Angiotensin II (Ang II) and γ-aminobutyric acid (GABA) have been suggested as novel mediators involved in the modulation of GI motility, and, interestingly, other studies have pointed out their connection with inflammatory conditions, suggesting that a detailed investigation of these mediators could lead to their identification as novel therapeutic targets in inflammatory disorders, including Inflammatory Bowel Disease. Angiotensin II,

the main effector of the renin-angiotensin system (RAS), has been previously reported to act in the gut microenvironment as modulator of water/electrolytes absorption, glucose transport and bicarbonate secretion, *via* its action on the specific AT1 and AT2 receptors. However, its role in the modulation of gut motility is increasingly emerging, as Ang II induces enteric smooth muscle contraction in the small intestine and colon, mostly *via* modulation of the enteric tachykinergic signaling. Moreover, a series of recent studies has also underlined the connection between Ang II and GI inflammation, as intestinal levels of Ang II are higher in CD patients, and antagonists of angiotensin receptors (especially AT1) displayed beneficial effect in animal models of IBD. Thus, novel studies about the role of Ang II in health and disease could disclose its potential as therapeutic target for the treatment of both IBD-related GI motor dysfunction and inflammation. Hence, in the first part of this thesis, we explored the role of Ang II as modulator of colonic motility in control animals and in an animal model of IBD. We firstly evaluated different inflammatory markers in the IBD model, in order to characterize the extent and severity of ongoing inflammation, demonstrating the appearance of pathological signs associated to IBD as clinical symptoms (weight loss, diarrhea), macroscopic tissue damage (i.e. evident ulcerations, bowel wall thickening), histological damages and a significant immune infiltrate in the bowel wall. We then compare the effect of Ang II on the colonic mechanical activity in control and inflamed animals, demonstrating that Ang II mediates contraction of enteric colonic smooth muscle both in control and IBD animals, but its contractile effect is reduced in the latter. AT1 receptors were the sole responsible for Ang II-mediated contraction in the control animals, whilst an influence of both AT1 and AT2 receptors have been observed in inflamed animals. Indeed, our experimental data suggest that reduced contraction to Ang II in experimental inflammation could be dependent on the inhibitory action of tonically activated AT2 receptors, counteracting the excitatory effect of

AT1 receptors. In detail, AT2 receptor would induce an increase in nitrergic signaling in the ENS, in turn resulting in a general depression of colonic contractile activity. We speculate that such effect could contribute to the observed reduction in colonic mechanical activity observed in IBD patients, and thus that pharmacological targeting of AT2 receptors would represent a novel strategy in the treatment of motor dysfunctions in IBD.

In the second part of this thesis, we focused on the definition of the role of the GABAergic system in the modulation of colonic mechanical activity in an animal model, as a potential starting point for a consequent exploration of its involvement in GI inflammation. Indeed, different reports have already established the participation of GABAergic pathways in the circuitry of ENS governing GI motility, disclosing its action as neuromodulator causing enteric smooth muscle contraction or relaxation acting on specific $GABA_A$, $GABA_B$ and $GABA_C$ receptors. However, the exact significance of GABAergic modulation of GI function is still not completely clear, especially in the distal part of the GI tract. GABAergic fibers have been suggested to be part of a network of interneurons controlling the release of non adrenergic non cholinergic mediators responsible for smooth muscle relaxation in the peristaltic reflex, as well as to participate in the modulation of acetylcholine (ACh) and substance P (SP) release allowing smooth muscle contraction in peristalsis. Our experimental data added a novel level to the picture of GABAergic pathways in the colon, demonstrating the fine and opposite modulation of cholinergic signaling by differential activation of $GABA_A$ and $GABA_B$ receptors depending on GABA concentration. Low concentrations of GABA (10-50 μM) acting on $GABA_A$ receptors induced circular muscle contraction *via* ACh release from cholinergic motor neurons, in turn promoting *in vitro* peristaltic activity. Interestingly, experiment using the selective $GABA_A$ receptor antagonist bicuculline suggested that a tonic activation of $GABA_A$ receptor occurs in physiological conditions, potentially sustaining peristalsis. Conversely,

higher GABA concentrations (500μM–1mM), caused $GABA_B$-dependent opposite effects, reducing ACh release *via* an indirect pathway involving cholinergic interneurons and thus causing an inhibition of *in vitro* peristaltic activity. The observation of concentration-dependent effect of GABA could result of importance in pathologic conditions characterized by modification in content and functionality of enteric mediators, as reported in Inflammatory Bowel Disease. In addition, a plethora of studies recently linked the GABAergic system to different inflammatory diseases, including rheumatoidis arthritis, encephalomyelitis and dermatitis, exploring its novel role as neuroimmune modulator influencing immune processes as cytokine production, proliferation and maturation *via* GABAergic receptors located on immune cells. Thus, the exploration and comparison of the role of GABA in physiological condition and gastrointestinal disorders, as Inflammatory Bowel Disease, could represent a novel frontier for the definition of its role both in the defective bowel motor patterns in IBD as well as in the modulation of the inflammatory event.

In conclusion, results present in this thesis suggest that the modulation of the colonic mechanical activity by Ang II and GABA would be of impact in the physiopathology of Inflammatory Bowel Disease, and represent a scientific rationale for a broader investigation of the role of these enteric mediators in gastrointestinal inflammation.

SOMMARIO

Le Malattie Infiammatorie Croniche Intestinali (MICI o IBD, Inflammatory Bowel Disease) sono gravi patologie gastrointestinali (GI), comprendenti il morbo di Crohn (CD) e la colite ulcerosa (UC), caratterizzate da una reazione infiammatoria cronica associata a progressivi danni tissutali e una serie di sintomi, che includono disfunzione dell'attività motoria GI, diarrea, sanguinamento rettale, malassorbimento, perdita di peso. Diversi fattori contribuiscono alla fisiopatologia delle IBD, compresa la suscettibilità genetica, fattori ambientali, aumento della permeabilità intestinale e, soprattutto, il verificarsi di una reazione immunitaria anomala del Tessuto Linfoide Associato all'Intestino (GALT) contro antigeni "innocui" derivanti dal microbiota intestinale. Data la complessa interazione di questi diversi fattori, la causa esatta delle Malattie Infiammatorie Intestinali è ancora sconosciuta, e il loro trattamento rappresenta tutt'oggi un problema clinico, dal momento che che le attuali strategie terapeutiche sono limitate a classici farmaci anti-infiammatori che non conducono alla risoluzione completa della patologia e sono spesso associati ad effetti collaterali. Su queste basi, la ricerca di nuovi fattori e meccanismi coinvolti nella fisiopatologia delle IBD risulta assolutamente necessaria per il miglioramento delle attuali terapie disponibili. Recentemente, diverse ricerche hanno comprovato il potenziale coinvolgimento nelle IBD di mediatori del sistema nervoso enterico (SNE), la complessa rete neuronale che controlla la maggior parte delle funzioni intestinali, dimostrando che il cambiamento nel contributo e ruolo di diversi mediatori enterici potrebbe portare sia alle alterazioni patologiche della motilità GI nelle IBD, nonché alla modulazione dell'evento infiammatorio locale, controllando l'attività delle cellule immunitarie del GALT.

Recentemente l'angiotensina II (Ang II) e l'acido γ-aminobutirrico (GABA) sono stati riconosciuti come nuovi mediatori coinvolti nella modulazione della motilità gastrointestinale, e, inoltre, altri studi ne hanno evidenziato la connessione con differenti

condizioni infiammatorie, suggerendo che una ricerca approfondita sul ruolo di tali mediatori potrebbe condurre alla loro identificazione come nuovi target terapeutici in condizioni infiammatorie, tra cui le Malattie Infiammatorie Croniche Intestinali.

L'Angiotensina II, principale effettore del sistema renina-angiotensina (RAS), è stata precedentemente identificata come modulatore dell'assorbimento di acqua/elettroliti, del trasporto del glucosio e della secrezione di bicarbonato nel microambiente intestinale, tramite la sua azione sui recettori specifici AT1 e AT2. Tuttavia, diversi studi stanno dimostrando sempre più nel dettaglio il suo ruolo emergente nella modulazione della motilità intestinale, capace di indurre contrazione della muscolatura liscia enterica nel piccolo e grande intestino, in particolare attraverso la modulazione del segnale tachichinergico enterico. Inoltre, una serie di studi recenti ha sottolineato il collegamento tra Ang II e infiammazione gastrointestinale, dato che i livelli intestinali di Ang II sono più elevati nei pazienti IBD e antagonisti dei recettori dell'angiotensina (in particolare AT1) inducono un miglioramento della condizione infiammatoria in modelli animali di IBD. Nuovi studi sul ruolo dell'Ang II in condizioni fisiologiche e patologiche potrebbero dunque rivelare il suo potenziale come target terapeutico per il trattamento delle IBD, sia nella disfunzione motoria associata alle IBD sia nell'evento infiammatorio.

Nella prima parte di questa tesi, il ruolo dell'Ang II come modulatore della motilità del colon è stato esplorato in animali controllo e in un modello animale di IBD. Inizialmente, sono stati valutati differenti marker infiammatori nel modello IBD, in modo da caratterizzare la severità dell'infiammazione in corso, dimostrando la comparsa di segni patologici associati alle IBD quali sintomi clinici (perdita di peso, diarrea), danno tissutale macroscopico (ulcerazioni evidenti, ispessimento della parete intestinale), danni istologici e una infiltrazione significativa di cellule immunitarie nel tessuto intestinale. Abbiamo quindi comparato gli effetti dell'Ang II sull'attività meccanica del colon in animali

controllo e infiammati. Inizialmente, è stato osservato che l'Ang II media contrazione della muscolatura liscia del colon sia negli animali controllo e infiammati, ma il suo effetto contrattile risulta ridotto in questi ultimi. I recettori AT1 sono risultati i soli responsabili della contrazione indotta dall'Ang II negli animali controllo, mentre l'influenza sia dei recettori AT1 e AT2 è stata evidenziata negli animali infiammati. Infatti, i nostri dati sperimentali suggeriscono che la riduzione della contrazione indotta dall'Ang II nel corso dell'infiammazione sperimentale potrebbe dipendere dall'azione inibitoria dei recettori AT2 i quali, tonicamente attivati, contrasterebbero l'effetto eccitatorio dei recettori AT1. Nel dettaglio, i recettori AT2 sarebbero associati ad un aumento del segnale nitrergico nel sistema nervoso enterico, con conseguente inibizione dell'attività contrattile del colon. È possibile ipotizzare che tale effetto osservato potrebbe contribuire alla riduzione dell'attività meccanica colon osservata nei pazienti IBD, e che dunque un trattamento farmacologico mirato sui recettori AT2 potrebbe rappresentare una nuova strategia terapeutica per le disfunzioni motorie nelle IBD.

Nella seconda parte di questa tesi, ci siamo concentrati sulla definizione del ruolo del sistema GABAergico nella modulazione dell'attività meccanica del colon in un modello animale, come potenziale punto di partenza per la caratterizzazione del suo potenziale coinvolgimento nel contesto dell'infiammazione intestinale. Diversi studi hanno già suggerito la partecipazione di pathway GABAergici nei circuiti del SNE responsabili della regolazione della motilità gastrointestinale, rivelando la sua azione come neuromodulatore causante contrazione o rilasciamento del muscolo liscio enterico mediante attivazione dei recettori specifici $GABA_A$, $GABA_B$ e $GABA_C$. Nonostante ciò, l'esatto contributo del sistema GABAergico della modulazione delle funzioni GI risulta ancora non completamente chiarita, soprattutto nella parte distale del tratto GI. Fibre neurali GABAergiche farebbero parte di una rete di interneuroni che controllerebbero il rilascio di

mediatori non adrenergici non colinergici responsabili del rilasciamento del muscolo liscio enterico nel riflesso peristaltico, e inoltre parteciperebbero nella modulazione del rilascio di acetilcolina (ACh) e sostanza P (SP) necessari per la contrazione della muscolatura liscia nella peristalsi. I nostri dati sperimentali aggiungono un nuovo livello nel quadro dei meccanismi GABAergici nel colon, dimostrando la capacità del GABA di modulare il segnale colinergico attraverso l'attivazione differenziale di recettori $GABA_A$ o $GABA_B$ in funzione della concentrazione di GABA. Basse concentrazioni di GABA (10-50 μM) agirebbero sui recettori $GABA_A$, inducendo contrazione del muscolo liscio circolare del colon attraverso il rilascio di ACh da motoneuroni colinergici, promuovendo in ultimo l'attività peristaltica indotta *in vitro*. Da notare che i dati sugli effetti dell'antagonista selettivo del recettore $GABA_A$, bicucullina, suggerirebbero una attivazione tonica dei recettori $GABA_A$ in condizioni fisiologiche, potenzialmente associata al sostenimento dell'attività peristaltica. Al contrario, concentrazioni più elevate di GABA (500μM - 1mM), causavano effetti opposti dipendenti dall'attivazione dei recettori $GABA_B$, riducendo il rilascio di ACh tramite un pathway neurale indiretto che coinvolge interneuroni colinergici, e provocando così inibizione della attività peristaltica *in vitro*. L'osservazione di un effetto concentrazione-dipendente del GABA potrebbe rivelarsi cruciale in condizioni patologiche caratterizzate da cambiamenti nei livelli e nella funzionalità di mediatori enterici, come riportato nelle Malattie Infiammatorie Croniche Intestinali. Inoltre, diversi studi hanno recentemente associato il sistema GABAergico a diverse condizioni infiammatorie, tra cui artrite reumatoide, encefalomielite e dermatite, esplorando il suo ruolo come modulatore neuroimmunitario capace di influenzare processi quali produzione di citochine, proliferazione e maturazione, attraverso recettori GABAergici situati sulle cellule immunitarie. Dunque, la ricerca e il confronto del ruolo del GABA in condizioni fisiologiche e patologiche gastrointestinali, quali le IBD, potrebbe

condurre alla definizione del suo ruolo sia nelle modificazioni patologiche dell'attività motoria intestinale nelle IBD che nella modulazione dell'evento infiammatorio correlato a queste patologie. In conclusione, i risultati esposti in questa tesi suggeriscono che la modulazione dell'attività meccanica intestinale da parte dell'Ang II e del GABA potrebbe contribuire alla fisiopatologia delle Malattie Infiammatorie Croniche Intestinali, e rappresentare una base razionale per un'indagine più ampia del ruolo di questi mediatori enterici nel contesto dell'infiammazione intestinale.

CHAPTER 1

Inflammatory Bowel Disease: pathogenesis, treatment and animal models

Inflammatory bowel diseases (IBD) are a group of chronic inflammatory disorders affecting the gastrointestinal (GI) tract, including Crohn's Disease (CD) and Ulcerative Colitis (UC). The etiology of these disorders is not yet entirely clear due to their multifactorial origin, with an interplay of contributing factors including genetic susceptibility, immune dysfunctions, environmental factors (i.e. infections, lifestyle) (Xavier & Podolsky, 2007; Fig.1). IBD have a peak onset between 15 and 30 years of age, although they can occur at any age. Both UC and CD have a bimodal distribution of age, with a second smaller peak that occurs in individuals aged between 50 and 70 years of age (Andres et al, 1999). Males and females are equally affected, although ulcerative colitis is slightly more common in males, while Crohn's disease is slightly more common in women (Loftus et al., 2003, Andres et al., 1999). IBD patients display a chronic GI inflammation with periodic flare-ups characterized by severe symptoms as rectal bleeding, severe diarrhea, abdominal pain, fever and weight loss.

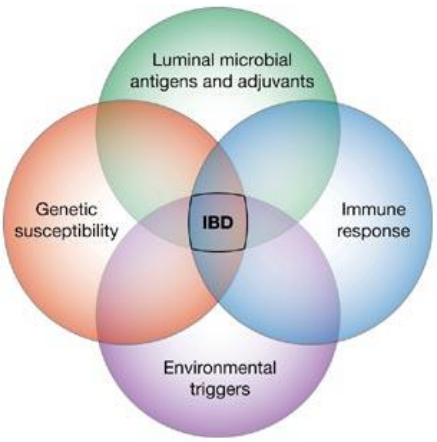

Figure 1: Multiple factors involved in IBD pathogenesis

The histological examination of biopsies obtained from patients with active disease reveals the presence of a large number of leukocytes, such as polymorphonuclear leukocytes, lymphocytes and monocytes within the intestinal wall. Coinciding with this inflammatory infiltrate, extensive intestinal lesions including ulcerations, edema, loss of goblet cells, bowel wall thickening occur (Hendrickson et al., 2002), progressively affecting the GI functions. Of note, patients with mild to moderate IBD displayed dysfunctions in intestinal motility, as a reduction of spontaneous contractions (Koch et al., 1988) and variations in the colonic transit (Reddy et al., 1991), even in phase of remission of the pathology. IBD-related motor dysfunctions importantly contribute to morbidity and quality of life issues of patients and, interestingly, several symptoms of IBD, including diarrhoea, malabsorption and weight loss, could be attributed to the alteration of motor function of the intestine (De Schepper et al., 2008). Changes in the neural signaling within the enteric nervous system (ENS), the semi-autonomous neuronal network primarily involved in the regulation of GI motility, together with enteric smooth muscle cells damage have been suggested as contributing factors in IBD-associated dysmotility (De Schepper et al., 2008; Mawe et al., 2015), although to date the exact cause of motor disorders in IBD are largely unclear.

Regarding the different factors involved in IBD physiopathology, genetics play a role in susceptibility as proven by epidemiological studies addressing IBD as genetically complex disorders (Hanauer, 2006). Several susceptible sites potentially associated with CD or UC have been identified. In particular, the gene NOD2 (nucleotide-binding oligomerization domain 2), also known as CARD15 (Caspase Activation and Recruitment Domain). was the first gene to be clearly associated with IBD (Hugot et al., 2001) and more than 60 mutations have been also identified, 3 of which have been linked to the development of Crohn's disease (Rowe, 2005). The product of this gene is a cytoplasmatic protein involved in the innate immune system, with a role in recognizing bacterial products; its mutation

would result in an altered intracellular processing of the bacterial products and consequently alteration in the immune response (Shih et al., 2008). Another factor contributing to the physiopathology of IBD is a defect in the intestinal mucosal barrier function, leading to increased permeability (Baumgart et al., 2007) associated with a greater adherence of the bacteria. In turn, abnormal exposure of enteric immune system to bacterial products may result in aberrant immune activation and inflammation (Peyrin-Biroulet et al., 2008; Sartor, 2008; Shih et al., 2008).

Accordingly, increasing evidence have demonstrated the disturbance in physiologic immune response in the gut as the most important factor in IBD, revealing an aberrant immune response to the autologous commensal microbiota as the major mechanism governing the sustained inflammation in IBD. The GI tract hosts the largest immune system in the body. The Gut-Associated Lymphoid Tissue (GALT) constituted a highly complex and fine regulated network, essential to properly face the continuous antigenic stimulation occurring in the GI tract (Koboziev et al., 2010; Fig.2). The immune activity within the GALT is essential to maintain tolerance to "harmless" antigens in the enteric milieu, i.e. food antigens and commensal microbiota antigens, as well as to ensure a proper immune response to the "harmful" antigens deriving from pathogens. Briefly, in absence of an infection, immune homeostasis is assured by a specialized enteric population of DCs ($CD103^+$ DCs) capable of inducing the differentiation of immunosuppressive T Regulatory cells (TRegs); TRegs activity sustains the tolerogenic state in the GALT avoiding the establishment of an improper inflammatory response to harmless stimuli mediated by aberrant activation of T helper lymphocytes (Thelper 1, Thelper 17, Thelper 2; Th1,Th17, Th2), primarily *via* secretion of immunosuppressive cytokines as Transforming Growth Factor (TGF)-β and IL-10 (Nutsch et al., 2012). On the contrary, a pathogenic infection triggers populations of DCs to orchestrate an immune response activating effector T-cells,

including Th1, Th2 and Th17 cells (Janeway et al., 2001), which release pro-inflammatory cytokines as TNF-α, IFN-γ, IL-5, IL-17. Hence, defects in these regulatory mechanisms could result in the occurrence of the chronic inflammatory event in IBD, which is accordingly associated with an improper T-cell over-reaction and a pathological and chronic increase in pro-inflammatory mediators, as TNF-α, IFN-γ and IL-5 (Strober & Fuss, 2011).

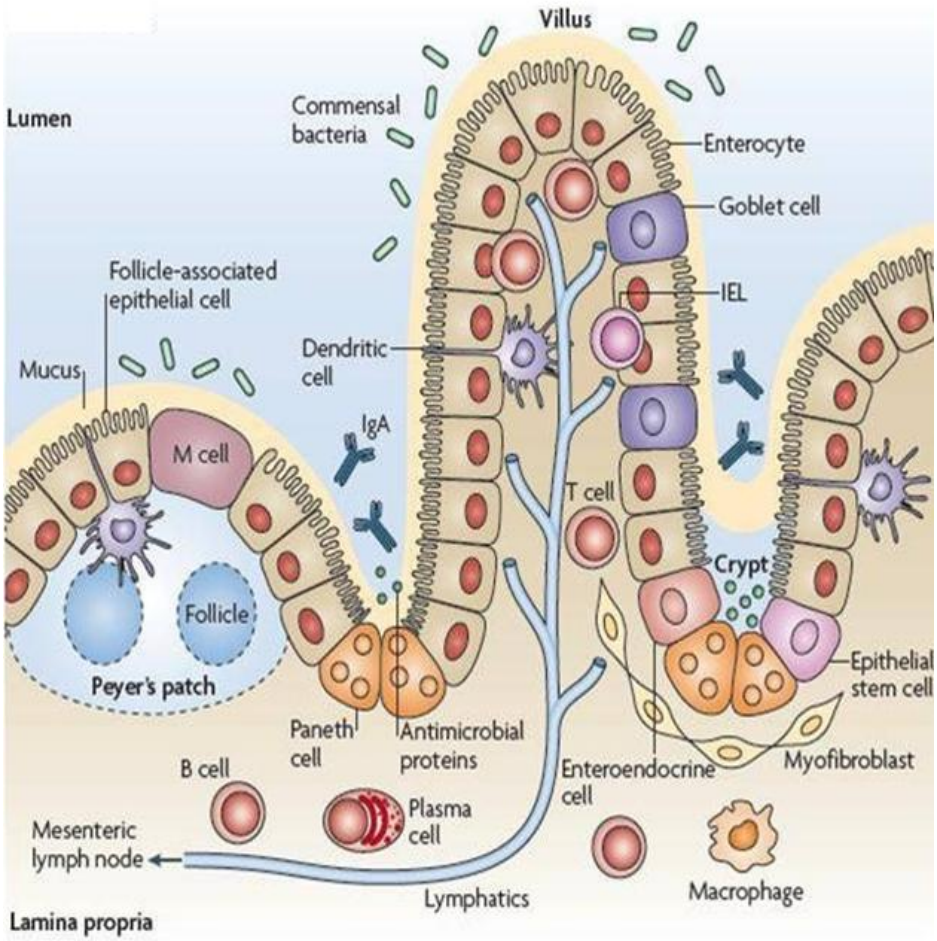

Figure 2: Schematic representation of the Gut-Associated Lymphoid Tissue.
Abbreviations: IEL: intraepithelial lymphocytes; IgA: Immunoglobulin A
(Adapted from Pereira et al., 2014)

The cell-mediated immune response occurring in IBD can follow two different pathways, characterizing the two forms of the disease (Fig. 3): a Th1 response associated with Crohn's disease, or a Th2 response associated with Ulcerative Colitis (Podolsky, 2002;

Fig.3). The activation of naive T cells is promoted by Antigen-presenting Cells (APCs), such as dendritic cells and macrophages, and the cytokines signaling influences the switching to Th1 or Th2 (Abreu, 2002). Overproduction of IL-12 shifts the immune response toward Th1 in Crohn's Disease. This response is characterized by an increased secretion of interferon-γ, TNF-α, IL-1β, and IL-6 (Abreu, 2002; Bouma et al., 2003), leading to transmural inflammation and tissue damages. On the other hand, activation of Th2 cells is associated with increased secretion of IL-4, IL-5, IL-10 and IL-13, associated with a mucosal inflammation. However, ulcerative colitis was recently linked with a pathological activity of a population of Natural Killer T cells, adding a novel layer of complexity to the exact definition of the Th2 response in UC (Strober & Fuss, 2011). It has been also suggested that inflammation in IBD may be due to a lack of TRegs (Boehm et al., 2012), leading to severe loss of tolerance to the commensal microbiota antigens, resulting in proliferation of immune cells and cytokine production. Indeed, IL-10 knockout mice spontaneously develop colitis, and treatment with TGF-β and IL-10 heals experimental colitis in animal model (Abreu, 2002).

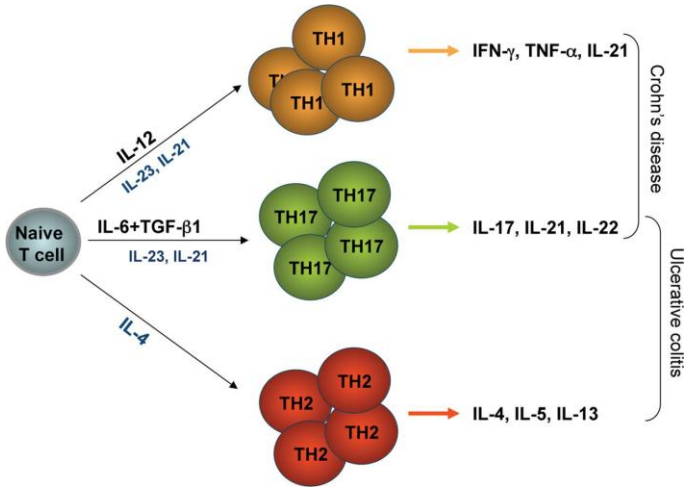

Figure 3: T helper cells and related cytokines involved in the physiopathology of Inflammatory Bowel Disease.

Currently there is no effective cure for IBD, and available therapy aims to subside chronic inflammation mainly *via* the use of classic anti-inflammatory drugs as 5-aminosalicylic acid and immunomodulators such as azathioprine, mercaptopurine, methotrexate (Randall et al., 2015). Also, monoclonal antibodies Infliximab and Adalimumab targeting TNF-α have reported to maintain remission of the symptoms in IBD patients (Furfaro et al., 2015). However, most of drugs for IBD treatment are temporarily effective and associated with different side effects, especially during long-term treatment, including bone marrow defects, liver toxicity, pancreatitis, opportunistic infections and lymphomas (Triantafillidis et al., 2011). Also, besides of drugs directly contrasting the inflammatory event, treatment of gastrointestinal motor disorders in IBD patients are largely neglected. As aforementioned, although intestinal motor dysfunctions are likely important contributors in IBD symptomatology, few researches addressed the exact cause of dysmotility in IBD, and treatment of motor abnormalities solely include classical drugs employed in the therapy for functional GI disorders (i.e. prokinetics). The definition of a strategy targeting the specific pathways underlying GI aberrant motor patterns in IBD, likely involving changes in the action of enteric mediators within the neuronal network of ENS, is almost overlooked (De Schepper et al., 2008). Thus, the identification of novel players in the physiopathology of IBD controlling both immune activity and intestinal motor defects could lead to the development of new effective therapies for IBD.

Over the years, the development of different experimental models of IBD has allowed a closer investigation of the early events, interactions between different components and identification of major immunological pathways in IBD (Kiesler et al., 2015; Fig.4). Animal models have increasingly provided important information for understanding the multifaceted inflammatory mechanism characterizing these pathologies, and represent important tools for the definition of novel therapeutic strategies. In general, an ideal animal

model of IBD should present some key features: the gut should have morphological alterations, inflammation, symptoms and signs, pathophysiology and clinical course similar or identical to those found in humans. However, this is rarely possible due to the complexity of the disease and the high genetic and environmental influences that determine a high variability in its onset and clinical course. Thus, no single model seems able to mimic the complexity of human IBD, but each model provides valuable insights into one or another major aspect of disease. Animal models of IBD are generally divided into four main classes (Mizoguchi, 2012): 1) genetically engineered models, especially mice with deletion of specific genes representing key IBD susceptibility genes (i.e. IL-10 knockout mice); 2) spontaneous-developing colitis models, as the C3H/HeJBir mouse; 3) chemically-induced models, containing numerous models in which colitis is induced by administration of a compound, as trinitrobenzenesulfonic acid (TNBS), dinitrobenzensulfonic acid (DNBS), dextran sulfate sodium (DSS), oxalozone; 4) the adoptive cell transfer models in immunocompromised animals, where transfer of naive T lymphocytes into T and B cell deficient mice induces severe colonic inflammation in the recipient, resembling IBD-like lesions.

Adoptive transfer models have provided numerous information to understand the adaptive immune mechanism involved in the pathogenesis of IBD. TNBS model has been used to assess and develop anti-IL-12p40 therapy that is currently applying to human IBD, and IL-10 knockout model has provided significant contributions for the role of probiotics in IBD (Sheil et al., 2006). Also, DSS model has been useful for dissecting the mechanism of inflammation-associated epithelial barrier dysfunction. Noticeably, recently, DNBS model have been used for investigation of the pathophysiology of intestinal motor dysfunctions in IBD (Antonioli et al., 2014a,b). Thus, the appropriate choice of a specific IBD model in experimental research represents a fundamental starting point to perform an accurate

analysis of novel therapeutic strategies addressing one or more of the multiple pathologic mechanisms associated with IBD (Fig.4). The combination of novel researches on animals model of IBD and patients would result in a deeper knowledge about innovative factors and pathways in IBD, in order to effectively understand the multifaceted etiology of these disorders.

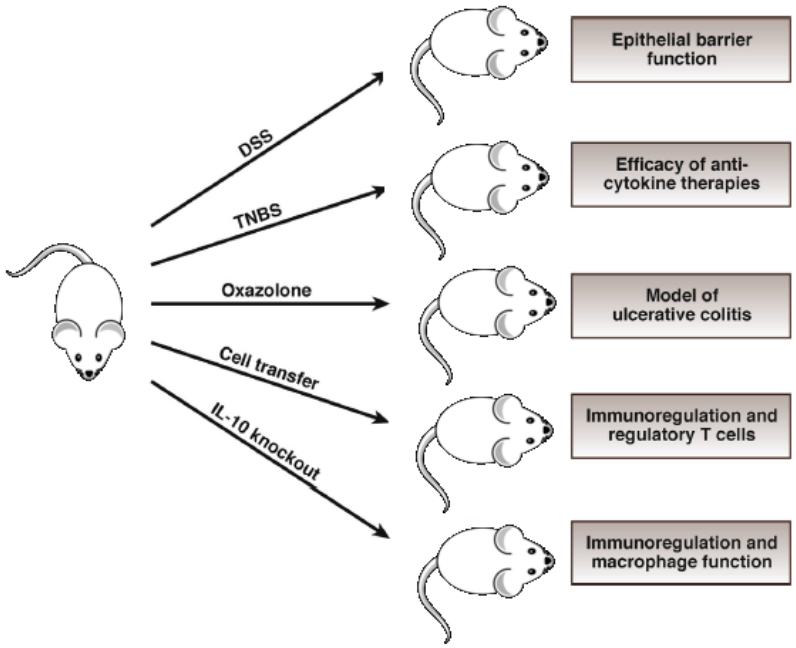

Figure 4: Schematic representation of main animal models of Inflammatory Bowel Disease, accompanied by major mucosal immune functions/therapies that are best addressed using these respective models.

CHAPTER 2

The enteric nervous system and Inflammatory Bowel Disease: Role of enteric mediators on bowel dysmotility and inflammation

Gastrointestinal (GI) functions, including secretion, regulation of local blood flow and motility are under the control of the enteric nervous system (ENS), a composite neuronal network within the gut wall, responsible for a semi-autonomous regulation of GI activity. The ENS is extended from the oesophagus to the rectum, and it is organized into two major plexi, the submucosal (Meissner's) plexus and the myenteric (Auerbach's) plexus (Goyal & Hirano, 1996) (Fig.5). Each plexus contains neuronal cells and enteric glial cells; neuronal cell bodies are clustered in ganglia, with axonal projection connecting the ganglia and innervating effectors, including smooth muscle cells, endocrine glands and vasculature. The submucosal plexus is deputed to the regulation of GI absorption, secretion and blood flow, whilst the Auerbach's plexus is primarily implicated in the fine modulation of GI motility, and it is located between the circular and longitudinal muscle layers of the gut wall (Johnson et al., 2012). Interestingly, 2/3 of ENS neurons are hosted in the myenteric ganglia, suggesting the great complexity of enteric circuitry modulating GI motility (Furness, 1987). Additionally, a mucosal plexus extending to the lamina propria and epithelium has been also reported, since ongoing studies provide evidence for a potential involvement of the ENS in modulating diverse mucosal functions as well as the function of immune cells belonging to the GALT (Rescigno et al., 2008; Genton & Kudsk, 2003). The ENS can autonomously ensure proper GI function through its complex organization including sensory neurons (intrinsic primary afferent neurons: IPANs), interneurons and motoneurons, realizing functional circuitries capable of generating stereotyped behaviours patterns, especially secretory and motor patterns as the peristaltic reflex (Hansen, 2003) However, a connection of the ENS with the central nervous system (CNS) is ensured by

extrinsic innervation, since both the sympathetic and the parasympathetic system influence the ENS activity ("the brain-gut axis") (Fig.5): sympathetic noradrenergic fibers inhibiting GI activity, and parasympathetic vagal and sacral fibers usually promoting GI function (Phillips & Powley, 2007). Also, extrinsic vagal and spinal afferents supply the CNS with information about gut discomfort or pain, electrolyte homeostasis and tissue integrity.

Given its complexity and a number of neuronal cells similar to the brain and the spinal cord, the ENS has been classically considered as the "the second brain" (Gershon, 1998). Such definition is sustained by the large number of different neurotransmitters (NTs) and mediators involved in ENS circuitry, including acetylcholine (ACh), serotonin (5-hydroxytryptamine: 5-HT), vasoactive intestinal peptide (VIP), tachykinins, calcitonin gene related peptide (cGRP), neuropeptide Y (NPY), nitric oxide (NO), pituitary adenylate cyclase-activating polypeptide (PACAP), somatostatin, encephalin and purines, as ATP and adenosine (Furness, 1994). Research efforts have successfully clarified the role of different ENS mediators, identifying for instance NTs released by excitatory motoneurons, including ACh and tachykinins, or by inhibitory motoneurons, including VIP, NO, ATP, PACAP. In addition, enteric sensory neurons are reported to mainly utilize ACh, tachykinins and cGRP. However, even more important is the great series of mediators released by interneurons, as ACh, NO, somatostatin, encephalin and many other, capable of opportunely modulate the activity of other enteric neurons in order to realize the physiologic neural integration required for a coordinated and proficient GI function (Bornstein et al., 2004).

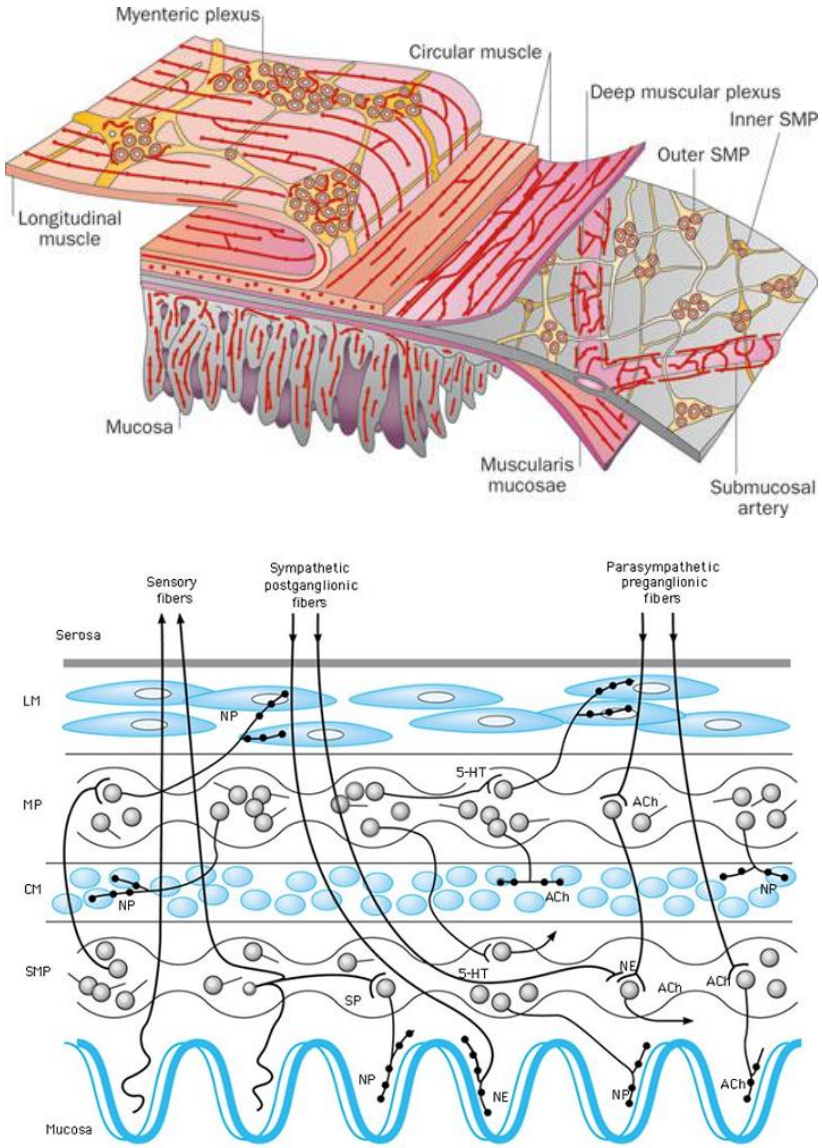

Figure 5: (Top) Organization of plexi of the Enteric Nervous System within the intestinal wall. **(Bottom):** Detail of some of the circuitry of the Enteric Nervous System, and its connections with sympathetic and parasympathetic systems.
Abbreviations: 5-HT, 5-hydroxytryptamine; ACh, Acetylcholine; CM, Circular Muscle; LM, Longitudinal Muscle; MP, Myenteric Plexus; NE, Norepinephrine; NP, Neuropeptides; SMP, Submucosal Plexus.

Different studies have reported the pathological changes affecting ENS during IBD, mostly resulting from action of inflammatory mediators as cytokines, arachidonic acid-derived metabolites and oxygen free radicals (Lomax et al, 2005). Major structural changes in the ENS include changes in the plexus architecture, hypertrophy and hyperplasia of the neural fibers and alterations of the cell body of neurons and enteric glial cells. Studies conducted in patients with CD, UC and control subjects showed that nerve fibers in the ileum and

colon appear dilated, empty, sometimes with large vacuoles in the vicinity of the cell membrane. These structural abnormalities are related to axonal damage and necrosis (Geboes & Collins, 1998). Immunohistochemical studies in patients suffering from Crohn's disease revealed an increase of neurons positive for the nitric oxide synthase (NOS) and vasoactive intestinal peptide (VIP), primarily suggesting the possibility of disturbed neural modulation of smooth muscle relaxation in the inflamed intestine (Geboes & Collins, 1998). Indeed, novel studies have underlined that structural changes in the ENS during IBD are likely accompanied with functional changes, including changes in the electrical properties and excitability of enteric neurons and modification in the contribution and role of the different enteric mediators constituting the network of the ENS (Mawe, 2015; Lakhan & Kirchgessner, 2010). Thus, both structural and functional pathological changes in the ENS could be responsible for the reported disturbance in GI motility in IBD patients, together with defects in enteric smooth muscle cells (Ohama et al., 2007). However, although the aberrant GI motility in IBD could prominently contribute to different major symptoms including diarrhoea, weight loss and malnutrition, the consequences of the IBD-related inflammatory event on GI motility have been currently largely overlooked. Studies in IBD patients and animal model of IBD reported that motility pattern is predominantly altered towards a diarrheic state, with a pathologic increase in stool frequency. In the colon, suppression of colonic Rhythmic Phasic Contractions (RPCs) and increased frequency of Giant Migrating Contractions (GMCs) have been reported (Sarna, 2010). Overall, a reduced colonic contractility and the decrease in spontaneous contractions seems mainly result in a diminished resistance to luminal transit, which, in combination with altered secretory activity, could thus lead to the observed diarroic condition. Of importance, dysmotility in IBD is not limited to the distal part of the GI tract; indeed, small intestinal contractility seems to be enhanced in human CD (Vermillion et al., 1993), whilst

defective gastric emptying was also observed (Kristinsson et al., 2007) (Table 1). Thus, it is believed that inflammation could modulate the whole ENS circuitry, inducing pathologic GI motor behaviour pattern along the entire GI tract. Pathological changes in motility thus exacerbate IBD by promoting diarrhea and generally impairing digestive function, likely leading to reduced nutrients absorption. Intriguingly, motility disturbance have been reported even during clinical remission in IBD, suggesting the existence of pathological long-lasting changes in bowel patterns (Bassotti et al., 2014).

Recent studies underlined the effective possibility that motility disturbance could derive from the action of different cytokines, major mediator of inflammation, on the gut neuromuscular apparatus. As an example, in the 2,4,6-trinitrobenzenesulfonic acid (TNBS) murine model of IBD, decreased contractility was attributable to the action of different cytokines, such as IL-1β, TNF-α and IL-12 (Kinoshita et al., 2006; Kisoyue et al., 2006). However, as aforementioned, accumulating evidence pointed out the effective importance of changes in expression of enteric mediators and neurotransmitters, as well as of their specific receptors, in the IBD-associated dysmotility. An increased number of serotonin (5-HT)-producing enterochromaffin cells (ECCs) has been revealed in animal models of IBD and IBD patients (Oshima et al., 1999; El-Salhy et al., 1997); 5-HT is strongly involved in the modulation of vagal afferent signaling stimulating motor reflexes, as well as within the circuitry of the ENS as trigger of intestinal peristalsis (Grider et al., 1996). Thus, changes in 5-HT levels in IBD could be associated with the disturbance in GI motility. Although increased 5-HT signaling in IBD would be expected to promote motility, experimental evidence have shown that increased 5-HT could paradoxically result in inhibition of propulsive activity, as a consequence of receptor desensitization (Linden et al., 2003).

In addition, increased levels of the enteric mediator substance P (SP), belonging to the family of tachykinins peptides and largely involved in the regulation of excitatory

signaling in the ENS, have been reported in the colonic tissues of IBD patients, accompanied by an increased expression of its specific receptor NK-1 (Renzi et al., 2000). Actually, a shift from a mainly cholinergic to a SP innervation in IBD has been observed, and the density of SP nerve fibers seem to correlate with the severity of IBD (Bernstein et al., 1993). Apart from the potential misbalanced neural excitatory signaling in the ENS due to the increased contribution of SP, a plethora of studies reported that SP is also able to stimulate cytokines production from endothelial cells, macrophages and mast cells (O'Connor et al., 2004), thus potentially contributing to the sustainment of inflammation and cytokine-induced motility dysfunction. Another series of researches underlined the increased Vasoactive Intestinal Peptide (VIP) concentration in colonic biopsies from CD patients as well as in colonic tissues isolated from animal model of IBD (Todorovic et al., 1996), as guinea-pig TNBS model (Linden et al., 2005) and dextran sulfate sodium (DSS) model in rat (Kishimoto et al., 1992). An increased VIP innervation was also observed (Bishop et al., 1980). VIP is an enteric mediator involved in the modulation of secretory responses and inhibitory motor neurons, mediating relaxation of intestinal smooth muscle cells together with other mediators as NO and PACAP. Intriguingly, the prior observation of augmented VIP-containing nerves in IBD patients was later accompanied by the demonstration of increased nitric oxide synthase and PACAP immunoreactivity (Belai et al., 1997), suggesting the possibility of an overall pathologic potentiation of inhibitory signaling in the ENS during inflammation. Accordingly, an increased non adrenergic non cholinergic innervation associated with impaired contractility have been proposed as a major responsible for GI dysmotility in UC (Tomita et al., 1998). Apart from this and other pioneer observations, the exact and specific contribution of the large number of enteric mediators in the physiopathology of defective intestinal motor activity is still a matter of investigation, and novel researches are needed to unravel the precise mechanisms

underlying the dysmotility, even in a translational perspective aiming to identify specific treatment for the IBD-associated gut motility dysfunction. Indeed, most of current therapeutic strategies for dysmotility in IBD have been extrapolated from general treatment of dysmotility in functional gastrointestinal disorders, overlooking the development of a therapy specifically targeting the effective pathways involved in the dysfunction.

Table 1. Intestinal motility disturbances described in patients with IBD

LOCAL

Colon:

- Decreased contractility (Snape et al., 1991)
- Reduction in spontaneous contractions (Boyer et al., 1997)
- Variation in colonic transit (Reddy et al., 1991)

Small intestine:

- Enhanced contractility (Vermillion et al., 1993)
- Increased orocaecal transit time (Tursi et al., 2003)

REMOTE

- Decreased gastric emptying in CD (Kohno et al., 2007)
- Gastroparesis in patients with inactive CD (Kristinsson et al., 2007)

Abbreviations: CD, Crohn's Disease; IBD, Inflammatory Bowel Disease.

However, the involvement of enteric mediators in the physiopathology of IBD could not be limited to the changes in bowel motor patterns; indeed, a series of recent studies have underlined the concept of the ENS as a source of a large amount of compounds potentially acting on immune cells and thus involved in the control of both physiologic immune response and GI inflammation, focusing their attention on the importance of the "neuroimmune dialogue" between the ENS and the intestinal mucosal immune system

(Genton et al., 2003). Previous studies identified neural mediators as a novel category of compounds capable of regulating immunity (Pacheco et al., 2012) (Fig.6). Immune cells possess different receptors for neurotransmitters, functionally coupled to the modulation of classic inflammatory processes including cytokine production, proliferation, chemotaxis, phagocytosis (Holzmann et al., 2012). As an example, dopamine (DA) has been reported to act both on dendritic cells (DCs) and T cells, with a pro- or anti-inflammatory role depending on its concentration and receptor subtype expressed (Pacheco et al., 2014). Also, a cholinergic anti-inflammatory pathway (CAIP) has been widely explored: in an event of uncontrolled inflammation, elevated levels of pro-inflammatory cytokines detected in the brain trigger a vagal-mediated reflex, resulting in an increase of ACh release in the spleen. In turn, ACh dampens cytokine production from splenic macrophages *via* activation of nicotinic α7 receptors (Tracey et al. 2007), preventing the pathologic consequences of uncontrolled inflammation.

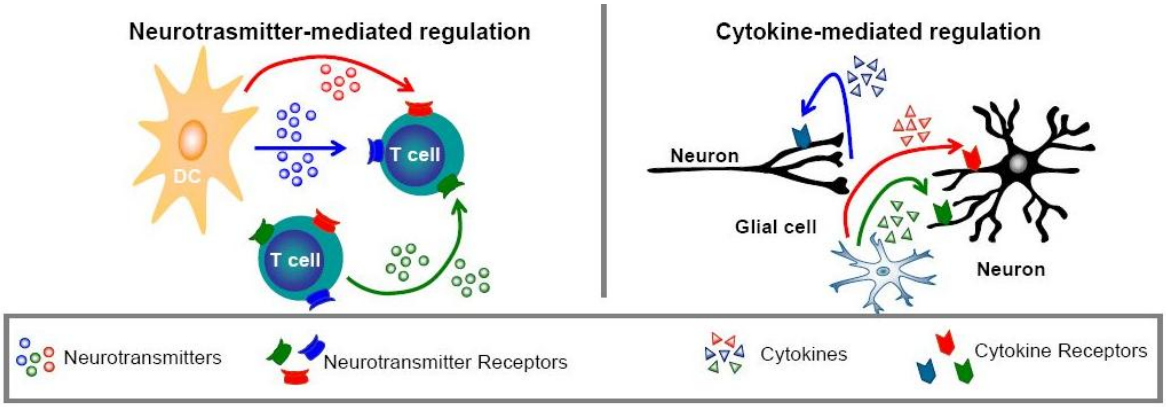

Figure 6: Novel view regarding common regulation of immune cells and neuronal cells activity by neurotransmitters and cytokines . Neurotransmitter-mediated regulation of immune cells (sx) and cytokine-mediated regulation of neuronal and glial cells (dx). Abbreviations: DC, Dendritic Cell.
(Adapted from Pacheco et al., 2012)

In 2014, Matteoli et al. extended the concept of CAIP, demonstrating the possibility of a vagal-dependent dampening of intestinal inflammation *via* an ENS-mediated control of enteric macrophages function. Indeed, the network of contacts between the ENS and the

intestinal immune cells constituting the GALT (Rescigno et al., 2008) effectively supports the existence of mechanisms of neuroimmune modulation even in the gut, suggesting the possibility of an effective functional cooperation between these two systems. In this context, a lot of studies reported major pro- or anti-inflammatory effects of enteric neuropeptides, as SP and neuropeptide Y (NPY) (Margolis & Gershon, 2009); SP induces cytokine production from immune cells via activation of NK-1 receptors (Derocq et al., 1996), and, accordingly, NK-1 antagonists were found to ameliorate inflammation in IBD animal model (Ursino et al., 2009). NPY could have a pro-inflammatory role modulating immune cells activity, and accordingly NPY knockout mice have been found to be resistant to DSS-induced colitis (Chandrasekharan et al., 2008). Also, a complex modulatory role of enteric purinergic mediators in the intestinal inflammatory event has been described (Antonioli et al., 2008), and serotonin deficiency results in amelioration of experimental gut inflammation (Ghia et al., 2009). Hence, unravelling the specific roles of the different ENS mediators on immune cells could open the way to a novel strategy for the treatment of GI inflammatory states, targeting the "neuro-immune dialogue" in the gut.

Overall, it is becoming increasingly clear that a more accurate characterization of the role of the ENS and enteric mediators could result in important novel findings clarifying the complex physiopathology of IBD, as well as in providing a major scientific rationale for improving the current therapeutic strategy for IBD, both targeting the inflammatory event and the GI mechanical dysfunction.

CHAPTER 3

Angiotensin II and the renin-angiotensin system (RAS)

Angiotensin II (Ang II) represents the major effector of the renin-angiotensin system (RAS), the principal modulatory system involved in the fine regulation of systemic blood pressure, as well as homeostasis of body fluids and electrolytes (Atlas, 2007) (Fig.7). Stimuli as low pressure detected by baroreceptors present in the juxtaglomerular cells of the renal arterioles, but also hyponatremia detected by cells of the macula densa in the distal tubule, trigger the initiation of the classic RAS pathway, *via* the first event of production and blood release of the enzyme renin by the renal juxtaglomerular apparatus. In turn, renin proteolyzes the angiotensigen, produced and released into the bloodstream by the liver. The product of this reaction is angiotensin I, a decapeptide, that by the action of the angiotensin-converting enzyme (ACE) loses two amino acids giving rise to the octapeptide Angiotensin II (Ang II). Ang II causes vasoconstriction and regulates blood pressure both directly, by modulating the absorption of water and sodium in the kidneys, and indirectly by stimulating the production and release of aldosterone from the adrenal glands, or centrally stimulating thirst pathways (Lavoie & Sigmund, 2003).

Apart from the regulation of blood pressure and water and electrolytes absorption, classic RAS has been reported to be involved in cardiac hypertrophy and in inflammation and fibrosis (Garg et al., 2012), especially through activation of the angiotensin type 1 receptors (AT1Rs). In recent years, different studies provided evidence for the final identification of two receptor subtypes for Ang II, namely the AT1Rs and the AT2Rs, whose functions appear to be antagonistic in most cases, as well as the characterization of an alternative RAS, where a counterpart of the ACE, called ACE-2, lead to the production of a different peptidic products, namely the Ang (1-7) and Ang (1-9) (Speth & Giese,

2013). Recent current investigations have showed that Angiotensin (1-7) effects include promotion of the release of NO and consequently vasodilation, inhibition of cell growt in the vessel wall, as well as anti-inflammatory, anti-fibrotic and neuroregenerative actions (Santos et al., 2000).

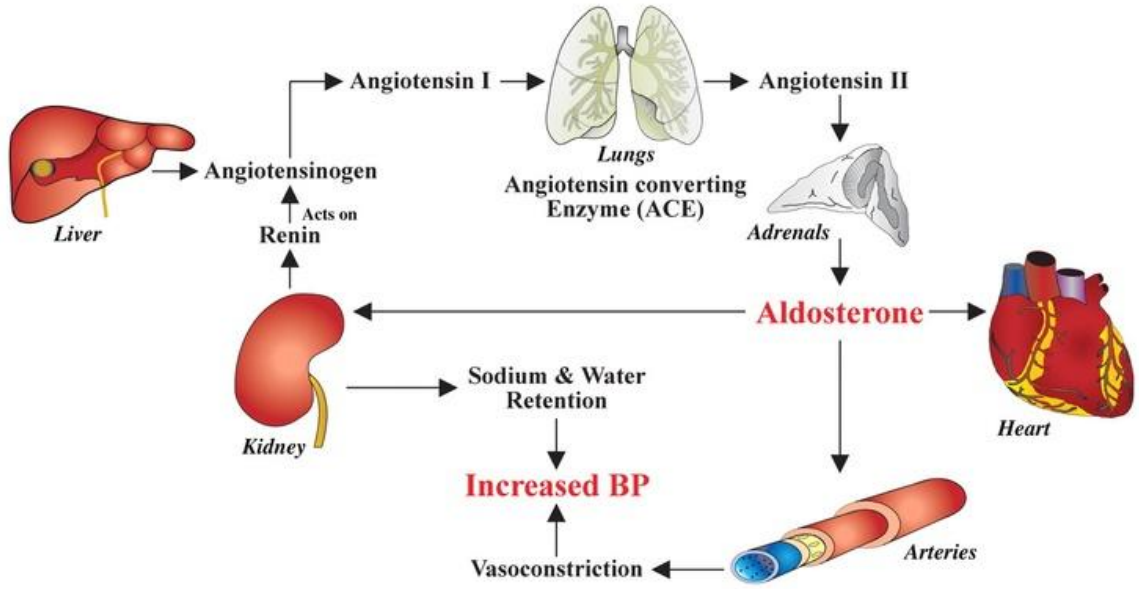

Figure 7: Schematic representation of the Renin-Angiotensin system (RAS).
Abbreviation: BP, Blood Pressure

Angiotensin II receptors

Angiotensin II carried out its functions by the activation of two subtypes of receptors, the AT1 and AT2 receptors (AT1Rs and AT2Rs) (De Gasparo et al., 2000) (Fig.8), which are seven transmembrane, G protein-coupled receptors (GPCR), with a sequence similarity of 30%. Most species express a single autosomal gene AT1, while rodents express two genes that are named AT1A and AT1B. AT1Rs are predominantly coupled to protein Gq/11, and they mediate signal transduction through the phospholipases A, C and D, the inositol phosphates, calcium channels, and a variety of serine/threonine and tyrosine kinases. AT2Rs are higly expressed during fetal development, while they are much less abundant in

adult tissues, although their upregulation has been observed in different pathological conditions. AT2Rs mediate signal transduction through serine and tyrosine phosphatases, phospholipase A2, nitric oxide and cyclic GMP (De Gasparo et al., 2000).

- **AT1 receptors (AT1Rs)**

The AT1Rs mediate most of classic responses to Ang II, as vasoconstriction, synthesis and release of aldosterone, and centrally induction of thirst (Speth & Giese, 2013). AT1R protein is constituted of 359 aminoacids and encoded by a single gene localized in chromosome 3. AT1Rs belong to the superfamily of GPCRs, thus the binding of Ang II induces a conformational change promoting the interaction with the G protein, which in turn modulate different effector systems. These latter include phospholipase C, D and A2, adenylate cyclase and ion channels. Over time, the AT1 receptor undergoes desensitization and regulation *via* internalization (Thomas et al., 1996). Responses induced by activation of the AT1Rs include smooth muscle contraction, secretion of aldosterone, neuronal activation, neurosecretion, ion transport (De Gasparo et al., 2000). In addition, these receptors could also regulate gene transcription and the expression of proteins that control the growth and cell proliferation in target tissues of Ang II. However, a chronic increase in levels of Ang II could also result in fibrosis and other pathophysiological changes in target organs possessing the AT1Rs (Robert et al., 1999). Intriguingly, activation of AT1Rs by Ang II could also induce pro-inflammatory pathways; stimulation of the AT1 receptor induces maturation of dendritic cells (DCs) and enhances the production of chemokines/cytokines, such as IL-6, IL-1α, and C-reactive protein (De Gasparo et al., 2000); increased expression of Toll-like receptor 4 (TLR-4) and reactive oxygen species (ROS) was also observed (Speth & Giese, 2013).

- **AT2 receptors (AT2Rs)**

AT2 receptor is a GPCR displaying an homology of 30% with the aminoacid sequence of the AT1R. AT2R consists of 363 aa and its gene is located on the X chromosome in humans, rats and mice. Its expression is high and ubiquitous in the fetus, then it starts to decrease after birth, even completely disappearing in some tissue as the skin. In other tissues, such as the adrenal gland and the heart, the AT2R level decreases to a certain low but still detectable point, and then persists for the rest of life (Speth & Giese, 2013). The characterization of the precise functions of this receptor are still ongoing, but it has been shown to mainly counteract the actions mediated by the AT1Rs. Indeed, activation of AT2R is vasodilatory, antiproliferative, pro-apoptotic and pro-differentiation, counteracting the vasoconstrictor, anti-proliferative and anti-apoptotic actions mediated by the AT1R (Fig. 6) (Csikos et al., 1998). AT2Rs activity is prominent in cells not undergoing growth, and AT2 receptor is not subject to desensitization and internalization, accordingly with its likely role of maintaining differentiated cells into a state of quiescence (Unger, 1999). As an example, activation of AT2Rs on neonatal hypothalamic neurons induces the serine/threonine phosphatase PP2A pathway, in turn inactivating Mitogen-activated protein kinase (MAPK) (Huang et al., 1999). This event leads to suppression of cell growth and induction of differentiation. The distribution of the AT2 receptors in various organs, such as the brain, heart, vascular tissue, adrenal, kidney, skin or intestine suggests a physiological role of AT2Rs in various body districts, but a plethora of studies also underlined its effective involvement in pathophysiological processes (De Gasparo et al., 2000), as inflammation and fibrosis, suggesting the necessity of increasing research to establish the AT2Rs as possible novel therapeutic targets in different disorders.

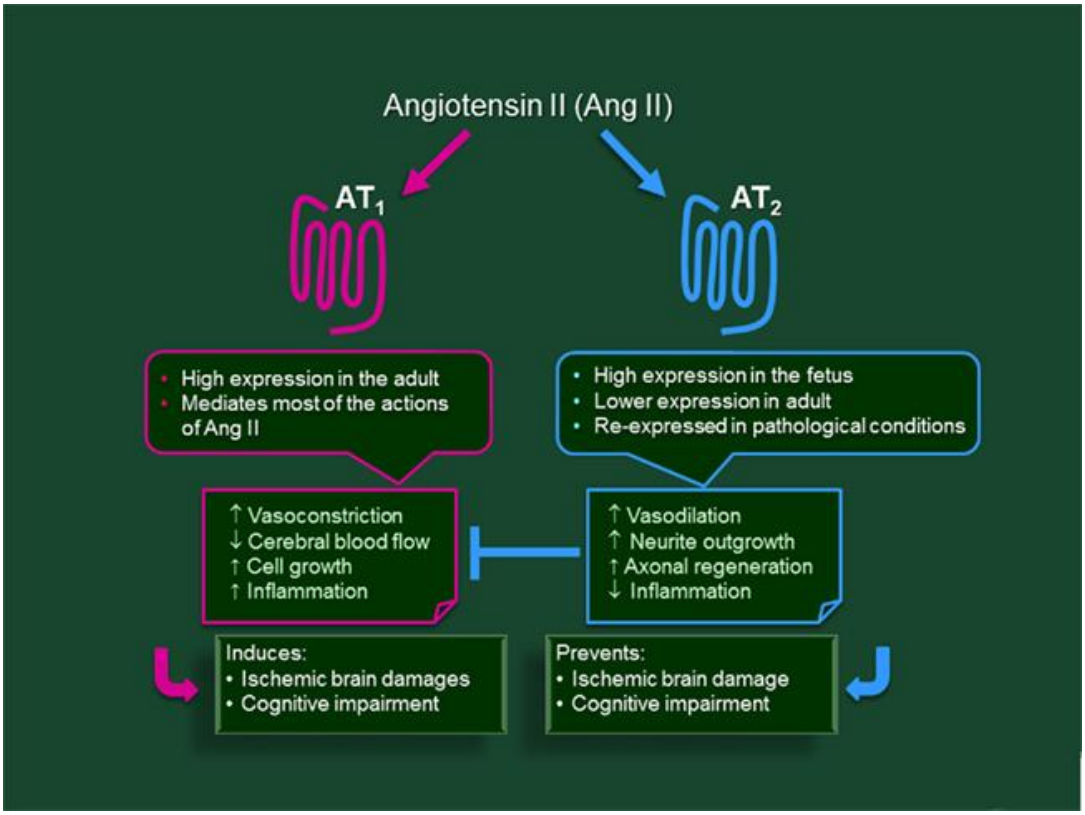

Figure 8: Major effects induced by activation of AT1 and AT2 receptors by Angiotensin II

The local RAS in the gastrointestinal tract: physiological and pathological roles

Differently from the prior assumption of the RAS as a sole endocrine system, generating peptides and enzymes released into the blood stream which then act on the target organs, recent studies have progressively demonstrated that most organs including the brain, kidneys, heart, liver, pancreas, reproductive organs, skin and intestine, constitutively express all components of the RAS, which could thus constitute local systems involved in tissue homeostasis with paracrine/autocrine function. As an example, the heart expresses renin, ACE, AT1R and AT2R, modulating myocytes proliferation (Urata et al., 1990) and the brain expresses renin, angiotensinogen, Ang II, Ang (1-7), AT1R, AT2R, locally regulating blood pressure, fluid and electrolyte balance, the thirst, the blood-brain barrier and neuronal pathways of learning and memory (Ganten et al., 1971). Even more relevant

could be the contribution of a local RAS within the GI tract; indeed, components of RAS including renin, angiotensinogen, ACE, AT1 and AT2 receptors have been identified in the intestine of animal model and humans (Hirasawa et al. 2002; Mastropaolo et al., 2015). In the small intestine, RAS seems to be involved in various processes as bicarbonate secretion, absorption of sodium, water, glucose and peptides, as well as in the regulation of motility (Garg et al., 2012). Indeed, Ang II induces contractile responses of longitudinal muscle of guinea-pig small intestine, *via* activation of neural AT1Rs, modulating release of ACh and substance P, and AT1R located on enteric smooth muscle cells (Hawcock & Barnes 1993). Subsequently, human studies also confirmed the role of AT1Rs in Ang II-induced contractions in the small intestine (Ewert et al., 2006). Regarding the colon, previous studies in our laboratory have demonstrated the presence of RAS components in mice and human colon, including renin, angiotensinogen, ACE, AT1Rs and AT2Rs (Mastropaolo et al., 2015). Ang II induced contractions of the colonic smooth muscle acting on AT1 receptors; interestingly, an interaction of Ang II with the tachykinergic signaling has been identified both in mice and humans. However, in mice, Ang II induces release of SP, in turn causing release of ACh through activation of neural NK-1 receptors; ACh is thus the final mediator causing smooth muscle contraction. On the other hand, in human colon, Ang II *via* the AT1Rs mediates the release of Neurokinin A (NKA) causing smooth muscle contraction acting on NK-2 receptors on smooth muscle cells. Although the expression of AT2 receptors has been reported in the GI tract of different animal species (Fändriks, 2010), Ang II-induced effect on GI motility seems to be dependent mainly on the activation of AT1Rs, suggesting that novel researches are needed to reveal the possible contribution of AT2 receptors in physiological or pathological conditions. Indeed, the local RAS in the small and large intestine is likely to play a physiological role within the neuronal network governing motility, but pathological changes of RAS signaling in the GI

tract, including changes in receptors expression, could influence GI motor disorders as well as motor activity in inflammatory conditions, as IBD.

In line with this hypothesis, a series of recent studies has also pointed out the possible involvement of RAS system in GI inflammation, especially in IBD. Mucosal levels of Ang II are higher specifically in CD patients (Jaszewski et al., 1990), and increased concentrations of Ang (1-7) and ACE2 have been also reported in IBD patients (Garg et al., 2014), suggesting the involvement of both the classic and alternative RAS in inflammation. Experimental studies revealed that inhibition of ACE or antagonists of angiotensin receptors (especially AT1Rs blockers) ameliorate inflammatory damages in murine colitis, reducing production of pro-inflammatory cytokines and increasing the levels of anti-inflammatory cytokines as IL-10 (Garg et al., 2012). Also, AT1a receptor-deficient mice displayed less severe experimental colitis than wild-type mice (Katada et al.. 2008). Thus, a in-depth examination of changes in classic and alternative RAS components could lead to the definition of RAS as a novel modulator of the inflammatory event, and thus a potential novel therapeutic target in IBD for the treatment of both inflammation and IBD-related GI motor dysfunction.

3.1
Different role for Angiotensin II receptors in the modulation of colonic motility in a murine model of Inflammatory Bowel Disease

3.1.1 Aim

Since novel investigations are needed to effectively disclose the possible of enteric RAS system in physiological and pathological conditions, in order to establish its potential as new possible target for treatment of IBD-associated GI motor dysfunction and inflammation, the aim of this study was to analyse and compare, *in vitro*, the possible effects of Ang II on colonic motor activity in control rats and in animal subjected to treatment with 2,4-dinitrofluorobenzenesulfonic acid (DNBS), as model for IBD. In particular, we characterize the different inflammatory markers in the DNBS model, the contractile activity, the effects of Ang II, the receptor subtypes involved, as well as their possible role in the dysmotility observed in DNBS-treated animals *versus* controls.

3.1.2 Materials & Methods

Animals

Sixteen female Wistar rats (weighing 200-300 g), obtained from Charles River Laboratories (Calco-Lecco, Italy) were used in the experiments. The animals were kept in the animal facility of the Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF), in a controlled environment (room temperature (22 ± 2°C) and humidity (60%) and 12h:12h day-night cycle), and fed with standard pellets and water *ad libitum* throughout the study. Animals were allowed to acclimatize to housing conditions for 1 week prior to experimentation. All experimental procedures were approved by Ministero della Sanità (Rome, Italy).

Colitis induction

Animals were randomly assigned to the control group and the colitis group (8 animals each). For the experimental induction of colitis, animals were fasted overnight and the following day, under light anaesthesia with isofluorane, a solution of 30 mg of 2,4-dinitrobenzensulfonic acid (DNBS) in 50% ethanol, for a total volume of 0.25 mL, was intracolonically instilled in each animal using a 8 cm plastic catheter (PE90). In control experiments, animals received 0.25 mL of saline solution (0.9% NaCl). The rats were sacrificed on the sixth day after the treatment with DNBS or saline.

Evaluation of inflammatory markers:

1) Disease Activity Index (DAI)

In the 6 days following treatment the weight and the fecal consistency of each animals were evaluated daily. These parameters were collected to assess a numerical index, the Disease Activity Index (DAI), as a combination of a Weight Loss % Score and Stool Consistency Score, in order to establish the severity of ongoing inflammation. The scores have been calculated daily according to the tables below:

Weight loss % Score
0: < 1%
1: 1-5%
2: 5-10%
3: 10-15%
4: >15%

Stool Consistency Score
0: normal
2: loose stool
4: diarrhea

Weight loss was calculated as the percent difference between the original body weight (equated as 100%) and the daily body weight. The final daily DAI Score resulted as a mean of the Weight loss % Score and the Stool consistency Score.

2) Analysis of macroscopic inflammatory damage

On the 6th day after the treatment with DNBS or saline, the animal was sacrificed and the colon removed, opened longitudinally and washed with Krebs solution, removing all the intraluminal contents. The weight and the length of the colon were registered, in order to calculate the weight/length ratio as indicator of colonic edema.

Then, macroscopic damage was scored as following:

- Presence and extension of tissue damage in the distal colon: 0, 1, 2, 3, 4, 5, 6 (normal aspect of mucosa, localised hyperemia with no ulcers, ulceration without hyperemia/bowel wall thickening, ulceration with hyperemia/bowel wall thickening at 1 site, two or more sites of ulceration with hyperemia/bowel wall thickening, major damage (necrosis) extended more than 1cm, major damage (necrosis) extended over 2 cm. If the damaged area covered more than 2 cm, the score was increased by 1 for each additional cm involved);
- Presence of adhesions between the colon and other organs: 0, 1, 2 (none, minor, major);
- Fecal consistency: 0, 1 (normal, diarrhea);
- Maximal thickness of the colonic wall (in mm);

The cumulative Score of the different parameters represents a numerical index determining the severity of macroscopic damage (Macroscopic Damage Score).

3) Analysis of microscopic inflammatory damage

A representative section of colon from each animal was fixed in 4% formaldehyde for 24h and embedded in paraffin for hematoxylin-eosin staining. Sections were observed under an optical microscope to evaluate and quantify the extent of inflammatory pathological changes, as following:

- Loss of mucosal architecture: 0, 1, 2, 3 (normal, mild, moderate, severe);
- Cell Infiltration: 0, 1, 2, 3 (absent, mild, moderate, severe);
- Muscle thickening: 0, 1, 2, 3 (absent, mild, moderate, severe);
- Presence of crypt abscesses: 0, 1 (absent, present);
- Depletion of globets cells: 0, 1 (absent, present);

The total sum of the scores represents a numerical index determining the severity of microscopic damage (Microscopic Damage Score).

4) Myeloperoxidase Assay

Myeloperoxidase (MPO) is an enzyme able to detoxify the cells from reactive oxygen species (ROS), present primarily in neutrophils and other cells of myeloid origin, and commonly used as a quantitative marker for establishing the degree of severity infiltration of immune cells during intestinal inflammation (Krawisz et al., 1984).

For each animal, a sample of tissue (around 100 mg) was removed from the area of major damage, snap frozen in liquid nitrogen and assayed within seven days using a the method described by Boughton-Smith et al. (1988). Before starting the MPO assay, samples were placed on ice for 15 minutes for unfreezing. The samples were then constantly kept in ice for the entire duration of the protocol, reported below:

1) Addition of detergent hexadecyl-trimethylammonium bromide (HTAB; 1mL/50 mg of tissue);

2) Homogenization of the tissue by Polytron at medium speed for about 20 s

3) Centrifugation at 6000g for 10 min at 4 °C

4) Collection of 35 μL of supernatant and loading of each sample in a cuvette with the addition of 1mL of solution of o-Dianisidine dihydrochloride and 250 μL of a solution of 30% hydrogen peroxide (H_2O_2).

5) Reading of the absorbance at the spectrometer λ = 450 nm (3 readings at 30-second intervals: 0-30-60 s).

Through a statistical software (GraphPad Prism version 4:00 for Windows, GraphPad Software, San Diego, California USA), a graph of the absorbance (A) as a function of time was designed, in order to extract the value Slope (Z), representing the change in absorbance over time. Considering the change in absorbance of $1.13*10^{-2}$ associated with 1 μmole of H_2O_2, total units of MPO will be equal to $Z/1.13*10^{-2}$. Since the ratio tissue/buffer is equal to 50 mg/ mL, then in 35 μL of supernatant there are 1.75 mg of tissue; consequently to obtain units MPO/mg, the previous value was divided by 1.75. Hence, the MPO was finally expressed in units MPO per mg of tissue, where one unit of MPO corresponds to the activity required to degrade a 1 μmole of H_2O_2 in one minute at room temperature.

Evaluation of the mechanical activity of colonic longitudinal muscle strips *in vitro:*

Longitudinal smooth muscle strips (10 mm in length) from the distal colon of control and DNBS-treated animals were prepared and suspended in the four channels of a vertical organ bath, each containing 10 mL of Krebs solution, oxygenated (95% O_2 and 5% CO_2) and maintained at a constant temperature of 37°C (Fig.9). The distal end of the strips was fastened to a small glass hook located inside of each channel, whilst the proximal end was connected, by means of a silk thread, to the hook of a isometric force transducer (FORT

125, Ugo Basile Biological Research Apparatus, Comerio VA, Italy), which allowed to supply as output, in proportion to the force applied on entry, a linear voltage with very low deflections. The mechanical activity was digitized by an A/D converter, displayed and recorded on a personal computer, using the PowerLab /400 system (Ugo Basile, Biological Research Apparatus, Italy), and finally analyzed using the program CHART4. The preparations were subjected to an initial tension of 500 mg and then left to equilibrate for at least 30 min, until stable spontaneous mechanical activity was observed.

Preparations from controls and DNBS rats were challenged with 10 μM carbachol (CCh) until stable responses were obtained. Increasing concentrations of Ang II were applied in a non-cumulative manner for approximately 5 min at regular 90 min intervals, to obtain concentration-response curves. In a second series of experiments, the effects of Losartan, selective AT1 receptor antagonist, and PD123319, AT2 receptor antagonist, were tested. In addition, tetrodotoxin (TTX), blocker of neural Na^+ voltage-gated ion channels, N^G-Nitro-L-arginine (L-NNA), an inhibitor of neural/endothelial nitric oxide synthase (nNOS/eNOS), or 1400W, an inhibitor of inducible NOS (iNOS), were tested on the response induced by a sub-maximal concentration of Ang II. Antagonists were left in contact with the tissue for at least 20 minutes before challenging the preparation with Ang II. Concentrations of the drugs used were determined from previous experiments (Mastropaolo et. al. 2013, 2015) and from literature.

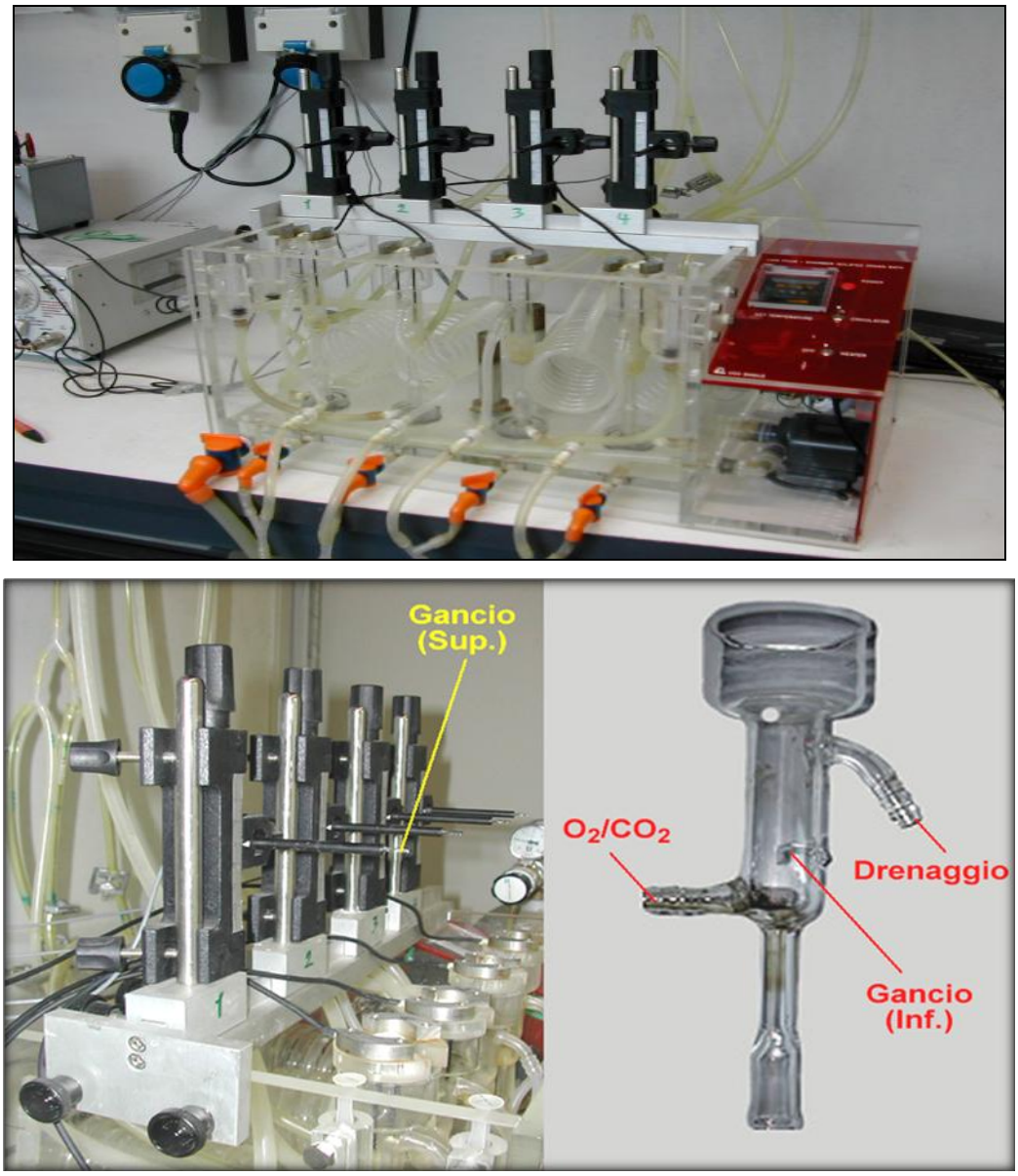

Figure 9: Organ bath system **(top)** and particular of an individual channel **(bottom)**

Solutions and drugs

Krebs solution consisted of (mM): NaCl=119; KCl=4.5; $MgSO_4$ = 2.5; $NaHCO_3$ = 25; KH_2PO_4 = 1.2; $CaCl_2$ = 2.5; and glucose = 11.1. Drugs used were: N-([3-(Aminomethyl)phenyl]methyl)ethanimidamide dihydrochloride (1400W), carbamylcholine chloride (carbachol, CCh), 2,4-Dinitrobenzenesulfonic acid (DNBS), isoproterenol (Iso), and Tetrodotoxin from Sigma-Aldrich Inc. (St Louis, MO, USA). Angiotensin II, 1-[[4-

(Dimethylamino)-3-methylphenyl]methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H imidazo[4,5-c] pyridine-6-carboxylic acid ditrifluoroacetate (PD123319), 2-Butyl-4-chloro-1-[[2′-(1Htetrazol-5-yl)-[1,1′-biphenyl]-4-yl]methyl]-1H-imidazole-5-methanol potassium salt (Losartan) and N^G-Nitro-L-arginine (L-NNA) from Tocris Bioscience (Bristol, UK). All drugs were dissolved in distilled water, except otherwise stated. Working solutions were then dissolved in Krebs solution.

Data analysis and statistical tests

All data are presented as means ± SEM: 'n' indicates the number of animals. Contractile responses induced by Ang II were reported as a percentage of the effect induced by 10 μM Carbachol (CCh). Ang II responses were fitted to sigmoid curves (Prism 4.0, Graph-PAD, San Diego, CA, USA), and EC50 values with 95% confidence limits (CLs) were determined.

Statistically significant differences were calculated by Student's t-test or by analysis of variance followed by Bonferroni's test, as appropriate. A p-value < 0.05 was considered statistically significant.

3.1.3 Results

Characterization of inflammatory markers in the DNBS rat model of IBD

1) Disease Activity Index

Animals subjected to treatment with DNBS showed in the following 6 days a progressive weight loss (Fig. 10) accompanied by diarroic condition. At day 6 after DNBS injection, Weight loss % Score and Stool consistency Score were 2.25 ± 0.8 and 3,5 ± 0.3, respectively (n = 8), resulting in a significant Disease Activity Index (DAI mean: 2.87 ± 0.2, n = 8, day 6 after DNBS, $p<0.05$ compared to controls) (Table 2). Control animals displayed no weight loss (Fig. 10) or changes in stool consistency.

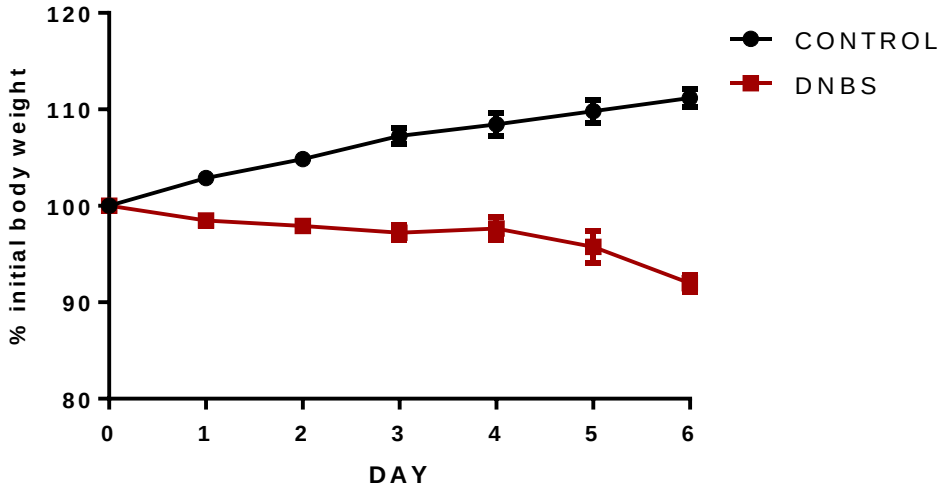

Figure 10: Body weight loss monitored during the days after saline (control animals, n=8) or DNBS injection (n=8), expressed as a percentage of body weight on day 0. Data are expressed as Mean ± SEM.

	DAI SCORE (DAY 6)
CONTROLS (N=8)	0 ± 0
DNBS (N=8)	2.87 ± 0.2*

Table 2: Disease Activity Index in saline and DNBS-treated animals at 6th day after treatment. Mean ± SEM. *p<0.05

2) Assessment of macroscopic inflammatory damage

On the 6th day after the treatment with DNBS, the distal colon appeared dilated, thickened, highly vascularized and it generally presented an area of major damage with obvious ulcerations or necrotic tissue (Fig.11a,b). Diffuse adhesions of the colon with other organs were also observed. No evident tissue damages, thickening of colonic wall, adhesions or other obvious pathological signs were observed in saline-treated animals (Fig.11c).

Figure 11. (a)

Figure 11. (b)

Figure 11. (c)

Figure 11: Macroscopic appearance of colon from a DNBS-treated animal (a) - (b) or saline-treated animal (c).

Colon weight/length ratio significantly increased in the DNBS-treated animals compared to controls, indicating significant tissue edema (mean 0,12±0,01 in controls *vs* 0,26 ±0,02 in DNBS animals, $p<0.05$) (Fig.12).

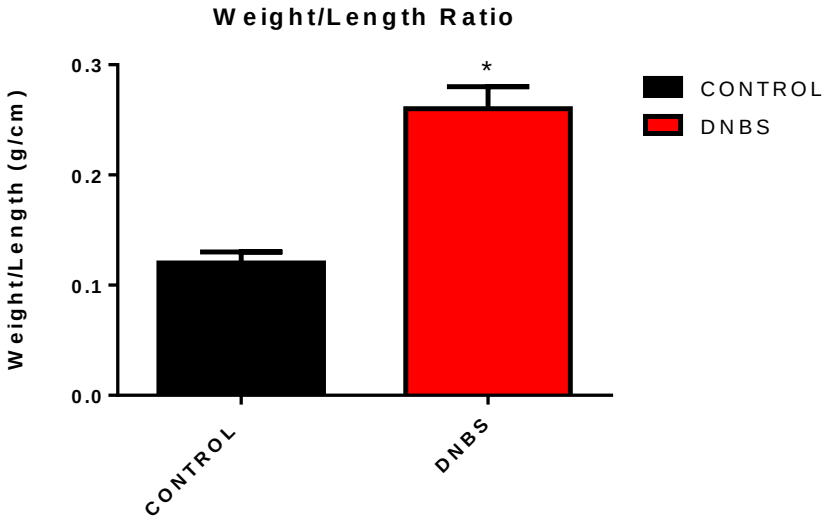

Figure 12: Colon weight/Length ratio in colitis *versus* control animals, expressed Mean ± SEM. *p<0.05

Evaluation of macroscopic damage in DNBS-treated animals results in a mean Macroscopic Damage Score of 11.3 ± 0.4 (n = 8, day 6 after DNBS) (Table 3).

	MACROSCOPIC DAMAGE SCORE
CONTROLS (N=8)	0 ± 0
DNBS (N=8)	11.3 ± 0.43*

Table 3: Macroscopic Damage Score in colitis *versus* control animals, expressed Mean ± SEM. *p<0.05

3) Evaluation of microscopic inflammatory damage

Histological examination showed in the samples of the colon from the DNBS group serious pathological changes characterized by infiltration of immune cells in the intestinal

wall, moderate to severe loss of mucosal architecture, depleted globet cells and edema (Fig. 13).

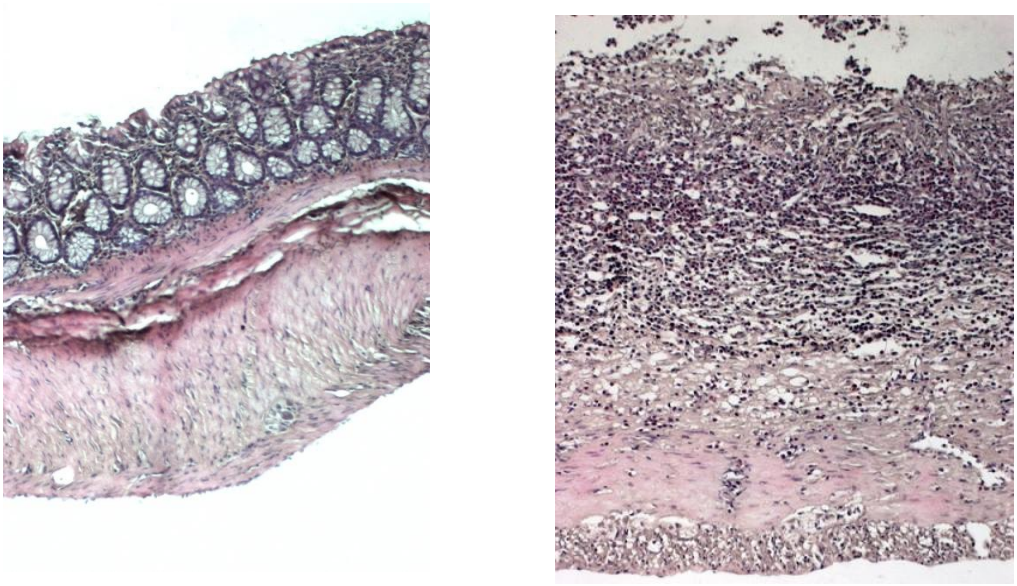

Figure 13: Microscopic apperance of colonic tissue in control (sx) *versus* colitis (dx) animals

The evaluation of the microscopic damages results in a mean Microscopic Damage Score of 4.6 ± 0.4 (n = 8, day 6 after DNBS) (Table 4).

	MICROSCOPIC DAMAGE SCORE
CONTROLS (N=8)	0 ± 0
DNBS (N=8)	4.6 ± 0.4*****

Table 4: Microscopic Damage Score in colitis *versus* control animals, expressed. Mean ± SEM. *p<0.05

4) Levels of Myeloperoxidase (MPO)

The samples of the colon of animals in the group DNBS showed a significant increase in the levels of myeloperoxidase (MPO) compared to control animals, indicating an extensive

neutrophilic infiltration caused by the treatment (mean: 30.8 ± 4.1 Units/mg, n = 4, day 6 after DNBS *versus* 5.75 ± 1.2 Units/mg, n = 4 in the control animals) (Fig.14).

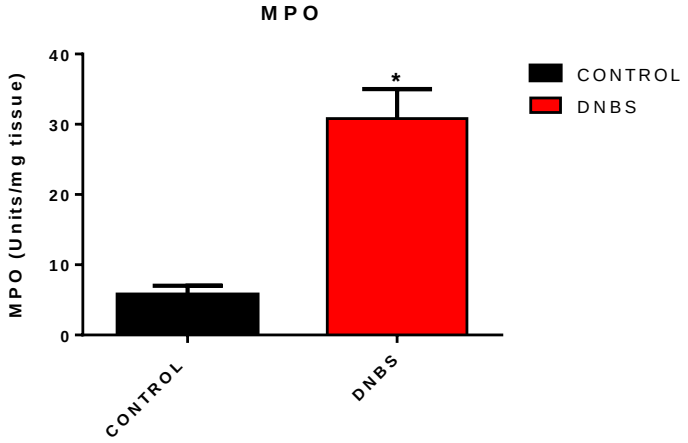

Figure 14: MPO levels in colitis *versus* control animals, expressed in Units of MPO/mg of tissue. Mean ± SEM. *p<0.05

Effect of Angiotensin II on the colonic mechanical activity in controls and DNBS-treated animals

Strips of longitudinal muscle of colon obtained from control rats and DNBS-treated rats, once mounted in the channels of the organ bath and after the equilibration time, developed a spontaneous contractile activity. However, the mechanical activity of the DNBS strips was characterized by contractions with an amplitude significantly lower than of preparations obtained from control rats (amplitude: 1073.2 ± 67.7 mg, n = 8 in controls and 231.1 ± 21.0 mg, n = 8, in DNBS rats; p<0.05) (Fig.15).

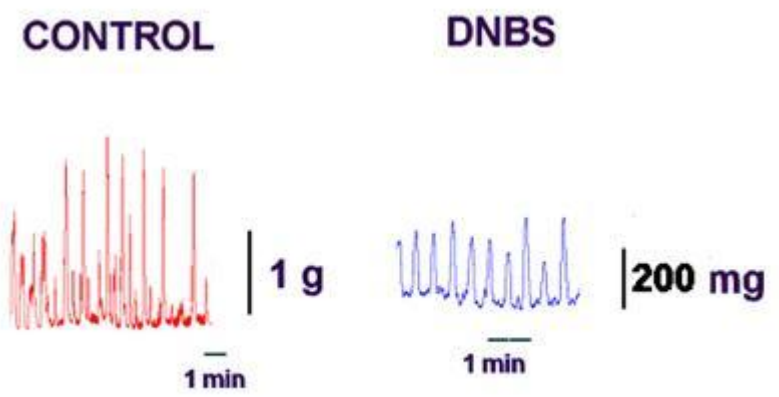

Figure 15: Original tracing showing the spontaneous mechanical activity of colonic longitudinal muscle strips from control and DNBS-treated animals

In addition, DNBS preparations had a significantly lower contractile and relaxant response, respectively to carbachol (CCh; 10 µM), muscarinic cholinergic receptor agonist, and Isoproterenol (Iso; 0.1 µM), β2-adrenergic receptor agonist, than control preparations, confirming an altered colonic motility in DNBS-treated animals (Fig.16).

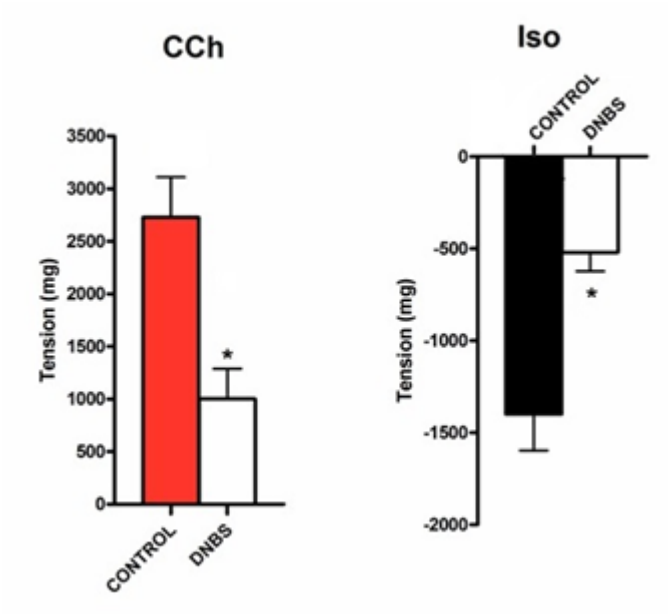

Figure 16: Histograms showing the effects of CCh (10 µM) and Iso (0.1 µM) in control (n = 8) and DNBS preparations (n = 8) . Data are expressed as mean ± S.E.M. *p < 0.05 when compared to the control.

The exogenous administration of Angiotensin II (3-300 nM) caused a concentration-dependent excitatory effect, increasing the basal tone of the colonic longitudinal muscle of both groups of animals (Fig.17). However, the response to Ang II in strips from DNBS rats was significantly reduced compared to controls, with a significant rightward shift of the dose-response curve (EC50 of 35.8 nM, 95% Cls 9.5-125.6 nM in controls; EC50 of 111.8 nM 95% Cls 59.4-217.9 µM in DNBS preparations) (Fig. 17-18).

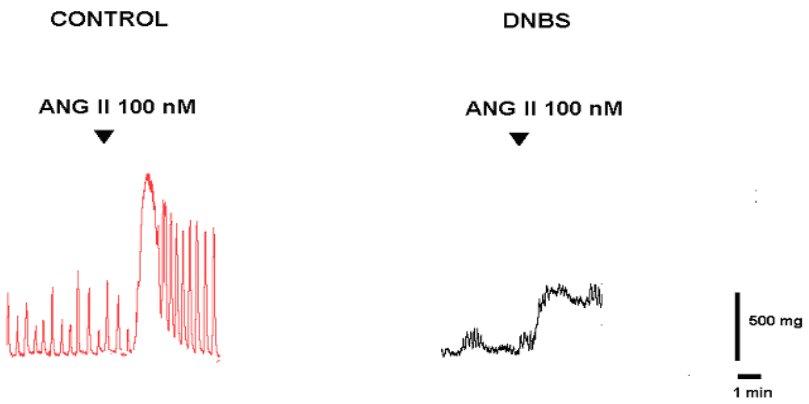

Figure 17 : Representative original tracing showing the excitatory effect induced by a submaximal dose of Ang II (100 nM) in control and DNBS-treated animals.

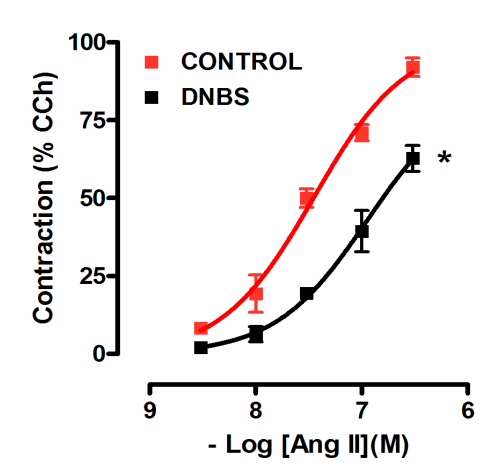

Figura 18 : Concentration-response curves of Ang II (3 -300 nM) in longitudinal muscle strips from colon of control rats and DNBS-treated rats. Data are reported as means ± SEM and expressed in % of the excitatory effect induced by carbachol 10 µM. *p<0.05 when the concentration-response curve was compared to that of controls.

In order to determine the receptors involved in the effects mediated by Ang II, samples were pre-treated with the AT1 receptor (AT1R) antagonist, Losartan, and the AT2 receptor (AT2R) antagonist, PD123319. Losartan (10 nM) antagonized the excitatory effects induced by Ang II in both preparations, shifting the concentration-response curve of Ang II to the right (Fig. 17) (controls: EC50 of 77.6 nM, 95% Cls 27.1-223.2 nM, n = 6, in the presence of Losartan; DNBS preparations: EC50 of 110.1 nM, 95% Cls 23.1-530.2 nM, n=5, in the presence of Losartan) (Fig.19). In contrast, pretreatment with the AT2 receptor antagonist, PD123319 (100 nM), had no effect on the control preparations but it led to a significant increase of the Ang II effects in DNBS preparations, increasing the maximum response of about 35% (Fig. 19) (EC50 of 29.1 nM, 95% Cls 12.2-70.0 nM, n=5, in presence of PD123319). Thus, during inflammation, a recruitment of inhibitory AT2 receptors would counteract the excitatory effect mediated by AT1 receptor, decreasing the response to Ang II.

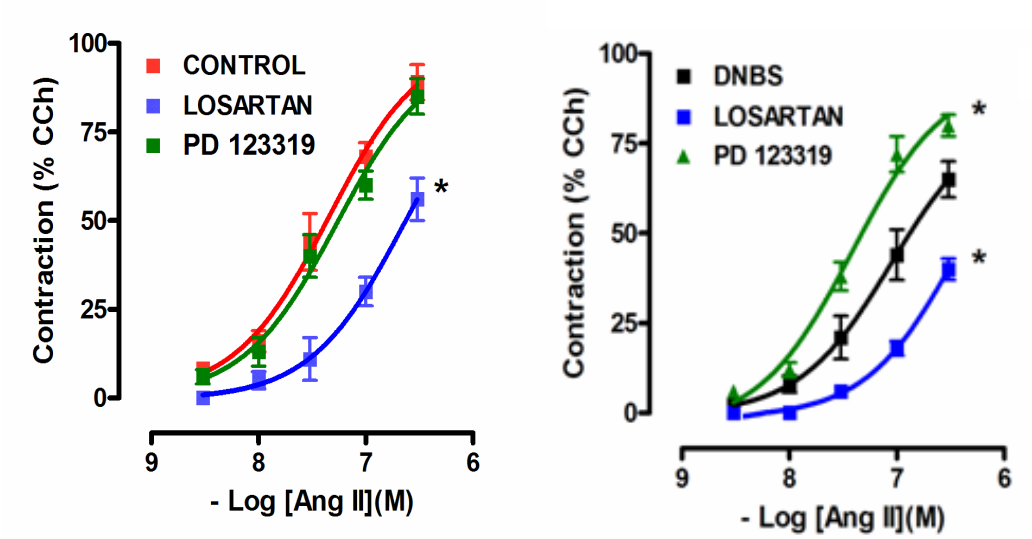

Figure 19: Concentration-response curves of Ang II (3-300 nM), alone or in the presence of Losartan (10 nM; n=5) or PD123319 (100 nM n=5) in preparations from controls (left) or DNBS-treated animals (right). The data are reported as means ± S.E.M. and expressed in % excitatory effect induced by carbachol 10 μM . *p<0.05 when the concentration-response curve was compared to that observed in control conditions .

In order to determine whether the receptors involved in the Ang II-induced effects were localized at the level of enteric neurons and/or at post-junctional level, samples were pre-treated with tetrodotoxin (TTX), blocker of neural Na^+ voltage-gated ion channels. TTX (1 μM) did not alter the excitatory effect induced by a submaximal dose of Ang II (100 nM) in strips obtained from control rats, while in DNBS preparations it significantly increased the contractile response to the same extent of the AT2 receptor antagonist (Fig.20). These observations indicate that AT1 receptors mediating Ang II excitatory effects are located at post-junctional level in the controls, whilst AT2 receptors, mediating inhibitory effects, seem to be located at pre-junctional level in DNBS preparations.

Since different evidence pointed out that effects of Ang II following activation of the AT2 receptors may be likely related to nitric oxide (NO), the effects of Ang II (100 nM) in DNBS preparations were tested in presence of L-NNA (10 μM), an inhibitor of neural/endothelial nitric oxide synthase (nNOS/eNOS) and 1400W (10 μM), an inhibitor of inducible NOS (iNOS). The effect of Ang II was increased in the presence of L-NNA, in a manner comparable to that observed in the presence of the AT2 receptor antagonist or TTX, while no increase was observed in the presence of 1400W (Fig.20). Co-administration of PD123319 and L-NNA or TTX did not determine additive effects, suggesting that the AT2 receptors would induce neural release of NO from inhibitory nerves.

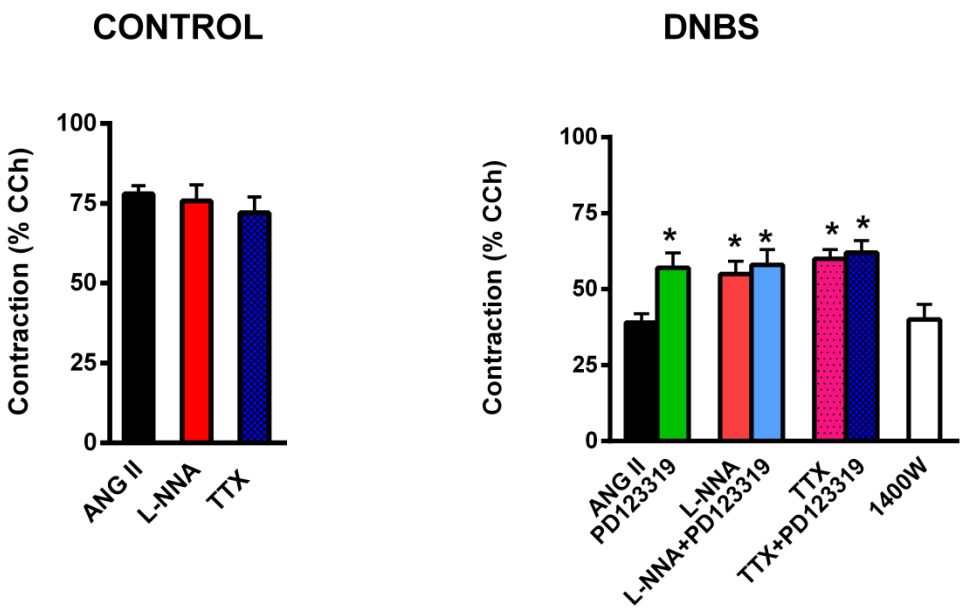

Figure 20: Histograms showing the effects of Ang II (100 nM) alone, and in the presence of L-NNA (10 μM), or TTX (1 μM) in preparations of the colon of control rats and in the presence of L-NNA (10 μM) , TTX (1 μM), 1400W (10 μM) or following co-administration of TTX and PD123319 (100 nM) or L-NNA and PD 123319 (100 nM) in DNBS rats. Data are mean ± SEM and expressed in % excitatory effect induced by carbachol 10 μM. * $p < 0.05$ compared to control.

Lastly, the administration of PD123319 (100 nM) induced *per se* a 40% increase of the amplitude of spontaneous contractions, suggesting a tonic activation and participation of AT2 receptors in the modulation of the spontaneous mechanical activity during inflammation. Noticeably, administration of PD123319 was able to increase also both the contractile and relaxant response respectively to CCh (1 μM ; from 1110 ± 78 mg to 1920 ± 252 mg in presence of PD123319, n=4) and Iso (0.1 μM; from 550 mg ± 42 to 835 ± 82 mg in presence of PD123319, n=4). This would indicate that, in our experimental conditions, the tonic activation of AT2 receptors could be partly responsible for inflammation-associated motility dysfunction.

However, treatment with PD123319 was not able to effectively restore the amplitude of spontaneous contractions observed in controls, as well as the contractile and relaxant response to CCh (1μM) and Iso (Fig.15-16), suggesting that AT2 receptor activation would

represent solely one of the multiple mechanisms leading to impaired muscle contractility in the course of inflammation.

3.1.4 Discussion and conclusions

Results from our experiments firstly demonstrated that a tonic activation of AT2 receptors on inhibitory neurons, in turn leading to NO release, could contribute to the general reduction of muscle contractility in the course of experimental GI inflammation.

Ang II was able to induce smooth muscle contraction of colonic longitudinal muscle strips both in control animals and animal subjected to treatment with DNBS, this latter inducing an inflammatory event resembling IBD as demonstrated by the evaluation of different inflammatory markers, including clinical signs (weight loss, diarrhea), macroscopic and microscopic tissue damages (ulcerations, bowel wall thickening, loss of physiological mucosal architecture) and extensive immune infiltrate. However, the effect of Ang II was lower in preparations obtained from DNBS-treated animals, suggesting modification in Ang II-mediated signaling during experimental inflammation. In control animals, the contractile effect of Ang II was dependent on AT1 receptors activation, as pre-treatment with Losartan, AT1 receptor antagonist, significantly antagonized Ang II effect, being not affected by pre-treatment with PD123319, AT2 receptor antagonist. This is line with our previous studies about an exclusive role of AT1 receptors in mediating excitatory effects of Ang II in the mice and human colon (Mastropaolo et al., 2013, 2015), as well as with other studies addressing AT1 receptors as the major effectors of Ang II-related effects (Speth & Giese, 2013).

In preparation obtained from DNBS-treated animals, the contractile effect of Ang II was also antagonized by the AT1 receptor antagonist, Losartan, indicating the involvement of AT1 receptors, but, interestingly, pre-treatment with the AT2 receptor antagonist,

PD123319, resulted in a significant increase of the amplitude of Ang II-mediated contractile effect. This observation suggest that, in experimental inflammation, tonic activation of AT2 receptors occurs, causing an inhibitory effect on Ang II-mediated contraction. The presence of AT2 receptors in the colon has been reported in human whole thickness preparations as well as specifically on epithelium, crypt, mesenchymal cells (Hirasawa et al., 2012; Mastropaolo et al., 2015). However, our previous experiments on human sigmoid colon did not reveal any involvement of AT2Rs in the modulation of colonic mechanical activity in physiological condition (Mastropaolo et al., 2015); accordingly, here we observed that AT2Rs were not involved in Ang II-induced contraction in preparation obtained by rats not subjected to experimental inflammation. The neural blocker TTX did not influence the Ang II-induced response in control samples and in inflamed animals in the presence of the AT2 receptor antagonist, indicating that AT1 receptors mediating Ang II excitatory effects are located at post-junctional level in both preparation. On the other hand, TTX increased the Ang II-dependent contractile response in preparation from inflamed animals to the same exent of the AT2 receptor antagonist, suggesting that AT2 receptors mediating inhibitory effects may to be located at pre-junctional level..

Thus, during experimental inflammation the tonic activation of neural AT2 receptors would induce inhibitory enteric neuronal signaling ultimately resulting in a reduction of Ang II contractile effect. Inhibitory signaling in the ENS is dependent on the action of different mediators, including for instance NO, VIP and PACAP (Furness, 1994); since NO was already identified as a downstream mediator of AT2 receptor signaling (Israel et al., 2000), we investigated the possible interplay between AT2 receptors and nitric oxide by using different nitric oxide synthase blockers, including L-NNA (nNOS/eNOS blocker) and 1400W (iNOS blocker). L-NNA, but not 1400W, was able to increase Ang II-mediated

contraction, at as similar level of PD123319; furthermore, the additive administration of L-NNA and PD123319 did not induce further increase of Ang II-related contractile effect, suggesting that activation of AT2 receptors and nitric oxide signaling could effectively constitute two steps of a unique pathway. Moreover, the lack of similar effect of 1400W underlined the likely neural source of NO, reinforcing the possibility of activation of AT2 receptors located on enteric inhibitory nitrergic neurons. Lastly, another major finding of our study was that AT2 receptor antagonist PD123319 was able *per se* to significantly increase the amplitude of spontaneous colonic contractions, as well as the contractile and relaxant response to the muscarinic agonist carbachol and β2-adrenergic agonist Isoproterenol in DNBS-treated animals, but not in controls. This would imply that inflammation-induced changes in the enteric microenvironment could include a tonic activation of AT2 receptors, in turn involved in a negative modulation of the spontaneous mechanical activity. Also, AT2 receptors would induce a general depression of smooth muscle contractile properties, changing the sensitivity of enteric smooth muscle both to muscarinic and adrenergic activation.

The observed activation of AT2Rs in the course of experimental colitis represents a crucial starting point both for a major comprehension of AT2R role and for the understanding of novel pathways associated with bowel inflammation. Indeed, the shift from sole AT1 receptors activation in physiological condition to AT1/AT2 receptors activation in inflammation suggest that the local RAS system in the GI tract undergoes substantial modifications. If such changes represent solely an inflammation-induced effect on the RAS system or a "defense" mechanism of the ENS to counterbalance inflammatory damages is currently difficult to clarify. Plasticity is a well-known powerful property of the ENS (Schäfer et al., 2009), capable of opportunely reorganizing enteric circuitry in the attempt to preserve physiological GI function and respond to pathological changes in the enteric

milieu. As aforementioned, the RAS system in the GI tract seems to be implicated in sodium and water reabsorption (Garg et al., 2012), and AT2 receptors activation has been reported to stimulate absorption of such nutrients in the rat jejunum (Jin et al., 1998). Intriguingly, the mechanism underlying AT2-induced modulation of absorption is believed to involve NO production (Schirgi-Degen & Beubler, 1995) as reported in the kidney (Siragy & Carey, 1996;1997). Considering that water/electrolyte absorption is impaired in GI inflammation, including in IBD patients (Head et al., 1969; Barkas et al. 2013), the increase in AT2 receptors signaling and the consequent increase in NO production could represent a compensatory mechanism aiming to maintain adequate absorption during inflammation. However, considering the possible whole consequences of AT2 signaling on GI functions, in our experiment we revealed that the occurring tonic AT2 receptor activation mainly result both in a general impairment of enteric smooth muscle contractility and in defective spontaneous phasic contractions, suggesting that the shift from AT1 to AT2 receptors contribution in the modulation of colonic motility in the contest of inflammation could represent a novel target for the development of specific therapy for bowel dysmotility in IBD. Potentiated nitrergic neurotransmission could account for Ang II-induced changes in contractility, in accordance with the hypothesis of an increase in non adrenergic non cholinergic signaling as a possible cause of UC-associated colonic motor dysfunction (Tomita et al., 1998). In addition, the disclosed increased participation of AT2 receptors in inflammation suggests that novel researches addressing the role of AT2 receptors in inflammatory pathways are needed. Indeed, most of researches have been currently focused on the potential pro-inflammatory role of AT1 receptors in IBD, and on the use of AT1 receptor antagonists to ameliorate colitis in animal model. However, our data revealed that the understanding of the consequences of increased AT2 receptor signaling in the ENS, coupled to the nitrergic signaling, could lead to the

definition of novel possible Ang II-induced modulatory pathways on immune activity and inflammation, allowing a future depict of the comprehensive involvement of the local RAS system in GI inflammation.

CHAPTER 4

4.1 GABA: γ-aminobutyric acid

γ-aminobutyric acid (GABA) is an aminoacid derivate widely considered as the main inhibitory neurotransmitter (NT) in the central nervous system (CNS) of mammals, responsible, together with glutamate, of finely regulating neuronal excitability, and thus involved in numerous CNS functions, as well as in the physiopathology of neurological diseases including epilepsy, anxiety disorders, schizophrenia, sleep disorders, drug and alcohol addiction (Fagg & Foster, 1983; Watanabe et al., 2002). In the CNS, GABA is primarily concentrated in the substantia nigra and globus pallidus, as well as in the hypothalamus, the periaqueductal gray matter and hippocampus (Petroff, 2002). The Purkinje cells in the cerebellum are also GABAergic neurons utilizing the inhibitory action of GABA for the fine control and coordination of complex motor functions (Person & Raman, 2012). Apart from its prominent action in the CNS, different studies have demonstrated the presence of GABA in different peripheral tissues of mammals, and organs such as the pancreas, fallopian tubes, uterus, ovary, testes, kidneys, liver, lungs, stomach and intestines (Erdo, 1992), driving research efforts into the investigation of the action of this neurotransmitter outside the CNS.

4.2 Synthesis and metabolism

GABA is synthesized from glutamic acid by the glutamic acid decarboxylase (GAD), using piridossalphosphate as cofactor (Rowley et al., 2012). Once released in the synapse, GABA can act on specific different ionotropic and metabotropic receptors localized in pre- or postsinaptyc nerve terminals, as long as its action is terminated by GABA transporters

(GATs) allowing reuptake of GABA in the axon terminals or glial cells. Subsequently, a GABA transaminase (GABA-T) converts GABA into a succinic semialdehyde. The amino group removed from GABA is transferred from the GABA-T to a molecule of α-ketoglutarate to form glutamic acid for the production of new GABA. Also, succinic semialdehyde can be oxidized by succinic semialdehyde dehydrogenase (SSADH) into succinic acid and can then enter the Krebs cycle.

4.3 GABA receptors

GABA interacts with three types of receptors: $GABA_A$, $GABA_B$ and $GABA_C$ receptors (Bormann, 2000). $GABA_A$ and $GABA_C$ receptors are ionotropic receptors belonging to the family of ligand-gated Cl^- channels, and mainly responsible for mediating the fast inhibitory activity of GABA. GABA-dependent chloride channel gating is generally inhibitory on a neuron by mediating inflow of chloride anions, in turn hyperpolarizing the neuronal membrane; however, under conditions of high intracellular chloride, as reported both in immature neurons and in enteric neurons, GABA-related outflow of chloride anions occur, inducing depolarization of the membrane potential (Xue et al., 2009; Liu et al., 2013). Such depolarizing action of GABA has been demonstrated as an important mechanism in brain development, as well as in the modulation of gastrointestinal functions (Perrot-Sinal et al., 2003; Krantis, 2000). $GABA_B$ receptors are heterodimeric metabotropic G protein-coupled receptors (GPCRs), negatively acting on presynaptic voltage-activated Ca^{2+}channels and positively acting on postsynaptic inwardly rectifying K^+ channels, classically mediating the long-term inhibitory action of GABA (Bormann, 2000).

4.3.1 $GABA_A$ receptors

- **Structure**

$GABA_A$ receptors are pentameric receptors composed of a combination of five subunits constituting a chloride channel (Sigel & Steinmann, 2012) (Fig.21). Each $GABA_A$ receptor subunit is composed of a extracellular N-terminal domain and four membrane-spanning domains (M1-M4), followed by a short extracellular C-terminal domain. A long intracellular loop between M3 and M4 is believed to be a possible a target for protein kinases as well as for anchoring to the cytoskeleton. $GABA_A$ receptor subunits have been classified into eight classes, including α, β, γ, δ, ε, θ, π and ρ; moreover, several isoforms codified by distinct genes have been reported: α (1-6), β (1-4), γ (1-3), ρ (1-3) (Barnard et al., 1998). The aminoacid homology between the different classes is about 30-40%, suggesting their common origin from an ancestral gene. The most common $GABA_A$ receptor contains two α, two β and one γ subunit (α2β2γ), rarely, subunit δ, ε e π can substitute γ. Although ρ subunits are classically listed as $GABA_A$ receptors subunits, they do not coassemble with the other $GABA_A$ subunits, but rather form homooligomery (Jembrek & Vlainic, 2015). Receptors exclusively formed by ρ subunits have been initially classified as $GABA_C$ receptors. Subsequently, because of the structural homology with $GABA_A$ receptors, the International Union of Pharmacology subcommittee on nomenclature recommended to consider $GABA_C$ receptors as a subtype of $GABA_A$ receptors, named $GABA_A$-ρ receptors. However, accumulating and ongoing studies strongly support the distinct pharmacology, function, genetic and localization of these receptors, questioning about the necessity of an effective distinction between $GABA_A$ and $GABA_C$ receptors (Enz, 2001). Since $GABA_A$ receptors are pentameric assemblies formed by a combination of numerous subunits, a large number of receptor subtypes could occur. This great heterogeneity could confirm the great importance of (A)-GABAergic

neurotransmission in the fine regulation of neuronal excitability, with the necessity of different $GABA_A$ receptor subtypes deputed to different functions. Indeed, ongoing studies recent successfully demonstrated the possibility of a separation of central effects of GABAergic agents, as sedation, anxiolysis and memory enhancement, *via* the use of subtype-selective $GABA_A$ agonists (Rudolph & Möhler, 2006); as an example, α2- or α3-containing $GABA_A$ receptors have been reported to specifically provide anxiolysis without sedation. Moreover, Seifi et al. (2014) recently described the complexity of the (A)-GABAergic system in mouse colon, since subtype-specific GABAergic agents displayed a wide range of effects on colonic contractility, likely related to the diverse subunit composition of $GABA_A$ receptors located on populations of enteric neurons. Hence, future scientific efforts are needed to establish the functional correlation between the different $GABA_A$ receptor subtypes and specific neuronal functions, also in view of developing novel $GABA_A$-related therapeutic agents.

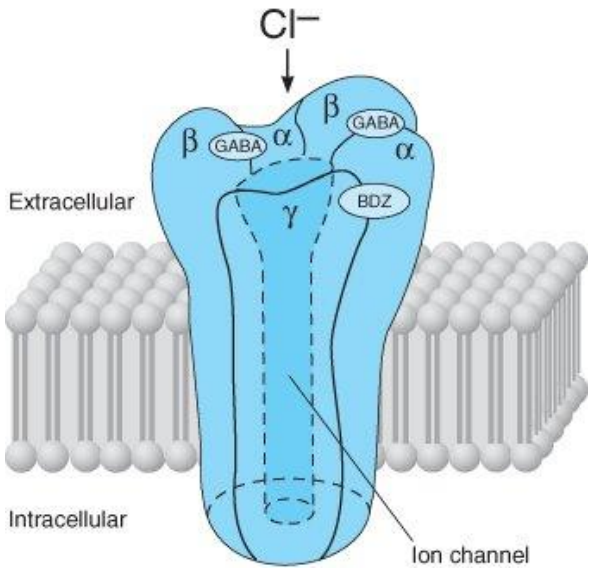

Figure 21: Schematic representation of a classic α2β2γ $GABA_A$ receptor.

- **Pharmacology**

The $GABA_A$ receptors are pharmacologically distinct from the other GABA receptors *via* their sensitivity to the action of drugs as bicuculline, acting as antagonist, and of muscimol, acting as agonist. The $GABA_A$ receptor binds two molecules of GABA at the interface between the α and the β subunits; once bound to GABA, the protein receptor changes conformation within the membrane, opening the pore and allowing chloride anions (Cl^-) to flow (Bormann, 2000). As aforementioned, in most of neurons activation of $GABA_A$ receptors results in inflow of chloride anions with an hyperpolarizing effect, reducing the neuronal excitability. However, in population of neurons sustaining elevated intracellular Cl^- concentration, as immature and enteric neurons, activation of $GABA_A$ receptors results in an outflow of chloride depolarizing the membrane potential and thus resulting in prominent excitatory effects. Apart the binding sites for GABA, $GABA_A$ receptors contain binding sites for several important drugs, including benzodiazepines, barbiturates, neurosteroids and ethanol, acting as allosteric modulators classically reinforcing the effect of GABA (Sieghart, 2015). Moreover, different subtypes of $GABA_A$ receptors are responsible for two forms of GABA-mediated inhibition, depending on the localization of the receptors (Farrant & Nusser, 2005). Synaptic $GABA_A$ receptors, localized on the postsynaptic membrane, mediate a classical temporary "phasic" inhibition, whilst extrasynaptic $GABA_A$ receptors, located outside the synapse, mediate a persistent "tonic" inhibition. Extrasynaptic $GABA_A$ receptors display an higher affinity for GABA compared to synaptic $GABA_A$ receptors, as well as a reduced desensitization; also, extrasynaptic receptors are insensitive to benzodiazepine, but high sensitive to barbiturates, suggesting they could be the key targets for anesthetics.

4.3.2 $GABA_B$ receptors

$GABA_B$ receptors are heterodimeric receptors pharmacologically sensitive to the antispastic baclofen (agonist) and phaclofen (antagonist). $GABA_B$ receptors are constituted by two different subunits containing seven transmembrane domains each: the GABAB1 subunit, bearing the binding site for GABA, and the GABAB2 subunit, coupled to a G-protein (Bettler et al., 2004) (Fig.22). Also, GABAB2 subunit is needed to mask an endoplasmic reticulum retention signal of GABAB1 and to enhance agonist affinity. $GABA_B$ receptors are the main responsible for GABA-mediated long term inhibition, through their dual presynaptic and postsynaptic localization:

- **Presynaptic GABAB receptors** negatively influence and control the release of GABA itself ($GABA_B$ autoceptors) or other NTs ($GABA_B$ heteroceptors; i.e. glutamate, noradrenaline, dopamine) through inhibition of voltage-gated Ca^{2+} channels (Lewis, 2010). Presynaptic $GABA_B$ autoceptors are thus involved in a negative feedback avoiding excessive release of GABA in the synapse, whilst presynaptic $GABA_B$ heteroceptors finely regulate the release of numerous neurotransmitters, permitting the maintenance of a balance between excitatory and inhibitory neurotransmission.
- **Postsynaptic GABAB receptors** positively regulate K^+ channels causing hyperpolarization of the postsynaptic membrane, and so reducing neuronal excitability (Gage, 1992).

Although $GABA_B$-dependent inhibitory effect on adenylate cyclase has been also reported (Dolphin, 1984), the functional significance of such latter action is still largely unclear.

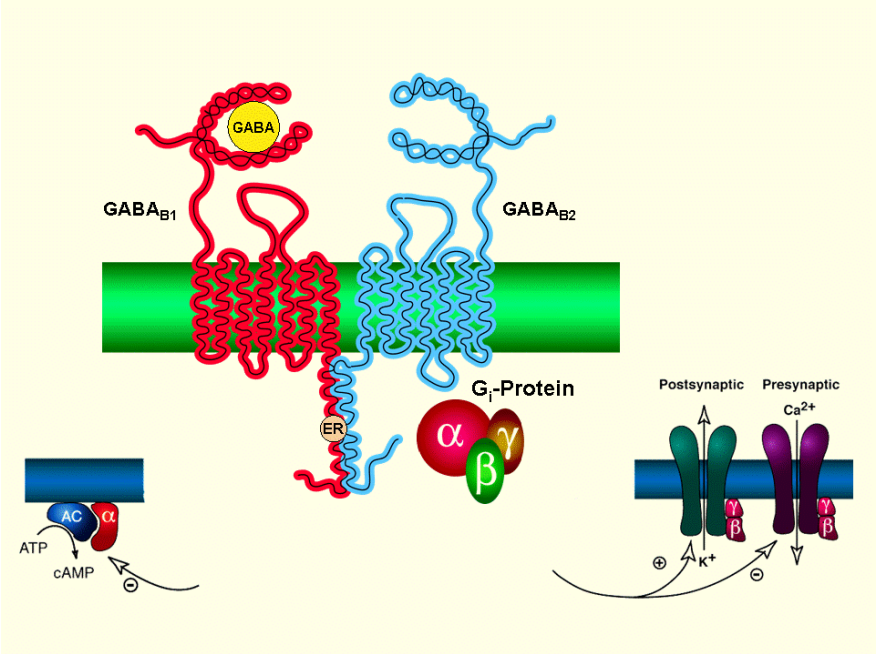

Figure 22: Schematic representation of the $GABA_B$ receptor and related major intracellular pathways

4.3.3 $GABA_C$ receptors

As aforementioned, ionotropic $GABA_C$ receptors are currently classified as a subclass of $GABA_A$ receptors, namely $GABA_{A-\rho}$ receptors. However, different studies suggest the necessity of a precise distinction of $GABA_A$ and $GABA_C$ receptors based on pharmacological properties, genetics and function (Enz, 2001). $GABA_C$ receptors are pentameric receptors pharmacologically sensitive to cis-4-aminocrotonic acid (CACA, specific agonist) and (1,2,5,6-Tetrahydropyridin-4-yl)methylphosphinic acid (TPMPA, specific antagonist). $GABA_C$ receptors are entirely composed of ρ subunits (ρ subunits: ρ1-ρ3), constituting chloride channels as $GABA_A$ receptors (Fig.23). Although $GABA_C$ receptors are primarily expressed in the retina, participating in retinal signal processing (Lukasiewicz et al., 2004), evidence for functional $GABA_C$ receptors in the spinal cord, pituitary, superior colliculus and the gut has been reported (Johnston et al., 2003), suggesting that a more detailed functional characterization of $GABA_C$ receptors in the should be recommended in order to disclose their effective role besides the retina.

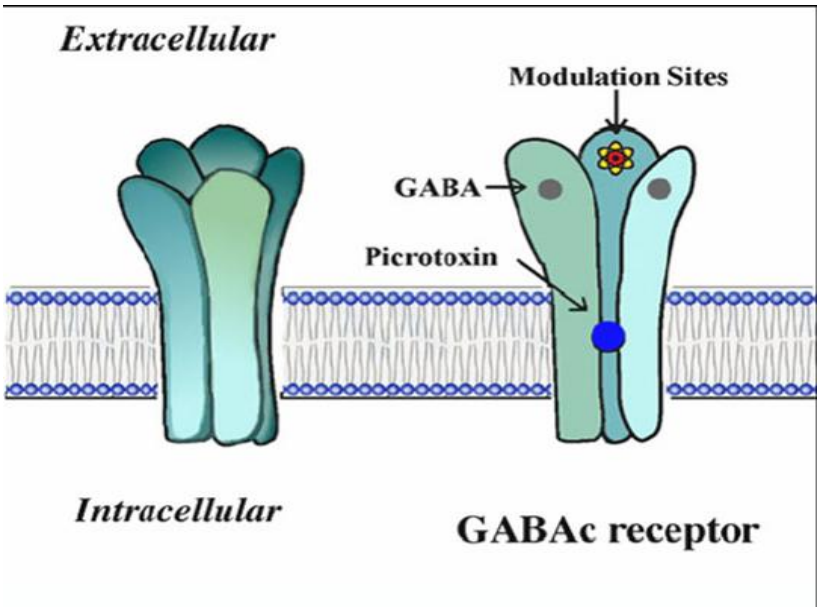

Figure 23: Schematic representation of the $GABA_C$ receptor

4.4 The enteric GABAergic system: focus on colonic motility and inflammation

Among the modulatory NTs in the ENS, a plethora of studies addressed γ-aminobutyric acid (GABA) as a potential powerful mediator involved in different enteric neural circuits underlying GI motility as well as submucosal and mucosal functions (Krantis, 2000; Hyland & Cryan, 2010; Auteri et al., 2015) (Fig.24). GABA meet all the criteria to be considered an enteric mediator, since GABA-synthesizing enzyme glutamate decarboxylase (GAD) has been found in the ENS, particularly in the myenteric plexus, as well as the degrading enzyme, GABA transaminase (GABA-T) (Tanaka, 1985; Williamson et al., 1995). High affinity membrane GABA transporters have been shown both on enteric neurons and enteric glial cells, and GABA release from enteric neurons is calcium- and tetrodotoxin-(TTX) dependent (Taniyama et al., 1982). However, to date the exact significance of GABAergic signaling in the GI tract is still incompletely understood. Regarding the GABAergic neuronal network in the gut, GABAergic neurons have been

reported in the myenteric, submucosal and mucosal plexi. In the myenteric plexus, GABA has been reported in somatostatin-, NO- and encephalin-containing neurons, whilst GABAergic cells in the submucosa and mucosa co-localize with NO or somatostatin (Krantis, 2000). GABA is also secreted by mucosal endocrine-like cells, likely G- and D-cells in the stomach, implying its potential as local paracrine/autocrine mediator (Krantis et al., 1994). In addition, a series of studies have demonstrated the presence of GABA receptors, $GABA_A$, $GABA_B$ and $GABA_C$ receptors, both in neuronal and non-neuronal cells in the entire GI tract (Poulter et al., 1999; Casanova et al., 2009). In particular, classical $\alpha 2\beta 2\gamma$ $GABA_A$ receptors are found both in myenteric and submucosal neurons, as well as in intestinal epithelial cells. $GABA_A$ receptors in the ENS were found to elicit depolarizing effects, since enteric neurons maintain prominent intracellular Cl^- concentration, possibly through the action of a sodium-potassium-chloride symporter (Xue et al., 2009). The depolarization induced by ionotropic $GABA_A$ receptor activation lead to an influence on NTs release from both excitatory and inhibitory neurons. Regarding metabotropic $GABA_B$ receptors, studies in the rat GI tract revealed distribution of $GABA_B$ receptors in submucosal and myenteric neurons, in the latter mainly on nitrergic neurons. However, a large amount of pharmacological evidence showed that the main action of metabotropic $GABA_B$ receptors in the ENS is the presynaptic inhibition of ACh release *via* inhibition of voltage-sensitive calcium channels (Marcoli et al., 2000). In addition, different rat GI epithelial cells from the stomach to the colon possess $GABA_B$ receptors (Davanger et al., 1994), supporting the possibility of GABA involvement in the regulation of gastric functions. Moreover, in the small intestine, many $GABA_B$-immunoreactive cells contain 5-HT, supporting their identification as enterochromaffin (EC) cells, and suggesting the involvement of $GABA_B$ receptors in local secretory and peristaltic reflexes (Krantis, 2000). Few studies focused on the localization and functional role of the $GABA_C$

receptors in the gut. $GABA_C$ receptor subunits have been found in the rat duodenum, ileum and colon, in IPANs and nitric oxide synthase (NOS)-immunoreactive neurons (Johnston et al., 2003). Interestingly, the specific $GABA_C$ agonist CACA was found to induce a promoting effect on the release of NO from inhibitory motoneurons in rodents (Zizzo et al., 2007). Thus, a more detailed characterization of $GABA_C$ receptors in the GI tract is needed to disclose their effective involvement in the modulation of GI functions. Regarding the GABAergic receptor system in humans, $GABA_B$ receptor subunits have been found in the lower esophageal sphincter (LES), stomach and small intestine (Calver et al., 2000; Torashima et al., 2009), whilst no study addressed the localization of ionotropic $GABA_A$ and $GABA_C$ receptors in the human GI tract. Thus, novel investigations addressing the enteric GABAergic receptors in humans are absolutely needed to disclose the effective role of GABA in the regulation of GI function.

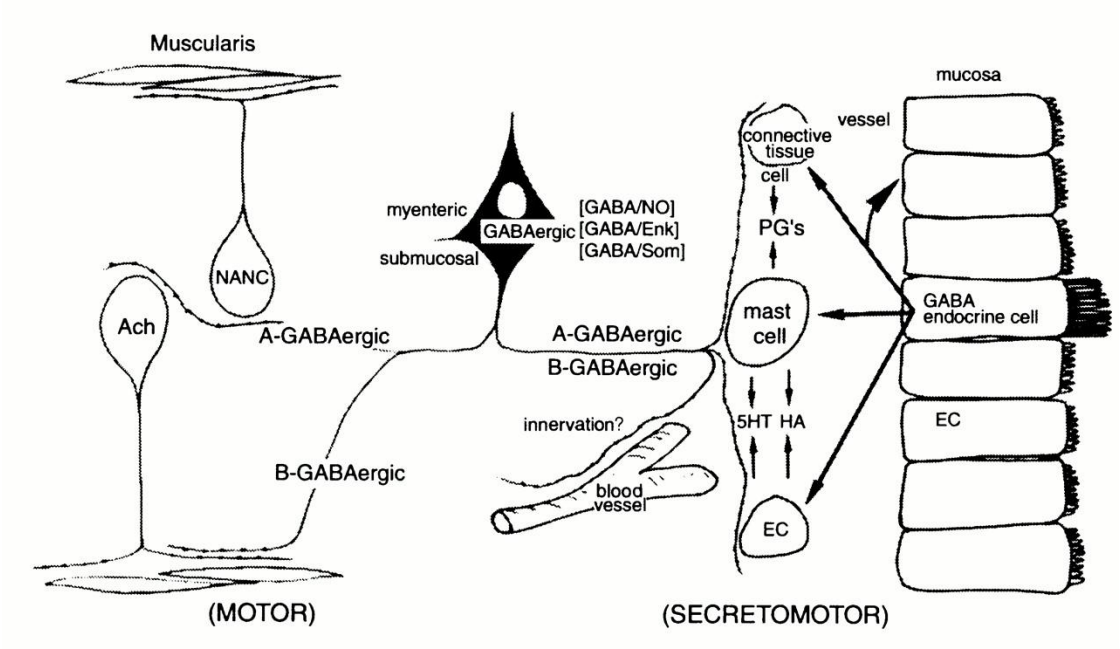

Figure 24: GABAergic innervation in the intestinal wall. ACh, cholinergic motor neurons; EC; Enterochromaffin cells; ENK, Enkephalin; NANC, non adrenergic non cholinergic motor neurons; 5-HT, 5-hydroxytryptamine; HA, histamine; EC, enterochromaffin cell; NO, Nitric Oxide; PG, prostaglandins. (Adapted from Krantis et al., 2000)

The involvement of enteric GABAergic signaling on GI motility has been extensively reported, disclosing a variety of action of GABA and GABAergic drugs in the different GI tract (Auteri et al., 2015). In the stomach, $GABA_A$ receptors have been linked to a facilitatory effect on non adrenergic non cholinergic (NANC) neurotransmission (Krantis et al., 1998), whilst activation of $GABA_B$ receptor has been related to a modulatory action of GABA on the vagal drive to the stomach, in turn influencing gastric tone and contractility (Andrews et al., 1987). In the small intestine, effects of GABA include the modulation of both excitatory and inhibitory signaling in the ENS, and, specifically, a fine regulation of ACh release in the ileum has been demonstrated (Roberts et al., 1993). However, even more interesting is the potential role of GABAergic pathways in the large intestine. Indeed, studies addressing the effects of GABA on the colonic mechanical activity were closely associated to its action in the modulation of the most important physiological colonic motor pattern, the peristalsis. Peristalsis is a reflex controlled by the ENS and induced by the mechanical and chemical stimuli determined by intestinal contents on the intestinal mucosa. These stimuli induce 5-HT release from enterochromaffin (EC) cells, in turn activating intrinsic sensory neurons involved in the initiation of specific enteric neural circuits (Grider, 2003). Indeed, peristalsis consists of a neural ascending excitation and descending inhibition, leading to a muscular contraction orally and relaxation aborally to the stimulated GI tract. The resulting rhythmic and coordinated motor behaviour allows the physiological propulsion of luminal contents along the colon. Ascending contraction depends on the release of ACh and substance P (SP) from enteric excitatory motor neurons, whilst descending inhibition is related to the muscular response to mediators released by enteric inhibitory motor neurons, namely NO, VIP, ATP and pituitary adenylate cyclase-activating peptide (PACAP). A complex network of enteric interneurons, including ACh-, VIP-, NO-, somatostatin- and opioids-containing neurons, is

implicated in modulating the peristaltic activity. Different researches reported that GABAergic neurons are likely involved in this ENS circuitry underlying the modulation of peristaltic activity, although its effective role in regulating the colonic mechanical activity are still a matter of debate. Both GABA and the selective $GABA_B$ agonist baclofen reduced peristaltic propulsion in the rabbit *via* the modulation of the cholinergic signal (Tonini et al., 1989), whilst $GABA_A$ receptor blockade induced an inhibitory effect on the peristaltic activity, both on ascending contraction and descending relaxation (Grider & Makhlouf, 1992). Subsequent studies demonstrated that GABA is able to activate a population of VIP/PACAP/NOS interneurons (Grider, 1998), in turn positively influencing the release of ACh and substance P from excitatory motor neurons mediating the ascending contraction. Also, GABA has been reported to be actively involved in a functional enteric network, comprising somatostatinergic and opioidergic neurons, allowing the descending relaxation *via* a promotion of neurotransmitters release from inhibitory motor neurons (Grider, 1994). Although these results already suggest the potential importance of GABAergic pathways in the modulation of colonic peristalsis, the effective significance of GABA signaling in the control of physiologic colonic mechanical activity is still unclear, and additional studies are needed to unravel the effective significance of GABA signaling on colonic motor patterns. In addition, a recent study by Seifi et al. (2014) demonstrated the complexity of the (A)-GABAergic system in mouse colon, since subtype-specific GABAergic agents displayed a wide range of effects on colonic contractility, likely related to the diverse localization and function of differently composed $GABA_A$ receptors on populations of enteric neurons. Drugs specifically activating α1-γ2-containing $GABA_A$ receptors or α4-containing $GABA_A$ receptors increased the force of spontaneous contractions, agonist at α2-γ2-containing $GABA_A$ receptors increased the frequency of contractions, whilst activation of α3-γ2- and α5-γ2-containing receptors was associated

with decreased force of spontaneous colonic contractions (Seifi et al., 2014). Such finding are of particular importance in a therapeutic view, since GABAergic subtype-specific drugs could be developed to possibly realize a precise desirable effect in the colonic environment avoiding unwanted actions. The precise characterization of GABAergic pathways in the regulation of colonic motility could thus lead to the identification of pathological modification of GABA signaling in GI motor disorders, including GI disturbances in IBD. Indeed, possible changes of GABAergic signaling in the course of inflammatory conditions are potentially linked to the disclosed role of GABA as one of the neuromodulators involved in the regulation of immune cell activity and inflammatory events, since GABA receptors were found on several immune cells, including DCs, macrophages and T cells (Jin et al., 2013). Although GABAergic fibers in the gut are mainly interneurons, occurring within the myenteric and submucosal plexus in animal models and humans, the presence of GABAergic neuronal cells ramifying within the mucosa, and so potentially functionally associated with immune cells in the GALT have been reported (Krantis, 2000). Moreover, GABAergic neurons in the submucosa are likely to be involved in a ENS circuitry modulating histamine release from mucosal mast cells (MacNaughton et al., 1996) supporting the presence of GABA-related enteric neuroimmune pathways. Intriguingly, agonists of $GABA_A$ receptors have been reported to dampen macrophage's cytokine production and to reduce T-cell proliferation, whilst $GABA_B$ agonists inhibited both TNF-α production from peripheral blood mononucleated cells and IL-6 and IL-12 release from microglia (Bjurstöm et al., 2008; Jin et al., 2013). Overall, these effects primarily support a possible anti-inflammatory action of GABA *via* the negative control of major inflammatory processes in different immune cells. Accordingly, *in vivo* studies revealed a positive action of GABA treatment on animal models of inflammation. For instance, GABA treatment ameliorates inflammation in a

mouse model of rheumatoid arthritis as well as in non-obese diabetic mice (Tian et al., 2004, 2011). Furthermore, $GABA_B$ agonists ameliorated dermatitis in mice (Duthey et al., 2010), and $GABA_A$ agonists improved experimental encefalomielitis (Bath et al., 2010). Although scarce investigations have addressed the possible action of GABA on GI inflammation, a pioneer study by Dudley et al. (2011) demonstrated that the anti-epileptic drug topiramate, possessing $GABA_A$ agonism properties, reduces macroscopic and microscopic GI inflammatory scores in the 2,4,6-Trinitrobenzenesulfonic acid (TNBS)-induced rat model of IBD. However, the mechanism underlying topiramate-induced effect on IBD inflammation remain unknown, and so further investigations are needed to reveal the potential involvement of GABA and GABAergic drugs in the IBD-related inflammation.

4.5
Opposite role played by $GABA_A$ and $GABA_B$ receptors in the modulation of peristaltic activity in mouse distal colon

4.5.1 Aim

As previously stated, enteric GABA signaling is believed to be involved in colonic peristalsis, although its effective contribution is far from being clear. Therefore, the aim of this study was to analyze, *in vitro*, the possible effect of GABA on the colonic mechanical activity, using as model the mouse distal colon. In particular, we studied the effects induced by GABA receptors recruitment in both isolated circular muscle preparations and whole colonic segments, in order to determine their influence both on the spontaneous and electrically-evoked colonic contraction and on the experimentally-induced peristaltic reflex, respectively.

4.5.2 Materials and Methods

Animals

Experiments were performed on adult male C57BL/6 mice obtained from Charles River Laboratories (Calco- Lecco, Italy). Animals were kept under environmentally controlled condition (ambient temperature 24 °C, humidity 60% and 12 h light/dark cycle) with food and water *ad libitum*. Procedures involving animals and their care were conducted in conformity the Italian D.L. no. 116 of 27 January 1992 and associated guidelines in the European Communities Council Directive of 24 November 1986 (86/609/ECC). The mice were euthanized using isoflurane anesthesia followed by cervical dislocation, and after laparotomy the colon was rapidly excised and placed in Krebs solution. Then, whole segments of distal colon (about 5 mm proximal to the anus) of approximately 3.5 cm

length or circular muscle strips (10 mm in length) were prepared and used for the different experiments.

In vitro measurement of colonic peristaltic activity

A modified Trendelenburg set-up (Figure 25a) was used to induce peristaltic activity in isolated colonic segments (Trendelenburg, 2006; Seerden et.al, 2007). Colonic segment was mounted horizontally in a 4 ml organ bath filled with warm and oxygenated Krebs solution (37 °C, 95% O2 and 5% CO_2) (Figure 25b). The aboral end was secured to an open, adjustable outlet that could be raised in height. The oral side of the segment was connected to a pressure transducer (Statham Mod. P23XL; Grass Medical Instruments, Quincy, MA, USA) for the recording of intraluminal pressure differences and to a perfusion pump allowing continuous intraluminal infusion of warm and oxygenated Krebs solution at a rate of 0.5 ml/min.

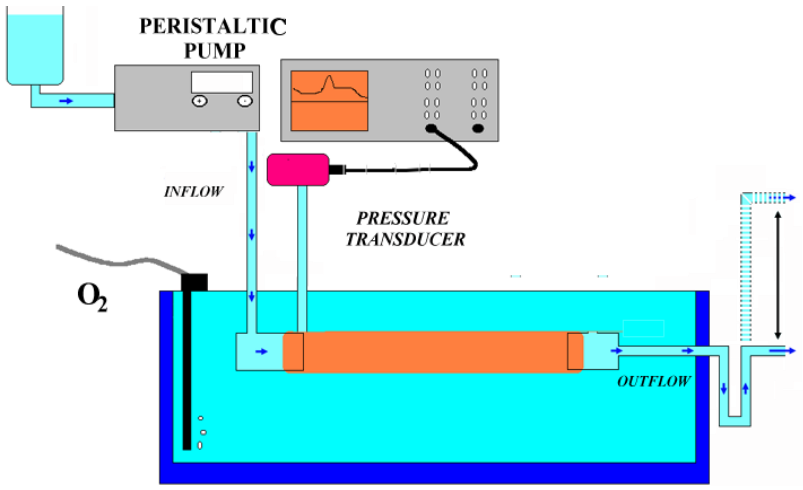

Figure 25 (a): Schematic representation of the Trendelenburg set-up for the *in vitro* analysis of peristaltic activity.

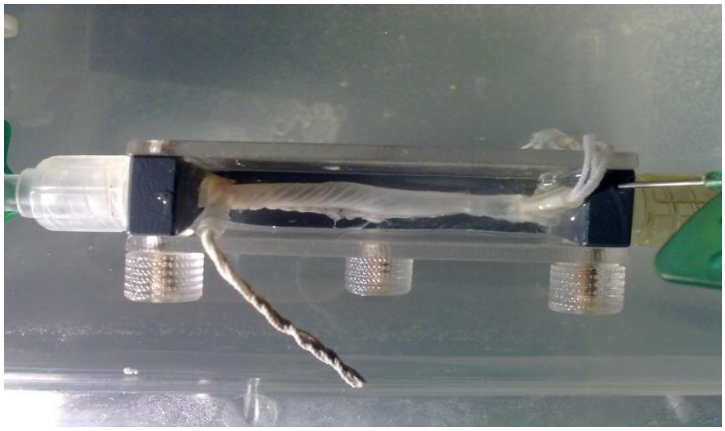

Figure 25 (b): Particular of colonic segment mounted in the Trendelenburg set-up

The tissue was allowed to equilibrate for about 20 min and then the outlet was gradually moved up to a height of 7.5 cm by increments of 2.5 cm every 20 min. The gradual distension of the colonic tissue resulted in rhythmic and repetitive peristaltic contractions propagating aborally, which were recorded by the pressure transducer at the oral side of the segment as cyclic pressure waves and recorded on ink-writer polygraph (Grass model 7D). Time control experiments showed that at an outlet-height of 7.5 cm pressure waves were highly reproducible persisting for several hours. In a first series of experiments, tissues were exposed to cumulative concentrations of GABA with an incubation time of 5 min per concentration. Then, using specific agonists and antagonists for the GABAergic receptor subtypes we evaluated the specific contribution of each receptor to the peristaltic activity. Each preparation was tested with a single agonist/antagonist, except when otherwise stated.

In vitro analysis of the mechanical activity of colonic circular muscle strips

Segments of distal colon were opened along the mesenteric border and pinned mucosa side up. The mucosa was removed by sharp dissection under a microscope and full-thickness muscular strips (10 mm in length) were cut in the direction of circular muscle and

suspended in a four-channel organ bath containing 10 ml of oxygenated (95% O_2 and 5% CO_2) Krebs solution maintained to 37 °C. The distal end of each strip was tied to an organ holder and the proximal end was secured with a silk thread to an isometric force transducer (FORT 25, Ugo Basile, Biological Research Apparatus, Comerio VA, Italy). Mechanical activity was amplified and digitized *via* an analog/digital interface (Quad Bridge and PowerLab/400, AD Instruments, Ugo Basile, Biological Research Apparatus, Comerio VA, Italy), prior being acquired onto a personal computer. The preparations were subjected to an initial tension of 500 mg and were allowed to equilibrate for at least 30 min. After the equilibration time, preparations were challenged with 10 μM carbachol (CCh) for 2 min, until stable responses were obtained. The contractile response to CCh (10μM) was 962.2± 87.7 mg, n=20. Electrical field stimulation (EFS) was applied from a Grass S88 electrical stimulator (Grass Instruments Co., Quincy, Mass, USA) through a stimulus isolation unit (SIU5) using direct coupling. Stimuli (0.5 ms, 10 V for 10 s) were delivered *via* a pair of platinum plate electrodes. 4 Hz frequency was chosen to specifically activate cholinergic neurons, whilst 32 Hz frequency was selected to elicit a non adrenergic non cholinergic (NANC) response in the presence of atropine and guanethidine (1 μM each). GABA or GABAergic receptor agonists were tested on EFS responses being applied for approximately 5 min at 20 min intervals. GABAergic antagonists were left in contact with the tissue at least for 30 min. Each preparation was tested with a single agonist/antagonist, except when otherwise stated. Concentrations of the drugs used were determined from literature (Zizzo et al., 2007; Rotondo et al., 2010).

Solutions and drugs

The following drugs were used: atropine sulfate, baclofen, bicuculline, cis-4-aminocrotonic acid (CACA), γ-aminobutyric acid (GABA), guanethidine monosulphate, hexamethonium

bromide, (1,2,5,6-tetrahydropyridin-4-yl) methylphosphinic acid hydrate (TPMPA), tetrodotoxin (TTX), all purchased from Sigma (Sigma-Aldrich, Inc., St. Louis, USA). Phaclofen was from Tocris (Tocris Cookson LtD., Avonmouth, UK). Bicuculline was dissolved in dimethyl sulphoxide (DMSO), phaclofen in 0.1 N NaOH and all the other drugs were dissolved in distilled water. The working solutions were prepared fresh on the day of the experiment by diluting the stock solutions in Krebs. The final volume of DMSO or NaOH in the organ bath did not exceed 0.1% and control experiments showed that they have no effect on the spontaneous contractile activity or on the peristaltic activity.

Data analysis and statistical tests

Colonic peristaltic activity was assessed for each colonic segment by quantifying the maximal amplitude (cm H_2O) and the interval (s) between the peaks of two successive contractions. The different parameters of peristalsis from five consecutive contractions were measured immediately prior to addition of drugs and during treatments. Contractile responses to EFS were expressed as a percentage of the contractile response produced by 10 µM CCh. All data are expressed as means±SEM. The letter n indicates the number of experimental animals. Statistical analysis was performed by means of Student′s t test or by means of analysis of variance followed by Bonferroni′s test, as appropriate. (PRISM, version 4.0; GraphPad software Inc., San Diego, CA, USA). A p value of less than 0.05 was regarded as significant.

4.5.3. Results

Effects of GABA on colonic peristaltic activity in mice

The gradual distension of distal colonic segments mounted in the Trendelenburg system induced a series of rhythmic peristaltic contractions propagating aborally with a mean pressure amplitude of 16 cm H_2O and a mean interval of 60 s (Fig. 26). Administration of GABA induced diverse effects on peristaltic activity depending on the concentration used. At a concentration range from 10 µM to 50 µM, GABA caused a significant increase in the amplitude of the peristaltic waves, but it did not significantly modify the interval of peristaltic waves (Fig.26). On the other hand, administration of GABA at concentration 500µM-1mM, induced a significant inhibition of peristaltic activity, reducing the amplitude and increasing the interval of peristaltic waves (Fig. 26).

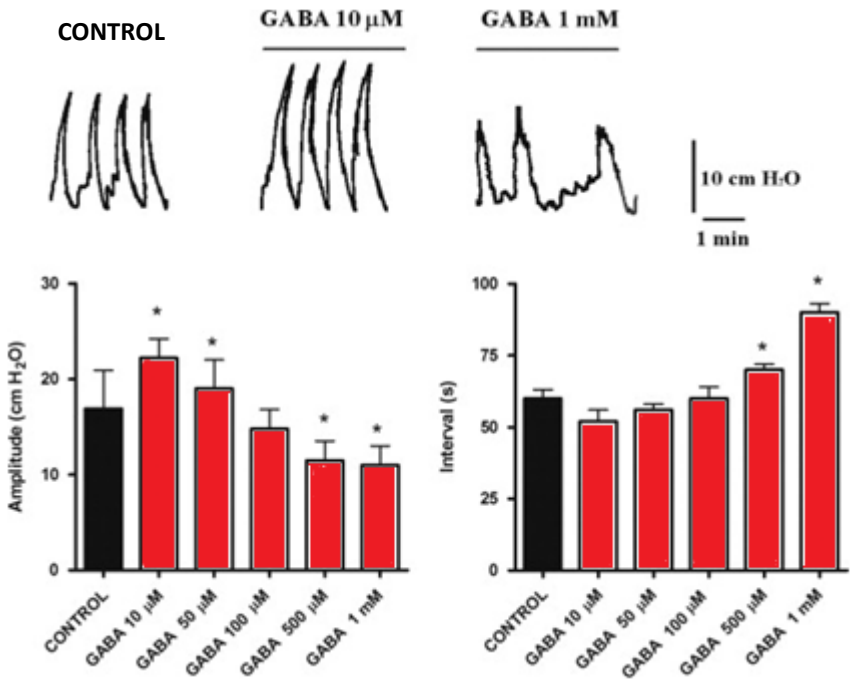

Figure 26: GABA effects on peristaltic pressure waves in mouse distal colon. (**Top)** Original tracings showing the distension-induced pressure waves in mouse distal colon in control condition or in the presence of GABA 10 µM and GABA 1 mM. **(Bottom)** Concentration-dependent effects of GABA (10 µM–1 mM) on the amplitude and interval of distension-induced colonic peristaltic activity in mouse distal colon. Results are expressed as means±S.E.M. (n=12). $^{*}P$<0.05 *versus* control.

Interestingly, the excitatory effect of low GABA concentrations were mimicked by administration of muscimol (100 µM) (Fig. 27a,b), a selective $GABA_A$-receptor agonist, and antagonized by pretreatment with bicuculline (10µM), a selective $GABA_A$-receptor antagonist. Intriguingly, bicuculline *per se* was able to reduce the amplitude of peristaltic waves (Fig. 27a). On the other hand, the inhibitory effect of higher concentration of GABA were mimicked by the selective $GABA_B$ receptor agonist baclofen (100 µM), and prevented by phaclofen (10 µM), $GABA_B$-receptor antagonist (Fig. 27a,b). Phaclofen *per se* showed no effect on peristaltic activity. $GABA_C$ receptor agonist CACA (100 µM) and $GABA_C$ receptor antagonist TPMPA (10 µM) did not influence the amplitude and interval of colonic peristaltic waves (Fig. 27a,b).

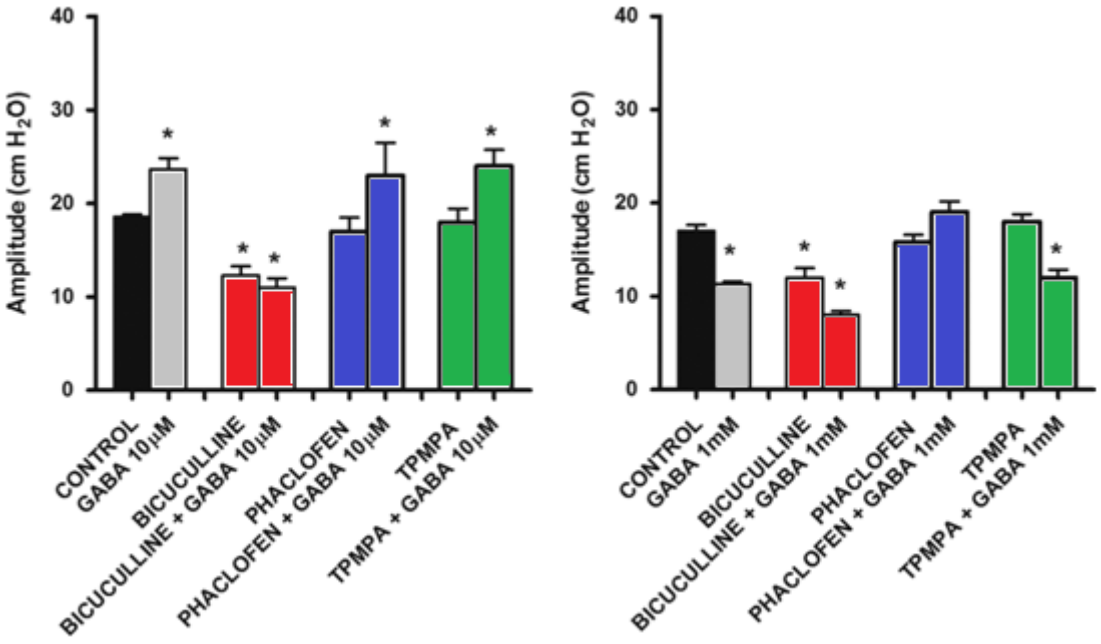

Figure 27 (a): Effects of GABA and of selective GABA receptor antagonists on peristaltic pressure waves in mouse distal colon. Histograms showing the effects induced by 10 µM GABA (left) or by 1 mM GABA (right) on the amplitude of colonic peristaltic activity in mouse distal colon in the absence or in the presence of bicuculline (10 µM, *n*=4 each), phaclofen (10 µM, *n*=4 each) and TPMPA (10 µM, *n*=3 each) $GABA_A$, $GABA_B$ and $GABA_C$ receptor antagonists respectively. Results are reported as means±S.E.M. The graphed values for the control bars are the means of the data obtained before each treatment. $^*P<0.05$ when compared to its own control.

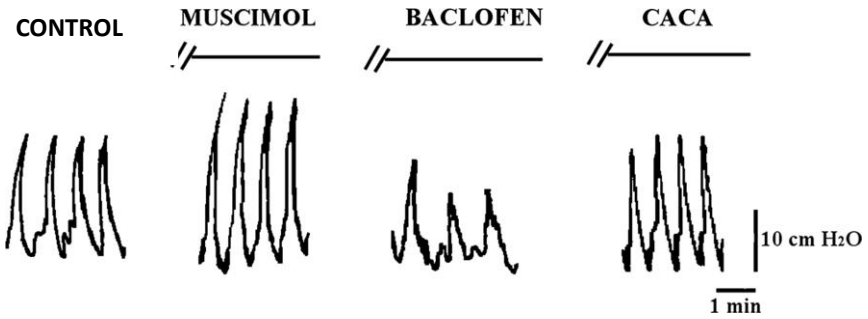

Fig. 27 (b): Effects of selective GABA receptor agonists on peristaltic pressure waves in mouse distal colon. Original tracings showing the effects induced by muscimol (100 μM), baclofen (100 μM) or CACA (100 μM), $GABA_A$, $GABA_B$ and $GABA_C$ receptor agonists respectively, on the distension-induced pressure waves in mouse distal colon.

Effect of GABA on spontaneous and electrically-evoked colonic circular muscle contractions in mice

Circular muscular strips isolated from mouse distal colon, once placed in the organ bath and after a period of stabilization, developed a spontaneous mechanical activity consisting of rhythmic contractions with an amplitude of 245.0±15.2 mg and a frequency of 4.4±0.3 cpm (contractions per minute) (n=20). Administration of GABA or GABAergic drugs did not influence the amplitude and frequency of spontaneous contractile activity (data not shown). Electrical field stimulation (EFS: 0.5 ms, 4 Hz, 10 V for 10 s) elicited a biphasic response: a low in amplitude transient muscular relaxation followed by a high in amplitude contraction (439.8± 31.0 mg, n=24), the latter depending on the release of ACh from enteric neurons (Zizzo et al., 2011). Treatment with GABA diversely influenced the neurally-evoked cholinergic contractions depending on the concentration utilized. GABA at 10–50 μM concentrations significantly increased the neurally-evoked cholinergic contractile responses to EFS, whilst in the range of 500 μM–1 mM GABA caused a significant decrease of the same responses (Fig. 28).

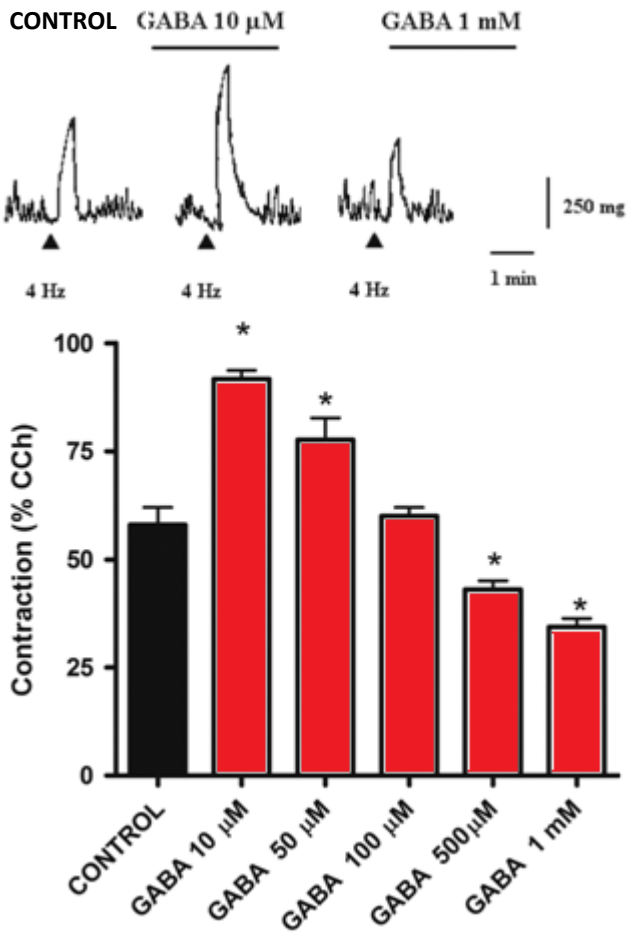

Figure 28: Effects of GABA on cholinergic electrically-evoked responses in the circular muscle of mouse distal colon. **(Top)** Original tracings showing the effects induced by 10 µM GABA or by 1 mM GABA on the cholinergic electrically-evoked responses in the circular muscle strips of mouse distal colon. Electrical field stimulation was delivered at 0.5-ms pulse, 4 Hz, 10 V for 10 s. **(Bottom)** Histogram showing the concentration-dependent effects induced by GABA (10 µM–1 mM) on the cholinergic responses to electrical field stimulation in the circular muscle strips of mouse colon. Data are means±S.E.M and are expressed as a percentage of the amplitude of contraction induced by 10 µM CCh taken as 100%. The graphed values for the control bars are the means of the data obtained before each treatment. ∗P<0.05 when compared to the respective own control.

GABA-induced excitatory effect was mimicked by muscimol (100 µM), the $GABA_A$ receptor agonist, and blocked by pretreatment of the samples with bicuculline (10 µM), the selective $GABA_A$ receptor antagonist, which also caused *per se* a reduction of the amplitude of the EFS-induced cholinergic contractions (Fig. 29-30). On the contrary, bicuculline did not antagonize the inhibitory effects induced by GABA 500 µM–1 mM. Indeed, such inhibitory effects were prevented by pre-incubation with the selective

$GABA_B$-receptor antagonist, phaclofen (10 μM), and mimicked by the $GABA_B$ receptor agonist baclofen (100 μM). Phaclofen *per se* was not able to affect neither the GABA-induced excitatory effects nor the neurally-evoked cholinergic contractions (Fig. 29-30). As for the induced peristaltic activity, $GABA_C$-receptor agonist and antagonist did not affect the responses (Fig. 29-30).

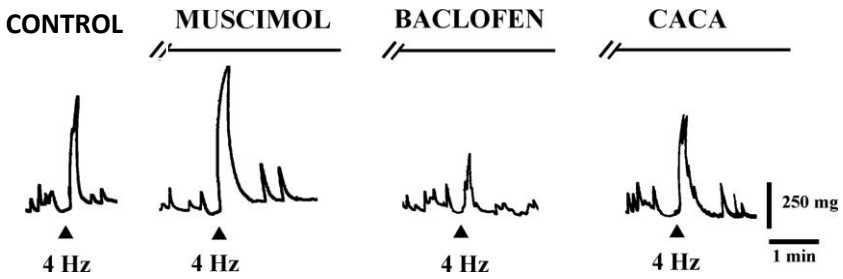

Figure 29: Effects of selective GABA receptor agonists on cholinergic electrically-evoked responses in the circular muscle of mouse distal colon. Original tracings showing the effects of muscimol (100 μM), baclofen (100 μM) or CACA (100 μM), $GABA_A$, $GABA_B$ and $GABA_C$ receptor agonists respectively, on neurally-evoked cholinergic contraction in circular muscle of mouse distal colon

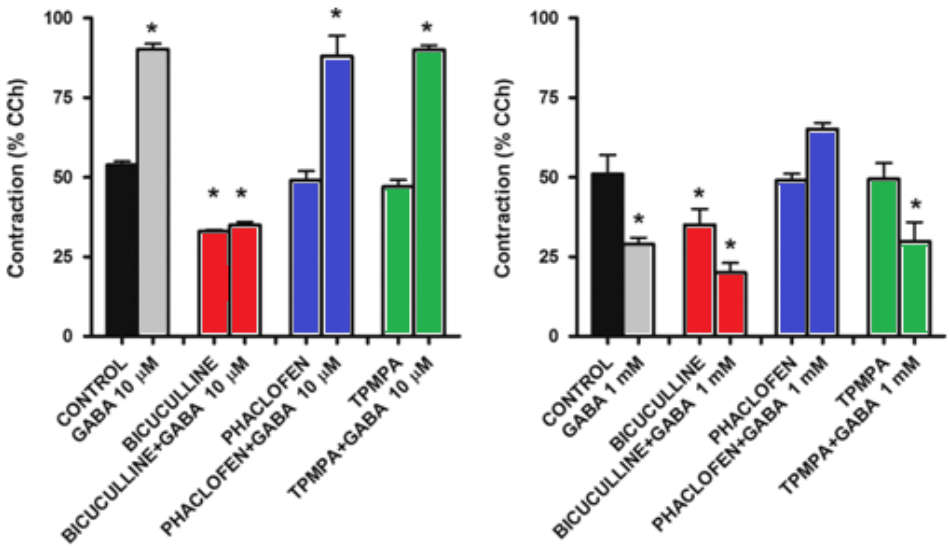

Figure 30: Histograms showing the effects induced by 10 μM GABA (left) or by 1 mM GABA (right) on the neurally evoked cholinergic contraction (0.5-ms pulse, 4 Hz, 10 V for 10 s) in the circular muscle of mouse colon in the absence or in the presence of bicuculline (10 μM, n=4 each), phaclofen (10 μM, n=4 each) and TPMPA (10 μM, n=3 each), GABAA, GABAB and GABAC receptor antagonists respectively. Results are reported as means±S.E.M and are expressed as a percentage of the amplitude of contraction induced by 10 μM CCh taken as 100%. ⋆P<0.05 when compared to the respective own control.

Pretreatment of the samples with hexamethonium (100 µM), nicotinic receptor antagonist, which *per se* did not modify the neurally-evoked cholinergic contractions, antagonized the inhibitory effects induced by high GABA concentrations, without affecting the excitatory effects induced by the lower ones (Fig. 31). The adrenergic blocker guanethidine (1 µM), (Fig. 31) did not influence neither the excitatory nor the inhibitory GABA-induced effects on the EFS-evoked cholinergic contractions.

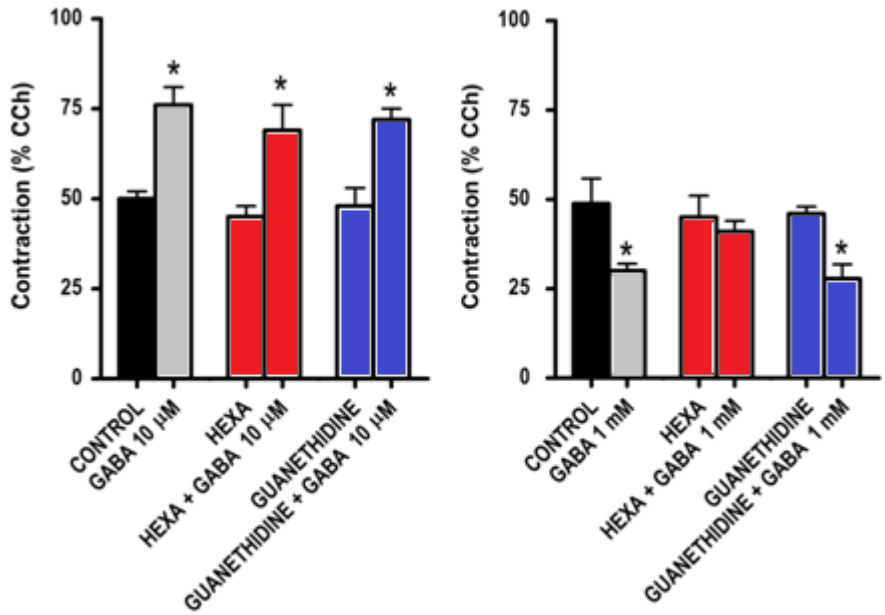

Figure 31: Histograms showing the effects induced by 10 µM GABA (left) or by 1 mM GABA (right) on the neurally evoked cholinergic contraction (0.5-ms pulse, 4 Hz, 10 V for 10 s) in the circular muscle of mouse colon in the absence or in the presence of the nicotinic receptor antagonist, hexamethonium (HEXA 100 µM, *n*=4) or of the adrenergic neuron blocking agent, guanethidine (1 µM, *n*=3). Results are reported as means±S.E.M and are expressed as a percentage of the amplitude of contraction induced by 10 µM CCh taken as 100%. $^{*}P<0.05$ when compared to its own control.

Moreover, in the presence of atropine and guanethidine, to induce non adrenergic non cholinergic (NANC) conditions, EFS was characterized by a more evident initial transient inhibitory phase followed by a rebound contraction, the latter with an amplitude at 32 Hz of 449.9±24.3 mg (n=6). GABA or GABAergic drugs failed to affect both the inhibitory phase of the EFS and the following NANC contractions (Fig. 32).

Finally, GABA or GABAergic drugs had no effect on the amplitude of the contractions induced by the muscarinic agonist carbachol (10 μM).

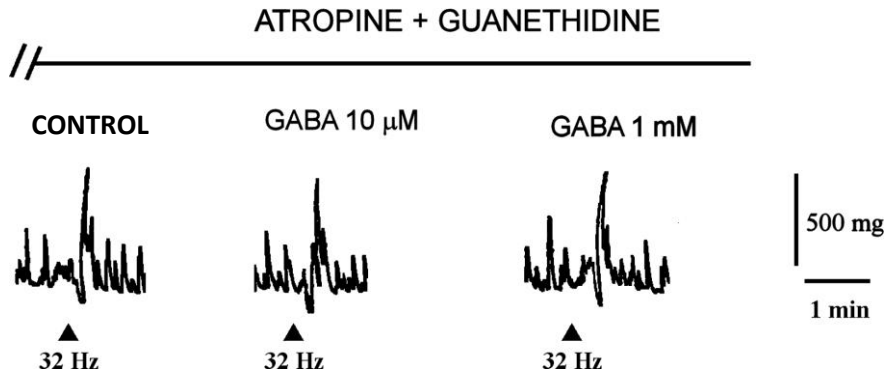

Figure 32: Original tracings showing the non-adrenergic, non-cholinergic (NANC) responses to electrical field stimulation (0.5 ms pulse, 32 Hz, 10 V for 10 s) in circular muscle of mouse distal colon in control conditions and in the presence of 10 μM GABA or 1 mM GABA. Note that GABA at any concentration affected neither the early inhibitory phase nor the following contraction.

4.5.4 Discussion and conclusions

Our experimental results indicates that GABA in mouse distal colon is able to modulate peristaltic activity *via* the regulation of ACh release from enteric excitatory cholinergic neurons, interacting with $GABA_A$ or $GABA_B$ receptors. Low GABA concentrations (10–50 µM) activate $GABA_A$ receptors resulting in an increase of ACh release and peristaltic activity. On the other hand, higher GABA concentrations (500 µM–1 mM) activate $GABA_B$ receptors, and their activation likely overrides $GABA_A$ receptors effects, ultimately reducing ACh release and peristaltic activity. As previously stated, controversial data are reported about the role of GABA in the regulation of colonic peristalsis. GABA and baclofen, the selective $GABA_B$ agonist, were found to inhibit peristaltic activity *via* a negative influence on the cholinergic signaling (Tonini et al., 1989), whilst blockade of $GABA_A$ receptors was reported to elicit an inhibitory effect on peristalsis acting both on the ascending contraction and descending relaxation *via* a negative action on ACh and VIP release, respectively (Grider & Makhlouf, 1992). Moreover, studies in rat distal colon have provided evidence for an in-depth contribution of GABAergic signaling in the neuronal network governing peristalsis. GABA activates a VIP/PACAP/NOS interneurons, in turn inducing the release of ACh and substance P from excitatory motor neurons allowing the ascending contraction (Grider, 1998). Also, an interplay between GABAergic somatostatinergic and opioidergic neurons seems to be required for a positive regulation of neurotransmitters release from inhibitory motor neurons related to descending relaxation (Grider, 1994). Our study integrated this previous findings underlining a possible contribution of GABA in the modulation of enteric cholinergic neurotransmission in the mouse colon. Our experimental data show that different concentration of GABA induced the recruitment of different GABA receptors subtypes, causing opposite effects on the peristaltic activity of mouse distal colon. Low GABA concentrations lead to activation of

$GABA_A$ receptors inducing an increase in the amplitude of the colonic peristaltic waves, being such effect mimicked by the $GABA_A$ agonist muscimol. The sensitivity of such effect to pre-treatment of sample with the selective $GABA_A$ receptor antagonist bicuculline, but not with $GABA_B$ or $GABA_C$ receptor antagonists, confirms the involvement of $GABA_A$ receptors in the observed action. Interestingly, bicuculline *per se* reduced the amplitude of peristaltic waves suggesting that GABAergic neurons could be physiologically involved in the peristaltic reflex, exerting a tonic excitatory effect *via* $GABA_A$ receptors likely sited on excitatory neural pathways. So, in contrast with data obtained in rabbit colon or guinea pig ileum where $GABA_A$ receptors were unlikely to play a major role in sustaining peristalsis (Tonini et al., 1989a and Tonini et al., 1989b), in mouse colon $GABA_A$ receptors seems of importance in mediating a tonic positive influence on colonic propulsive activity. On the other hand, high GABA concentrations could activate $GABA_B$ receptors, causing a decrease in peristaltic activity. Inhibitory mechanisms associated with activation of $GABA_B$ receptors have been reported in other animal species, including humans (Gentilini et al., 1992; Hyland and Cryan, 2010).

To in-depth investigate, in our experimental setting, the enteric network possibly influenced by GABAergic signalling, nerve-evoked responses to electrical field stimulation were studied in circular muscle strips isolated from mouse distal colon. Data from such experiments indicate that GABA specifically modulate the amplitude of the electrically-evoked cholinergic contractile responses, whilst it did not influence the non-adrenergic non-cholinergic excitatory and inhibitory responses. In addition, GABA and GABAergic drugs did not affect carbachol-induced contractions, suggesting no changes in the sensitivity of muscle cells to muscarinic activation and consequently implying that GABA-related effects on cholinergic signaling involve prejunctional mechanisms. Once more, GABA recruited different receptors in relation to the concentration: $GABA_A$ receptors

were activated by low concentration of GABA (10–50 μM), causing an enhancement of electrically-induced cholinergic contractions (and so ACh release). On the contrary, $GABA_B$ receptors are activated in the presence of higher GABA concentrations (500 μM–1 mM), inducing a depression of cholinergic contraction (e.g. a reduced ACh release). Hence, regulation of cholinergic activity seems the mechanism by which GABA regulates peristaltic activity in mouse colon, influencing the contractile activity of colonic circular muscle. Of note, the excitatory effects of GABA are not modified by pre-treatment with the ganglionic blocker hexamethonium, nicotinic receptor antagonist, suggesting that $GABA_A$ receptor activation likely involves a direct regulation of cholinergic motoneurons. Instead, GABA-induced inhibitory effects on the cholinergic activity are hexamethonium-sensitive, implying the activation of a more complex network involving neuronal nicotinic receptors and so cholinergic interneurons. Finally, the effects of GABA were not modified by guanethidine, indicating no influence of GABA on adrenergic sympathetic terminals. However, we cannot completely exclude the hypothesis that GABA may also indirectly regulate ENS signaling, and then peristaltic activity, *via* modulation of the release of enterochromaffin cells-derived mediators such as serotonin, as previously reported in guinea-pig small intestine (Schworer et al., 1989). Thus, further studies are needed to solve this issue. Despite the effects of GABA and GABAergic drugs on the response to electrical stimulation of enteric nerves, spontaneous mechanical activity was not significantly affected by the same drugs, producing no effect neither on the basal tone nor on the amplitude and frequency of spontaneous contractile activity.

Regarding the sensitivity of GABA receptor to different concentration of GABA, some evidence reported that $GABA_A$ receptor could be more sensitive than $GABA_B$ receptors to activation by GABA. For instance, saturation binding studies outer neocortical layers of rat yielded a dissociation constant for $GABA_A$ receptors of about 90 nM and for $GABA_B$

receptors of about 340 nM (Chu et al., 1990). Thus, we can speculate that both $GABA_A$ and $GABA_B$ receptors are activated at 500 μM – 1 mM GABA concentrations, but the effect of $GABA_B$ receptor could completely dominate or override $GABA_A$ receptor effects on cholinergic transmitter release, in turn resulting in a final inhibitory action. Lastly, previous experiments in our laboratory have reported the presence of functional $GABA_C$ receptor in mouse duodenum mediating inhibitory responses to GABA (Zizzo et al., 2007). However, in the present experiment in the mouse colon, as in the stomach (Rotondo et al., 2010), we did not reveal any involvement of $GABA_C$ receptors supporting a region-specific localization and function of the different GABA receptor subtypes. In conclusion, the present work provides evidence for a functional role of GABA in mouse colonic peristalsis, displaying a tonic facilitatory effect of GABA *via* activation of $GABA_A$ receptors likely located on excitatory nerve pathways. Changes in enteric GABA concentration as by local production from commensal intestinal microbiota (Barrett et al. 2012) or potentially in intestinal inflammatory diseases commonly associated with modification in ENS signaling, as IBD (Mawe, 2015; Lakhan & Kirchgessner, 2010) could result in the activation of $GABA_B$ receptors and in turn in a negative influence on colonic motility. Thus, novel researches addressing possible changes of the enteric GABA concentration and GABAergic signaling in IBD could reveal the possible contribution of GABA in the observed dysfunction of colonic motor activity in these pathologies. In addition, given the previously described immunomodulatory actions of GABA (Jin et al., 2013), it could be possible to speculate that modification in the GABAergic signaling would result in modulation of immune activity in IBD. Thus, the investigation of the effect of GABA and GABAergic drugs in the contest of IBD is a necessary step to clarify the whole potential contribution of enteric GABA in the physiopathology of IBD.

CHAPTER 5

General discussion

Inflammatory bowel diseases (IBD), including Crohn's disease and ulcerative colitis, are invalidating inflammatory disorders affecting the GI tract, characterized by a chronic inflammatory reaction associated with relapse alternated to periods of clinical remission. A plethora of factors is believed to be involved in initiation and sustainment of symptoms, including genetic susceptibility, altered intestinal permeability, changes in intestinal microbiota, pathological alteration of immune pathways (Hanauer, 2006). Ongoing researches are also demonstrating the complex interplay among these factors, reinforcing the definition of IBD as composite multifactorial pathologies and suggesting that increasing scientific efforts are needed to clarify the physiopathology of IBD in order to develop effective therapeutic strategies.

Keeping in mind this objective, novel insights are emerging from studies addressing the involvement of the enteric nervous system (ENS), "the brain in the gut" responsible for the fine modulation of most GI functions, in IBD (Di Giovangiulio et al., 2015). Indeed, the large amount of neurotransmitters and mediators constituting part of the ENS network are strictly involved in intestinal homeostasis, ensuring physiological GI processes as secretion, motility, regulation of local blood flow, nutrient absorption, pain signaling. Importantly, after that increasing evidence pointed out the possibility of modulation of immunity by neural mediators ("nerve-driven immunity", Pacheco et al., 2012), parallel researches have addressed the possible existence of neuroimmune pathways in the ENS (Genton, 2003). Indeed, nerve fibers of the ENS have been reported to extend throughout the intestinal lamina propria and epithelium, being thus potentially functionally associated with immune cells belonging to the Gut-Associated Lymphoid Tissue (GALT). Starting from this bases, several enteric mediators including neuropeptides, serotonin and ACh

have been reported to influence intestinal inflammation (Oshima et al., 1999; Vu et al., 2014), with a pro- or anti-inflammatory actions, suggesting the ENS as a source of substances potentially involved in the modulation of local immune processes in physiologic or pathologic conditions. Concerning IBD, the investigation of the link between IBD symptoms and ENS mediators represent an entire new field potentially leading to the definition of novel effective therapies for these severe disorders. Indeed, changes in signaling of enteric mediators could contribute both directly to the inflammatory event in IBD or indirectly to the impairment of different GI functions, especially the defects in intestinal motor patterns which are likely associated with severe IBD symptoms, as diarrhea, weight loss and malabsorption.

Different studies have provided evidence for Angiotensin II (Ang II) and γ-aminobutyric acid (GABA) as novel enteric mediators involved in the ENS signaling for the control of bowel motility, as well as in several major processes including secretion and absorption for Ang II and gastric acid secretion, pain signaling and colon carcinogenesis for GABA (Garg et al., 2012; Auteri et al., 2015). Interestingly, both mediators have been recently linked also to the control of inflammatory events (Katada et al., 2008; Jin et al., 2013), suggesting that an in-depth investigation on such substances could lead to the comprehension of their contribution in intestinal inflammatory disorders, as Inflammatory Bowel Disease, and, in turn, to their identification as potential novel pharmacological targets. Regarding Ang II, the experimental data presented in this study pointed out that IBD-related inflammation is associated with changes in the functional role of Ang II in the modulation of colonic mechanical activity. First, Ang II induced contractile effects in colonic muscle strips from both controls and animal model of IBD, but the amplitude of Ang II-induced contraction was significantly lower in the latter. In addition, in control animals, contractile effects were solely the result of activation of AT1 receptors, whilst, in preparations from inflamed

animals, AT1 receptors were still responsible for Ang II-mediated contraction, but the observation that the AT2 receptor antagonist, PD123319, significantly increase the amplitude of Ang II-mediated contractile effects lead to the hypothesis that tonic activation of AT2 receptors in the course of inflammation causes an inhibitory effect on Ang II-mediated contraction. Experimental results suggest that the inhibitory effect would be related to activation of AT2 receptors located on inhibitory nitrergic neurons in the ENS, in turn counteracting the AT1-dependent excitatory effect. The observation of changes in Ang II-mediated effect between controls and inflamed animals could represent a major finding, suggesting an effective participation of Ang II and the local renin-angiotensin system (RAS) in the GI tract in IBD physiopathology. In addition, considering that the effective role of AT2 receptors is still largely unclear, the specific activation of AT2 receptors solely in experimental inflammation points out and confirm previous studies on the connection between AT2 receptors and inflammatory conditions (Sabuhi et al., 2011). In particular, our study underlined that tonic activation of AT2 receptors would participate in inflammation-associated inhibition of GI motility, likely via a pathological promotion of nitrergic signaling in the ENS. Experiments using the AT2 receptor antagonist, PD123319, demonstrated that tonically-activated AT2 receptors dampen the amplitude of colonic phasic contractions, reduce the Ang II-induced contractions of enteric smooth muscle and, interestingly, decrease the contractile and relaxant responses to the muscarinic agonist carbachol and β2-adrenergic agonist Isoproterenol. This latter observations imply that AT2 receptor signaling could also induce a dysfunction of smooth muscle contractile properties, although the exact mechanism underlying such modification requires novel investigation. Thus, novel researches could reveal that pharmacological targeting of AT2 receptors would represent a novel therapeutic strategy aiming to restore normal GI motor patterns in IBD, extending the current knowledge about IBD physiopathology. Also, exploration of the

comprehensive role of AT2 receptors could reveal their involvement in the modulation of immune cell activity and inflammation, reinforcing the current evidence about the role of Ang II as inflammatory mediator in the GI tract (Garg et al., 2014).

Regarding GABA, the results of our study in mouse colon showed that, in physiological conditions, GABA could act as modulator of the cholinergic signaling required for peristalsis and, particularly, a dual action of GABA coupled to activation of $GABA_A$ or $GABA_B$ receptors have been disclosed. Indeed, low GABA concentrations (10-50 μM) induced the activation of $GABA_A$ receptors, promoting ACh release from cholinergic motor neurons in turn responsible for colonic circular muscle contractions. Accordingly, *in vitro* peristalsis experiments revealed a promoting effect of low GABA concentration, as well as of the selective $GABA_A$ agonist muscimol, on the amplitude of experimentally-induced colonic peristaltic waves. Of note, administration of bicuculline, the selective $GABA_A$ receptor antagonist, inhibited *per se* both ACh release and *in vitro* peristalsis, suggesting that a tonic activation of $GABA_A$ receptor could physiologically occur, inducing a facilitatory effect on cholinergic signaling and sustaining peristalsis. On the other hand, higher GABA concentrations activate $GABA_B$ receptors, causing opposite effects: particularly, they reduced ACh release *via* an indirect pathway likely involving cholinergic interneurons, in turn resulting in a diminished EFS-induced cholinergic contractions of colonic circular muscle strips. Accordingly, such high GABA concentrations, as well as the selective $GABA_B$ receptor agonist baclofen, reduced the amplitude and increased the interval of peristaltic waves. Actually, we can speculate that high GABA concentrations could activate both $GABA_A$ and $GABA_B$ receptors, but the inhibitory effect of $GABA_B$ receptors would override the excitatory effect of $GABA_A$ receptors, resulting in the observed inhibitory action. Interestingly, the modulation of enteric cholinergic signaling in the ileum by the opposite effect of $GABA_A$ (increase of

ACh release) and $GABA_B$ (decrease of ACh release) receptors activation have been pointed out by different research groups (Roberts et al., 2003; Takeda et al., 1989), whilst the possible modulation of the cholinergic signaling by GABA in the colon was not entirely clear. Indeed, effect of GABA in the colon has been primary linked to the modulation of NANC signaling, being likely involved in enteric neuronal network promoting the release of NANC mediators required for circular muscle relaxation (Grider 1994). However, the involvement of GABA in the excitatory cholinergic and tachykinergic input for longitudinal muscle contraction in peristalsis has been also suggested by studies in rat colon (Grider, 1998). Our study integrates these previous data demonstrating the fine modulation by GABA of the cholinergic signaling required for colonic circular muscle contraction in peristalsis. $GABA_A$ receptors seems to be tonically activated by low GABA concentrations in physiological conditions, promoting peristalsis through a direct excitatory effect on ACh release from cholinergic motor neurons. On the contrary, an increase in enteric GABA would activate $GABA_B$ receptors within a more complex enteric neuronal network, ultimately decreasing cholinergic signaling and inhibiting peristalsis. Such changes in GABA-mediated action depending on the concentration and GABA receptor subtypes activated would be of importance in pathological conditions; especially, since different researches have reported the possibility of GABA production from commensal microbiota (Barrett et al., 2012), it is possible to speculate that in pathological conditions associated with changes in the composition and function of intestinal microflora, as Inflammatory Bowel Disease (Hold et al., 2014), potential modifications in microbiota-derived GABA would influence the ENS circuitry and in turn GI motility. Especially, our data pointed out that increased GABA content would negatively influence colonic mechanical activity, dampening the peristaltic reflex. Thus, an in-depth

investigation of the enteric GABAergic system in IBD should be recommended to reveal its potential contribution in IBD-associated dysmotility.

Interestingly, GABA has been recently identified as a neuroimmune modulator capable of influencing the activity of immune cells by binding to specific GABA receptors on their surfaces (Jin et al., 2013). Accordingly, GABA and GABAergic agents have been reported to counteract inflammation in rheumatoid arthritis, experimental encefalomielitis and dermatitis animal model (Tian et al., 2011; Bhat et al., 2010; Duthey et al., 2010). However, pro-inflammatory action of GABA has been also described in psoriasis, (Nigam et al., 2010) suggesting that novel investigations are needed to determine the effective immunomodulatory properties of the GABAergic system. Regarding GI inflammation, it has been shown, *via* metabolomic analysis, that disease recurrence and luminal levels of GABA are correlated in Clostridium difficile (Cd)-induced colitis (Dann et al., 2014 abstract). Also, administration of GABA in drinking water seems to exacerbate Cd-related inflammation, altering immune response to the infection. In parallel, the same group showed that administration of GABA in drinking water could also worsen colitis induced in mice by administration of dextran sulfate sodium (DSS), an established model of IBD (Dann et al. 2015, abstract). In contrast, Dudley et al. (2011) demonstrated that in the TNBS rat model of IBD, oral treatment with topiramate, an anti-epileptic drugs possessing $GABA_A$ agonistic properties, ameliorate macroscopic and microscopic inflammation-related damages. Thus, these pioneer studies suggest that characterization of the role of GABA and its receptors in the contest of intestinal inflammation should be recommended also for exploring the immunomodulatory properties of the GABAergic system.

In conclusion, results present in this thesis indicate that modification in the local RAS and Ang II role in the ENS circuitry could contribute to the colonic dysmotility associated with Inflammatory Bowel Disease, and represent a potential novel pharmacological target for

IBD treatment. An investigation on the immunomodulatory role of Ang II in IBD, especially associated with AT2 receptors activation, could represent the next step for a whole comprehension of the function of local RAS in intestinal inflammation. In addition, the disclosed role of enteric GABA as modulator of cholinergic signaling involved in the colonic peristaltic activity would represent the scientific rationale for exploration of its function in the contest of inflammation-induces defective colonic motor pattern. Once more, pioneer studies in literature (Dann et al., 2014, 2015; Dudley et al., 2011) suggest that investigation of the potential immunomodulatory properties of GABA in GI inflammation should be also recommended, in order to depict its whole function in intestinal inflammatory disorders.

REFERENCES

- Abreu MT. The pathogenesis of inflammatory bowel disease: translational implications for clinicians. Curr Gastroenterol Rep.2002; 4: 481–489.
- Andres PG, Friedman LS. Epidemiology and the natural course of inflammatory bowel disease. Gastroenterol Clin North Am. 1999; 28: 255–281.
- Andrews PL, Bingham S, Wood KL. Modulation of the vagal drive to the intramural cholinergic and non-cholinergic neurones in the ferret stomach by baclofen. J Physiol. 1987;388:25-39.
- Antonioli L, Fornai M, Colucci R, Ghisu N, Tuccori M, Del Tacca M, et al. Pharmacological modulation of adenosine system: novel options for treatment ofinflammatory bowel diseases. Inflamm Bowel Dis 2008;14(4):566–74.
- Antonioli L, Fornai M, Awwad O, Giustarini G, Pellegrini C, Tuccori M, Caputi V, Qesari M, Castagliuolo I, Brun P, Giron MC, Scarpignato C, Blandizzi C, Colucci R. Role of the A(2B) receptor-adenosine deaminase complex in colonic dysmotility associated with bowel inflammation in rats. Br J Pharmacol. 2014;171(5):1314-29.
- Antonioli L, Giron MC, Colucci R, Pellegrini C, Sacco D, Caputi V, Orso G, Tuccori M, Scarpignato C, Blandizzi C, Fornai M. Involvement of the P2X7 purinergic receptor in colonic motor dysfunction associated with bowel inflammation in rats. PLoS One. 2014;9(12):e116253.
- Atlas SA. The renin-angiotensin aldosterone system: pathophysiological role and pharmacologic inhibition. J Manag Care Pharm. 2007 Oct;13(8 Suppl B):9-20.
- Auteri M, Zizzo MG, Serio R. The GABAergic System and the Gastrointestinal Physiopathology. Curr Pharm Des. 2015;21(34):4996-5016.
- Barkas F, Liberopoulos E, Kei A, Elisaf M. Electrolyte and acid-base disorders in inflammatory bowel disease. Ann Gastroenterol. 2013;26(1):23-28.
- Barnard EA., et al. "International Union of Pharmacology. XV. Subtypes of γ-aminobutyric acidA receptors: classification on the basis of subunit structure and receptor function." Pharmacological reviews 1998; 50.2: 291-314.

- Barrett E, Ross RP, O'Toole PW, Fitzgerald GF, Stanton C. γ-Aminobutyric acid production by culturable bacteria from the human intestine. J Appl Microbiol. 2012;113(2):411-7.
- Bassotti, G., Antonelli, E., Villanacci, V., Salemme, M., Coppola, M., & Annese, V. Gastrointestinal motility disorders in inflammatory bowel diseases. World journal of gastroenterology: WJG 2014: 20(1):37.
- Baumgart DC, Carding SR. Inflammatory bowel disease: cause and immunobiology. Lancet. 2007;369:1627-1640.
- Belai A, Boulos PB, Robson T, Burnstock G. Neurochemical coding in the small intestine of patients with Crohn's disease. Gut. 1997;40(6):767-74.
- Bernstein CN, Robert ME, Eysselein VE. Rectal substance P concentrations are increased in ulcerative colitis but not in Crohn's disease. Am J Gastroenterol. 1993;88(6):908-13.
- Bettler, B., Kaupmann, K., Mosbacher, J., and Gassmann, M.. Molecular structure and physiological functions of GABAB receptors. Physiol. Rev 2004; 84, 835–867
- Bhat R, Axtell R, Mitra A, Miranda M, Lock C, Tsien RW, et al. Inhibitory role for GABA in autoimmune inflammation. Proc Natl Acad Sci U S A 2010;107(6):2580–5.
- Bishop AE, Polak JM, Bryant MG, Bloom SR, Hamilton S. Abnormalities of vasoactive intestinal polypeptide-containing nerves in Crohn's disease. Gastroenterology. 1980;79(5 Pt 1):853-60
- Bjurstöm H, Wang J, Ericsson I, Bengtsson M, Liu Y, Kumar-Mendu S, Issazadeh-Navikas S, Birnir B. GABA, a natural immunomodulator of T lymphocytes. J Neuroimmunol. 2008;205(1-2):44-50.
- Boehm F, Martin M, Kesselring R, Schiechl G, Geissler EK, Schlitt HJ, Fichtner-Feigl S.Deletion of Foxp3+ regulatory T cells in genetically targeted mice supports development of intestinal inflammation. BMC Gastroenterol. 2012;12:97.
- Bormann J. The 'ABC' of GABA receptors. Trends Pharmacol Sci. 2000;21(1):16-9
- Bornstein JC, Costa M, Grider JR. Enteric motor and interneuronal circuits controlling motility. Neurogastroenterology & Motility 2004;16(Suppl 1): 34-8.

- Boughton-Smith, N.K., Wallace, J.L., Whittle, B.J., 1988. Relationship between arachidonic acid metabolism, myeloperoxidase activity and leukocyte infiltration in a rat model of inflammatory bowel disease. Agents Actions 25, 115–123.
- Bouma G, Strober W. The immunological and genetic basis of inflammatory bowel disease. Nat Rev Immunol. 2003; 3: 521–533.
- Boyer JC, Guitton C, Pignodel C, Cuq P, Moussu P, Pouderoux P, Christen MO, Balmes JL, Bali JP. Differential responsiveness to contractile agents of isolated smooth muscle cells from human colons as a function of age and inflammation. Dig Dis Sci. 1997;42(11):2190-6.
- Calver AR, Medhurst AD, Robbins MJ, et al. The expression of GABA(B1) and GABA(B2) receptor subunits in the cNS differs from that in peripheral tissues. Neuroscience. 2000;100(1):155-70.
- Casanova E, Guetg N, Vigot R, , et al. A mouse model for visualization of GABA(B) receptors. Genesis. 2009;47(9):595-602
- Chandrasekharan B, Bala V, Kolachala VL, Vijay-Kumar M, Jones D, Gewirtz AT, Sitaraman SV, Srinivasan S. Targeted deletion of neuropeptide Y (NPY) modulates experimental colitis. PLoS One. 2008 Oct 1;3(10):e3304.
- Chu, D.C.,Albin,R.L.,Young,A.B.,Penney,J.B.,.Distribution and kinetics of GABAB binding sites in rat central nervous system: a quantitative autoradiographic study.Neuroscience1990; 34: 341–357.
- Csikos, T., Chung, O. and Unger, T. (1998). Receptors and their classification: focus on angiotensin II and the AT2 receptor. J. Human Hypertens. 12, 311-318
- Dann S, Eaves-Pyles T, Peniche A, Choudhury B, Feng H and Savidge T. Microbiota-derived GABA exacerbates Clostridium difficult-associated intestinal inflammation and suppresses development of protective immunity (MPF1P.768). The Journal of Immunology May 1, 2014vol.192 (1 Supplement) 66.7
- Dann S, Kelly H, Suarez D, Hoppe D, Banks M, Peniche A. Luminal-GABA promotes inflammation in mouse models of colitis (MUC5P.756). The Journal of Immunology May 1, 2015vol.194 (1 Supplement)138.14
- Davanger S, Hjelle OP, Babaie E, Larsson LI, Hougaard D, Storm-Mathisen J, Ottersen OP. Colocalization of gamma-aminobutyrate and gastrin in the rat antrum: an immunocytochemical and in situ hybridization study. Gastroenterology. 1994;107(1):137-48.

- De Gasparo M, Catt KJ, Inagami T, (2000) The angiotensin II receptors. Pharmacol Rev
- De Schepper HU, De Man JG, Moreels TG, Pelckmans PA, De Winter BY. Review article: gastrointestinal sensory and motor disturbances in inflammatory bowel disease – clinical relevance and pathophysiological mechanisms. Aliment Pharmacol Ther. 2008;27(8):621-37.
- Derocq JM, Ségui M, Blazy C, Emonds-Alt X, Le Fur G, Brelire JC, Casellas Effect of substance P on cytokine production by human astrocytic cells and blood mononuclear cells: characterization of novel tachykinin receptor antagonists. P.FEBS Lett. 1996;399(3):321-5.
- Dolphin, A. C. GABA B receptors: has adenylate cyclase inhibition any functional relevance? Trends in Neurosciences 7.10 (1984): 363-364.
- Dudley JT, Sirota M, Shenoy M, Pai RK, Roedder S, Chiang AP,et al. Computational repositioning of the anticonvulsant topi-ramate for inflammatory bowel disease. Sci Transl Med 2011, http://dx.doi.org/10.1126/scitranslmed.3002648.
- Duthey B, Hübner A, Diehl S, Boehncke S, Pfeffer J, Boehncke WH. Anti-inflammatory effects of the GABA(B) receptor agonist baclofen in allergiccontact dermatitis. Exp Dermatol 2010;19(7):661–6
- El-Salhy M, Danielsson A, Stenling R, Grimelius L. Colonic endocrine cells in inflammatory bowel disease. J Intern Med. 1997 Nov;242(5):413-9.
- Enz R. GABA(C) receptors: a molecular view. Biol Chem 2001;382(8):1111–22.
- Erdo SL.GABA outside the CNS. 1992. Pag.22-25
- Ewert S, Spak E, Olbers T, Johnsson E, Edebo A, Fändriks L. Angiotensin II induced contraction of rat and human small intestinal wall musculature in vitro. Acta Physiol (Oxf). 2006;188(1):33-40.
- Fagg, G.E. and Foster, A.C. Amino acid neurotransmitters and their pathways in the mammalian central nervous system. Neuroscience 1983; 9:701-719.
- Fändriks L. The angiotensin II type 2 receptor and the gastrointestinal tract. J Renin Angiotensin Aldosterone Syst. 2010;11(1):43-8.

- Farrant M, Nusser Z. Variations on an inhibitory theme: phasic and tonic activation of $GABA_A$ receptors Nature Reviews Neuroscience 2005; 6:215-229
- Frigo GM, Galli A, Lecchini S, Marcoli M. A facilitatory effect of bicucullineon the enteric neurones in the guinea-pig isolated colon. Br J Pharmacol1987;90:31–41.
- Furfaro, F., Bezzio, C., Ardizzone, S., Massari, A., de Franchis, R., & Maconi, G. Overview of Biological Therapy in Ulcerative Colitis: Current and Future Directions. Journal of gastrointestinal and liver diseases 2015, 24(2), 203-213.
- Furness JB, Costa M. The enteric nervous system. Edinburgh: Churchill Livingstone, 1987.
- Furness JB. Gastrointestinal neurotransmitters. Baillieres Clin Endocrinol Metab. 1994;8(1):51-76.
- Gage P.W. 1992. Activation and modulation of neuronal K+ channels by GABA.Trends Neurosci. 15(2):46-51.
- Ganten D, Minnich JL, Granger P, et al. Angiotensin-forming enzyme in brain tissue. Science 1971; 173: 64–5.
- Garg M, Burrell LM, Velkoska E, Griggs K, Angus PW, Gibson PR, Lubel JS. Upregulation of circulating components of the alternative renin-angiotensin system in inflammatory bowel disease: A pilot study. J Renin Angiotensin Aldosterone Syst. 2015;16(3):559-69.
- Gentilini G, Franchi-Micheli S, Pantalone D, Cortesini C, Zilletti L. GABAB receptor-mediated mechanisms in human intestine in vitro. Eur J Pharmacol. 1992;217(1):9-14.
- Genton L, Kudsk KA. Interactions between the enteric nervous system and the immune system: role of neuropeptides and nutrition. The American Journal of Surgery 2003; 186: 253–8.
- Gershon MD. The second brain. New York: HarperCollins Publishers, 1998
- Ghia JE, Li N, Wang H, Collins M, Deng Y, El-Sharkawy RT, Côté F, Mallet J, Khan WI. Serotonin has a key role in pathogenesis of experimental colitis. Gastroenterology. 2009;137(5):1649-60.

- Goyal RK, Hirano IH. The Enteric Nervous System. N Engl J Med 1996; 334:1106-1115.
- Grider JR, Kuemmerle JF, Jin JG. 5-HT released by mucosal stimuli initiates peristalsis by activating 5-HT4/5-HT1p receptors on sensory CGRP neurons. Am J Physiol. 1996 May;270(5 Pt 1):G778-82.
- Grider JR. Interplay of somatostatin, opioid, and GABA neurons in the regulation of the peristaltic reflex. Am J Physiol – Gastrointest Liver Physiol 1994;267:G696–701.
- Grider JR. Neurotransmitters mediating the intestinal peristaltic reflex in the mouse. J Pharmacol Exp Ther. 2003;307(2):460-7. Epub 2003 Sep 9.
- Grider JR. Regulation of excitatory neural input to longitudinal intestinal muscle by myenteric interneurons. Am J Physiol 1998;275:G973–8.
- Hanauer SB. Inflammatory bowel disease: Epidemiology, pathogenesis, and therapeutic opportunities. Inflammatory Bowel Diseases, 2006; 12: s3-9
- Hansen MB. Neurohumoral Control of Gastrointestinal Motility. Physiol. Res. 2003; 52: 1-30.
- Hawcock A. B, Barnes, J. C. Pharmacological characterization of the contractile responses to angiotensin analogues in guinea-pig isolated longitudinal muscle of small intestine. Br.J.Pharmacol. 1993; 108:1150-1155.
- Head, L. H., Heaton, J. W., & Kivel, R. M. Absorption of water and electrolytes in Crohn's disease of the colon. Gastroenterology 1969; 56(3), 571-579.
- Hendrickson BA, Gokhale R, Cho JH. Clinical aspects and pathophysiology of inflammatory bowel disease. Clin Microbiol Rev. 2002;15(1):79-94.
- Hirasawa K, Sato Y, Hosoda Y, Yamamoto T, Hanai H. Immunohistochemical localization of angiotensin II receptor and local renin-angiotensin system in human colonic mucosa. J Histochem Cytochem. 2002 Feb;50(2):275-82.
- Hold, G. L., Smith, M., Grange, C., Watt, E. R., El-Omar, E. M., & Mukhopadhya, I. Role of the gut microbiota in inflammatory bowel disease pathogenesis: what have we learnt in the past 10 years?. World journal of gastroenterology: WJG 2014; 20(5), 1192
- Holzmann B. Nerve-Driven Immunity: The Effects of Neurotransmitters on Immune Cells, Functions and Disease. In: Nerve-Driven Immunity:

Neurotransmitters and Neuropeptides in the Immune System. Vienna: Springer, 2012.

- Huang XC, Richards EM and Sumners C. Mitogen activated protein kinases in rat brain neuronal cultures are activated by angiotensin II type 1 receptors and inhibited by angiotensin II type 2 receptors. J Biol Chem 1996; 271:15635–15641
- Hugot JP, Chamaillard M, Zouali H, et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease.Nature. 2001; 411: 599–603.
- Hyland NP, Cryan JF. A gut feeling about GABA: focus on $GABA_B$ receptors. Frontiers in pharmacology 2010; 1:124
- Israel, A., Sosa, B. & Gutierez, C.I. Brain AT(2) receptor mediate vasodepressor response to footshocks: role of kinins and nitric oxide. Brain Res Bull 2000; 51, 339–343.
- Janeway CA Jr, Travers P, Walport M, et al. Immunobiology: The Immune System in Health and Disease. 5th edition. New York: Garland Science, 2001.
- Jaszewski R, Tolia V, Ehrinpreis MN, et al. Increased colonic mucosal angiotensin I and II concentrations in Crohn's colitis. Gastroenterology 1990; 98: 1543–8.
- Jembrek MJ, Vlainic J. GABA Receptors: Pharmacological Potential and Pitfalls. Curr Pharm Des. 2015;21(34):4943-59.
- Jin XH, Wang ZQ, Siragy HM, Guerrant RL, Carey RM. Regulation of jejunal sodium and water absorption by angiotensin subtype receptors. Am J Physiol. 1998;275(2 Pt 2):R515-23.
- Jin Z, Mendu SK, Birnir B. GABA is an effective immunomodulatory molecule. Amino Acids. 2013;45(1):87-94.
- Johnson L, Ghishan F, Kaunitz J, Merchant J, Said H, Wood J. Physiology of the Gastrointestinal Tract, Two Volume Set. 5th edition. San Diego, CA, USA: Elsevier Science, 2012
- Johnston GA, Chebib M, Hanrahan JR, Mewett KN. GABAC receptors as drug targets. Curr Drug Targets CNS Neurol Disord 2003;2(4):260–8.
- K. Geboes & S. Collins. Structural abnormalities of the nervous system in Crohn's disease and ulcerative colitis. Neurogastroenterol. Motil. 1998; 10:189–202
- Katada K, Yoshida N, Suzuki T, Okuda T, Mizushima K, Takagi T, Ichikawa H, Naito Y, Cepinskas G, Yoshikawa T. Dextran sulfate sodium-induced acute

colonic inflammation in angiotensin II type 1a receptor deficient mice. Inflamm Res. 2008;57(2):84-91.

- Kiesler P, Fuss IJ, Strober W. Experimental Models of Inflammatory Bowel Diseases. Cell Mol Gastroenterol Hepatol. 2015;1(2):154-170.
- Kinoshita, K., Hori, M., Fujisawa, M., Sato, K., Ohama, T., Momotani, E. and Ozaki, H. Role of TNF-alpha in muscularis inflammation and motility disorder in a TNBS-induced colitis model: clues from TNF-α-deficient mice. Neurogastroenterol. Motil., 2006; 18: 578–588.
- Kishimoto S, Kobayashi H, Shimizu S, Haruma K, Tamura T, Kajiyama G, Miyoshi A:Changes of colonic vasoactive intestinal peptide and cholinergic activity in rats with chemical colitis. Dig Dis Sci 1992, 37:1729-1737
- Kiyosue, M., Fujisawa, M., Kinoshita, K., Hori, M. and Ozaki, H. Different susceptibilities of spontaneous rhythmicity and myogenic contractility to intestinal muscularis inflammation in the hapten-induced colitis. Neurogastroenterol. Motil., 2006; 18: 1019–1030.
- Koboziev I., Karlsson F. and Grisham M. Gut-associated lymphoid tissue, T cell trafficking, and chronic intestinal inflammation. Annals of the New York Academy of Sciences 2010; 1207: 86-93
- Koch TR, Carney JA, Go VLW, Szurszewski JH. Spontaneous contractions and some electrophysiologic properties of circular muscle from normal sigmoid colon and ulcerative colitis. Gastroenterology 1988; 95: 77–84.
- Kohno N, Nomura M, Okamoto H, Kaji M, Ito S. The use of electrogastrography and external ultrasonography to evaluate gastric motility in Crohn's disease. J Med Invest. 2006 Aug;53(3-4):277-84.
- Krantis A, Mattar K, Glasgow I. Rat gastroduodenal motility in vivo: interaction of GABA and VIP in control of spontaneous relaxations. Gastrointest Liver Physiol 1998;38:G897–903.
- Krantis A, Tufts K, Nichols K, Morris GP. [3H]GABA uptake and GABA localization in mucosal endocrine cells of the rat stomach and colon. J Auton Nerv Syst. 1994;47(3):225-32.
- Krantis A. GABA in the Mammalian Enteric Nervous System. News Physiol Sci. 2000;15:284-90.

- Krawisz JE, Sharon P, Stenson WF. Quantitative assay for acute intestinal inflammation based on myeloperoxidase activity. Assessment of inflammation in rat and hamster models. Gastroenterology.1984;87:1344–1350.
- Kristinsson JO, Hopman WP, Oyen WJ, Drenth JP. Gastroparesis in patients with inactive Crohn's disease: a case series. BMC Gastroenterol. 2007;7:11.
- Lakhan SE, Kirchgessner A. Neuroinflammation in inflammatory bowel disease. J Neuroinflammation. 2010;7:37.
- Lavoie JL, Sigmund CD. Minireview: overview of the renin-angiotensin system--an endocrine and paracrine system. Endocrinology. 2003;144(6):2179-83
- Lewis, S. Synaptic transmission: A closer look at presynaptic GABAB receptors. Nature Reviews Neuroscience 2010, 11(10), 664-665.
- Linden DR, Couvrette JM, Ciolino A, McQuoid C, Blaszyk H, Sharkey KA, Mawe GM:Indiscriminate loss of myenteric neurons in the TNBS-inflamed guinea-pig distal colon. Neurogastroenterol Motil. 2005;17(5):751-60.
- Linden DR, Chen JX, Gershon MD, Sharkey KA, Mawe GM. Serotonin availability is increased in mucosa of guinea pigs with TNBS-induced colitis. Am J Physiol Gastrointest Liver Physiol. 2003 ;285(1):G207-16.
- Liu S, Ji T, Ren W, Qu MH, Zhu JX, Wood JD. Role of Na-K-2Cl symporter in GABA-evoked excitation in rat enteric neurons. FASEB J 2013;27:1160.5[meeting abstract supplement]
- Loftus EV Jr, Sandborn WJ. Epidemiology of inflammatory bowel disease. Gastroenterol Clin North Am. 2003; 31: 1–20.
- Lomax A. E., Fernández E., Sharkey K. A. Plasticity of the enteric nervous system during intestinal inflammation. Neurogastroenterol. Motil. 2005; 17: 4–15.
- Lukasiewicz PD, Eggers ED, Sagdullaev BT, McCall MA. GABAC receptor-mediated inhibition in the retina. Vision Res. 2004;44(28):3289-96.
- MacNaughton WK, Pineau BC, Krantis A. gamma-Aminobutyric acid stimulates electrolyte transport in the guinea pig ileum in vitro. Gastroenterology 1996; 110: 498–507.
- Marcoli M, Scarrone S, Maura G, Bonanno G, Raiteri M. A subtype of the gamma-aminobutyric acid(B) receptor regulates cholinergic twitch response in the guinea pig ileum. J. Pharmacol. Exp. Ther. 2000; 293: 42-7.

- Margolis KG, Gershon MD. Neuropeptides and inflammatory bowel disease. Curr Opin Gastroenterol. 2009 Nov;25(6):503-11.
- Mastropaolo M, Zizzo MG, Auteri M, Caldara G, Liotta R, Mulè F, Serio R. Activation of angiotensin II type 1 receptors and contractile activity in human sigmoid colon in vitro. Acta Physiol (Oxf). 2015;215(1):37-45.
- Mastropaolo M, Zizzo MG, Mulè F, Serio R. Angiotensin II contractile effects in mouse colon: role for pre- and post-junctional AT(1A) receptors. Acta Physiol (Oxf). 2013;207(2):337-45. doi: 10.1111/apha.12041.
- Matteoli G, Gomez-Pinilla PJ, Nemethova A, Di Giovangiulio M, Cailotto C, van Bree SH, Michel K, Tracey KJ, Schemann M, Boesmans W, Vanden Berghe P, Boeckxstaens GE. A distinct vagal anti-inflammatory pathway modulates intestinal muscularis resident macrophages independent of the spleen. Gut. 2014;63(6):938-48.
- Mawe GM. Colitis-induced neuroplasticity disrupts motility in the inflamed and post-inflamed colon. J Clin Invest. 2015;125(3):949-55.
- Mizoguchi A. Animal models of inflammatory bowel disease. Prog Mol Biol Transl Sci. 2012;105:263-320.
- Nigam R, El-Nour H, Amatya B, Nordlind K. GABA and GABA(A) receptor expression on immune cells in psoriasis: a pathophysiological role. Arch Dermatol Res. 2010;302(7):507-15.
- Nutsch KM, Hsieh CS. T cell tolerance and immunity to commensal bacteria. Curr Opin Immunol. 2012;24(4):385-91.
- O'Connor TM, O'Connell J, O'Brien DI, Goode T, Bredin CP, Shanahan F. The role of substance P in inflammatory disease. J Cell Physiol. 2004;201(2):167-80.
- Ohama T, Hori M, Ozaki H. Mechanism of abnormal intestinal motility in inflammatory bowel disease: how smooth muscle contraction is reduced? J Smooth Muscle Res. 2007;43(2):43-54.
- Oshima S, Fujimura M, Fukimiya M. Changes in number of serotonin-containing cells and serotonin levels in the intestinal mucosa of rats with colitis induced by dextran sodium sulfate. Histochem Cell Biol. 1999;112(4):257-63.
- Pacheco R, Contreras F, Zouali M. The dopaminergic system in autoimmune diseases. Frontiers in immunology 2014;5:117.

- Pacheco R, Prado C, Contreras F. Cells, molecules and mechanisms involved in the neuro-immune interaction. INTECH Open Access Publisher, 2012. DOI: 10.5772/48367.
- Pereira C, Araújo F, Granja PL, Santos HA, Sarmento B.Targeting membrane transporters and receptors as a mean to optimize orally delivered biotechnological based drugs through nanoparticle delivery systems. Curr Pharm Biotechnol. 2014;15(7):650-8.
- Perrot-Sinal TS, Auger AP, McCarthy MM. Excitatory actions of GABA in developing brain are mediated by l-type Ca2+ channels and dependent on age, sex, and brain region. Neuroscience. 2003;116(4):995-1003.
- Person L, Raman IM. Synchrony and neural coding in cerebellar circuits Front Neural Circuits. 2012; 6: 97.
- Petroff OA. GABA and glutamate in the human brain. Neuroscientist. 2002;8(6):562-73.
- Peyrin-Biroulet L, Desreumaux P, Sandborn WJ, et al. Crohn's disease: beyond antagonists of tumour necrosis factor. Lancet. 2008;372:67-81.
- Phillips RJ, Powley TL. Innervation of the gastrointestinal tract: Patterns of aging. Autonomic Neuroscience 2007; 136 (1): 1-19
- Podolsky DK. Inflammatory bowel disease. N Engl J Med. 2002; 347: 417–429.
- Poulter MO, Singhal R, Brown LA, Krantis A. GABA(A) receptor subunit messenger RNA expression in the enteric nervous system of the rat: implications for functional diversity of enteric GABA(A) receptors. Neuroscience 1999;93(3):1159–65.
- Randall, C. W., Vizuete, J. A., Martinez, N., Alvarez, J. J., Garapati, K. V., Malakouti, M., & Taboada, C. M. From historical perspectives to modern therapy: a review of current and future biological treatments for Crohn's disease. . Therapeutic Advances in Gastroenterology, 2015 Vol. 8(3) 143–159.
- Reddy S.N., Bazzocchi G., Chan S., et al. Colonic motility and transit in health and ulcerative colitis. Gastroenterology 1991; 101: 1289–97.
- Renzi D, Pellegrini B, Tonelli F, Surrenti C, Calabrò A. Substance P (neurokinin-1) and neurokinin A (neurokinin-2) receptor gene and protein expression in the healthy and inflamed human intestine. Am J Pathol. 2000;157(5):1511-22.

- Rescigno M. Don't forget to have a "second brain". Mucosal Immunology 2008; 1: 328–9.
- Robert V, Heymes C, Silvestre JS, Sabri A, Swynghedauw B, Delcayre C. Angiotensin AT1 receptor subtype as a cardiac target of aldosterone: role in aldosterone-salt-induced fibrosis. Hypertension. 1999;33(4):981-6
- Roberts DJ, Hasler WL, Owyang C. GABA mediation of the dual effects of somatostatin on guinea pig ileal myenteric cholinergic transmission. Am J Physiol 1993;264(5 Pt1):G953–60.
- Rotondo A, Serio R, Mulè F. Functional evidence for different roles of GABAA and GABAB receptors in modulating mouse gastric tone. Neuropharmacology. 2010;58(7):1033-7.
- Rowley NM, Madsen KK, Schousboe A, Steve White H. Glutamate and GABA synthesis, release, transport and metabolism as targets for seizure control. Neurochem Int. 2012;61(4):546-58.
- Rudolph U, Möhler H. GABA-based therapeutic approaches: $GABA_A$ receptor subtype functions. Curr Opin Pharmacol. 2006;6(1):18-23.
- Sabuhi R, Ali Q, Asghar M, Al-Zamily NR, Hussain T. Role of the angiotensin II AT2 receptor in inflammation and oxidative stress: opposing effects in lean and obese Zucker rats. Am J Physiol Renal Physiol. 2011;300(3):F700-6.
- Santos RA, Campagnole-Santos MJ, Andrade SP. Angiotensin-(1-7): an update. Regul Pept. 2000; 91: 45-62.
- Sarna SK. Colonic Motility: From Bench Side to Bedside. San Rafael (CA): Morgan & Claypool Life Sciences; 2010. Colonic Motility Dysfunction. Available from: http://www.ncbi.nlm.nih.gov/books/NBK53473/
- Sartor R.B. Microbial influences in inflammatory bowel diseases. Gastroenterology, 2008;134:577–594.
- Schäfer KH, Van Ginneken C, Copray S. Plasticity and neural stem cells in the enteric nervous system. Anat Rec (Hoboken). 2009;292(12):1940-52.
- Schirgi-Degen A., Beubler E. Significance of nitric oxide in the stimulation of intestinal fluid absorption in the rat intestine in vivo. Br. J. Pharmacol. 1995; 114:13–18.

- Schwörer H, Racké K, Kilbinger H. GABA receptors are involved in the modulation of the release of 5-hydroxytryptamine from the vascularly perfused small intestine of the guinea-pig. Eur J Pharmacol. 1989;165(1):29-37.
- Seerden TC, De Man JG, Holzer P, Van den Bossche RM, Herman AG, Pelckmans PA, De Winter BY. Experimental pancreatitis disturbs gastrointestinal and colonic motility in mice: effect of the prokinetic agent tegaserod. Neurogastroenterol Motil. 2007 Oct;19(10):856-64.
- Seifi M, Brown JF, Mills J, Bhandari P, Belelli D, Lambert JJ, Rudolph U, Swinny JD. Molecular and functional diversity of GABA-A receptors in the enteric nervous system of the mouse colon. J Neurosci. 2014;34(31):10361-78.
- Sheil B, MacSharry J, O'Callaghan L, O'Riordan A, Waters A, Morgan J, Collins JK, O'Mahony L, Shanahan F. Role of interleukin (IL-10) in probiotic-mediated immune modulation: an assessment in wild-type and IL-10 knock-out mice. Clin Exp Immunol. 2006 May;144(2):273-80.
- Shih DQ, Targan SR, McGovern D. Recent advances in IBD pathogenesis: genetics and immunobiology. Curr Gastroenterol Rep. 2008;10:568-575.
- Sieghart W. Allosteric modulation of GABAA receptors via multiple drug-binding sites. Adv Pharmacol. 2015;72:53-96.
- Sigel E, Steinmann ME. Structure, function, and modulation of GABA(A) receptors. J Biol Chem. 2012;287(48):40224-31.
- Siragy H. M., Carey R. M. The subtype-2 (AT_2) angiotensin receptor regulates renal guanosine 3′,5′-monophosphate and AT_1 receptor-mediated prostaglandin E_2 production in conscious rats. J. Clin. Invest. 1996; 97:1978–1982.
- Siragy H. M., Carey R. M. The subtype-2 (AT_2) receptor mediates renal production of nitric oxide in conscious rats. J. Clin. Invest 1997. 100:264–269
- Snape WJ Jr, Williams R, Hyman PE. Defect in colonic smooth muscle contraction in patients with ulcerative colitis. Am J Physiol. 1991;261(6 Pt 1):G987-91.
- Speth RC,. Giese MJ. Update on the Renin-Angiotensin System. Journal of Pharmacology & Clinical Toxicology 2013; 1(1): 1004.
- Strober W, Fuss IJ. Proinflammatory cytokines in the pathogenesis of inflammatory bowel diseases. Gastroenterology. 2011 May;140(6):1756-67.

- Takeda T, Taniyama K, Baba S, Tanaka C. Putative mechanisms involved in excitatory and inhibitory effects of somatostatin on intestinal motility. Am J Physiol. 1989;257(4 Pt 1):G532-8.
- Tanaka C. gamma-Aminobutyric acid in peripheral tissues. Life Sci. 1985; 37(24): 2221-35.
- Taniyama, K, Kusunoki M, Saito N, Tanaka C. Release of gammaaminobutyric acid from cat colon. Science 1982; 217: 1038-40.
- Thomas WG, Thekkumkara TJ, Baker KM. Molecular mechanisms of angiotensin II (AT1A) receptor endocytosis. Clin Exp Pharmacol Physiol Suppl. 1996;3:S74-80.
- Tian J, Lu Y, Zhang H, Chau CH, Dang HN, Kaufman DL. -Aminobutyric acidinhibits T cell autoimmunity and the development of inflammatory responsesin a mouse Type 1 diabetes model. J Immunol 2004;173:5298–304.
- Tian J, Yong J, Dang H, Kaufman DL. Oral GABA treatment downregulates inflammatory responses in a mouse model of rheumatoid arthritis. Autoimmunity. 2011;44(6):465-70.
- Todorovic V, Janic B, Koko V, Micev M, Nikolic JA, Ratkovic M, Leposavic G, Jankovic T, Knezevic-Usaj S, Milicevic Z. Colonic vasoactive intestinal polypeptide (VIP) in ulcerative colitis--a radioimmunoassay and immunohistochemical study. Hepatogastroenterology. 1996;43(9):483-8.
- Tomita R, Munakata K, Tanjoh K. Role of non-adrenergic non-cholinergic inhibitory nerves in the colon of patients with ulcerative colitis. J Gastroenterol. 1998;33(1):48-52
- Tonini,M.,Crema,A.,Frigo,G.M.,Rizzi,C.A.,Manzo,L.,Candura,S.M.,Onori,L.,.An in vitro study of the relationship between GABA receptor function and propulsive motility in the distal colon of the rabbit. Br.J.Pharmacol. 1989a; 98,1109–1118.
- Tonini,M.,De,P.G.,Onori,L.,Manzo,L.,Rizzi,C.A.,Crema,A. The role of $GABA_A$ receptor function in peristaltic activity of the guinea-pig ileum: a comparative study with bicuculline,SR95531 and picrotoxinin.Br.J.Pharmacol. 1989b; 97,556–562.
- Torashima Y, Uezono Y, Kanaide M, Ando Y, Enjoji A, Kanematsu T, Taniyama K. Presence of GABA(B) receptors forming heterodimers with GABA(B1) and

GABA(B2) subunits in human lower esophageal sphincter. J Pharmacol Sci. 2009;111(3):253-9.

- Tracey KJ. Physiology and immunology of the cholinergic antiinflammatory pathway. Journal of Clinical Investigation 2007; 117(2): 289-9
- Trendelenburg P. Physiological and pharmacological investigations of small intestinal peristalsis. Naunyn Schmiedebergs Arch Pharmacol. 2006 May;373(2):101-33.
- Triantafillidis, J. K., Merikas, E., & Georgopoulos, F. Current and emerging drugs for the treatment of inflammatory bowel disease. Drug design, development and therapy 2011; 5:185.
- Tursi A, Brandimarte G, Giorgetti G, Nasi G. Assessment of orocaecal transit time in different localization of Crohn's disease and its possible influence on clinical response to therapy. Eur J Gastroenterol Hepatol. 2003;15(1):69-74.
- Unger T. The angiotensin type 2 receptor: Variations on an enigmatic theme. J Hypertens 1999; 17:1775–1786.
- Urata H, Kinoshita A, Misono KS, Bumpus FM, Husain A. Identification of a highly specific chymase as the major angiotensin II-forming enzyme in the human heart. J Biol Chem, 1990; 265: 22348–57.
- Ursino MG, Vasina V, De Ponti F. Protection from DNBS-induced colitis by the tachykinin NK(1) receptor antagonist SR140333 in rats. Eur J Pharmacol. 2009;603(1-3):133-7.
- Vermillion DL, Huizinga JD, Riddell RH, Collins SM. Altered small intestinal smooth muscle function in Crohn's disease. Gastroenterology. 1993;104(6):1692-9.
- Vu JP, Million M, Larauche M, Luong L, Norris J, Waschek JA, Pothoulakis C, Pisegna JR, Germano PM. Inhibition of vasoactive intestinal polypeptide (VIP) induces resistance to dextran sodium sulfate (DSS)-induced colitis in mice. J Mol Neurosci. 2014;52(1):37-47.
- Watanabe M, Maemura K, Kanbara K, Tamayama T, Hayasaki H. GABA and GABA receptors in the central nervous system and other organs. Int Rev Cytol. 2002;213:1-47.
- Weiner HL, da Cunha AP, Quintana F, Wu H. Oral tolerance. Immunol Rev. 2011;241(1):241-59.

- Williamson S, Faulkner-Jones BE, Cram DS, Furness JB, Harrison LC. Transcription and translation of two glutamate decarboxylase genes in the ileum of rat, mouse and guinea pig. J. Auton. Nerv. Syst. 1995; 55 (1-2): 18-28.
- Xavier RJ, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease." Nature 2007 448.7152: 427-434.
- Xue H, Liu S, Ji T, Ren W, Zhang XH, Zheng LF, Wood JD, Zhu JX. Expression of NKCC2 in the rat gastrointestinal tract. Neurogastroenterol Motil. 2009;21(10):1068-e89.
- Zizzo MG, Mulè F, Serio R. Functional evidence for GABA as modulator of the contractility of the longitudinal muscle in mouse duodenum: role of GABA(A) and GABA(C) receptors. Neuropharmacology. 2007;52(8):1685-90.
- Zizzo, M. G., Mulè, F., Mastropaolo, M., Condorelli, D. F., Belluardo, N., & Serio, R. Can guanine-based purines be considered modulators of intestinal motility in rodents?. European journal of pharmacology 2011; 650(1), 350-355.

LIST OF PUBLICATIONS

Michelangelo Auteri

Publications in ISI Journals:

- **Auteri M**., Zizzo MG., Serio R. "The GABAergic system and the gastrointestinal physiopathology". Current Pharmaceutical Design 2015; 21(34):4996-5016.

- Mastropaolo M., Zizzo MG., **Auteri M**., Caldara G., Liotta R., Mulè F., Serio R. Activation of Angiotensin II type 1 receptors and contractile activity in human sigmoid colon in vitro. Acta Physiol (Oxf). 2015 Sep;215(1):37-45.

- **Auteri M.,** Zizzo MG., Serio R. "GABA and GABA receptors in the gastrointestinal tract: from motility to inflammation" Pharmacological Research 2015 Mar;93:11-21

- **Auteri M.,** Zizzo MG., Mastropaolo M., Serio R. "Opposite role played by $GABA_A$ and $GABA_B$ receptors in the modulation of peristaltic activity in mouse distal colon". European Journal of Pharmacology 2014 May 15; 731:93-99

- Mastropaolo M., Zizzo MG., **Auteri M**., Mulè F., Serio R. "Arginine vasopressin, via activation of post-junctional V1 receptors, induces contractile effects in mouse distal colon" Regulatory Peptides 2013 Nov 10; 187:29-34.

- Farro G., Gomez-Pinilla PJ.; Di Giovangiulio M; Stakenborg N; **Auteri M**; Thijs T; Depoortere I; Matteoli G; Boeckxstaens GE. Smooth muscle and neural dysfunction contribute to different phases of postoperative ileus. *In revision for publication on* Neurogastroenterology & Motility

- Zizzo MG., Cavallaro G., **Auteri M**., Caldara G., Amodeo I., Mastropaolo M., Nuzzo D., Di Carlo M., Fumagalli M., Mosca F., Mulè F., Serio R. Postnatal

development of the dopaminergic signalling involved in the modulation of intestinal motility in mice. *In revision for publication on* Pediatric Research

Proceedings (International Congresses) :

- Zizzo MG., **Auteri M**., Mastropaolo M., Serio R. "Role for D1-like and D2-like dopamine receptors in the modulation of intestinal motility in mice" Abstract in NeuroGASTRO 2015 meeting, Istanbul, 4-6 giugno 2015

- Zizzo MG., **Auteri M.,** Caldara G., Serio R. "Differential recruitment of Angiotensin II receptors in the modulation of rat colonic contractile activity in experimental inflammation" Abstract in NeuroGASTRO 2015 meeting, Istanbul, 4-6 giugno 2015

- Zizzo MG., **Auteri M**., Mastropaolo M., Serio R. "Opposite effects of dopamine on the mechanical activity of longitudinal and circular muscles in human colon" Abstract in NeuroGASTRO 2015 meeting, Istanbul, 4-6 giugno 2015

Proceedings (National Congresses):

- Zizzo MG., **Auteri M.,** Caldara G., Serio R. Role of renin-angiotensin system in colonic dysmotility associated with bowel inflammation in rats. Meeting Ricerca di base, interdisciplinare e traslazionale in ambito Biologico e Biotecnologico, Palermo (PA), 17-18 dicembre 2015

- Zizzo MG., **Auteri M.,** Mastropaolo M., Serio R. Pharmacological characterization of dopamine effects on the mechanical activity of longitudinal and circular muscles in human colon. Meeting Ricerca di base, interdisciplinare e traslazionale in ambito Biologico e Biotecnologico, Palermo (PA), 17-18 dicembre 2015

- Mastropaolo M, Zizzo MG, Caldara G, **Auteri M**, Serio R. "Maternal high fat diet consumption during pregnancy and lactation: impact on intestinal

morphology and function in preweaning offspring". Meeting Ricerca di base, interdisciplinare e traslazionale in ambito Biologico e Biotecnologico, Palermo (PA), 17-18 dicembre 2015

- **Auteri M**., Zizzo MG., Mastropaolo M., Serio R. " Novel evidences for a role of dopamine as modulator of intestinal motility: a study on mouse distal colon" Abstract e Speaker in Meeting Ricerca di base, interdisciplinare e traslazionale in ambito Biologico e Biotecnologico, Palermo (PA), 26-27 giugno 2014

- **Auteri M**. "The enteric nervous system: from motility to inflammation" Speaker invitato in Second Galveston-Palermo Meeting: Advances in biomedicine and neuroscience, Palermo (PA), 13 Marzo 2014

- Zizzo MG.,Mastropaolo M.,**Auteri M**., Serio R. "Postnatal maturation of serotonin signaling system in mouse duodenum" Abstract in 86° Congresso Nazionale della Società Italiana di Biologia Sperimentale, Palermo (PA), 24-25 ottobre 2013

- Zizzo MG., Mastropaolo M., **Auteri M**., Mulè F.,Serio R. "Postnatal development of 5-Hydroxytryptamine (5-HT) signaling system in the mouse" Abstract in 64° Congresso Nazionale della Società Italiana di Fisiologia, Portonovo (AN), 18-20 settembre 2013

- **Auteri M.**, Zizzo MG., Mastropaolo M., Serio R. "GABA & "Little Brain": Ruolo emergente nel controllo della motilità intestinale" Abstract in Meeting Biotecnologie: ricerca di base, interdisciplinare traslazionale in ambito biomedico, Palermo (PA), 27-28 giugno 2013

- **Auteri M.,** Zizzo MG., Mastropaolo M., Serio R. "In medio stat virtus": concentration-dependent effects of GABA on colonic motor patterns in mice" Abstract e Speaker in Annual Meeting of Young Researchers in Physiology, Anacapri (NA), 21-24 maggio 2013