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New Insights into Molecular Mechanisms of Enteric Neuro-Epithelial Pathology: From Damage to Protection

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Abstract

Backgrond: Functional gastrointestinal disorders (FGID) are chronic conditions characterized by symptoms for which no overt organic cause can be found. They affect up to 20% of western populations with evidence suggesting they are equally common in the Third World (Spiller 2005). Although symptoms are generally mild or moderate, a small subset of cases shows severe manifestations, i.e. nausea, vomiting, bloating, abdominal distension, intractable constipation and chronic pain, with such an intensity hampering normal feeding and compromising considerably patients' quality of life. In addition, this subset of patients may also have recurrent intestinal sub-occlusive episodes, which occur in the absence of demonstrable mechanical causes, leading to numerous hospitalizations as well as useless and potentially harmful surgical interventions. This condition is referred to as chronic intestinal pseudo-obstruction (CIPO), a rare and intractable chronic digestive disease with symptoms and signs of intestinal obstruction without demonstrable mechanical cause. CIPO can result from derangements affecting the integrity of a variety of regulatory cells/tissues, i.e. smooth muscle cells, the interstitial cells of Cajal (ICC) (pace-makers of gut motility), and neurons (either intrinsic – the enteric nervous system- or extrinsic nerve pathways).

Aims and experimental studies: The present thesis was conceived to provide a thorough account of the molecular defects underlying CIPO and highlight new strategies of neuro-epithelial protection to prevent enteric neurodegeneration. The experimental studies are based on genetic analysis of CIPO patients and characterization of RAD21 in the ENS of human and mouse intestine. Other studies are focus on 5-hydroxytryptamine-4 (5-HT4) receptor particularly studying whether the activation of colonic epithelial 5-HT4 protects against experimentally induced inflammation of the mouse colon.

Conclusion: My thesis focuses on both mechanisms of neurodegeneration and neuro/epithelial protection as a paradigm to better understand both pathophysiology of severe conditions such as CIPO, and related targeted therapeutic options.

LIST OF ABBREVIATIONS

5-HT *serotonin*

ACTG *actin G2*

AF *activity front(s)*

AFUs *Arbitrary fluorescence units*

APOB48 *Apolipoprotein B48*

BUPA *bursts of uncoordinated phasic activity*

BWA *Burrows-Wheeler Aligner; v0.6.2*

CAID *Chronic Atrial Intestinal Dysrhytmia*

CCT *Cytosolic Chaperonin Containing TCP-1*

CdLS *Cornelia de Lange syndrome*

ChAT *choline acetyl transferase*

CIPO *Chronic intestinal pseudo-obstruction*

DAI *disease activity index*

DAPI *4,6-diamidino-phenylindole,dihydrochloride*

DMEM *Dulbecco's modified Eagle's medium*

DMSO *dimethyl sulfoxide*

dpf *days post fertilization*

DSS *dextran sodium sulfate*

EC *enterochromaffin cells*

ECs *endothelial cells*

EMSA *electromobility shift assays*

EN *enteral nutrition*

ENS *enteric nervous system*

FBS *fetal bovine serum*

FGID *Functional gastrointestinal disorders*

FITC *fluorescein isothiocyanate*

FLNA *filamin A*

FVM *Familial Visceral Myopathy*

GATK *Genome Analysis Toolkit*

GFAP *glial fibrillary acid protein*

GI *gastrointestinal*

HDS *histological damage score*

hpf *hours post fertilization*

hpi *post bead injection*

IBD *inflammatory bowel disease*

IBS *Irritable Bowel Disease*

IBS-C *constipation predominant IBS*

IBS-D *diarrhea-predominant IBS*

ICC *interstitial cells of Cajal*

IJP *inhibitory junction potential*

IP *intraperitoneal*

IR *Immunoreactivity*

LCLs *lymphoblastoid cell lines*

LES *lower esophageal sphincter*

MGS *Mungan syndrome*

MNGIE *Mitochondrial Neurogastrointestinal Encephalomyopathy*

MO *morpholino*

MOI *multiplicity of infection*

NCAM *neuronal cell adhesion molecule*

nNOS *neuronal nitric oxide synthase*

NPY *Neuropeptide Y*;

NT *non-targeting*

PBS *phosphate buffered saline*

PDGFRα *Platelet-derived growth factor receptor-α*

POLG1 *polymerase gamma*

PPN *partial parenteral nutrition*

qRT-PCR *Real-time quantitative RT-PCR*

ROH *Runs Of Homozygosity*

rtPCR *reverse transcriptase polymerase chain reaction*

SNP *single nucleotide polymorphism*

SPUPA *sustained periods of uncoordinated phasic activity*

SRB *sulforhodamine B*

TNBS *trinitrobenzene sulfonic acid*

TPN *total parenteral nutrition*

TYMP *thymidine phosphorylase*

UES *esophageal sphincter*

WES *Whole exome sequencing*

INTRODUCTION

Chapter I

5. MORPHO-FUNCTIONAL ORGANIZATION OF THE ENTERIC NERVOUS SYSTEM

The enteric nervous system (ENS) shows the unique feature of being the largest and most complex division of the peripheral nervous system (PNS). The ENS is "*brain-in-the-gut*" since it is capable of controlling a vast array of digestive functions independently from the central nervous system (CNS). Within the ENS, nerve cells and glia are organized in plexuses, either non-ganglionic (boundles of nerve fibers) or ganglionic, i.e., myenteric (Auerbach's) and submucosal (Meissner's) ganglia, which are interconnected by nerve bundles (Figure 1). An intact ENS is essential for body homeostasis, while, in contrast, abnormalities of ENS cause digestive disorders. Thus, a properly functioning gastrointestinal (GI) tract requires a normally developed and operative ENS in order to control motility, secretion/absoprtion, blood flow, cross-talk with the immune system as well as plays a role in the maintenance of epithelial barrier. Another noteworthy aspect pertains to the interplay between the ENS and the gut microbiota, i.e. the trillion of germs dispersed throughout the lumen of the gut, mainly concentrated in the most distal segments.

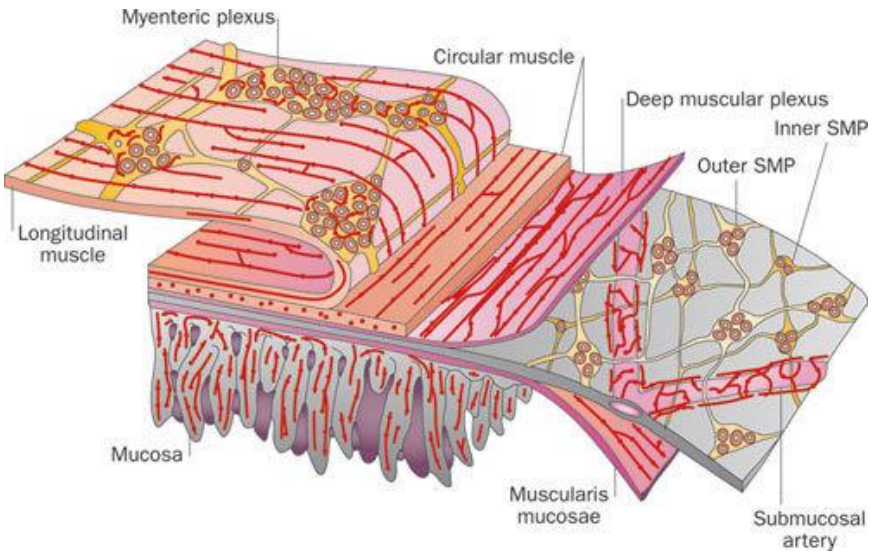

Figure 1. Organization of the ENS of human and medium–large mammals.

The ENS has ganglionated plexuses, the myenteric plexus between the longitudinal and circular layers of the external musculature and the SMP that has outer and inner components. Nerve fiber bundles connect the ganglia and also form plexuses that innervate the longitudinal muscle, circular muscle,

muscularis mucosae, intrinsic arteries and the mucosa. Innervation of gastroenteropancreatic endocrine cells and gut-associated lymphoid tissue is also present, which is not illustrated here. Abbreviations: ENS, enteric nervous system; SMP, submucosal plexus. Modified from Furness, J.B. The Enteric Nervous System (Blackwell Oxford, 2006).

The GI tract is a continuous tube that runs from the mouth to the anus. The alimentary canal includes the mouth, pharynx, esophagus, stomach, small intestine (consisting of duodenum, jejunum, and ileum) and large intestine. It is within this intricate organ that a number of coordinated processes are necessary for digestion and absorption. Muscular sphincters compartmentalize the bowel, dividing it into functionally distinct regions with different luminal environments. The upper esophageal sphincter (UES) maintains the highest resting pressure of all sphincters, preventing air from entering the esophagus. It consists of striated muscle, is under control of the swallowing center in the medulla, and relaxes during swallowing to permit food to enter the esophagus. The lower esophageal sphincter (LES) separates the esophagus and the stomach and consists of smooth muscle that relaxes during swallowing. It functions to coordinate the passage of food into the stomach after swallowing and prevent the reflux of gastric contents, including acid, into the esophagus. A defective LES can contribute generate symptoms such as heartburn. The pyloric sphincter separates the stomach from the duodenum, and its resting pressure contributes to regulate gastric emptying and prevent duodenal-gastric reflux. Reflux of bile acids and digestive enzymes can lead to gastritis, ulcer formation, and risk of perforation. The ileocecal sphincter is a valve-like structure that separates the ileum and cecum, preventing back flux of colonic contents into the ileum. A subgroup of patients with irritable bowel syndrome can experience bloating, bowel distension and pain partly due to bacterial overgrowth in the small intestine. Finally, the internal (smooth muscle) and external (skeletal muscle) anal sphincters control elimination of waste products. A constant detection of luminal contents allows for ingested material to be transported caudally at a physiological rate, allowing each region of the gut to perform their respective function. Smooth and skeletal (in the esophagus and anus) muscle contractions are thus coordinated into activity patterns, such as segmentation (small intestine) or haustration (colon) that grind, mix, and propel aborally the ingested food. Secretory mechanisms exert a pivotal role in order to maintain an appropriate pH and a regulated concentrations of electrolytes, enzymes, and mucous. The pH of the highly acidic chyme in the stomach reaches

1.5 to 3.5. At this pH, the hydrogen ion concentration is around 3 million times that of the arterial blood. Secretion of hydrogen carbonate ion into the lumen of the duodenum by the exocrine pancreas neutralizes the acidic chyme delivered from the stomach to duodenum. These changes are necessary to promote digestion, absorption, and detoxification of ingested materials. A continuously regenerated semipermeable epithelial barrier separates the lumen from the internal milieu of the body. This barrier promotes absorption, but also avoid the leakage of indispensable molecules into the intestinal lumen as well as the transit of digestive enzymes, toxins, and germs into the body from the lumen. Clearly, this highly organized structure of the GI tract and its related physiological functions require a sophisticate degree of regulation and coordination, provided by the ENS. Thus, since the ENS contributes significantly to body homeostasis and it is not surprising that any noxae perturbing ENS maintenance and integrity can result in a variety of disorders some of them so severe to be life threatening and / or hinder significantly the patients' quality of life.

5.1 Developmental aspects

During ENS development, precursors emigrate to the gut from the vagal and sacral neural crest. The population as a whole, if not each cell, is pluripotent and diverges to give rise to enteric neurons and glia. Notably, there is little cell death within the bowel and neurons are generated in the appropriate numbers. After development, maintenance of the mature ENS is a balanced phenomenon, in which the process of apoptotic cell death causes extensive neuronal loss that is complemented by an equally active process of neurogenesis. Gliogenesis also occurs. In contrast to the developmental period, neuronal precursor cells of the mature bowel do not express SOX10, although they continue to express nestin and p75NTR (Figure 2).

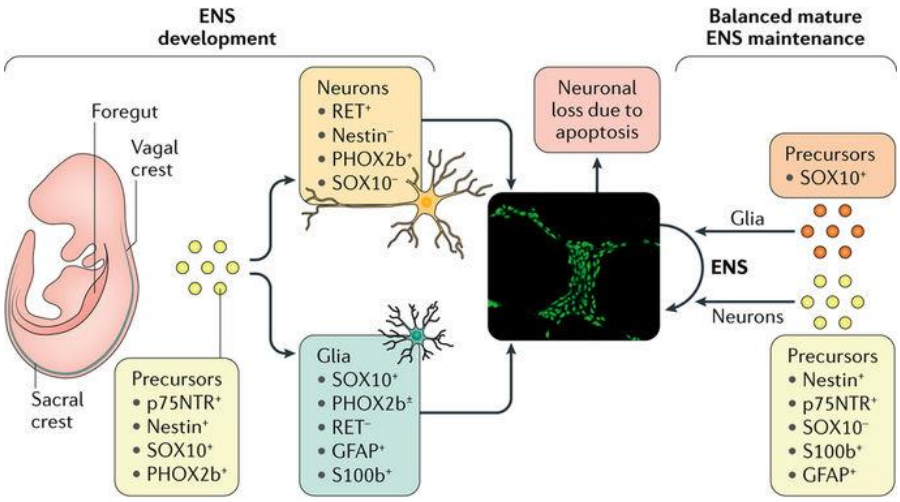

Figure 2. The dynamic life history of enteric neurons and glia.

This scheme summarizes the dynamic life history of enteric neurons and glia. Modified from Rao M. et al 2017.

The development of the ENS has been investigated in a wide array of animal models, including mice, chickens (Goldstein and Nagy, 2008), zebrafish (Shepherd and Eisen, 2011) and invertebrates (Copenhaver, 2007). Consistent evidence acquired over many years of research in this field indicates that all the neurons and glia of the ENS arise from neural crest-derived cells that migrate into the developing GI tract during development. The majority of these arise from the vagal enteric neuronal crest cells (ENCCs), which originate in the caudal hindbrain region of the neural tube, opposite somites 1-7 (Yntema and Hammond, 1954; Le Douarin and Teillet, 1973). During development, these vagal ENCCs migrate into the foregut (developing esophagus and stomach), and colonize the developing GI tract in a rostral to caudal wave (Young and Newgreen, 2001). In the developing mouse, ENCCs reach the foregut at E9.5, enter the midgut (developing small intestine) at E10.5, and reach the anal end of the colon at E14.5 (Kapur et al. 1992) (Figure 3).

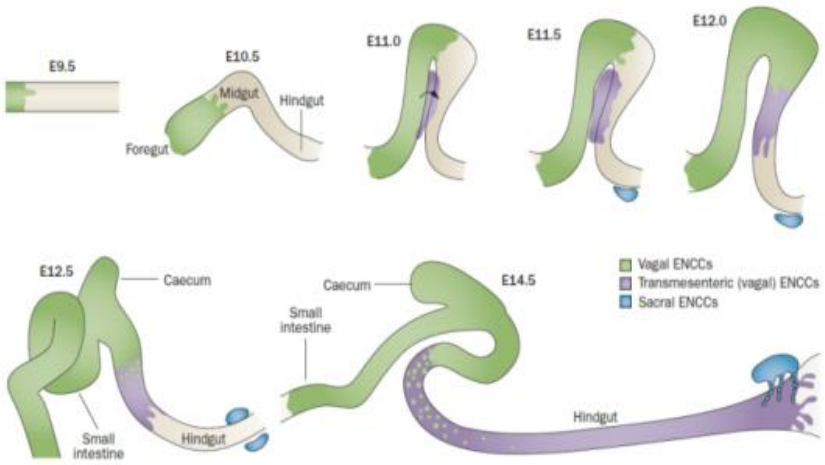

Figure 3. **Migratory pathways of enteric neuronal cell crests (ENCCs) through the mouse gut during embryonic life.** ENCCs enter the foregut around developmental stage E9 and migrate caudally within the gut mesenchyme. At E11.0–E11.5, there is a transient apposition of the midgut and postcaecal gut, and a subpopulation of vagal ENCCs (shown in purple) takes a short-cut across the mesentery to colonize the colon. The transmesenteric cells give rise to the ENS in a large part of the colon. Vagal ENCCs reach the anal end of the gut around E14.5. Cells derived from the sacral neural crest (shown in blue) emigrate to the vicinity of the hindgut around E11.5, but then undergo a waiting period and enter the distal hindgut around E13.5 along with nerve fibres arising from extrinsic neurons.7 As the gestation period for a mouse is ~19 days, colonization of the gut by ENCCs takes >25% of the gestation period. Abbreviations: ENCCs, enteric neural crest-derived cells; ENS, enteric nervous system. From Obermayr et al. 2013.

This is the longest migration of cells during embryonic development, and takes place over approximately 3 weeks in human foetal development (Wallace and Burns, 2005). Sacral ENCCs also contribute to a small population of neurons and glia in the colon (Le Douarin and Teillet, 1973; Pomeranz and Gershon, 1990; Serbedzija et al. 1991a; Burns and Le Douarin, 1998; Wang et al. 2011). Differentiation of neurons takes place before ENCCs have reached the anal end of the gut. In the developing mouse embryo, around 10-15% of ENCCs in the midgut express panneuronal markers at E10.5 (Baetge and Gershon, 1989; Young et al., 1999). These neurons are located along the entire length of the colonized gut, including at the migratory wavefront (Young et al., 1999).

The early events of ENS development, such as migration and proliferation, have been extensively examined in several studies (Gershon, 1981; Young and Newgreen, 2001; Anderson et al., 2006; Heuckeroth and Pachnis, 2006; Heanue and Pachnis, 2007; Young, 2008; Laranjeira and Pachnis, 2009; Gershon, 2010; McKeown et al., 2012; Sasselli et al., 2012b). A failure of ENCCs to reach the anal end of the colon results in Hirschsprung disease (HSCR), a congenital disease characterized by the lack of enteric ganglia for a variable length throughout the gut. The aganglionic segment of the intestine causes a functional obstruction since the affected region cannot generate peristalsis and therefore propel its contents. Complete colonization of the GI tract requires complex coordination of ENCC proliferation, survival, migration, and differentiation. These events are regulated by a number of key factors including: 1) soluble factors released from the gut mesenchyme; 2) the expression of extracellular matrix molecules; 3) expression of cell surface receptors by ENCC; and 4) the expression of transcription factors by ENCCs that regulate downstream events. The two main signaling pathways involved are the glial cell line derived neurotrophic factor (GDNF) – Ret / GDNF family receptor α1 (GFRα1) and the endothelin 3 (ET-3) – endothelin receptor B (EDNRB) pathway. GDNF and ET-3 are produced by the gut mesenchyme and ENCCs express Ret, and its GFRα1 co-receptor, as well as EDNRB (Heanue and Pachnis, 2007). There are also genetic interactions between many of these factors (Wallace and Anderson, 2011). Mice that lack GDNF, Ret, or GFRα1 have complete aganglionosis of the small and large intestines (Schuchardt et al., 1994; Pichel et al., 1996; Sanchez et al., 1996; Enomoto et al., 1998), whereas mice that lack ET-3 or EDNRB only have aganglionosis of the terminal colon (Baynash et al., 1994; Hosoda et al., 1994). The next sections will detail the critical step occurring during development of the ENS, namely cell migration of vagal and sacral component; proliferation and survival; development of the two main ganglionated (myenteric and submicosal) plexuses; and, finally, development of enteric neuron subtypes.

5.1.1 Cell migration. Many factors are important for control of the migration of vagal ENCCs. The migratory behavior of these ENCCs have been examined in several time-lapse imaging studies using intact preparations of embryonic gut (Young et al., 2004; Druckenbrod and Epstein, 2005; Druckenbrod and Epstein, 2007; Wang et al., 2011). ENCCs migrate in chains and cell-cell interaction is important for regulating this behaviour (Young et al., 2004; Druckenbrod and Epstein, 2005). The typical paradigm how to study migration mechanisms

involved changes / modification of signaling systems (Anderson et al., 2006; Breau et al., 2009; Druckenbrod and Epstein, 2009; Zhang et al., 2012b). In some cases delays into the timetable of ENCC migration can result in colonic aganglionosis. For example, the aneural hindgut of older embryos (E16.5) is less permissive to ENCC migration than that of younger embryos (E11.5, Hotta et al., 2010). This can be due to several factors, such as changes in the expression of certain attractive and repulsive factors (e.g. increased laminin expression in the colon between E13.5 and E14.5) (Druckenbrod and Epstein, 2009); and the same factors can exert different effects on ENCCs at different ages (e.g. GDNF has a proliferative effect on ENCCs from E12.5 gut, but this effect is reduced on ENCCs from older embryonic gut) (E14.5 - E16.5) (Chalazonitis et al. 1998). The increased laminin expression is thought to prevent ENCC migration through the colon at E14.5. Delays in ENCC migration would result in colonic aganglionosis at birth, as observed in Ednrb$^{-/-}$, β1 integrins conditional knockout, and Ret 9/- hypomorphic mice (Breau et al. 2006; Uesaka et al. 2008; Druckenbrod and Epstein, 2009). However, more recent studies in L1$^{-/y}$, Tcof1 +/1 and Gdnf +/1 mice have shown that although ENCC migration is delayed, the cells can still reach the anal end of the colon (Anderson et al. 2006; Flynn et al. 2007; Barlow et al. 2012) and therefore no aganglionosis occurs at birth. In the case of Tcof1 +/- mice, there is increased proliferation of ENCCs at the wavefront of migration at E11.5, and also decreased neuronal differentiation, thereby promoting the migration of ENCCs through the hindgut (Barlow et al. 2012). Therefore, finely tuned processes involving ENCC proliferation, survival, migration and differentiation, along with that of the microenvironment of the mesenchyme are required for complete colonization of the hindgut.

In addition to the most prominent component (i.e. vagal), also another neural crest component conytibutes to form the ENS, i.e. the sacral NCCs. These precursor cells migrate out from the neural tube at later stages, E9-9.5, than the vagal component at least in the mouse (Serbedzija et al. 1991b). They then pause at the nerve of Remak (which is an extension of the pelvic plexus) in chick embryos (Burns and Le Douarin, 1998) and the pelvic ganglia in mouse embryos (Kapur, 2000) for several days, prior to migrating into the hindgut. In both chick and mouse embryos, the sacral NCCs enter the gut (hence ENCCs) after the entry of vagal NCCs into the hindgut (Burns and Le Douarin, 1998; Kapur, 2000). However, there is a small period of time when the two populations are distinct as the sacral NCCs enter the caudal hindgut prior to its colonization by vagal ENCCs (Druckenbrod and Epstein, 2005). The presence of

vagal NCCs is not necessary for the migration of sacral ENCC into the gut as sacral NCC derived neurons are found in the gut when vagal ENCCs are ablated (Burns et al. 2000; Burns and Le Douarin, 2001) and in transgenic animal models, where vagal-derived ENCCs are absent from the gut (Durbec et al. 1996; Cacalano et al. 1998; Anderson et al. 2006a; Erickson et al. 2012). Derivatives of the sacral ENCCs in the ENS account for up to 17% of neurons in the distal hindgut, and cannot completely colonize the colon to compensate the absence of vagal NCC derivatives (Burns and Le Douarin, 1998; Burns et al. 2000; Erickson et al. 2012). The developmental potential of sacral ENCCs is reduced in comparison to vagal ENCCs, as transplantation of vagal ENCCs to the level of pre-migratory sacral NCCs results in increased colonisation of the hindgut (Burns and Le Douarin, 2001; Burns et al., 2002). The increased potential of vagal ENCCs appears to be due to increased expression of Ret in comparison to sacral ENCCs (Delalande et al. 2008). The migratory behavior of sacral ENCCs has been observed in *in vitro* co-cultures where a piece of pelvic ganglion was apposed to a segment of aneural hindgut (Wang et al., 2011). The donor sacral ENCCS appear to migrate along nerve fibers that had extended into the gut from the pelvic ganglion donor tissue (Burns and Le Douarin, 1998; Wang et al., 2011). Sacral NCCs contribute to the ENS of the colon, more the myenteric, rather than submucosal, plexus (Burns and Le Douarin, 2001). Overall, the physiological effect of ablation of sacral ENCCs remains unknown.

5.1.2 Proliferation and survival. Proliferation of ENCCs is important for producing sufficient numbers for colonizing the gut. ENCCs proliferate as they migrate towards (Barlow et al. 2008) and through the gut (Gianino et al. 2003; Young et al., 2005). Reducing the number of vagal NCCs results in incomplete colonization of the GI tract (Yntema and Hammond, 1954; Petersvandersanden et al., 1993; Burns et al. 2000), and a mininum number of NCCs is required for full colonisation (Barlow et al. 2008). The density of ENCCs impacts directly on their migratory behavior. For example, when the ENCC population at the wavefront is decreased, the distance that they migrate also decreases in direct proportion to the number of remaining cells (Young et al. 2004; Druckenbrod and Epstein, 2005).

A model of "frontal expansion" has been proposed, where proliferation of ENCCs near the migratory wavefront drives the invasion of ENCCs into the uncolonized gut (Simpson et al. 2007). In these experiments, increased proliferation of cells near the wavefront was detected

in gut organ cultures *in vitro* (Simpson et al. 2007); however, this increased proliferation has not yet been detected *in vivo* (Young et al. 2005; Barlow et al. 2012), except in the zebrafish gut, which like cultured explants of gut, do not grow while being colonised by ENCCs (Olden et al. 2008). In the mouse, the proportion of dividing ENCCs is not significantly different near the wavefront in comparison to the colonized regions of the gut (Young et al. 2005). Furthermore, there does not appear to be distinct proliferative zones within the GI tract, with dividing ENCCs and neurons located throughout the colonized gut (Young et al. 2005; Walters et al. 2010; Barlow et al. 2012). It seems likely that while high proliferation rates are required at the wavefront to drive migration into the uncolonized regions (Simpson et al. 2007), high proliferation rates are also required behind the wavefront in vivo to maintain the density of cells while the gut is growing rapidly in length.

Regulation of cell death is also important during ENS development (Enomoto, 2009; Chalazonitis et al. 2012). Prior to entering the foregut, there is apoptotic death of vagal ENCCs en route to the gut, which is important for regulating ENCC numbers (Wallace et al. 2009). Once they enter the gut, very little ENCC or neuronal cell death has been detected (Gianino et al. 2003; Kruger et al. 2003; Wallace et al. 2010), which is, as indicated above, very different from many other parts of the developing nervous system. One study has found that conditional loss of GFRα1 signaling in mice at E12.5 – E15.5 in embryonic development results in neuronal cell death in the colon and subsequently, colonic aganglionosis at birth (Uesaka et al. 2007). However, cell death in these mice did not involve common cell death executors, i.e. caspase-3 or caspase-7, or the proapoptotic protein Bax, nor did dying cells exhibit the morphological features of apoptosis such as chromatin compaction and mitochondrial pathology (Uesaka et al. 2007), raising the possibility that this form of cell death has not been detected in previous experiments using traditional techniques of TUNEL or caspase staining. Instead, cell death was detected by abnormal nuclear shapes using electron microscopy (Uesaka et al., 2007). The death of Sox10-expressing ENCCs has been detected in mice without defects in signaling pathways, using live imaging of ENCCs that express the fluorescent protein Venus in their nucleus (Corpening et al. 2011). In these cells, death was observed as fragmentation of the nucleus, and was identified in a small percentage of Sox10-expressing ENCCs at all different regions along the colonised gut (Corpening et al. 2011). A very recent study has also reported a decrease in the number of Hu-expressing

neurons per ganglion in the rat antrum between 4-12 weeks after birth suggesting significant postnatal neuronal cell death in the antrum (Baudry et al. 2012).

5.1.3 Development of the myenteric and submucosal plexuses. In the small intestine of both chickens and mice, ENCCs first congregate in the outer mesenchyme, forming the future myenteric plexus (MP), while the submucosal plexus (SMP) develops several days later, from a secondary migration of cells from the MP (Gershon et al. 1980; Pham et al. 1991; Kapur et al. 1992). In mice, neurons can be detected in the presumptive SMP of the small intestine at E15.5 (Jiang et al. 2003). The secondary migration of ENCCs into the SMP is dependent on netrin-deleted in colo-rectal cancer (DCC) signaling. Netrins are expressed in mucosa of the developing small intestine, while DCC, a netrin receptor, is expressed by ENCCs. Netrin$^{-/-}$ mice lack submucous ganglia, indicating that netrin acts as a chemoattractant to a sub-population of myenteric ENCCs (Jiang et al. 2003). Recently, GDNF is also an important chemoattractant for the migration of ENCCs from the presumptive MP to the SMP (Uesaka and Enomoto, 2012). In the embryonic mouse, the circular muscle does not differentiate until after the colonisation of the hindgut at E14.5 (McKeown et al. 2001). As the ENCCs migrate down the hindgut, they are dispersed through the presumptive circular muscle layer. As the circular muscle differentiates, the ENCCs condense into a single layer on the serosal surface of the circular muscle, thus forming the MP (McKeown et al., 2001). Neurons are present in the location of the presumptive SMP of the colon at E18.5, but are sparsely distributed, and colonic submucosal ganglia are not evident until post-natal day (PD)3 (McKeown et al. 2001).

Vagal NCCs do not express any neuronal markers en route to the foregut (Anderson et al. 2006a), and differentiation takes place as ENCCs migrate through the GI tract. In the developing mouse, ENCCs that express neuronal markers are present in the foregut at E9.5, and in the midgut at E10.5 (Baetge and Gershon, 1989). These cells express a variety of panneuronal markers such as Hu (Fairman et al. 1995), neuronal class III β-tubulin (TUJ; Fairman et al. 1995; Conner et al. 2003), neurofilament-M (NF-M; Payette et al. 1984), protein gene product 9.5 (PGP9.5; Young et al. 2002) and microtubule associated protein 2 (MAP2; Baetge et al. 1990). Enteric neurons at E10.5 and E11.5 are also known as the "transiently catecholaminergic" population of ENCCs as all ENCCs that express pan-neuronal

markers also express tyrosine hydroxylase (TH) at these ages (Baetge and Gershon, 1989; Young et al. 2002). By E12.5, TH expression is downregulated and is only present in a subpopulation of enteric neurons. The embryonic transiently catecholaminergic neurons are different from the TH positive dopaminergic neurons of the adult ENS (Li et al. 2011). It does not appear to be any distinct neurogenic zones in the ENS as these early enteric neurons are dispersed amongst the undifferentiated ENCCs, including at the migratory wavefront of ENCCs (Young et al. 1999; Young et al. 2002). The differentiation of ENCCs into neurons impacts on the colonization of the GI tract by ENCCs, which is vital to ENS development. ENCCs migrate through the gut as chains and lay down a network of ENCCs. Time lapse imaging experiments have shown that the chains are maintained for many hours after their initial formation (Young et al. 2004). Through development, cells aggregate together to form distinct ganglionic clumps with interconnecting neurite fiber strands. The formation of ganglia proceeds in a rostral-to-caudal progression (Epstein et al., 1991). In the mouse, enteric ganglia start to become distinct at E13 as revealed by increased separation between groups of cells (Young et al. 1998b). An increase in the number of neurons per ganglion has been described through embryonic development (Tanano et al. 2005). Changes in the morphology of ganglia have been described in the early postnatal development of the rat (Schafer et al. 1999). In the duodenum, MP at birth appeared more densely packed with cells than those of older postnatal ages, whilst in the colon, distinct ganglia were not present until P7 (Schafer et al., 1999).

The neuronal cell adhesion molecule (NCAM) and its polysialylated form, PSA-NCAM, are important in ganglion formation. Immunohistochemical studies have shown that both NCAM and PSA-NCAM are present in developing enteric neurons, and PSA-NCAM is important for the clustering of enteric neurons *in vitro* (Faure et al. 2007). Regulation of NCAM expression and polysialylation involve bone morphogenic protein 4 (BMP4) and the transcription factor Hand2 (Faure et al. 2007; Lei and Howard, 2011). Inhibition of BMP4 signaling results in the formation of smaller ganglia in chick embryos, and also in a reduced neurite fasciculation in mouse embryos (Goldstein et al. 2005; Fu et al. 2006). The clustering of neurons *in vitro* is dependent on the presence of BMP4 signaling (Faure et al. 2007). The formation of enteric ganglia is disrupted in a conditional Hand2 mutant, and the organized network structure of the ganglia is absent in these mice (Lei and Howard, 2011). This is thought to be due to decreases in the regulation of NCAM expression in the ENS of these mice (Lei and Howard, 2011). In

β1 integrin mutant mice, there is also abnormal ganglia formation, however, this does not appear to be as severe as that of the Hand2 mutant, as distinct ganglia are still present (Breau et al. 2006). In the β1 integrin mutant, ENCCs aggregate together during migration, leaving large cell-free space, which lead to the formation of an abnormal ganglia network (Breau et al. 2006). The expression of cell adhesion molecules, including that of NCAM, did not appear to be disrupted in the β1 integrin mutants (Breau et al. 2006). Mathematical modeling of ganglia formation has suggested that threshold criteria for ENCC proliferation, differentiation, and cell-cell adhesion are required for the normal formation of enteric ganglia (Hackett-Jones et al. 2011).

Likewise enteric neurons, also glial cells originate from the same neural crest progenitors, which migrate and colonize the GI tract. Upon colonization of the embryonic gut, neural-crest derived progenitors mature into neurons and glia via Hedgehog/Notch pathway (Gershon and Rothman 1991; Ruhl 2005). In the mouse ENS, the development of enteric glia occurs later than that of enteric neurons (Young et al. 2003). Cells immunoreactive for S100b are present only in the duodenum at E14.5 (Young et al. 2003), and cells expressing GFAP are present at E16.5 (Rothman et al. 1986). Prior to this, the precursors of enteric glia maintain Sox10 expression, which is downregulated in neurons (Young et al. 2003). Expression of B-FABP, which is restricted to glial precursors and not common precursors of both neurons and glia, is present in the foregut and midgut at E11.5 (Young et al. 2003). The molecular pathways influencing the development of enteric glia are not well understood. Sox10 is essential for the generation of enteric glia (Britsch et al. 2001; Paratore et al. 2001). Notch and Foxd3, which indirectly regulate the expression of Sox10, are therefore also important for gliogenesis in the ENS (Okamura and Saga, 2008; Mundell et al. 2012; Wahlbuhl et al. 2012). One pathway has been identified whereby signaling through the ErbB3 receptor has been found to promote glial differentiation (Chalazonitis et al. 2011a). The ErbB3 receptor is expressed by the developing enteric glia and regulated by Sox10 (Britsch et al. 2001). Glial growth factor 2 (GGF2) is a ligand of ErbB3 expressed by the enteric mesenchyme and promotes glial differentiation (Chalazonitis et al. 2011). BMPs, which promote neurogenesis, also promote gliogenesis via this pathway as they promote the expression of ErbB3 (Chalazonitis et al. 2011). In the adult ENS, enteric glia has been shown to play many different roles in regulating GI function (Furness, 2006; Neunlist et al. 2014). One recent study has shown that they act as adult neural

stem cells of the ENS and can differentiate to enteric neurons in response to injury (Laranjeira et al. 2011).

5.1.4 Development of enteric neuron subtypes. The age at which a neuronal precursor exits the cell cycle is defined as the birthdate of that neuron. In the developing CNS, it is thought that neuronal precursors exit the cell cycle prior to expressing neuronal markers, hence, neurons are "postmitotic". In contrast to this, neurons in sympathetic ganglia (Rothman et al. 1978) and the developing ENS continue to divide after initiating their expression of pan-neuronal markers (Teitelman, 1981; Young et al. 2005). However, there does not appear to be division of enteric neurons that express subtype specific markers (Young et al. 2005), hence, enteric neurons appear to exit the cell cycle prior to their subtype differentiation. However, further investigation is required as there is currently no reliable immunohistochemical method for detecting cholinergic enteric neurons during embryonic development.

5-hysroxytryptamine (5-HT) / serotoninergic and choline acetyltransferase (ChAT) / cholinergic neurons are "born" at E8, which is prior to the entry of vagal NCCs into the gut (Pham et al. 1991). Several other subtypes begin to be born at E10, including VIP, NYP, enkephalin and CGRP neurons (Pham et al. 1991). A subsequent study showed that NOS, CALB and GABAergic neurons were born from E12.5 to P1, however, E12.5 was the earliest birthdate examined (Chalazonitis et al. 2008). In both studies, the birthdates of neurons in the MP were found to be earlier than those of submucous plexus neurons (Pham et al. 1991; Chalazonitis et al. 2008). In the third study, the birth of VIP neurons was examined in chick embryos (Epstein et al. 1992). So far, no correlation between the time of cell cycle exit and the first immunohistochemical detection of its neurochemical phenotype has been determined. This length of time appears to vary for different neuronal subtypes.

5.2 Structural features

The ENS is a complex neuronal network, which runs along the length of the GI tract, from the esophagus to the anus. The entire structure of the ENS is arranged into ganglia, aggregates of neuronal cell bodies and glial cells, connected by interganglionic strands in order to form two plexuses: the myenteric (Auerbach's) and the submucosal (Meissner's) (Furness 2012; Costa

and Brookes 2008). The outer myenteric plexus is located between the longitudinal and circular smooth muscle layers and is (although not exclusively) involved in the control of gut motor patterns. The inner submucosal plexus resides within the submucosa and is involved with local fluid secretion and absorption (Furness et al. 1990; Brehmer et al. 1998). Taken together the network formed by the two ganglionated plxuses is constituted by about 100 million neurons in the guinea pig (in humans likely 400-500 millions) and even more supporting cells (enteric glia) outnumbering from 3 to 5 times enteric neurons (Furness 2006).

5.2.1 Myenteric plexus (MP). The MP forms a network around the gut, which extends from the upper esophagus to the internal anal sphincter. The MP is embedded within the two layers of the muscularis externa, i.e. the circular and longitudinal coats, and composed by a neuronal cell bodies and related nerve processes (Furness 2006). The MP shows numerous differences in its morphological organization, among GI segments of different species (Vittoria et al. 2000; Domeneghini et al. 2004; Chiocchetti et al. 2009b). A subserosal ganglionated plexus has been identified in the cattle abomasum (Vittoria et al. 2000) and in the human sigmoid colon (Crowe and Burnstock 1990). However, in all other human colonic levels examined (ascending, transverse and descending colon) the subserosal plexus showed no ganglia but only nerve fibers (Ibba-Manneschi et al. 1995). Generally, in the MP, ganglia are bigger than the SMP in terms of number of neurons, and are linked by interconnecting strands (or primary strands), which constitute the so-called primary plexus (Furness 2006). The MP are located in parallel to the circular muscle layer, although this feature can vary among species (Irwin 1931); primary interconnecting strands show longitudinal course, an organization that seems to be a distinctive feature of the MP in most small and large mammals (Scheuermann et al. 1986; Gabella 1987; Santer and Baker 1988; Pearson 1994; Furness 2006; Freytag et al. 2008; Bodi et al. 2009). The other two components of MP are the secondary and tertiary plexuses (Furness 2006). The secondary plexus is constituted by fine bundles of nerve fibers running parallel to the circular muscle layer and primary plexus (Balemba et al. 1999; Furness 2006). The tertiary plexus is made by thin interconnecting strands (the smallest in size) supplying the longitudinal muscle layer (Scheuermann et al. 1986; Balemba et al. 1999; Furness 2006).

5.2.2 Submucosal plexus (SMP). The SMP is well developed in the small and large intestine, while only a few submucous ganglia can be found in the esophagus (third inferior part) and stomach particularly in large mammals, including humans (Lefebvre et al. 1995; van Ginneken et al. 1996; Teixeira et al. 2001; Izumi et al. 2002; Furness 2006). The interconnecting strands of the SMP are usually thin and SMP ganglia are small. The SMP is organized in a single layer in small mammals (Timmermans et al. 2001), whereas it shows a multilayered (two or three layers) organization in large mammals (Brehmer et al. 2010). In these species two different ganglionated plexuses can be identified, namely the internal submucous plexus and the external submucous plexus (Schabadasch 1930; Gunn 1968; Messenger and Furness 1990; Timmermans et al. 1992a; Timmermans et al. 1992b; Pearson 1994; Timmermans et al. 1997). External and internal SMP are separated by a thin connective tissue layer (Gunn 1968; Christensen and Rick 1987; Hoyle and Burnstock 1989; Balemba et al. 1998) and by submucosal blood vessels (Balemba et al. 1998). The external and internal SMPs appear different among the investigated species. Generally, they can be distinguished on the basis of their location, architecture, meshwork density, size and form of the ganglia, and light microscopic appearance (Stach 1977b; Stach 1977c; Stach 1977a; Stach 1978; Scheuermann and Stach 1984). The external SMP shows the most irregularly organized nerve meshwork, while the internal meshwork is smaller and more regular compared to the external SMP (Gunn 1968; Scheuermann et al. 1987; Hoyle and Burnstock 1989; Timmermans et al. 1990; Balemba et al. 1999). Additionally, the two compartments of SMP neurons also show differences in the content of neurochemical messengers / transmitters, being the external SMP more similar to that of myenteric neurons (Scheuermann W.D. 1998; Hens et al. 2000). In addition to the mucosa, some neurons of the external SMP also supply the muscular layer (Sanders and Smith 1986; Furness et al. 1990; Timmermans et al. 1994b; Timmermans et al. 1997; Porter et al. 1999; Timmermans et al. 2001). The internal SMP neurons supply the mucosa and only a small number have projections to the muscle layers (Porter et al. 1999; Timmermans et al. 2001). Thus, in conclusion the external and internal SMP neurons overlap in terms of functional control of fluid movements, local blood flow and sensory functions, while the external SMP can also affect gut motility (Timmermans et al. 1990).

5.2.3 Neurochemistry of enteric neurons. Enteric neurons are able to synthesize and release about 30 different mediators / bioactive substances that may act as messengers, i.e.

neurotransmitters, neuromodulators and neuropeptides (Table 1). Studies over the years have clearly shown that each subclass of enteric neurons can be characterized on the basis of the combination of messengers. This property is known as *neurochemical coding* and turned to be a feature of the ENS that is maintained through most animal species (Costa et al. 1996). Primary neurotrasmitters exert the same role in different species and along the GI tract. These substances include acetylcholine (ACh) and tachykinins in enteric excitatory motor neurons, and nitric oxide (NO), vasoactive intestinal polypeptide (VIP) and others messengers in inhibitory motor neurons. Inhibitory motor neurons may be immunohistochemically identified by the presence of the neuronal nitric oxide synthase (nNOS), the neuronal isoform of the enzyme synthesizing NO, the primary neurotransmitter released by inhibitory motor neurons, while excitatory motor neurons may be immunohistochemically identified by the presence of the synthesizing enzyme choline acetyltransferase (ChAT). Secondary neurotransmitters or modulators include substances which may vary among different groups of neurons depending on the GI tract and the species considered (Furness 2006) (Table 1). Research performed in the last thirty years has shown that classification of the several classes of enteric neurons is the result of a combination of various technical approaches and criteria. These features include: neuronal shape; histochemical and immunohistochemical staining; projections; and electrophysiological and functional properties.

Type of neuron	Primary transmitter	Secondary transmitters, modulators	Other neurochemical markers
Enteric excitatory muscle motor neuron	ACh	Tachykinin, enkephalin (presynaptic inhibition)	Calretinin, γ-aminobutyric acid
Enteric inhibitory muscle motor neuron	Nitric oxide	VIP, ATP or ATP-like compound, carbon monoxide	PACAP, opioids
Ascending interneuron	ACh	Tachykinin, ATP	Calretinin, enkephalin
ChAT, NOS descending interneuron	ATP, ACh	ND	Nitric oxide, VIP
ChAT, 5-HT descending interneuron	ACh	5-HT, ATP	ND
ChAT, somatostatin descending interneuron	ACh	ND	Somatostatin
Intrinsic sensory neuron	ACh, CGRP, tachykinin	ND	Calbindin, calretinin, IB4 binding
Interneurons supplying secretomotor neurons	ACh	ATP, 5-HT	ND
Noncholinergic secretomotor neuron	VIP	PACAP	NPY (in most species)
Cholinergic secretomotor neuron	ACh	ND	Calretinin
Motor neuron to gastrin cells	GRP, ACh	ND	NPY
Motor neurons to parietal cells	ACh	Potentially VIP	ND
Sympathetic neurons, motility inhibiting	Noradrenaline	ND	NPY in some species
Sympathetic neurons, secretion inhibiting	Noradrenaline	Somatostatin (in guinea pig)	ND
Sympathetic neurons, vasoconstrictor	Noradrenaline, ATP	Potentially NPY	NPY
Intestinofugal neurons to sympathetic ganglia	ACh	VIP	Opioid peptides, CCK, GRP

Table 1. Multiple transmitters of functionally distinct enteric neurons that control digestive function.

Abbreviations: 5-HT, 5-hydroxytryptamine; ACh, acetylcholine; CCK, cholecystokinin; ChAT, choline acetyltransferase; CGRP, calcitonin gene-related peptide; GRP, gastrin releasing peptide; ND, not determined; NPY, neuropeptide Y; NOS, nitric oxide synthase; PACAP, pituitary adenylyl-cyclase activating peptide; VIP vasoactive intestinal peptide. From Furness 2006.

5.2.4 Neuronal shape. Enteric neurons were categorized by Dogiel according to their shape using the methylene blue staining technique (Dogiel 1899). Myenteric and submucosal neurons were morphologically classified in three types, referred to as Dogiel type I, II and III. After Dogiel, many other authors proposed additional classifications based on individual neuronal morphology revealed by silver impregnation technique, immunoreactivity for neurofilaments and / or other markers (Stach 1989; Brehmer et al. 1999; Stach 2000; Brehmer

et al. 2002; Brehmer et al. 2004b). Taken together, these studies confirmed and extended the Dogiel classification from three up to seven types of neurons in addition to 'mini-neurons'. The morphological classifications of enteric neurons has been underestimated (Brehmer 2006), however, the possibility to correlate the morphological appearance of a given neuron to its neurochemical code, as well as bioelectrical / functional properties has also refueled the importance of studying the morphology of the enteric nerve cells.

5.2.5 Functional aspects. From a functional standpoint, enteric neurons can be divided in motor neurons, interneurons, intrinsic primary afferent neurons (IPANs) (Costa and Brookes 1996; Furness 2000), and intestinofugal afferent neurons (IFANs) (Furness 2003) (Figure 4).

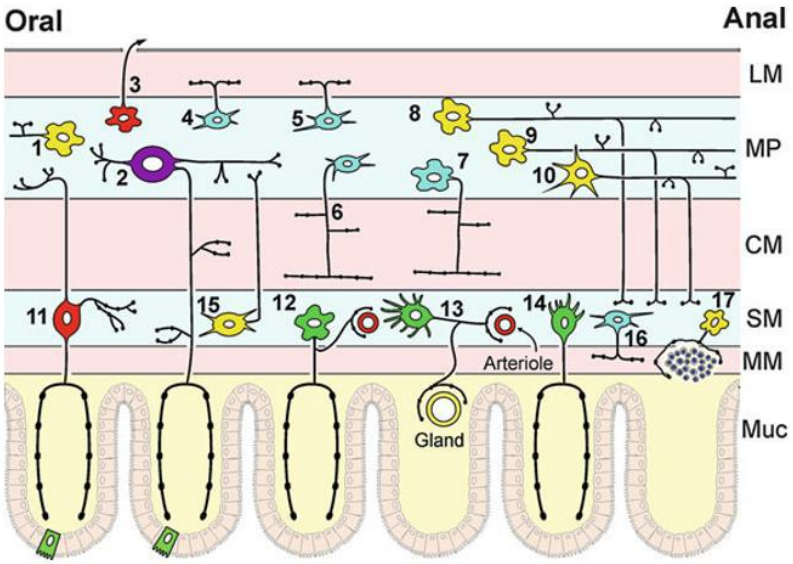

Figure 4. Types of neurons in the small intestine. The types of neurons in the small intestine, all of which have been defined by their functions, cell body morphologies, chemistries, key transmitters, and projections to targets. Neuron Types: Ascending interneurons (1); Myenteric intrinsic primary afferent neurons (IPANs) (2); Intestinofugal neurons (3); Excitatory longitudinal muscle motor neurons (4); Inhibitory longitudinal muscle motor neurons (5); Excitatory circular muscle motor neurons (6); Inhibitory circular muscle motor neurons (7); Descending interneurons (local reflex) (8); Descending interneurons (secretomotor and motility reflex) (9); Descending interneurons (migrating myoelectric complex) (10); Submucosal IPANs (11); Non-cholinergic secretomotor/ vasodilator neurons (12); Cholinergic secretomotor/vasodilator neuron (13); Cholinergic secretomotor (non-vasodilator) neurons (14); Uni-axonal neurons projecting to the myenteric plexus (15); motor neuron to the muscularis

mucosa (16); innervation of Peyer's patches (17). Abbreviations: LM longitudinal muscle, MP myenteric plexus, CM circular muscle, SM submucosal plexus, Muc mucosa. From Furness 2012.

Motor neurons. The category of motor neurons includes excitatory and inhibitory neurons directed to the GI musculature; secretomotor / vasodilator neurons are able to regulate mucosal secreting cells and vasodilation / vasoconstriction of the intestinal vasculature. Furthermore, another subset is represented by secretomotor neurons and neurons innervating entero-endocrine cells (Furness et al. 2000). The motor neurons innervating the smooth muscle of digestive tract are located within the MP of rodents (Wilson et al. 1987; Song et al. 1998) and within myenteric and external and internal submucous plexuses of large mammals [MP > external SMP > internal SMP] and humans [MP > external SMP > internal SMP] (Sanders and Smith 1986; Timmermans et al. 1990; Timmermans et al. 1994b; Porter et al. 1999; Hens et al. 2001). These neurons could be distinguished in circular muscle motor neurons, longitudinal muscle motor neurons, and motor neurons innervating the muscularis mucosae. According to the peristaltic reflex, the excitatory motor neurons are especially localized aborally to the innervated circular and longitudinal muscle (Brookes et al. 1991; Michel et al. 2000), while the inhibitory motor neurons are generally localized orally to the innervated circular and longitudinal muscle (Brookes et al. 1992; Brookes et al. 1996; Brookes et al. 1997; Pfannkuche et al. 1998; Yuan and Brookes 1999; Michel et al. 2000).

Interneurons. These are the enteric neurons with the longest projections. These cells, which have been identified with certainty only in the guinea-pig (Costa and Brookes 2008), mouse, rat, and human are mainly localized in the MP. Interneurons form long functional chains of ascending and descending elements through which information may travel for short or long distances (Pompolo and Furness 1993). Interneuron projections extend up to 14 mm anally and up to 136 mm orally in guinea pig small intestine (Song et al. 1997). It is worth noting that interneurons may also function also as mechanoceptors (Stebbing and Bornstein 1996; Spencer et al. 2006; Dickson et al. 2007; Smith et al. 2007). So far, four types of interneurons have been identified in the guinea-pig small intestine: one ascending and three types of descending interneurons. The ascending interneurons are MP Dogiel type I cholinergic neurons (Furness 2000; Brookes 2001) and may contain also calretinin, SP, neurofilament triplet protein (NFP), and enkephalin (Brookes et al. 1997). Descending MP interneurons (5% of all ENS cells in guinea-pig) are phenotypically cholinergic neurons further distinct into

three types based on their immunoreactivity for NOS/VIP, SOM and 5 hydroxytryptamine (5HT) (Portbury et al. 1995; Song et al. 1997; Brookes 2001). nNOS/VIP/ChAT/immunoreactive Dogiel type I interneurons can contain also neuropeptide Y (NPY) (Uemura et al. 1995), gastrin releasing peptide (GRP), bombesin (BN) (Brookes, 2001) and the enzyme alkaline phosphatase (Song et al. 1994). SOM/ChAT immunoreactive Dogiel type III MP neurons (Portbury et al. 1995; Song et al. 1997) project to other MP ganglia and also to SMP ganglia (Brookes 2001). Also, 5HT/ChAT immunoreactive Dogiel type I MP neurons send their projections to MP and SMP and seem to have significant roles in excitatory pathways regulating both motility and secretion (Neal and Bornstein 2007).

Intrinsic primary afferent neurons (IPANs). The IPANs are the first neurons of the reflex pathways in the intestine (Furness et al. 2004a). They are involved in the control of physiological functions as motility, blood flow and secretion, being responding to several stimuli, such as distention, luminal chemistry and mechanical stimulation of the mucosa (Furness 2006). IPANs have typical electrophysiological properties. In fact, they have broad action potentials that are related to both sodium and calcium currents and are followed by early and late (slow) after hyperpolarizing potentials (AHPs) (Furness et al. 2004a). Their targets are represented by mucosa and other MP and SMP neurons (Bornstein et al. 1989; Kirchgessner et al. 1992; Song et al. 1992; Evans et al. 1994). Cell bodies of multi-axonal IPANs are 10-30% of neurons in SMP and MP ganglia of the small and large intestine; no IPANs are detected in the esophagus and they are rare in the stomach, where motility is primary controlled by vagal efferents (Furness et al. 2014). All the IPANs of guinea-pig and other species intestine show Dogiel type II morphology (non-dendritic, multi-axonal type II neurons) (Kirchgessner et al. 1992; Kunze et al. 1995; Bertrand et al. 1997; Kunze et al. 1998; Kunze et al. 1999). A large percentage (82-84%) of myenteric IPANs of the guinea-pig ileum expresses IR for the calcium-binding protein calbindin (CALB) (Furness et al. 1988; Iyer et al. 1988; Costa et al. 1996) and almost all MP and SMP IPANs express cytoplasmic NeuN-IR (Chiocchetti et al. 2003; Van Nassauw et al. 2005). Furthermore, all of them show ChAT-IR (Steele et al. 1991; Li and Furness 1998). Notably, only 30% of submucosal IPANs of the guinea-pig ileum appear to be IR for CALB (Iyer et al. 1988; Song et al. 1991; Quinson et al. 2001), and that CALB is not confined to the IPANs since it is also localized in 50% of submucosal calretinin-IR secretomotor / vasodilator neurons (Quinson et al. 2001). Many researchers studied CALB-IR also in other species, with the aim to establish whether CALB

could be considered an IPAN marker. In the pig small intestine, CALB cannot be considered markers of IPANs, being mainly localized in interneurones and intestinofugal neurons (Brehmer et al. 2004a; Brown and Timmermans 2004). Dénes and Gábriel (Denes and Gabriel 2004) described CALB-IR myenteric neurons in rabbit small intestine. These cells showed Dogiel type I morphology, ChAT-IR, and were identified as interneurons. Also in the mouse colon, CALB cannot be considered a good marker of IPANs, while the anti-CGRP antibody is considered the most specific marker of these cells (Furness et al. 2004b). Non-dendritic multiaxonal type II neurons involved in mucosal innervations have been demonstrated also in porcine (Hens et al. 2000) and human (Hens et al. 2001) small intestine. Unlikely from guinea-pig, porcine and human (and mouse) IPANs express CGRP, which has been considered a specific marker of type II neurons in these species (Scheuermann et al. 1986) (Scheuermann et al. 1991; Timmermans et al. 1992a; Balemba et al. 1998; Hens et al. 2000; Wolf et al. 2007).

Intestinofugal primary afferent neurons (IFANs). Intestinofugal neurons represent a unique subset of enteric neurons with their cell body located in the myenteric ganglia and projections giving off the intestinal wall (Szurszewski et al. 2002). Most of them show a Dogiel type I morphology, whereas a minority have Dogiel type II features (Lomax et al. 2000; Ermilov et al. 2003). IFANs act as mechanoceptors, being able to detect changes in volume and to respond to the stretching (but not to the tension) of the smooth muscle cells (Crowcroft et al. 1971; Szurszewski and Weems 1976; Kreulen and Szurszewski 1979). Once activated, IFANs usually release Ach in the in the prevertebral ganglion (PVG) thereby evoking nicotinic fast post synaptic potentials (Szurszewski 1994). A subset of IFANs responds to colonic distention by releasing gamma-amino butyric acid (GABA), which facilitate Ach release from cholinergic IFANs in PVG. Because of IFANs activation, the response of the reflex entero-PVG circuitry is the release of noradrenaline by sympathetic neurons in GI wall. This effect modulates smooth muscle contraction or myenteric neuron activity (Parkman et al. 1993; Ma and Szurszewski 1996; Walter et al. 2016). The functional significance of this reflex arc is to counteract large increases in tone and intraluminal pressure during filling (Szurszewski et al. 2002).

5.2.6 Enteric glia. The existence of accompanying non-neuronal cells (hence the term glia) dates back to the beginning of the 19th century with the first glial cells were described by Robert Remak who detailed very elegantly 'special cellular elements' while reporting in his thesis (published in 1838) on nerve fibers and their surrounding sheats, later on called Schwann cells. However, the term "glia" (in the ancient Greek *γλία* language means "glue") was introduced by Virchow (1856). Virchow used for the first time the word glia describing the neuroglia in the CNS. He thought neuroglia was a kind of connective tissue and found that this tissue also contained cellular elements. We owe the first drawings of a star-shaped glial cell to Otto Deiters (1865).

Concerning the ENS, the first description of nucleated satellite cells around nerve cell bodies of the enteric ganglia was given by Dogiel (1899), and many other investigators have studied these cells in the ganglia and nerve strands of the gastrointestinal plexuses and referred to them as 'Schwann cells' (Stöhr 1952). The term 'enteric glial cell' was used for the first time by Giorgio Gabella in his original and extremely accurate research made along 10 years of work in (Gabella 1971 to 1981). Other studies, which have used immunohistochemical markers to locate enteric glia, revealed that these cells are common in the ganglia and nerve fiber bundles. Enteric glia express glial fibrillary acidic protein (Jessen and Mirsky 1980, 1983) and the S-100 Ca^{2+}-binding protein (Ferri et al. 1982b), both of which are typical of CNS astrocytes. Glial cells in other autonomic ganglia do not contain glial fibrillary acidic protein (Jessen and Mirsky 1983). Electron microscopy studies also showed that what it was intended as "enteric neuronal satellite cells" actually are glial cells closely reminiscent of astrocytes of the CNS and distinct from Schwann cells of other peripheral ganglia or nerve trunks (Gabella 1971, Gabella 1972, Cook & Burnstock 1976, Gabella 1981, Komuro et al. 1982). Specifically, enteric glial cells partly surround nerve cell bodies and axons in the ganglia, leaving bare large areas of neuronal membrane at the surface of ganglia; also, their processes contain bundles of gliofilaments and are surrounded by a single basement membrane (none of these aspects are detectable in Schwann cells). There is a marked difference in the glial cell-to-axon relationship between small (guinea pig, rat) and large mammals (such as cat or human). In small mammals, glial cell processes fail to penetrate in the interstitium between nerve cell bodies as well as axons in the neuropil (Gabella 1972a, Komuro et al. 1982). In fact, many nerve processes show direct membrane-to-membrane contacts with each other; the glial cells only separate them into groups and rarely form a

sheath around an individual axon. In contrast, in enteric ganglia of human and monkey, axons are separated from one another by intervening glial cell processes (Baumgarten et al. 1970).

Compared with other peripheral glial cells (e.g., Schwann cells), enteric glial cells do not form basal laminae and they ensheath nerve bundles and not individual axons (Bannerman et al. 1986). In addition to the previously mentioned glial fibrillary acidic protein (GFAP) and S100b, other available immunohistochemical markers for glial cell labeling in the adult gut include and Sox (SRY-box) 8/9/10, the first two being the most frequently used (Ruhl 2005; Hoff et al. 2008). Recently, marker expression analysis showed that the majority of glia in the myenteric plexus co-express GFAP, S100b, and Sox10 (De Giorgio et al. 2012). However, a considerable fraction (up to 80%) of glia outside the myenteric ganglia, did not show labeling for these markers. The alternative combinations of markers reflect dynamic gene regulation rather than lineage restrictions, revealing an extensive heterogeneity and phenotypic plasticity of enteric glial cells (Boesmans et al. 2015) (Figure 5).

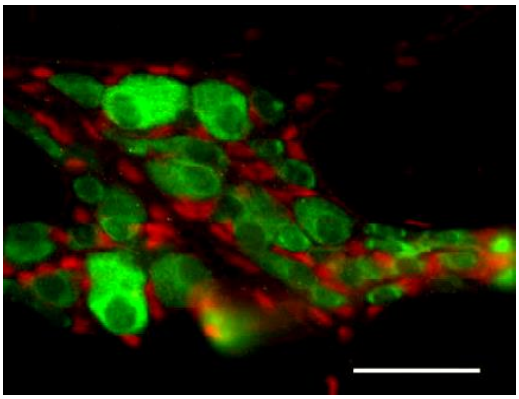

Figure 5. Differential expression of HuC/D and Sox10 in enteric glia of the myenteric plexus of the ileum. (a) Confocal photomicrograph showing double immunostaining for HuC/D (green fluorescence) a panneuronal marker and Sox10 (red) positive nuclei of glial cells. Note the presence of many Sox10-positive glial cells surrounding HuC/D labeled myenteric perikarya reflecting the structural and functional interplay of these two cell types in the ENS. Calibration bar= 25 µm. From De Giorgio et al. 2012.

Enteric glial cells have long been thought to exert a mere mechanical property by supporting neurons. Many evidence, however, indicates that these cells exhibit a number of functions,

ranging from support to neurotransmission to enteric neuronal maintenance and survival (Gulbransen and Sharkey 2012; Neunlist et al. 2013; Neunlist et al. 2014; Boesmans et al. 2015). In fact, glial cells are involved in many crucial tasks, such as synthesis of neurotransmitter precursors, uptake and degradation of neuroligands (i.e., detoxification of glutamate and g-aminobutyric acid), and expression of neurotransmitter receptors, thereby contributing to neuron-glia cross talk and neurotransmission (Neunlist et al. 2013). Furthermore, glial cells exhibit immunological properties (Ruhl 2005; Neunlist et al. 2008; Da Silveira et al. 2011), participate in epithelial barrier functions (Steinkamp et al. 2003; Neunlist et al. 2007; Savidge et al. 2007; Savidge et al. 2007; Bouchard et al. 2008; Van Landeghem et al. 2011) and evoke neuroprotection (Neunlist et al. 2014) (Figure 6). In addition, enteric glial cells have neurogenic potential being capable of generating enteric neurons in response to injury (Joseph et al. 2011; Laranjeira et al. 2011). Recent works also demonstrated that enteric glial cells can respond to neurotransmitters by changes in intracellular Ca2+, such as purinergic (ATP) (Gomes et al. 2009; Gulbransen and Sharkey 2009; Gulbransen et al. 2010) serotonergic and cholinergic signaling mechanisms (Boesmans et al. 2013).

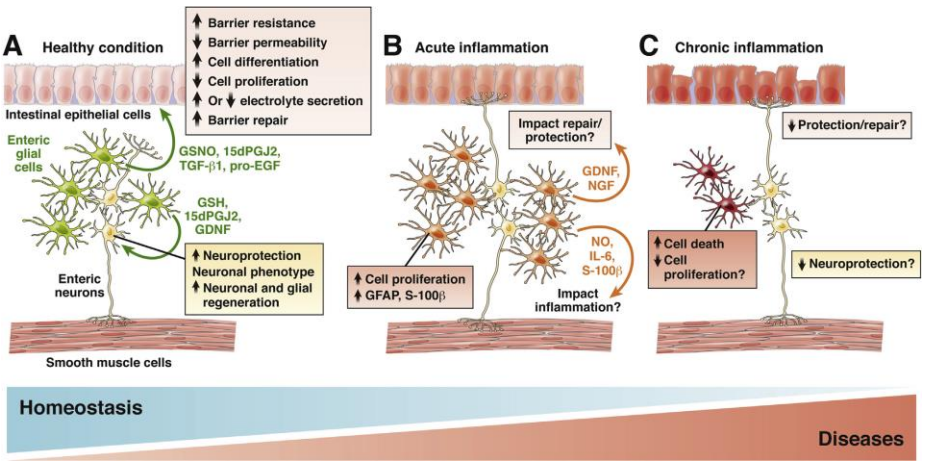

Figure 6. Enteric glial cells are central regulators of gut homeostasis and can play a role in gut diseases. (A) In normal conditions, glia control a number of neuronal aspects, i.e. neuroprotection, neuromediator expression, or neuronal renewal via liberation of different mediators. In addition, glia exhibit a key role in intestinal epithelial barrier integrity through the release of function-specific messengers (gliomediators). Taken together these features indicate that enteric glia possess protective and reparative properties in the gastrointestinal tract. (B) In pathological conditions, such as inflammation or bacterial stimulation, a phenomenon, known as reactive enteric gliosis (similar to astrogliosis in the brain) can occur. This contributes to the development of intestinal inflammation, but

also participate in protection/repair of intestinal epithelial barrier/neuronal lesions evoked by these mechanisms. (C) Enteric glia death (induced by specific viruses or pathogens) or abnormal enteric gliosis could contribute to neuronal degeneration or barrier dysfunctions observed in some chronic intestinal or extraintestinal diseases. From Neunlist et al. 2014.

Chapter II

6. ENTERIC NEUROPATHIES

6.1 General characteristics

Several GI disorders can result from enteric neuropathies, including both primary and secondary forms. These neuropathies have been grouped as congenital or developmental neuropathies; sporadic and acquired neuropathies; neuropathies associated with other disease states and iatrogenic or drug-induced neuropathies (Furness, 2012). Any damage, noxae or dysfunction involving the main effectors or the control system of the gut physiology may be responsible for the onset of a wide array of pathological conditions involving some lethal or seriously disabling manifestations (Schemann and Grundy 1999; Knowles et al. 2013). Pathologies that involve the ENS include dysmotilities such as achalasia, CIPO and Hirschsprung's disease (De Giorgio and Camilleri 2004). A view of the major milestones in ENS neuropathology over the years has been reported in Figure 7.

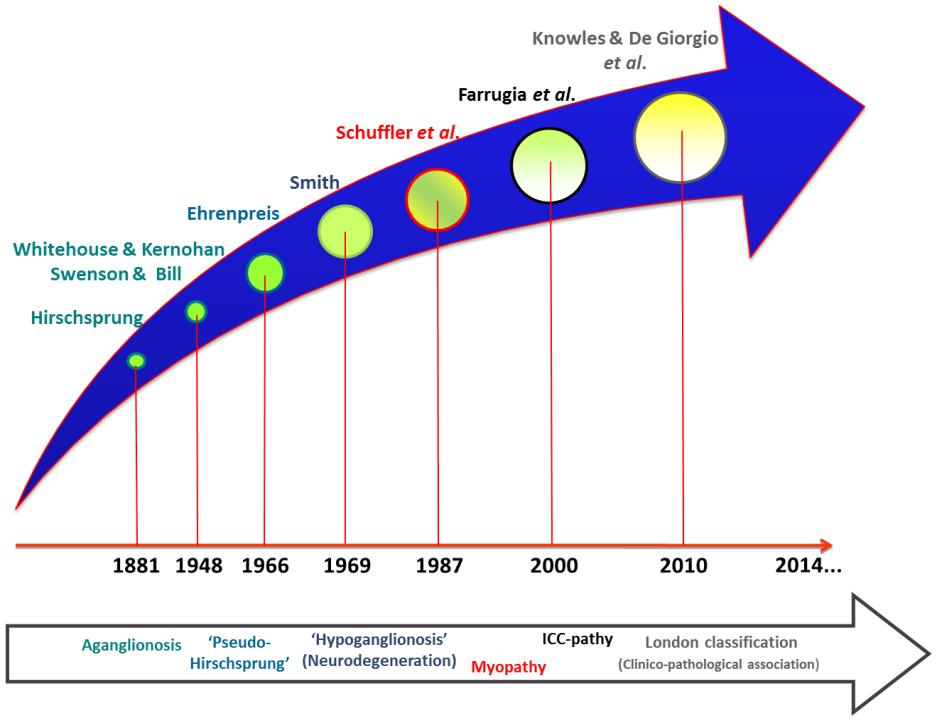

Figure 7. Synopsis summarizing some of the major milestones in ENS neuropathology over the years, i.e. from Hirschsprung's seminal description of congenital aganglionosis of the colon up to the London classification. Colors shown here couple the author(s) (above the curvilinear arrow) with the corresponding neuropathological acquisition (reported in the horizontal arrow). From De Giorgio et al, 2014)

6.2 Primary neuropathies

Enteric neuropathies are classified as primary in the case that the ENS is the major target of the disease process. Most are termed as 'idiopathic' to denote the poor aetiological understanding of these conditions. Among primary neuropathies are: Hirschsprung's disease, idiopathic achalasia, idiopathic gastroparesis and CIPO.

Hirschsprung's disease is characterized by an absence of enteric neurons (aganglionosis) in terminal regions of the gut, leading to tonic contraction of the affected segment, intestinal obstruction and massive distension of the proximal bowel (megacolon).Constipation is the dominant presenting symptom in Hirschsprung's disease. The cardinal feature of Hirschsprung's disease is an absence of enteric nerve cell bodies in the distal part of the colon and in the rectum. The extent of the defect varies from patient to patient. The absence of enteric nerves results in the inability of the distal gut to pass meconium and later, feces. Various genetic mutations, including in the receptor tyrosine kinase RET gene (Luo et al 1993) and in the endothelin receptor-B gene (ENDR-B) are associated with the familial Hirschsprung's disease (Robertson et al 1997). Loss of interstitial cells of Cajal has also been reported in this disease but other studies report a normal complement of interstitial cells of Cajal (Rolle et al 2002). Recent study by Chakravarti et al. observed that for most multigenic disorders, clinical manifestation (penetrance) and presentation (expressivity) are likely to be an outcome of genetic interaction between multiple susceptibility genes. As compared with wild-type mice intestinal gene expression, loss of Ret in null homozygotes led to differential expression of ~300 genes (Chakravarti et al. 2017). Corrent studies identified 28 de novo mutations in 21 different genes. Eight of the *de novo* mutations were identified occur in RET, the main HSCR gene, but the remaining 20 mutations reside in genes not reported in the ENS. These genes are also expressed in human and mouse gut and/or ENS progenitors. Importantly,

the encoded proteins are linked to neuronal processes shared by the central nervous system and the ENS (Gui et al. 2017).

Idiopathic achalasia is an esophageal motility disorder characterized by a failure of the esophageal smooth muscle to generate effective peristalsis and by a failure of the lower esophageal sphincter to relax completely during swallowing (Goldblum et al. 1994). Several neuropathologic findings have been described including injury of inhibitory nitric oxide producing nerves, degenerative changes of the vagus nerve and an occasional presence of cytoplasm inclusions in the dorsal motor nucleus of the vagus nerve and myenteric plexus. Since the initiating factor(s) and mechanisms leading to these characteristic features are currently unknown (Kraichely et al 2006), further studies are currently ongoing on the identification of gene abnormalities highlights novel signaling pathways involved in the neuro-interstitial cell of Cajal changes underlying achalasia (Bonora et al 2017 under review).

The pathophysiology of idiopathic gastroparesis is varied and includes smooth muscle fibrosis, loss of enteric nerves, extrinsic neuropathy and loss of ICC (Zarate et al 2003). Recent data suggest a significant role for loss of nNOS expression in enteric nerves and loss of organized ICC networks as dominant cellular defects in diabetic gastroparesis. It is unknown if these findings are applicable to other causes of gastroparesis.

CIPO indicates a condition in which affected subjects show failure of the propulsive forces of intestinal peristalsis to overcome the natural resistances to flow. Myogenic and neurogenic altered mechanisms contribute to CIPO (De Giorgio et al. 2011). Concerning CIPO-related neuropathies, there are evidences of neuronal degeneration and loss, with or without ganglionitis due to lymphocytic and eosinophilic infiltrate (De Giorgio and Camilleri 2004).Since this thesis is focused on this pathological condition a deep overview will be presented in next chapter.

6.3 Secondary neuropathies

Secondary neuropathies are defined as pathological conditions in which the ENS is not the primary target of the disease. The classification of this neurophaties can be as degenerative and inflammatory, based on the mechanisms underlying the enteric neuronal pathology. Degenerative neuropathies include diabetes mellitus (DM), while inflammatory neuropathies

include paraneoplastic enteric neuropathy and Chagas disease (Knowles et al. 2013). Symptoms depend on the type of nerves affected, more frequentely there are described abdominal pain, heartburn, dysphagia, post prandial fullness, nausea, diarrhoea and constipation. In diabetic gastroparesis several key cell types are affected by diabetes. Etiology or risk factors have not been clearly identified. Failure to sustain elevated heme oxygenase-1 (HO1) expression is associated with delayed gastric emptying in diabetic mice and polymorphisms in the HO1 gene (HMOX1) are associated with worse outcomes in other diseases (Gibbons et al 2017). Recent study showed that longer poly-GT repeats in the HMOX1 gene are more common in African Americans with gastroparesis. Nausea symptoms are worse in subjects with longer alleles (Gibbons et al 2017).

Chapter III

7. CHRONIC INTESTINAL PSEUDO-OBSTRUCTION (CIPO)

7.1 General features

Chronic intestinal pseudo-obstruction (CIPO), a rare and potentially life-threatening disorder with unknown prevalence and incidence, (Amiot et al. 2009; Di Nardo et al. 2017) is viewed typically as an insufficiency of the intestinal peristalsis that mimicks a sub-occlusive disease in the absence of mechanical obstructions (De Giorgio et al. 2004; Stanghellini al. 2007). The severity of the clinical presentation and the limited understanding of the disorder contribute to poor quality of life and increased mortality (Mann et al. 1997). In addition, there are no specific biochemical or molecular biomarkers of CIPO, hindering further a correct diagnosis.

CIPO can result from derangement affecting the integrity of a variety of regulatory cells/tissues, i.e. smooth muscle cells (effectors of contractility / relaxation), the interstitial cells of Cajal (ICC) (pace-makers of gut motility and regulators of neuronal input to smooth muscle cells), and neurons (either intrinsic – the enteric nervous system or extrinsic nerve pathways) (Di Nardo et al. 2017). In some cases, CIPO is associated with inflammation, predominantly of the enteric ganglia, which exhibit inflammatory neuropathy (De Giorgio et al. 2011). Based on the type of cellular pathology, CIPO has been classified into two major categories: visceral neuropathies and visceral myopathies. Neuromuscular and ICC abnormalities observed in CIPO may be “secondary” to a number of recognized diseases (e.g, metabolic, paraneoplastic, or neurologic disorders), or “idiopathic” in nature (De Giorgio et al. 2007). Enteric neurodegenerative abnormalities and immune-mediated changes may occur in gut specimens of patients with neuropathic CIPO. Inflammatory neuropathies are characterized by a dense inflammatory infiltrate characterized by CD3 positive (composed of both CD4 and CD8) lymphocytes almost invariably confined to the myenteric plexus (hence the term of lymphocytic myenteric ganglionitis). The close apposition of CD3 lymphocytes to myenteric neurons provides the basis to neuro-immune interactions targeting and affecting ganglion cell structure and survival (Antonucci et al. 2008). Indeed, experimental evidence indicates that inflammation/immune activation in the gastrointestinal tract can profoundly affect both morphology and function of the ENS.

7.2 Genetic aspects

From a genetic perspective, X-linked, autosomal dominant and recessive forms have been identified with mutations in filamin A (*FLNA*) (Clayton-Smith et al. 2009; Gargiulo et al. 2007), actin G2 (*ACTG2*) (Lehtonen et al. 2012), thymidine phosphorylase (*TYMP*) (Nishino et al. 1999) / polymerase gamma (*POLG1*) (Giordano et al. 2009) and more recently in *SGOL1* (Chetaille et al. 2014). However, the underlying genetic alterations and molecular mechanisms remain unknown in most CIPO cases. Deglincerti et al mapped a locus in a large consanguineous family segregating an autosomal recessive form of CIPO (Deglincerti et al. 2007; Mungan et al. 2003). In the affected family members, the major clinical feature was represented by CIPO, in addition to megaduodenum, long-segment Barrett esophagus, and cardiac abnormalities of variable severity (OMIM 611376; Mungan syndrome, MGS).

Specifically, the main mutations known involve several genes:

- FLNA (Xq28 chromosome)

The FLNA gene is involved in a form of X-linked POIC; encoding for filamin A, a protein that binds actin-binding protein. Actin filaments are connected by filamina A to allow the orthogonal branching within the cell. The protein is involved in cytoskeletal remodeling allowing variations in cell shape and migratory processes. Studies of an Italian family have identified a hemizigosis mutation in in the FLNA gene in affected males. The deletion of two bases in exon 2 identified a premature stop codon in position 103. In the presence of this mutation there is an excess in the formation of "constriction rings", structures important for the contractility of the cells. The shorter FLNA form may be responsible for cytoskeletal abnormalities by highlighting its role in the structure and function of enteric neurons (Gargiulo et al, 2007).

• ACTG2A (chromosome 2p13.1)

The actins are highly conserved proteins involved in various types of cell motility and cytoskeletal maintenance. Three types of actins have been identified: actin α, β and γ. Actin γ-2 is the protein encoded by the ACTG2A gene and represents an isoform expressed in the smooth muscle at the enteric level producing the SMA protein. The R148S mutation of

ACTG2 was observed in Familial Visceral Myopathy (FVM), a form of myopathic pseudo-obstruction with autosomal dominant transmission (Lehtonen et al, 2012; Matera et al, 2016) and appears to be associated with *de novo* sporadic POIC cases. $ACTG2^{R148S}$ variant is abnormally incorporated into actin filaments, with less actin-myosin II interaction and less stress fiber formation. This variant also appears to reduce the ability to interact with the actinic chaperonin CCT (Cytosolic Chaperonin Containing TCP-1), imposing correct actinic folding. In conclusion, R148S mutation interferes with the assembly and acto-myosin function of the intestinal musculature of FVM patients and the alteration in intestinal propulsion observed in FVM appears to be closely correlated with the decrease in the actin function at the enteric level. Through immunohistochemistry, accumulation of ACTG2 in the intestinal circular muscles, not found in the *muscularis mucosae*, can be observed in patients with FVM.

• TYMP (chromosome 22q13.32)

The gene encodes Thymidine Phosphorylase (TP), a protein with enzymatic activity capable of converting thymidine nucleosides and deoxyuridine into thymine and uracil. Mutations in TYMP lead to a reduction of TP activity with consequent toxic accumulation of nucleosides in the plasma of patients with Mitochondrial Neurogastrointestinal Encephalomyopathy (MNGIE). At mitochondrial level there are alterations such as multiple deletions and mitochondrial DNA depletion. MNGIE is characterized by peripheral neuropathy, chronic progressive external ophthalmoplegia, leukoencephalopathy and multiple mtDNA deletions in skeletal muscle. Thymidine phosphorylase also appears to play a role in the inhibition of glial cell proliferation (Hirano et al, 1999). Recent studies, conducted by our research group, have shown that in the liver there is a high expression of TP, suggesting that the transplant of this organ can be a valid therapeutic strategy (De Giorgio et al, 2016; Boschetti et al. , 2014).

• POLG1 (15q26.1)

The gene encodes the catalytic subunit of the mitochondrial genome polymerase.

The mitochondrial DNA polymerase is heterodimeric consisting of a homodimer of regulatory subunits plus a catalytic subunit. Mutations in this gene lead to multiple deletions in mtDNA

with clinical features such as skeletal myopathy and visceral dysmotility which are described in hypoperistalsis and CIPO. mtDNA depletion seems to be the likely cause of a visceral myopathy causing hypoperistalsis and intestinal pseudo-obstruction. Based on these findings, the external layer of *muscularis propria* is confirmed as the most susceptible point of the gastrointestinal tract to develop mtDNA depletion, possibly because of the constitutive low abundance of mtDNA within smooth muscle cells at this site. (Giordano et al, 2009).

• SGOL1 (chromosome 3p24.3)

The protein encoded by this gene is a component of the cohesin complex involved in the regulation of the cohesion of the sister chromatids which acts by protecting the centromeric from the cleavage during the mitotic phase. Mutations in homozygosity of the SGOL1 gene cause the Chronic Atrial Intestinal Dysrhytmia (CAID), which is chronic intestinal atrial dysrhythmia. Dysrhythmias are part of disorders involving pacemaker activity, both at cardiac and intestinal levels. In 17 patients with CAID was observed the coexistence of a pathological phenotype both at intestinal and cardiac levels. Specifically, the heart level disorder manifested as Sick Sinus Syndrome syndrome; instead the intestinal level it manifested as CIPO for the involvement of Cajal and SNE cells (Chetaille et al, 2014).

Whole Exome Sequencing (WES) analysis identified new genes mutated in CIPO. In order to identify the genetic defects in CIPO patients negative for RAD21 mutations, we selected a subset of CIPO cases associated with an enteric hypoganglionosis. We identified novel *de novo* missense variants in the genes B3GAT2 and SMC3 in two cases, and a missense variant in SCN11A in a patient with CIPO and small fibers neuropathy with severe abdominal pain. B3GAT2 encodes for the beta-1, 3-glucuronyltransferase 2 enzyme, involved in the synthesis of a complex sugar moiety present in neuronal cell adhesion molecules implicated in migration and adhesion (Gouveia et al, 2012). The protein encoded by SMC3 is a RAD21-binding protein and part of the cohesin complex. Mutations in SMC3 have been also described in the Cornelia de Lange syndrome (Deardoff et al, 2007), but the missense change in the CIPO patient maps to a different region. SCN11A encodes for the Nav1.9 sodium channel subunit. Since null scn11a mice show perturbed gut motility, SCN11A mutations may cause an intestinal gut dysmotility also in humans (Copel et al, 2013). We designed a targeted resequencing panel and identified several variants including a premature stop codon in

B3GAT2, and various rare/novel missense changes in SCN5A, SCN9A, SCN10A, SCN11A and TRPA1 (10.7% of patients) in additional CIPO patients (89 Italian and 29 Swedish patients, the latter recruited in collaboration with G. Lindberg and M. D'Amato). (Table 2).

PATIENT ID	DIAGNOSIS	VARIANTS	POLYPHEN-2	MAF ExAc
ITAtarget_1	CIPO(M)	**ACTG2, p.Arg257Cys**		Novel
SWEtarget_2	CIPO (F)	**B3GAT2, p.Lys95X** (rs7535544542)		MAF:0.00000825 Allele frequency: 1/121180
ITAtarget_4	CIPO (F)	**SCN5A, p.Val2016Met**	Probably damaging	Novel
ITAtarget_5	CIPO, SFN (F)	**SCN9A, p.Gly616Arg** (rs201338643)	Benign (SIFT: Tolerated)	MAF: 6.627e-05 Allele frequency: 8/120722
		SCN9A, p.Pro610Thr (rs41268673)	Benign (SIFT: Tolerated)	MAF:0.005261 Allele frequency: 496/94286
ITAtarget_8	CIPO (F)	**SCN9A, p.Asn1245Ser** (rs141268327)	Probably damaging	MAF:0.005262 Allele frequency: 496/94286
ITAtarget_11	CIPO (M)	**SCN10A, p.Arg351Cys**	Probably damaging	Novel
SWEtarget_1	CIPO (F)	**SCN10A, p.Asp1769Ala** (rs377121469)	Probably damaging	MAF: 7,415e-05 Allele frequency: 9/121382
ITAtarget_7		**SCN10A, c.1867+8C>T** (rs370354229)	/ (Splice region)	MAF:0.0001422 Allele frequency: 17/119532
ITAtarget_3	CIPO (M)	**SCN10A, p.Pro991Leu** (rs138413438)	Probably damaging	MAF: 0.000892 Allele frequency: 108/121082
SWEtarget_3	$Na_v1.5$ (LoF)	**SCN11A, p.Lys419Asn** (rs150269814)	Probably damaging	MAF: 0.000725 Allele frequency:88/121382
ITAtarget_12	CIPO (M)	**SCN11A, p.Arg889Lys**	/	Novel
ITAtarget_9	CIPO (F)	**SCN11A, p.Gly481Glu** (rs13059805)	Benign (SIFT: Tolerated)	MAF:0.03075 Allele frequency: 3704/120442
		SCN11A, p.Cys625Tyr	Possibly damaging	MAF:1.67e-05 Allele frequency: 2/119790
ITAtarget_10	CIPO (M)	**TRPA1,**	Probably damaging	Novel

		p.Arg458Cys (rs374228201))		
ITAtarget_2	CIPO/SFN (F)	**TRPA1, p.Asp477Asn** (rs61753711)	/	Novel
ITAtarget_6	CIPO (M)	**TRPA1, p.Try840Ser**	/	Novel

Table 2

Table 2: Analysis of variants identified in n= 118 CIPO patients (89 Italian cases (69 F, 20 M, age range=7-70 yrs, mean=35.2); 29 Swedish cases (24 F, 5 M, age range=20-68 yrs, mean=46.0.)

7.3 Cohesin complex molecules: The RAD21

RAD21 **(**8q24.11 cromosome) is a transcription factor and a key central component of the multi-protein cohesin complex (Xu et al. 2011) (Figure 8), which functions to protect chromosome separation during the metaphase–anaphase transition of mitosis (Guacci et al. 2007). RAD21 is ubiquitously expressed in many tissues (Faure et al. 2012) and its critical role emerged from mouse knock-out, which showed a lethal early embryonic phenotype (Biswas et al. 2016). Studying a familial cluster of CIPO patients, we have recently demonstrated a novel loss-of-function mutation in RAD21, which was associated with enteric neuropathy and severe gut dysmotility in the affected family members. The CIPO-causing RAD21 mutation was found to alter the expression of related genes, including RUNX1 and APOB. Moreover, the RAD21 loss-of-function mutation was associated with a markedly reduced number of enteric neurons, thus implying a neurogenic origin of the gut dysmotility reported in the affected family (Deglincerti et al 2007, Bonora et al. 2015).

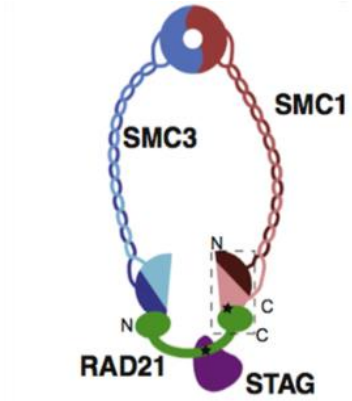

Figure 8. Cohesin complex Stucture: the structure include SMC1 and SMC3 subunits that linked proteins with ATP domain at one end. Togheter with RAD21 and STAG formed a large ring structure that can surround the sister chromatids. ATPase domains are needed to load cohesin to DNA. RAD21 is also able to link different sites within the genome, regulating the transcription of some genes; due to this characteristic it is involved in various processes linked to tumorigenesis (modified from Deardorff et al, 2012).

The distribution of RAD21 in the enteric nervous system, the intrinsic neural network controlling gastrointestinal (GI) physiology and homeostasis (De Giorgio et al. 2007, Rao et al. 2017), remains unknown. The mutation of RAD21 p.622 Ala> Thr is associated with a clinical phenotype that includes insufficient intestinal peristalsis and severe subocclusive episodes. Delay in intestinal motility and decrease in enteric neurons has also been demonstrated *in vivo* using a Danio rerio (zebrafish) model. In this model was observed the consequences produced by the silencing of RAD21 orthologues, Rad21, using splice blocking morpholino (MO), which allows block the splice sites of the pre-mRNA of interest.

Chapter IV

8. THE SEROTONERGIC SYSTEM IN THE GUT

8.1 5-hydroxytryptamine (5-HT) receptors

Serotonin receptors are classified into seven types, 5-HT1 through 5-HT7. Each type can have subtypes (A, B, etc.). These receptors are localized in the brain and in peripheral organs but their distribution is not homogeneous. The majority of 5-HT receptors are postsynaptic, with some exceptions, most notably 5-HT1A and 5-HT1B that are mainly presynaptic and modulate serotonin release. The signaling pathways to which these receptors are coupled are known, but it is has not been possible to link direct clinical effects systematically to their stimulation. Serotonin receptors are coupled to G proteins except 5-HT3 receptors which are receptor-channels, also called ionotropic receptors, which, in the activated state, are open and permeable to sodium and potassium cations. SERT 5-HT transporter, represents established targets for many pharmacological agents that affect brain function, including psychostimulants, antidepressants and neurotoxins (Figure 9).

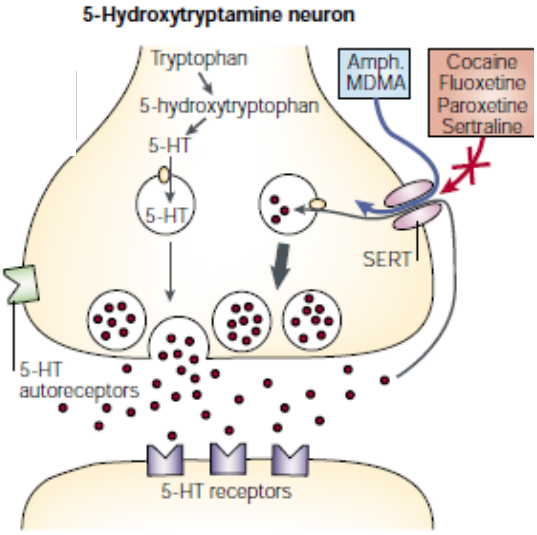

Figure 9. Schematic representation 5-HT synaptic terminals. Monoamine transporters are localized to perisynaptic sites, where they are crucial for the termination of monoamine transmission and the maintenance of presynaptic monoamine storage. Several selective pharmacological agents acting at each monoamine transporter are shown. Amph., amphetamine; 5-HT, 5-hydroxytryptamine; MDMA, 3,4-methylenedioxymethamphetamine; SERT, 5-HT transporter (modified from Torres et al. 2003).

The sensitivity of monoamine transporters, for substrates and inhibitors, has been examined in brain preparations and in heterologous systems with recombinant transporters. Actually, in the scenario of GI disorders, some the most interesting targets for pharmacological intervention are the 5-HT receptors that are known to affect gut motor function, in particular the ones belonging to the 5-HT1, 5-HT2, 5-HT3, 5-HT4, and 5-HT7 subtypes (De Ponti 2004).

The current knowledge on the serotonin system in the GI leads to conclude that the modulation of gut-derived serotonin and the serotonin pathways represents a significant and promising way of treating patients with GI dysmotility conditions.

The focus in gastroenterology has been on 5-HT3 and 5-HT4 receptors (Table 3), although in I present two studies on 5HT4. 5-HT4 receptors are also highly expressed in the colonic epithelium, where they appear to be expressed by all epithelial cells (Hoffman et al. 2012). Epithelial 5-HT4 receptors mediate a variety of responses, including 5-HT release, chloride secretion and goblet cell degranulation, as well as enhanced propulsive motility and reduced visceral hypersensitivity by intraluminal administration of a 5-HT4 agonist (Hoffman et al. 2012). 5-HT4 receptor agonists have prokinetic and anti-nociceptive actions and have been used to treat constipation-predominant irritable bowel syndrome (Mawe et al. 2013), but the functions of these receptors are still being elucidated, and they likely include protective actions. Furthermore, 5-HT4 receptors also appear to play a role in the development and (Li et al. 2011) survival of enteric neurons, since neuronal density is reduced in the intestines of 5-HT4 knockout mice.

	$5HT_{1A}$	**$5HT_{1B-D}$**	**$5HT_{2A}$**	**$5HT_{2B}$**	**$5HT_{3}$**	**$5HT_{4}$**	**$5HT_{7}$**	**$5HT_{1P}$[a]**
Receptor distribution	Enteric neurons	Enteric neurons; smooth muscle(?)	Enteric neurons; smooth muscle; enterocytes	Enteric neurons; smooth muscle	Enteric neurons	Enteric neurons; smooth muscle; enterocytes	Enteric neurons; smooth muscle	Enteric neurons; smooth muscle
Funtional effects[b]	<Trasmitter release (presynaptic action)	>Trasmitter release; relaxes gastric fundus	>Trasmitter release; contracts muscle; >secretion	>Trasmitter release (?); contracts muscle	Causes fEPSPs in some neurons >Trasmitter release >secretion; Activates IPANs	>Trasmitter release (presynaptic action);relaxes muscle; >secretion Activates IPANs; Inhibits 5HT release from EC cells	Mediates slow EPSPs; >Trasmitter release; Relaxes muscle	Mediates slow EPSPs; >Trasmitter release; Relaxes muscle; Activates IPANs

Table 3. 5-HT receptor subtypes in the gut:5-HT1P receptors are the only 5-HT receptors that have not yet been cloned. They are not included in the IUPHAR nomenclature of 5-HT receptors. B fEPSP, fast excitatory postsynaptic potential; upward arrow, increase; downward arrow, decrease.

8.2 Serotonin synthesis and secretion

5-HT belongs to the monoamine neurotransmitter family. Transepithelial signaling, involving pressure- or nutrient-induced secretion of paracrine messengers, such as serotonin 5-HT, initiates secreto-motor reflexes (Bertrand, 2004; Gershon and Tack, 2007). Most of 5-HT (~95%) is located in enterochromaffin (EC) cells, while a minimal component is contained in interneurons (~2%) of the ENS. Serotonin biosynthesis is dependent on two enzymes: tryptophan hydroxylase 1 (TPH1) present in EC cells; and TPH2 expressed in neurons. Interestingly, TPH2, but not TPH1, knock-out mice, showed a decreased myenteric neuronal density and proportions of dopaminergic and GABAergic neurons, thus implying that 5-HT, further to its functional properties, may exert an important role on neuronal survival in at least a subset of enteric neurons (Wood, 2011). Uptake, mediated primarily by the serotonin transporter (SERT) in enterocytes and neurons, terminates the action of released 5-HT, while an array of receptors widely expressed in the ENS triggers its biologic actions (Tonini, 2005; Galligan and Parkman, 2007; Beattie and Smith, 2008). In the gut, SERT mediate the reuptake into epithelial cells, platelets and other cells. SERT is able to binds 5-HT and internalizes the molecule through the cellular membrane; it is present both in the mucosa and enteric nerves. As consequence, SERT represents a critical inactivating mechanism for the serotonin signal and in maintaining the 5-HT homeostasis (Figure. 10) (Wade et al. 1996).

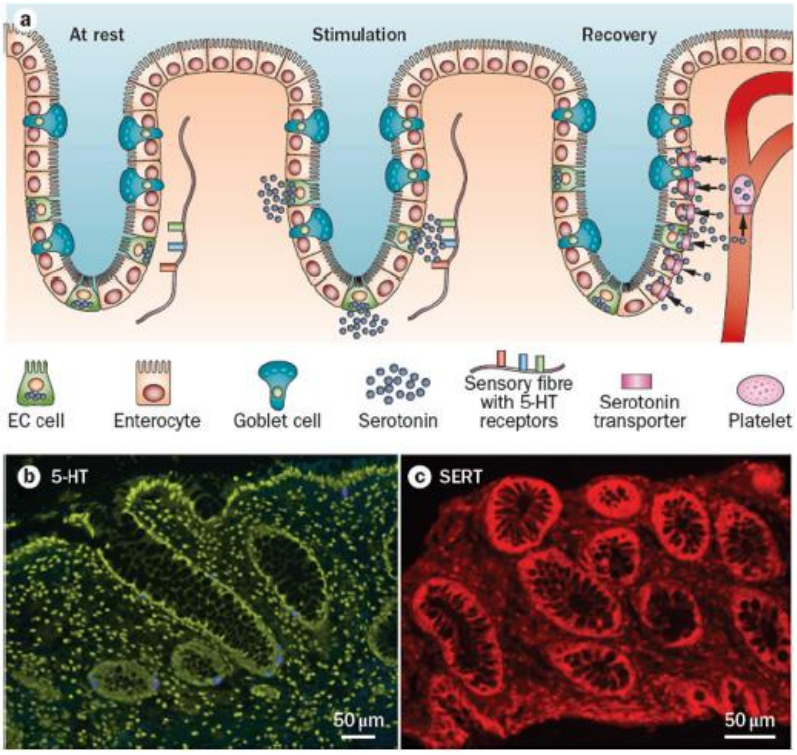

Figure 10. 5-HT signaling in the gut. a) The sequence of events involved in 5-HT signaling in the gut. At rest, 5-HT is synthesized by enterochromaffin cells. Upon mechanical or chemical stimulation, 5-HT is released into the interstitial space of the lamina propria and binds to receptors on nearby nerve fibers. 5-HT signaling is terminated during the recovery phase: 5-HT is transported by serotonin transporter into epithelial cells where it is enzymatically degraded, or it enters the blood stream where it is transported into platelets and stored for future use. **b;c)** Immunostaining for 5HT and SERT in a human rectal biopsy specimen demonstrating that essentially all cells in the colonic glands are SERT-immunoreactive. (Modified from Mawe and Hoffman 2013).

8.3 Serotonin influences gut physiology

For decades, it has been widely believed that serotonin has a major role in regulating GI function (Gershon and Tack 2007). This belief is based on a huge number of different findings. These include the well known observation that most of the body's serotonin is synthesized and stored in the intestine, the presence of many different serotonin receptors within the intestinal wall and very large amount of observations of altered intestinal behavior following treatment with exogenous agonists and antagonists. However, despite a mountain of evidence, the actual roles of serotonin in the GI tract have been maddeningly difficult to identify. Reasons for this failure include that there are both neural and mucosal sources of serotonin within the gut and the widespread and overlapping distribution of specific serotonin receptor subtypes. Various 5-HT4 agonists have been developed to treat gastrointestinal disorders characterized by altered motility (Waeber C et al. 1994), including the 5-HT4 full agonist, prucalopride, which significantly improves symptoms and quality of life of patients with severe chronic constipation with excellent safety and tolerance profile (Tack J et al. 2012). Interestingly, 5-HT4 receptors have also been shown to modulate neuronal survival and neurogenesis (Gershon MD et al. 2007). Indeed, in the central nervous system, 5-HT4 has been shown to be involved in synaptic plasticity crucial to cognitive processes and memory consolidation (EWaeber C et al. 1994). Recent findings also provide evidence for a role of 5-HT4 in mediating neurogenesis and synaptic plasticity in the ENS (Takaki M et al. 2014). Furthermore, 5- HT4-/- mice showed loss of enteric neurons and reduced size of the surviving neurons at 1 month in addition to delayed gastrointestinal transit (Liu MT et al. 2009). Finally, in vitro treatment with 5-HT4 agonists increased the number of enteric neurons developing from precursor cells and/or surviving in culture, supporting neurogenetic properties mediated by this receptor subtype (Liu MT et al. 2009). In this study, we tested the

hypothesis that the 5-HT4 full agonist, prucalopride, exerted a protective effect against neuronal injury using an established model of oxidative stress and different types of human neuronal cell lines, primary human enteric neurospheres (Bondurand N et al. 2003) and human submucosal neuron whole mount preparations obtained from colonic biopsies (Lebouvier T et al. 2010). We demonstrated that 5-HT4 activation stimulated a specific protective response in the different neuronal cells analyzed, including human enteric neurons. Our findings open new avenues in the treatment of gastrointestinal disorders and homeostasis, especially in relation to neurodegeneration often underlying severe gut dysmotility.

EXPERIMENTAL STUDIES

Chapter VI

10. Mutations in *RAD21* Disrupt Regulation of APOB in Patients with Chronic Intestinal Pseudo-obstruction

Modified from

"Mutations in *RAD21* Disrupt Regulation of APOB in Patients with Chronic Intestinal Pseudo-obstruction"

Elena Bonora, Francesca Bianco, Lina Cordeddu, Michael Bamshad, Ludmila Francescatto, Dustin Dowless, Vincenzo Stanghellini, Rosanna. Cogliandro, Greger Lindberg, Zeynel Mungan, Kivanc Cefle, Tayfun Ozcelik, Sukru Palanduz, Sukru Ozturk, Asuman Gedikbasi, Alessandra Gori, Tommaso Pippucci, Claudio Graziano, Umberto Volta, Giacomo Caio, Giovanni Barbara, Mauro D'Amato, Marco Seri, Nicholas Katsanis, Giovanni Romeo and Roberto De Giorgio.

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Abstract

Background & Aims—Chronic intestinal pseudo-obstruction (CIPO) is characterized by severe intestinal dysmotility that mimicks a mechanical sub-occlusion with no evidence of gut obstruction. We searched for genetic variants associated with CIPO to increase our understanding of its pathogenesis and indentify potential biomarkers.

Methods—We performed whole-exome sequencing of genomic DNA from patients with familial CIPO syndrome. Blood and lymphoblastoid cells were collected from patients and controls (individuals without CIPO); levels of mRNA and proteins were analyzed by quantitative reverse transcription PCR, immunoblot, and mobility shift assays. cDNAs were

transfected into HEK293 cells. Expression of *rad21* was suppressed in zebrafish embryos using a splice-blocking morpholino (*rad21a* MO). Gut tissues were collected and analyzed.

Results—We identified a homozygous mutation (p.622, encodes Ala>Thr) in *RAD21* in patients from a consanguineous family with CIPO. Expression of *RUNX1*, a target of RAD21, was reduced in cells from patients with CIPO compared with controls. In zebrafish, suppression of *rad21a* reduced expression of *runx1*; this phenotype was corrected by injection of human *RAD21* mRNA, but not with the mRNA from the mutated p.622 allele. *rad21a MO* zebrafish had delayed intestinal transit and greatly reduced numbers of enteric neurons, similar to patients with CIPO. This defect was greater in zebrafish with suppressed expression of *ret* and *rad21*, indicating their interaction in regulation of gut neurogenesis. The promoter region of *APOB* bound RAD21 but not RAD21 p.622 Ala>Thr; expression of wild-type RAD21 in HEK293 cells repressed expression of *APOB*, compared with control vector. The gut-specific isoform of APOB (APOB48) is overexpressed in sera from patients with CIPO who carry the RAD21 mutation. APOB48 is also overexpressed in sporadic CIPO in sera and gut biopsies.

Conclusions—Some patients with CIPO carry mutations in *RAD21* that disrupt the ability of its product to regulate genes such as *RUNX1* and *APOB*. Reduced expression of *rad21* in zebrafish, and dysregulation of these target genes, disrupts intestinal transit and development of enteric neurons.

Keywords

sporadic and familial chronic intestinal pseudo-obstruction; intestinal motility; animal model; genetic analysis

Methods

Patients and controls

An additional 21 Italian and 12 Swedish sporadic patients with idiopathic CIPO were included in the study (eight males and 25 females; mean age: 38.6+/−16.6 years). In Table 1 the major clinical characteristics of these patients are described. 500 Turkish controls were recruited at the Universities of Ankara and Istanbul; 240 controls of European ancestry were recruited at the University of Bologna. All data from patients and controls, including the informed consents, were handled in accordance with local ethical committee's approved protocols and in compliance with the Helsinki declaration.

High-Throughput SNP genotyping and Whole Exome Sequencing Analysis

400 ng of genomic DNA from peripheral blood was used for high-throughput SNP genotyping on Illumina Infinium HD Assay Gemini platform (Illumina, San Diego, CA, USA), according to manufacturer's protocol. Genotypes were converted into PLINK format with custom scripts. PLINK v1.07 (http://ngu.mgh.harvard.edu/~purcell/plink/) was used to isolate individual Runs Of Homozygosity (ROH) that showed > 1 Mb overlap Mb overlap between the three affected siblings.

RAD21 mutation screening in idiopathic CIPO cases

PCR primers for human RAD21 (NM_006265.2) were designed with Primer3 v4.0 (http://primer3.ut.ee) and are available on request. Genomic DNA extracted from peripheral blood was amplified according to the following PCR conditions: 30 ng of DNA, 2.5 mM MgCl2, 0.5 mM dNTPs, 0.5 μM primers, 5% DMSO in a final volume of 20 ml using the 2X KAPA Fast Taq Polymerase Master mix (KAPA Biosystems, MA, US). Forty cycles were carried out as follows: 95°C 1', 95°C 15'', 58°C 1', 72°C 1', with a final extension of 30'' at 72°C. PCR products were purified onto Millipore PCR clean-up plates and directly sequenced on both strands using the BigDye v1.1 kit (Life Technologies, Carlsbad, CA, USA). Electropherograms were visualized with Chromas version 2.0 and Sequencer version 4.7.

RAD21 cDNA transfection into HEK293 cells

Human RAD21 cDNA inserted in pCMV6 vector in frame with DDK epitope was purchased from OriGene (OriGene, Rockville, MD, USA). The mutation corresponding to p.622

Ala>Thr was inserted by site-directed mutagenesis using the QuickChange XL mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) according to manufacturer's instructions. The insertion of the changes was verified by direct sequencing. 3x105 HEK293 human renal cells (ATCC, UK) were plated for transfection of the different plasmids using liposomes according to manufacturer'

instructions (Lipofectamine, Life Technologies). Protein and RNA extraction was performed after 48 hrs from transfection.

Gene expression analysis

Total RNA from 1.5 ml fresh blood was extracted with the QIAGEN Blood Total RNA kit (QIAGEN, Venlo, Limburg, Netherlands). Total RNA from lymphoblastoid or transfected cells was extracted with RNeasy kit (QIAGEN). Total RNA from 1.5 ml fresh blood was extracted with the QIAGEN Blood Total RNA kit (QIAGEN, Venlo, Limburg, Netherlands). Total RNA from 5x106 LCLs or HEK293 cells, cultured according to standard protocols, was extracted with the RNeasy kit (QIAGEN). 1µg of DNase I-treated RNA was used for reverse transcription with random hexamers using the Multiscribe RT system (Life Technologies) at 48°C for 40′ in a final volume of 50 µl. qRT-PCR was performed with SYBRGreen, 0.5 µM primers, in an ABI 7500 Real-Time PCR System (Life Technologies). All target genes were normalized with the corresponding endogenous

control (beta-actin) using the ΔΔCt comparative method. PCR primers are available on request.

Zebrafish functional assays

A published *ret* MO was used.18 To measure *rad21a* MO efficiency, total mRNA was extracted from control and MO injected embryos, reverse-transcribed and the site targeted by the MO was PCR amplified (Figure S1).

Microgavage

Control and rad21 MO injected embryos were developed to 5dpf. Zebrafish larvae were anesthetized in Tricaine (Sigma), mounted in 3% methylcellulose and injected with fluorescent beads into the mouth as described Cocchiaro JL et al. 2013).

Electromobility shift assay (EMSA)

$2x10^6$ LCLs were rinsed in cold PBS-Na_3VO_4 0.2 mM, pelleted by centrifugation at 1600xg and lysed in 400 µl of Buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1mM EDTA, 1 mM DTT, 1 mM Na_3VO_4 and a protease inhibitors cocktail) for 15 min in ice. 25 µl of 10% NP-40 were added and the samples were centrifuged at 16000xg 30 sec. The nuclear pellets were resuspended in 50 µl Buffer C (20 mM HEPES pH 7.9, 0.4 mM KCl, 0.1mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM Na_3VO_4 and a protease inhibitors cocktail), incubated on ice for 45 min, and centrifuged at 16000xg 10 min at 4°C.10 µg of nuclear extracts, 20 fmol of biotin-labelled probes, 2 µg of sheared salmon sperm DNA, 2.5% glycerol, 5 mM KCl and 5 m$MgCl_2$ were incubated at room temperature for 30 min in 20 µl. Cold probes were added at a concentration 200X. The nuclear complexes were resolved by non-denaturing electrophoresis on 4% polyacrilammide gel, transferred onto nitrocellulose membrane at 380 mA 30 min at 4°C, crosslinked via UV binding 15 min at room temperature. For supershift assay, nuclear extracts were incubated at the same conditions as reported above, but the complexes were immunoprecipitated 40 min at 4°C with 1.5 µg of rabbit anti-RAD21 antibody (Abcam) before the EMSA assays. Complexes were resolved under non-denaturing conditions using a 3.5% polyacrylamide gel. EMSA shift was revealed with the LightShift Chemiluminescent EMSA Kit (Thermo Scientific Inc., IL, US). The used biotin-labelled probes are the following:

hAPO_AC2 5′-
CGGAGTTGTCAAGGCGGGGGCTGCAGGCAGAGGGCGCTAAAGAGCCCA

GGATGGCCGGG-3′ (chr11:116,662,104-116,662,163, hg19);

hAPOB_c1 5′-
CAGAGCACTGAAGACGCTTGGGGAAGGGAACCCACCTGGGACCCAGCCC
CTGGTGGCTGCGGCTGCAT-3′ (chr2:21,267,106-21,267,173, hg19) ;

hAPOB_c2 5′- CATTCCCACCGGGACCTGCGGGGCTGAGTGCCCTTCT-3′

(chr2:21,266,910-21,266,946, hg19).

Immunoprecipitation and western blotting

2×10^6 LCLs were used for immunoprecipitation assays. Crude sera of patients were diluted in PBS. Serial dilutions for cases and controls were performed (1:5, 1:10, 1:100, Figure S2A). 2x106 LCLs were lysed in 50 mM HEPES, 1 mM EDTA, 10% glycerol, 1% Triton X- 100, 150 mM NaCl in presence of protease inhibitors (Roche Diagnostics, Basel, Switzerland) and phosphatase inhibitors (Inhibition Cocktails 2 and 3, Sigma, St. Louis, MO, US). Pre-clearing was performed with rabbit IgG (Millipore, Billerica, MA, US) 1 hr at 4°C. Immunoprecipitation assays were performed at 4°C using 0.8 µg rabbit anti-SMC1 or anti-RAD21 antibody/reaction (Sigma Prestige) on Protein GSepharose (Sigma). Proteins were separated by SDS gel electrophoresis, transferred onto nitrocellulose membrane (GE Healthcare, Little Chalfont, UK) and subjected to western blotting with the Western Breeze kit (Life Technologies). Proteins extracted from sera of patients were diluted 1:5 in PBS, quantified with Lowry method and Coomassie Blue gel staining, separated by SDS gel electrophoresis, and transferred onto nitrocellulose membrane (GE Healthcare). Primary antibodies used were the following: goat anti-APOB48 1:250 (Santa Cruz, Texas; USA) and goat anti-APOB 1:1000 (Dako, Santa Clara, CA, US); rabbit anti-APOB100 (Abcam, Cambridge, UK) 1:200; mouse anti-GAPDH (Abcam) diluted at 1:5000. Bands were visualized by the ECL method (GE Healthcare).

Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded adult human colon tissues (patients routinely operated for uncomplicated colon cancers commonly used as controls for immunohistochemical experiments) according to protocols validated in our laboratory. Briefly, tissue sections were treated to remove paraffin embedding by three sequential washes in xylene and graded ethanol. Antigen unmasking was performed by heating sections at 95°C in 10 mM Sodium citrate buffer, pH 6.0, for 10 min and subsequent cooling at room temperature for 30 min. To reduce endogenous peroxidases, tissue sections were treated with an ad hoc blocking kit (GeneTex Inc., Irvine, CA; USA) and incubation for 16 h at 4°C with

primary antibodies. The following primary antibodies were used: anti-human APOB48 (Santa Cruz) and antihuman APOB diluted 1:100 (Abcam); anti-human RAD21 diluted 1:250 (Sigma Prestige), anti-human APOBEC1 diluted 1:100 (Creative Diagnostics, Bristol, UK). Incubations with the corresponding blocking peptides or with the secondary antibodies only were performed as negative controls (Figures S2a, b). Fluorescent secondary antibodies were diluted 1:300 in Triton-X and incubated 60 min at room temperature, before subsequent dehydration and visualization under a LEICA DMLB fluorescent microscope. DAB staining was performed according to standard protocols.

Quantitative evaluation of ganglion cells

We counted the number of neurons after immunostaining with a rabbit polyclonal anti human neuronal specific enolase (NSE) antibody 1:400 (Millipore, Germany). For each section, 25-28 high power (40x) microscopic fields, along the neuromuscular ridge of the myenteric ganglia, were captured to assess NSE immunoreactive neuronal cell bodies / ganglion.22 The same procedure was performed for the counts of neurons in the submucosal ganglia. Sections were examined using a Leica microscope equipped with a digital camera. Quantitative evaluation of neuron number in myenteric and submucosal ganglia was performed according to Ganns D et al. 2006.

Statistical analysis

Case-control association study for SNP rs72105712 was performed using Haploview 4.0 (http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/). Statistical analysis of quantitative differences was performed using the Student's t-test from the GraphPad package (http://graphpad.com/quickcalcs/). Fluorescent cell count was performed with ImageJ (http://rsbweb.nih.gov/ij); $\chi 2$ tests were calculated using the dedicated option from GraphPad.

Results

Identification of a novel RAD21 mutation in CIPO

We performed a combined single nucleotide polymorphism (SNP)-genotyping/next generation-sequencing approach in a consanguineous CIPO pedigree of Turkish origin (Figure 1A), where we previously mapped a linkage locus with a multipoint LOD score=5.019. 14 High-throughput SNP-genotyping in the family and detection of Runs of Homozygosity (ROH) by PLINK (http://ngu.mgh.harvard.edu/~purcell/plink/) confirmed the locus,14 by identifying two regions of extended homozygosity: 91,878,147-113,307,176; 116,713,296-124,956,205. We then performed WES on genomic DNA from two affected individuals (IV-9 and IV-11, Figure 1A). We filtered data for variants that were a) homozygous in our patients; b) rare (MAF <1%) in public databases (EVS, dbSNP); and c) predicted computationally to be pathogenic. We found a single variant fulfilling all these conditions inside our linkage interval on chromosome 8 (Figure 1; Figure S3A). This homozygous allele affects the coding change c. 1864 G>A in *RAD21* (NM_006265.2), and is predicted to generate a missense substitution p.622 Ala>Thr (Figure 1B). Mutation Taster (http://www.mutationtaster.org/) and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/)21 analysis predicted this change to be "disease causing" and "damaging", respectively (Mutation Taster p= 0.9999; PolyPhen-2 HumDiv score: 0.999, sensitivity: 0.14; specificity: 0.99; HumVar score: 0.993; sensitivity: 0.47; specificity: 0.96), and the variant was absent from public databases (dbSN: www.ncbi.nlm.nih.gov/SNP/, 1000 Genomes: www.1000genomes.org/, ESP: http://evs.gs.washington.edu/EVS/) and from 1000 control chromosomes of Turkish origin analyzed by our group. To test the candidacy of this allele, we performed segregation analysis on all available family members: all three affected sibs carried the change in a homozygous state and all carriers of the risk haplotype (including the parents) were heterozygous (Figure 1A).

To evaluate whether *RAD21* mutations were also prevalent in other idiopathic CIPO cases, we screened 21 Italian and 12 Swedish individuals with pseudo-obstruction, defined by clinical, manometric and radiological examination (Table 1). We did not identify any mutation in *RAD21* coding region, although a 1bp-indel was detected with high frequency in the upstream region (m.a.f= 0.364; g.11788122-11788123 ins(C)); rs72105712 (dbSNP137)). Since no allele frequencies were known for this SNP, we investigated the frequency in a control group of European ancestry (N= 240); we found no differences between cases and controls (m.a.f.= 0.36 in cases; 0.34 in controls; $\chi 2$= 0.08, p-value= 0.77). To test the candidacy of *RAD21*

further, we evaluated its expression in blood and lymphoblastoid cell lines (LCLs) obtained from one homozygous patient and several controls, including one unaffected wild-type brother. Real-time quantitative RT-PCR (qRT-PCR) showed that *RAD21* expression in the affected individual was comparable to that in controls, either in blood (Figure S3B) or in LCL cDNA (Figure S3C). The mutant protein was also expressed in LCLs in amounts comparable to wild-type cells (Figure S3D; upper panel). Likewise, testing the interaction of RAD21 with SMC1, one of its known partners, (Panigrahi AK et al. 2012) co-immunoprecipitation experiments showed that the mutant protein still retains SMC1-binding activity (Figure S3D, middle panel).

Since one of the main target genes activated by RAD21 is *RUNX1* (Horsfield et al. 2007, Marsman et al. 2014); we investigated whether mutated RAD21 could hamper its transcription activity: indeed, the affected individual's LCLs showed significantly reduced *RUNX1* expression compared to controls (Figure 1C). Similarly, transfection of mutant *RAD21* cDNA in HEK293 cells and

subsequent RT-qPCR revealed a significant decrease in *RUNX1* expression ($p=0.0028$, Student's t-test) compared to cells transfected with wild-type *RAD21* cDNA (Figure S3E).

Rad21a suppression causes downregulation of runx1 expression and loss of enteric neurons in vivo

To test the hypothesis that RAD21 is necessary for enteric development and to assay the pathogenic potential of the newly discovered allele in a physiologically relevant *in vivo* system, we investigated RAD21 in zebrafish development.

Given our *in vitro* observations on the role of RAD21 on *RUNX1* expression and its downregulation in patient's cells, we asked whether *RAD21* can replicate this defect *in vivo* and whether the patient's mutation has the expected effect. We identified (by reciprocal BLAST) the sole ortholog of *RAD21* and we injected zebrafish embryos with a *rad21a* splice blocking morpholino (MO) (at 1–2 cell stage, Figure S2A,B). Embryos were fixed at 14hpf (hours post fertilization) and stained with a *runx1* probe. During zebrafish development, *runx1* is expressed in the posterior lateral plate mesoderm (PLM). We observed defects in *runx1* expression patterns recapitulating prior studies, Horsfield et al. 2007, and Marsman et

al. 2014 with *runx1* expression either partially or completely absent in *rad21a* morphants (Figures 2A–C). *Runx1* expression was rescued by co-injection of MO with human WT *RAD21 mRNA*. In contrast, co-injection of MO and mRNA encoding the p.622 Thr allele was not able to rescue *runx1* expression, indicating that the RAD21 mutation has a loss-of-function effect *in vivo* (Figure 2D).

To test whether we could recapitulate the CIPO phenotype seen in *RAD21* mutant patients, including a severe impairment of gut motility and marked hypoganglionosis (Deglincerti et al. 2007) we characterized the zebrafish gut. Subsequent to MO injection, embryos were allowed to develop to 5dpf, at which time the digestive system has developed (Ng et al. 2005). Notably, we saw no appreciable *runx1* expression in the gut at this stage of development (data not shown), suggesting an earlier onset of the enteric phenotype. Control and MO embryos were fed fluorescent beads through microgavage, a technique that allows to determine the rate of intestinal motility as a function of time (Cocchiaro et al. 2013). After eight hours post bead injection (hpi), embryos were divided into 1–4 zones based on anatomical landmarks (Figure 3A) and the presence of fluorescence in each segment was scored. Consistent with the CIPO phenotype, *rad21a* morphants showed delayed food transit along the gut (Figure 3B). Moreover, staining of enteric neurons along the gut with antibodies against HuC/D showed a significant depletion of enteric neurons (Figure 3C). Quantitative analysis of the zebrafish gut (Figure 3D) at 4 (upper panel) and 5 (lower panel) dpf stages revealed that *rad21* morphants had a marked reduction of HuC/D-immunolabeled enteric neurons compared to controls, suggesting a neurogenic cause of the observed motility defects.

Notably, previous reports on *rad21a* morphants and *rad21*nz171 mutants have shown significantly reduced expression of *ascl1a*, a neuronal marker. Ascl1 is also a transcription factor required for the development of serotonergic neurons (Pattyn et al. 2004). An ASCL1 mutation has been reported previously in Haddad syndrome, a condition encompassing congenital central hypoventilation syndrome and Hirschsprung's disease (MIM 209880).

These data, the similarity of *rad21a* morphants to the hypoganglionic phenotype observed in a mouse model for the *ret* mutation C620R (Carniti et al. 2006; Yin et al. 2007) raised the possibility that RAD21 and RET might act synergistically during gut neurogenesis. We therefore performed epistasis analysis by co-injecting *rad21a* and *ret* MOs at subeffective doses, at which each MO alone was indistinguishable from control embryos. We observed

strong epistasis on the innervation of the gut; co-injection of the two genes phenocopied the phenotype of high dose *rad21* MOs (Figures 3E, F). At the same time, overexpression of RET did not rescue *rad21* morphants, or vice versa, suggesting that the two genes act on the same process but not directly in the same pathway. This observation is consistent with previous studies that showed RET to be induced by NGF in a runx1-independent manner (Luo et al. 2007).

The mutant RAD21 p.622 Ala>Thr does not bind to RAD21-binding elements in Apolipoprotein B promoter

Recent data have shown that RAD21/CTCF binding sites are present in the apolipoprotein A1/C3/A4/A5 gene cluster on chromosome 11 and that altered binding of these factors to these sites dysregulates apolipoprotein expression (Mishiro et al. 2009). We therefore performed electromobility shift assay (EMSA) experiments to test whether the mutant RAD21 protein could retain its binding affinity for the above-mentioned sites, in particular for the AC2 site (Mishiro et al. 2009). We observed no differences between the nuclear extracts from wild-type and mutant LCLs in binding to this site (Figure S3F). Next, we investigated if the nuclear extracts from wild-type and mutant LCLs could show differences in binding to human *APOB* promoter (Carlsson et al 1989). *In silico* analysis with MatInspector identified two binding sites for RAD21/CTCF (hAPOB_c1, chr2:21,267,137-21,267,173, matrix score: 0.862; hAPOB_c2, chr2:21,266,910-21,266,945, matrix score: 0.807) which maps to the two regulatory regions of the proximal *APOB* promoter (Carlsson et al 1989). EMSA assays performed with probes corresponding to the two sites showed a specific shift only in the presence of wild-type nuclear extracts (Figure 4A). Moreover, specific supershifts with an anti-RAD21 antibody were detectable only in wild-type, but not in mutant, nuclear extracts (Figure S4A). Transfection of the plasmids carrying either wild-type or mutant RAD21 cDNAs in frame with the DDK-epitope, in HEK293 cells, and subsequent RT-qPCR analysis of *APOB* expression revealed that wild-type RAD21 overexpression reduced *APOB* levels compared to the empty vector ($p=0.0098$; Student's t-test). In contrast, overexpression of the mutant protein had no effect, similarly to the empty-vector transfections (Figures 4B, C). These data suggest that RAD21 might act as a repressor of APOB.

Apolipoprotein B (APOB) is overexpressed in CIPO patients

APOB transcript generates two different proteins, APOB48 and APOB100 isoforms, both present in serum (Black et al. 2007). Since our data suggested a RAD21-regulated expression of APOB, we analyzed sera from the CIPO patient homozygous for *RAD21* mutation (IV-9), and from wild-type controls. We detected an elevated expression of APOB48 in the patient, whereas no significant differences could be appreciated for APOB100 (Figure 5A and Figure S5A). To understand whether APOB overexpression was unique to this patient or whether it might represent a more generalized phenomenon, we evaluated sera from RAD21 mutation-negative idiopathic CIPO patients and from control subjects. APOB48 showed an increase in CIPO patients (Figure 5B and Figure S5B). Moreover, compared to CIPO, sera derived from 12 patients with functional bowel disease, namely seven diarrhea-predominant IBS (IBS-D), four constipation predominant IBS (IBS-C) and one with alternating bowel IBS (IBS-C/D), did not show APOB48 overexpression (Figure 5C and Figura 11 Figure S5C). Furthermore, western blot analysis on the sera of patients with other gastrointestinal disorders, i.e. anorexia nervosa and mechanical intestinal obstruction, did not identify any APOB48 increase.

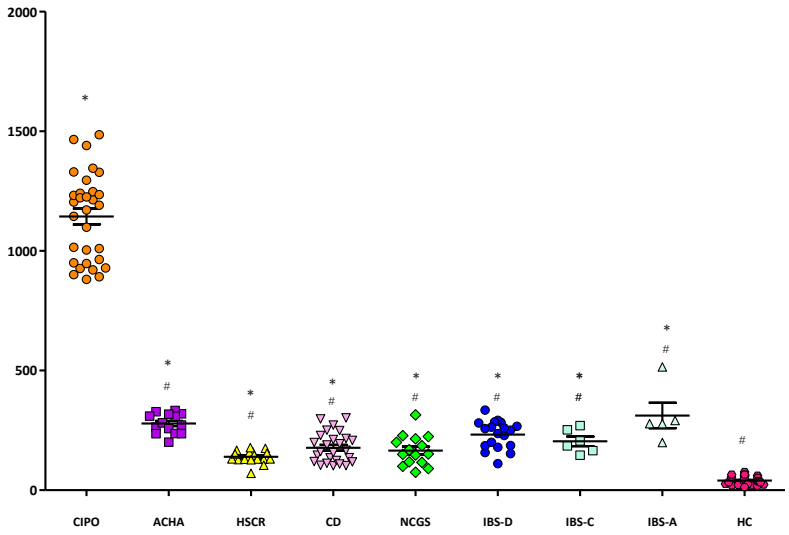

Figure 11: **APOB relative expression (A.U.) of sera's patients.** Chonic intestinal Pseudo-Ostruction (CIPO), Achalasia (ACHA), Hirschsprung disease (HSCR), Celiac Desease (CD), Non Celiac Gluten Sensitivity (NCGS), Irritable Bowel Syndrome with Diarrhea (IBS-D), Irritable Bowel Syndrome with Constipation (IBS-C), Irritable Bowel Syndrome - Alternating (IBS-A) and Healthy Controls (HC).

Data are expressed as single values. Horizontal lines represent mean values. * P<0 .0001 *vs.* CIPO (simple contrast analysis of variance, ANOVA). #P<0.001 vs. HC (simple contrast ANOVA).

Immunohistochemical analysis of gut biopsies (mainly ileum) of sporadic CIPO patients revealed APOB48 expression in myenteric neurons and in cells of the *lamina propria*, reminiscent of immunocytes (Figure 5). Consistent with the results obtained in the sera, the APOB48 signal was increased markedly in the biopsies of CIPO cases compared to controls and IBS cases (Figures 5D i-iv-vii). The quantitative analysis of immunolabeled cells in the *lamina propria* of eight CIPO patients (five Italian and three Swedish) indicated a significant increase in the number of APOB48-positive cells compared to the other individuals (controls n=3;IBS patients n=3) (32.9±9.2% vs 7.2±2.5% cases vs controls; p=0.0012, Student' t test; 32.9±9.2% vs 5.6±1.5% CIPO cases vs IBS cases; p=0.0008; CIPO cases (8) vs all (6), p=0.0001; Figures 5Dv-vi-vii). In addition, quantitative analysis performed in the gut biopsies of sporadic CIPO patients with a marked increased in APOB48 revealed a significant reduction in the number of neuron specific enolase (NSE)-labeled myenteric ganglion cell bodies / gaglion compared to control specimens (CIPO cases 22.71± 8.10 vs controls 48.65±13.80; p-value=0.0039, Student's t-test; Figure 5E). No significant differences were observed for neurons in the submucosal plexus between CIPO cases and controls (CIPO cases 5.61 ± 0.39 vs controls 8.36 ±2.96; p-value=0.1079, Student's t-test). We observed RAD21 staining in multiple tissues and throughout different components of the gut, as shown previously (www.proteinatlas.org), although no differences were appreciated between CIPO and controls (Figure S6A i-iv). Expression of APOBEC1, the gut-specific RNA editing enzyme responsible of the formation of APOB48 isoform, was also investigated; we observed similar immunostaining in control and CIPO tissue biopsies (Figure S6B i-ii).

Discussion

This study provides *in vitro* and *in vivo* evidence that a novel homozygous mutation in *RAD21* is associated with a syndromic form of CIPO (Deardorff MA et al. 2012). RAD21 is also a transcriptional regulator that binds to many sites in the genome (Parelho V et al. 2008). In concordance with the key role(s) of RAD21 in regulating cell division, altered expression and somatic loss-of-function mutations have been reported in different cancers (Cuadrado A

et al. 2012; Kon A et al. 2013). Furthermore, heterozygous germline mutations in cohesin subunits, i.e. RAD21, SMC1, STAG, or in regulators of cohesin, e.g. NIPBL, cause a broad spectrum of disorders referred to as cohesinopathies (namely Cornelia de Lange syndrome, CdLS, OMIM 122470), characterized by facial dysmorphisms, growth retardation, developmental delay and/or intellectual disability, and multiorgan involvement, including musculoskeletal malformations ranging from brachyclinodactyly to severe reduction defects (de Lange C et al. 1933, Ireland M et al. 1993, Jackson L et al. 1993, Opitz JM et al. 1985).

We observed a similar loss-of-function phenotype for two missense CdL mutations (p.376Pro>Arg, p.585Cys>Arg) and for our CIPO variant in RAD21 (p.622Ala>Thr). However, in the consanguineous family studied herein the heterozygous carriers of the *RAD21* mutation did not show sign of CdLS (Deglincerti A et al. 2007). Previously described patients with CdLS and different *RAD21* heterozygous mutations did not show gastrointestinal abnormalities such as CIPO (Minor A et al. 2014). It is worth noting that the mutations observed in CdLS and in our CIPO patients map to different RAD21 domains, potentially suggesting that specific mutational mechanisms in RAD21 can lead to different clinical entities in humans. Consistent with this notion, the CIPO-causing mutations do not appear to abolish the ability of RAD21 to bind to SMC1, whereas it does attenuate its transcriptional repressive role of other targets, such as APOB. Recently, however, a mutation in *SGOL1*, another cohesin protein, has been associated to severe gut and cardiac dysrhythmia in the absence of other congenital abnormalities or cohesinopathies (Chetaille P et al.2014).

RAD21 plays an important role in the development, survival and maintenance of epithelial cells and neurons of the gastrointestinal tract and mice heterozygous for a *Rad21* null mutation exhibit gastrointestinal defects following X-ray irradiation (Xu H et al. 2010). Our zebrafish studies suggest that *rad21* is essential for enteric neuron development: *rad21a* morphants recapitulate the CIPO phenotype, as they show a delayed transit along the gut and a significant depletion of enteric neurons. This latter finding is reminiscent of an enteric neuronal hypoganglionic phenotype observed in heterozygous *ret*C620R/+ mice16 and shares similarities with the histopathology of some CIPO patients (Deglincerti A et al. 2007, Carniti C et al. 2006, Yin L et al. 2007). The similarity of the *rad21* suppression phenotype in zebrafish to the neuronal defects of heterozygous *ret*C620R/+ mice prompted us to test whether those two genes might interact genetically. We showed that RET and RAD21 interact epistatically during differentiation or maintenance of enteric neurons. However, the failure of

the two genes to rescue the gut innervation phenotype suggests that they activate different molecular pathways.

Our previous studies identified Apolipoprotein B (*Apob*) as a target of RET signaling in Neuro2a cells, a murine model of enteric nervous system development. Moreover, in ret+/C620R mice that have an enteric neuronal hypoganglionic phenotype16 reminiscent of the histopathology observed in some CIPO patients, (Carniti C et al. 2006, Luo W et al 2007) *Apob* was markedly overexpressed. APOB is a major constituent of the plasma lipoprotein, and in mammals is synthesized in two different tissues, i.e. liver and intestine. Two different proteins derive from the APOB transcript by a RNA-editing enzyme, APOBEC1: the full-lenght APOB100, a large protein of 512 kDa synthesized by the liver (which does not express APOBEC1), essential for triglyceride-rich VLDL formation; and the gut-specific isoform APOB48, formed via the RNA-editing process and co-linear with the N-terminal half of APOB100. APOB48 has a key role in chylomicron assembly and transport in the intestine (Black DD et al. 2007).

APOB expression is regulated by a strong promoter in the proximal upstream region, containing several positive regulatory elements, including one within the noncoding exon 1, but also a negative regulatory element between bases −261 and −129.31 In this study we identified two RAD21-binding sites in APOB proximal promoter, i.e. one overlapping the negative element, and the other partially overlapping the exon 1 positive regulatory element. Only nuclear extracts from wild-type cells form a specific complex with either region, whereas no complex is observed in the presence of mutant RAD21. This suggests that RAD21 may act as a repressor of APOB transcription, associating to its negative regulatory element and competing with the transcriptional activators that bind to positive regulatory element.

Finally, we found that, compared to controls, gut-specific APOB48 levels were increased in the serum of the RAD21-mutated CIPO patient. Interestingly, sera from the sporadic CIPO patients, negative for RAD21 mutations, also showed consistently elevated APOB48 levels as compared to either IBS patients or healthy controls. We observed a variable expression of APOB100 in both controls and CIPO patients, in line with the fact that its regulation depends on different factors, including cholesterol and insulin levels (Watts GF et al. 2009). Notably, CIPO patients did not show evidence of altered lipid metabolism, i.e. total cholesterol and HDL levels were within the normal range.

APOB48 overexpression was further corroborated by the data on gut biopsies of CIPO patients: compared to controls, APOB48 immunoreactivity was significantly increased in cells (with morphological features of immunocytes) distributed throughout the *lamina propria* and in myenteric neurons of CIPO patients. Interestingly, a recent study identified a specific neuron-macrophage crosstalk in regulating gut motility (Muller PA et al. 2014). These data bear implications to the pathogenesis of functional bowel disorders, such as IBS. However, our study did not show any change in APOB expression in patients with IBS, suggesting that other molecular pathways can be also involved in patients with a more prominent gut dysfunction such as those with CIPO.

Furthermore, we found a significant decrease in the mean number of myenteric neurons /ganglion in CIPO tissues exhibiting a high APOB immunoreactivity. This finding is reminiscent of severe hypoganglionosis14 in the patients who were found to carry the RAD21 p.622Ala>Thr homozygous mutation in this study.

Based on our data, APOB48 expression (at serum and tissue level) was homogeneously increased in sporadic and familial CIPO and therefore making a possible correlation between this marker and the degree of neuronal loss / symptom severity undetectable. Further studies are eagerly awaited to clarify whether the correlation between APOB expression levels and neuronal / clinical CIPO phenotype exists.

It is currently unclear the pathophysiological significance of the generalized APOB overexpression observed in sporadic CIPO, where no mutation of RAD21 were identified. Our previous studies suggested a role for APOB in the molecular pathways required for enteric neuron development and survival (Evangelisti C et al. 2012). Therefore, APOB48 increase may be activated as a compensatory effect to an abnormal / defective enteric nervous system occurring in CIPO. We still do not know the precise temporal origin of the defect that leads to CIPO in patients with RAD21 mutations. The observed transcriptional downregulation of *runx1*, which is likely relevant in early neuronal progenitors, possibly in the crest, argues for a migratory defect. At the same time, the observed loss of APOB suppression might be more relevant to neuronal progenitors in the gut itself (Figure 6). Further studies of patients and conditional mouse mutants will be required to understand the potential relative contribution of different sites to CIPO pathology. Likewise, further research will be required to elucidate the factors contributing to the specific APOB48 overexpression

and its clinical value in the management of CIPO patients. Nonetheless, our data inform the molecular aspects underlying the pathogenesis of CIPO and lead to the identification of a candidate biomarker, namely APOB48 overexpression, for this severe, disabling gut dysmotility disorder.

Figures:

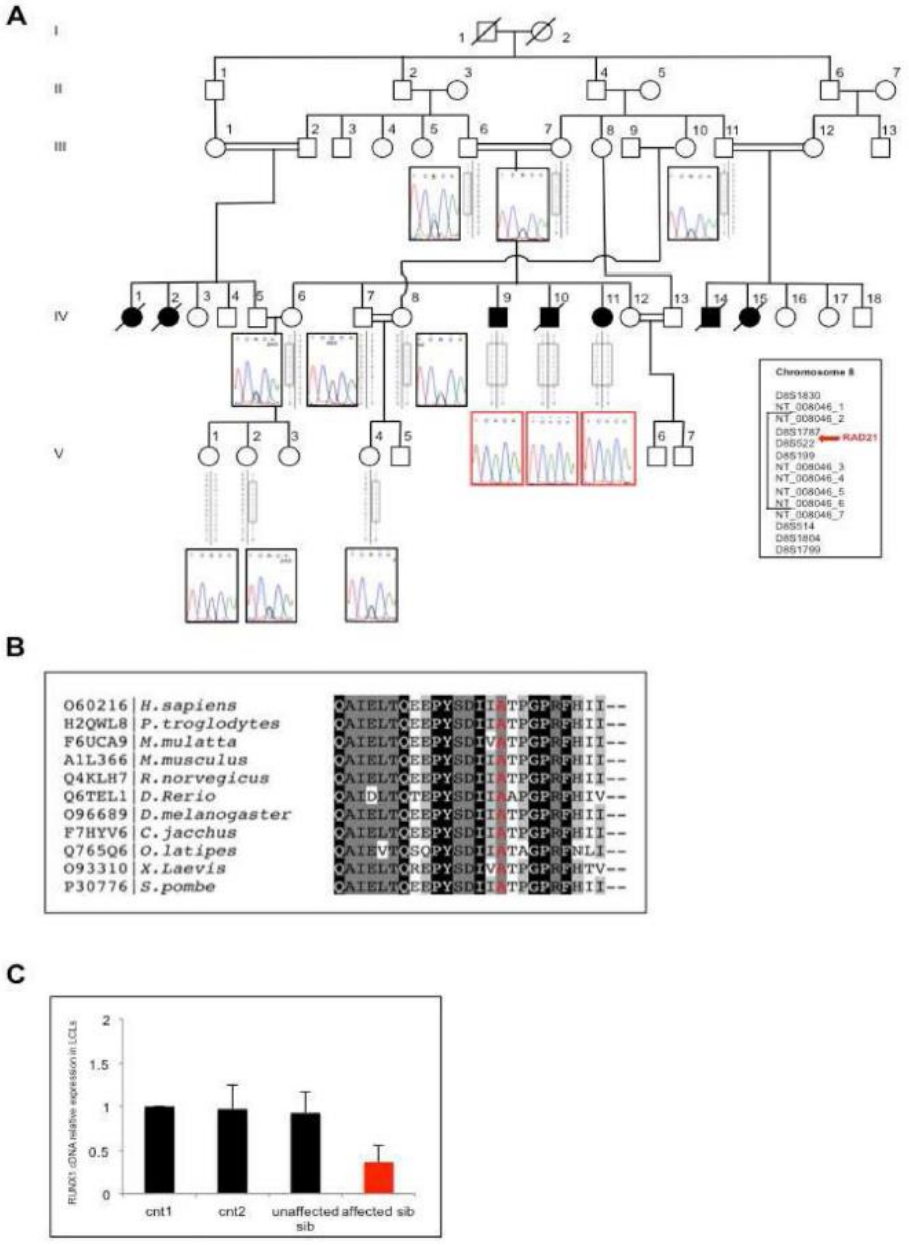

Figure 1. Identification of a novel homozygous mutation in RAD21 (**A**) Pedigree of the Turkish consanguineous family showing the segregation of the RAD21 mutation in the available members. For the homozygous patients, electropherograms are boxed in red. Grey boxes represent the haplotypes derived from microsatellite analysis. (**B**) RAD21 conservation across species surrounding the position p.622Ala (highlighted in red).(**C**) RUNX1 expression in controls and patient's LCLs (IV-9).

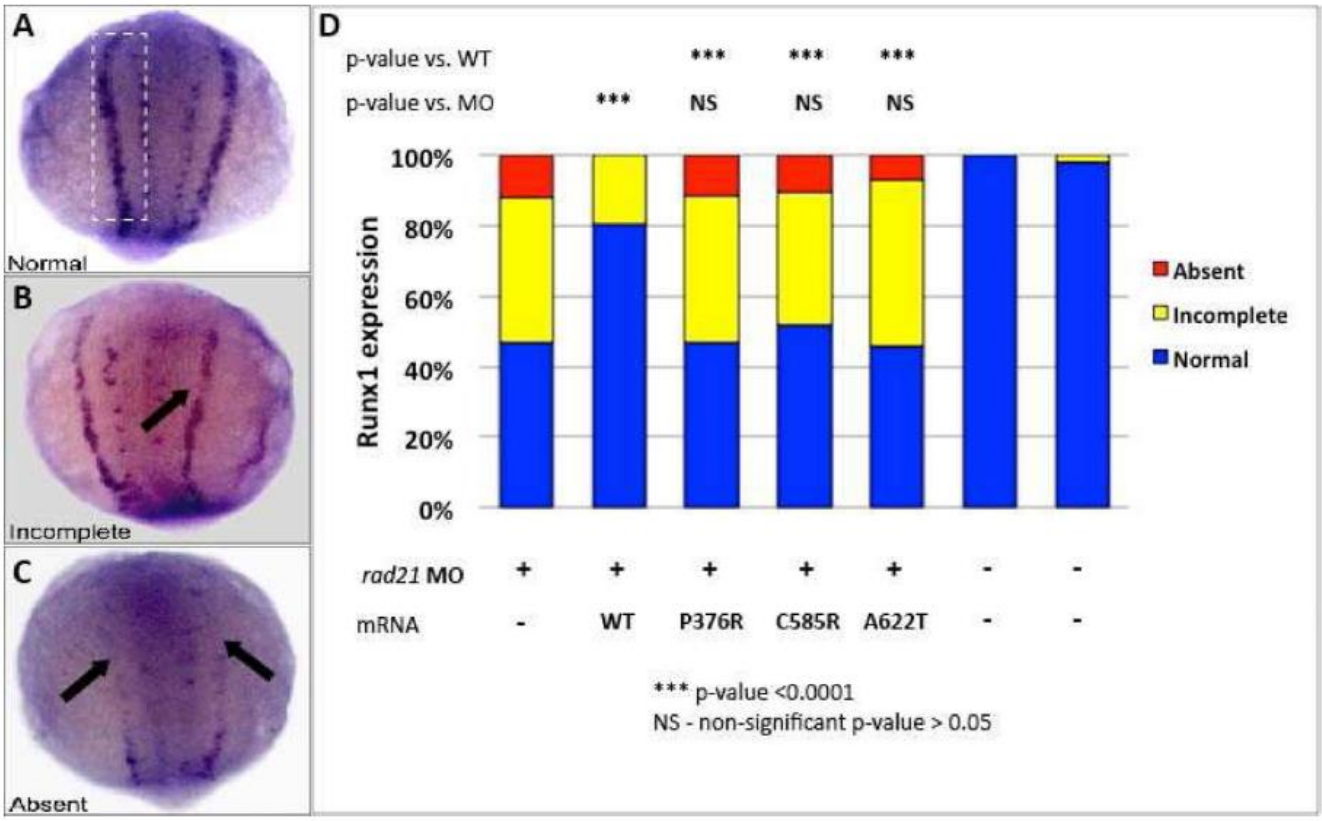

Figure 2. (A–D) RAD21 A622T affects runx1 expression in zebrafish embryos runx1 expression in the PLM in rad21a morpholino injected embryos rescued with wild type or mutants RAD21. Morpholino injected embryos were scored according to runx1 expression in the PLM as normal (A), partial (B), or absent (C). rad21a morphants could be rescued by wild type human RAD21 mRNA, while mutations previously reported in patients with "cohesinopathy" (P376A, C585R) and A622T can not rescue this phenotype.

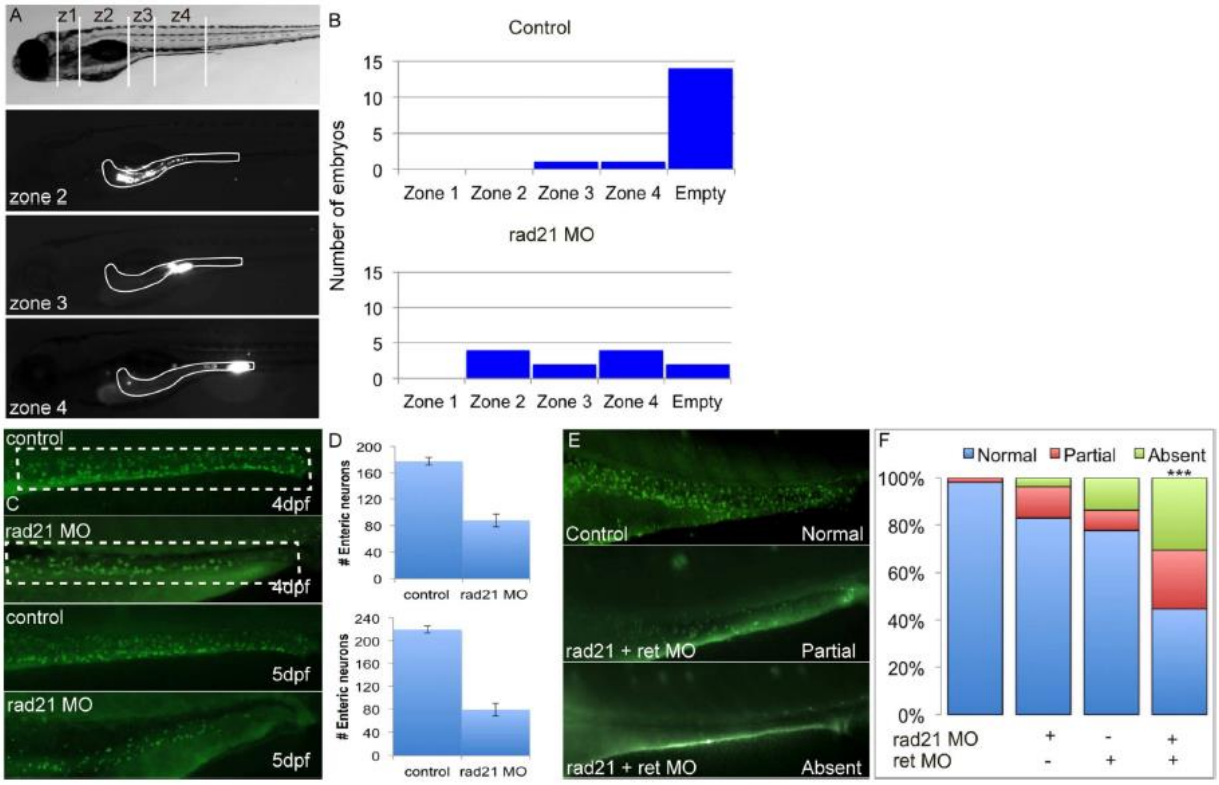

Figure 3. Gut dysmotility is caused by enteric neuronal loss in zebrafish embryos To assess gut motility in zebrafish larvae, we injected fluorescent beads into the mouth and recorded the rate of gut motility versus time. (A) Lateral view of 5dpf zebrafish larvae. Representative images of injected larvae show (after eight hours post injection, hpi) fluorescent beads in different gut compartments (zone 1–4). (B) In control embryos, most of the fluorescent beads have exited the gut by 8hpi, while rad21 MO injected embryos have reduced gut motility. (C-D) Compared to control larvae, rad21 morphants have a significant reduction of enteric HuC/D immunoreactive neurons at 4dpf (D; upper panel) and 5dpf (D; lower panel). (E) rad21 and ret interaction during the ENS development. Combination of suboptimal doses of rad21 and ret MOs causes a significant decrease in HuC/D enteric neurons, while embryos injected with suboptimal doses of rad21 MO or ret MO causes loss of HuC/D enteric neurons, not statistically significant (F).

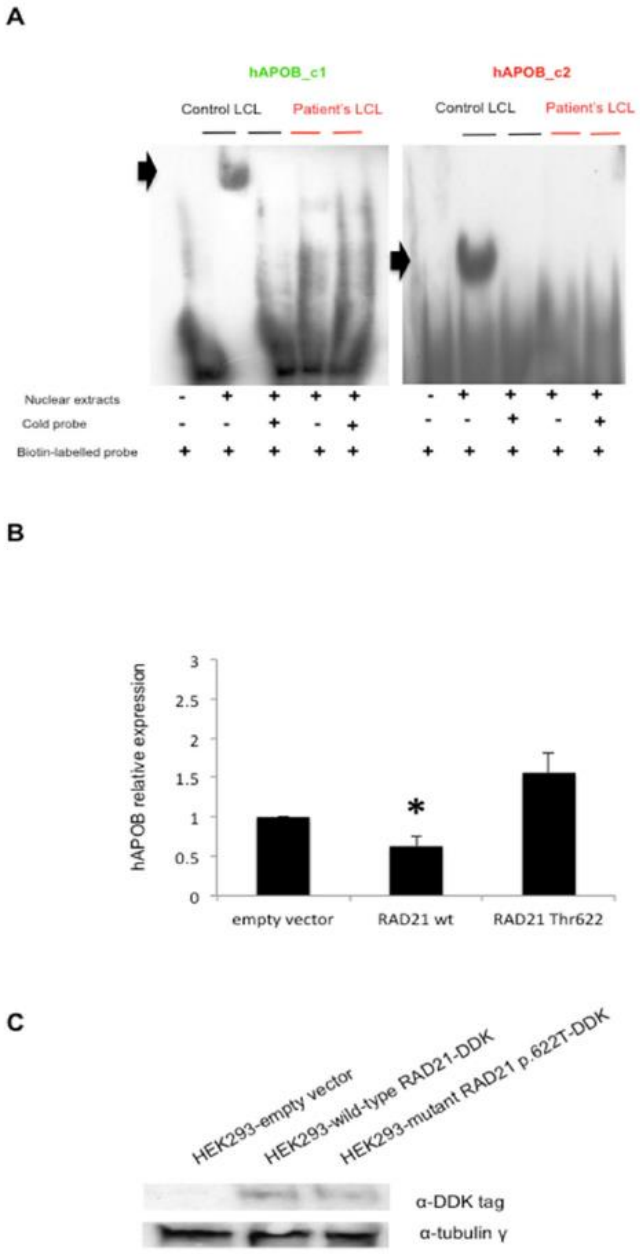

Figure 4. RAD21 regulates APOB overexpression (A) EMSA on human APOB promoter containing two putative binding sites for RAD21:hAPOB_c1 (green), hAPOB_c2 (red). EMSA analysis for hAPOB_c1 and _c2 regions biotin-labelled probes: only the nuclear extracts from wild-type (control) LCL) show a specific gel-shift (black arrows). (B): RT-qPCR for APOB expression in HEK293 cells transfected either with empty, RAD21 wild-type, RAD21 mutant p.622 Thr vectors. Data represent the mean values of three independent transfection experiments. Bars represent the standard deviation; black asterisk indicates the significant p-value (see in text). (C) Western blotting analysis showing the expression of recombinant wild-type and mutant RAD21 inframe with DDK tag in transfected HEK293.

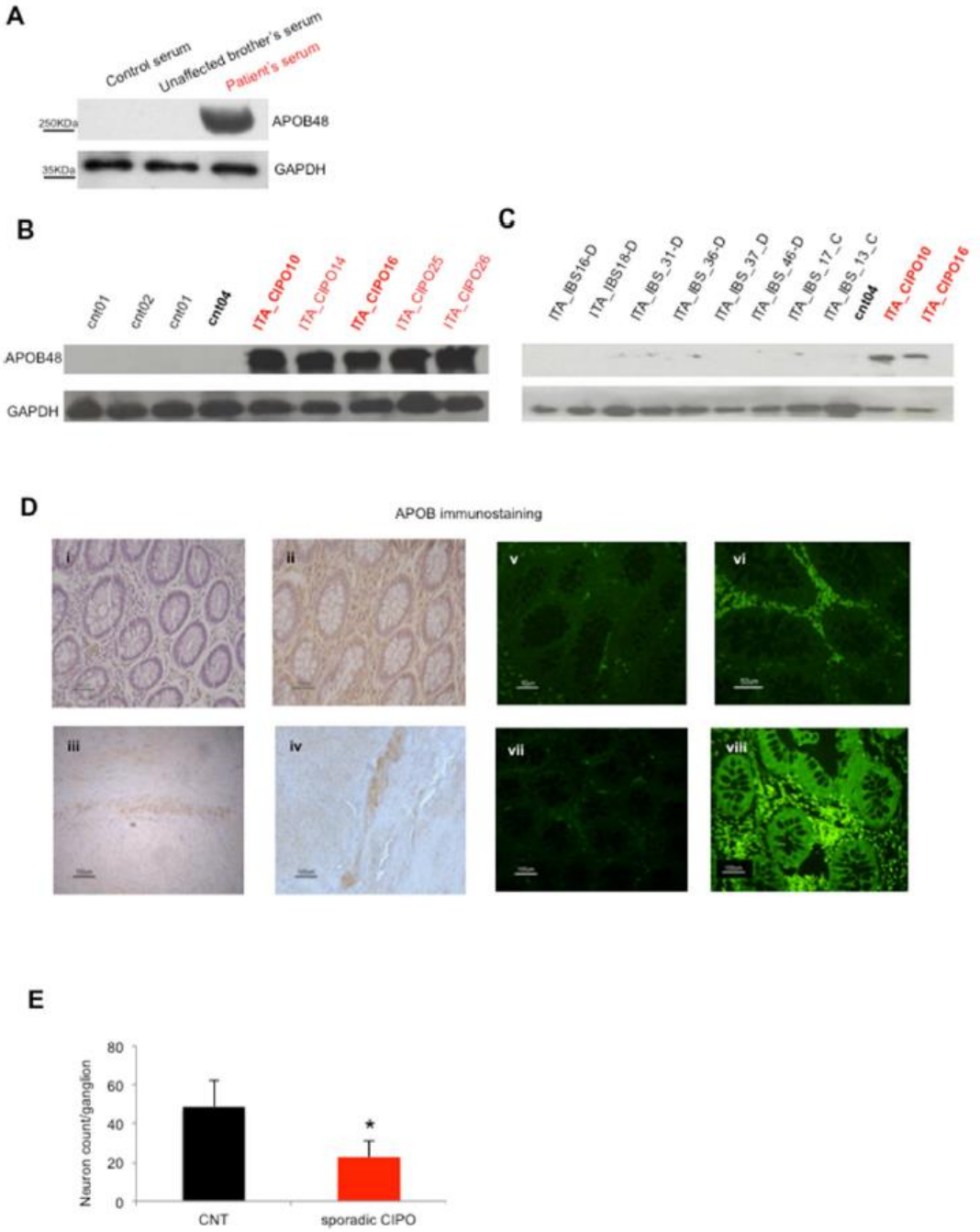

Figure 5. APOB overexpression is specific for CIPO patients (A)Western blotting analysis showing APOB48 expression in the patient carrying the homozygous RAD21 mutation (third lane), compared to control sera (upper panel); GAPDH was used as internal loading control (lower panel). (B) Western blotting analysis showing APOB overexpression in the sera of CIPO patients compared to controls (upper panel). (C) Western blotting analysis for APOB48 in IBS samples vs controls (in black) and CIPO patients (in red). Samples loaded in duplicates/triplicates are marked in bold (as index of reproducible blotting). IBS-C: constipation predominant irritable bowel syndrome; IBS-D: diarrhea predominant irritable bowel syndrome.(D) Immunohistochemistry and immunofluorescence staining for APOB in controls (D-i, iii,v), IBS cases (D-vii) and CIPO patients (D-ii, iv, vi). Representative

figures (D-i, ii) illustrate immunostaining of immunocyte-like cells distributed throughout the mucosa,including the lamina propria and the myenteric plexus (D-iii, iv). Note that the density ofAPOB immunoreactive immunocyte-like cells in the lamina propria was higher in CIPO patients (D-vi) vs. controls or IBS cases. (E) Histogram showing the significant decrease in the number of neurons per ganglion in the myenteric plexus observed in CIPO, compared to control tissue biopsies.

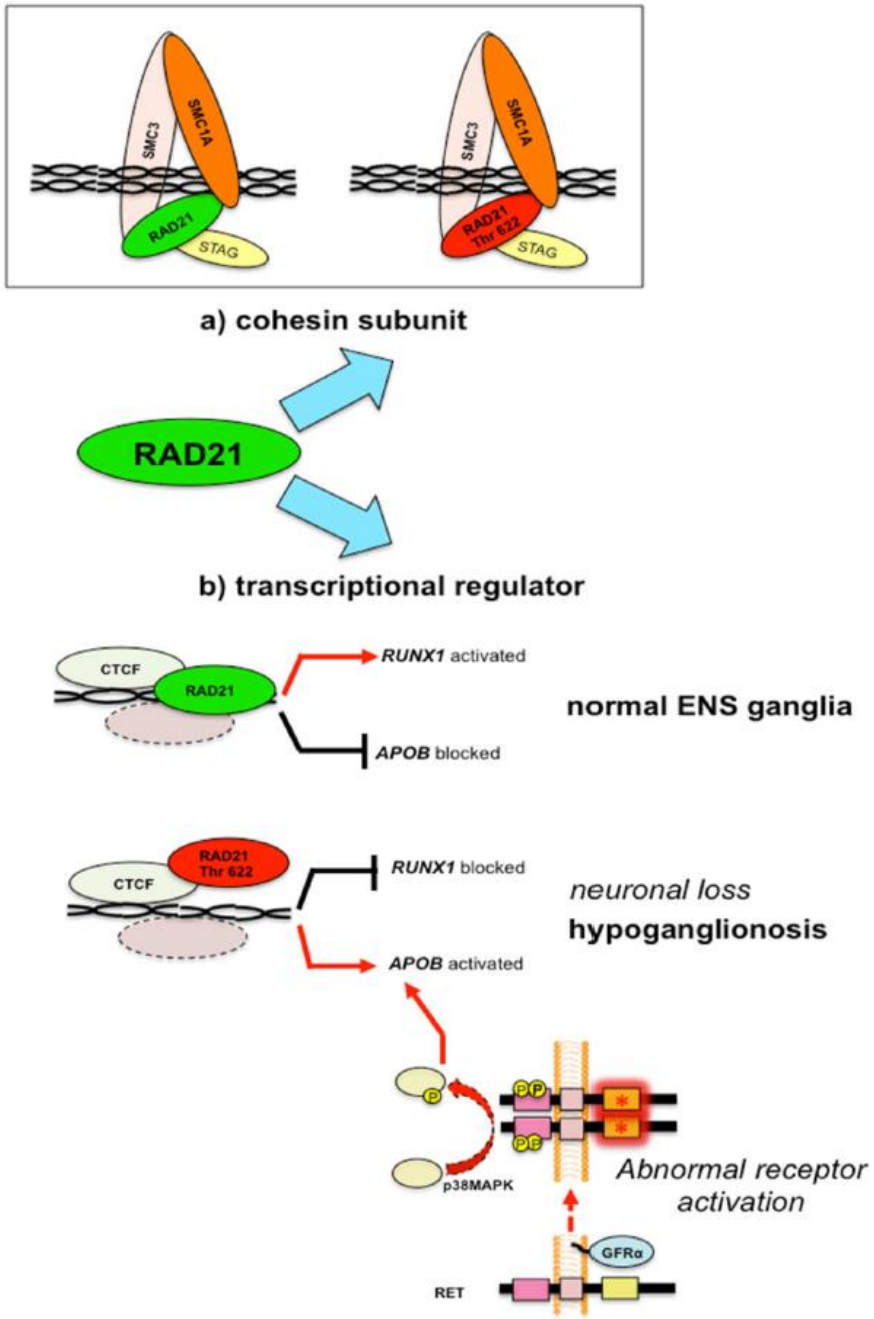

Figure 6. Model of RAD21 functioning as cohesin complex (A) or as transcription factor (B) (A) RAD21 belongs to the cohesin complex regulating chromosomal replication. RAD21 p.622Thr does not alter SMC1A subunit-binding. (B) Wild-type RAD21 promotes RUNX1 and represses APOB expression. RAD21 p.622Thr downregulates RUNX1 expression, derepresses APOB expression and leads to ENS neuron loss with resultant hypoganglionosis. Alternative pathways, including RET abnormal activation (e.g. the p.620 Cys>Arg mutation [red asterisk] in heterozygous mice) can lead to APOB overexpression, which is phenotypically associated with hypoganglionosis.

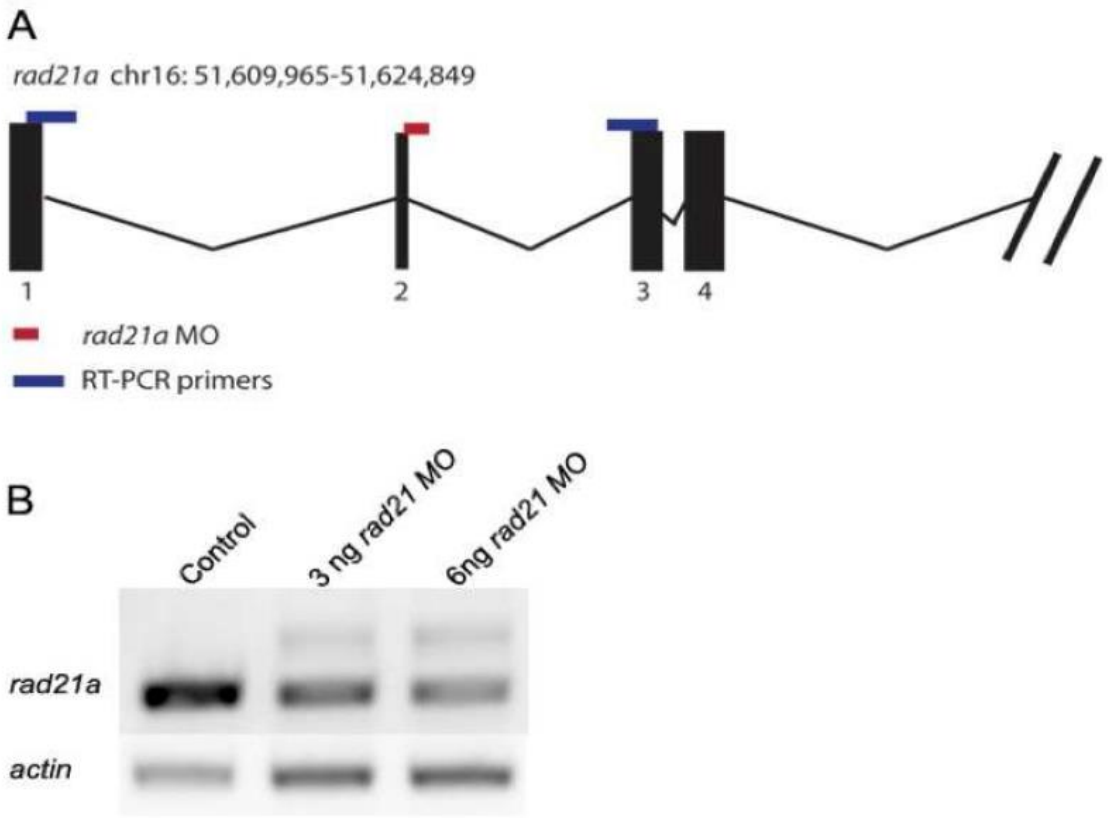

Figure S1. RT-PCR analysis of knockdown efficiency for splicesite targeted *rad21* Morpholino. (**A**) Schematic of zebrafish *rad21*. A splice blocking MO (red bar) was designed against the second exon-intron junction. PCR primers (blue bar) designed against exon 1 and exon 3 were used to test MO efficiency. (**B**) In control embryos, there is a single amplicon, while in *rad21* MO, there are two amplicons, demonstrating the efficiency of the MO used.

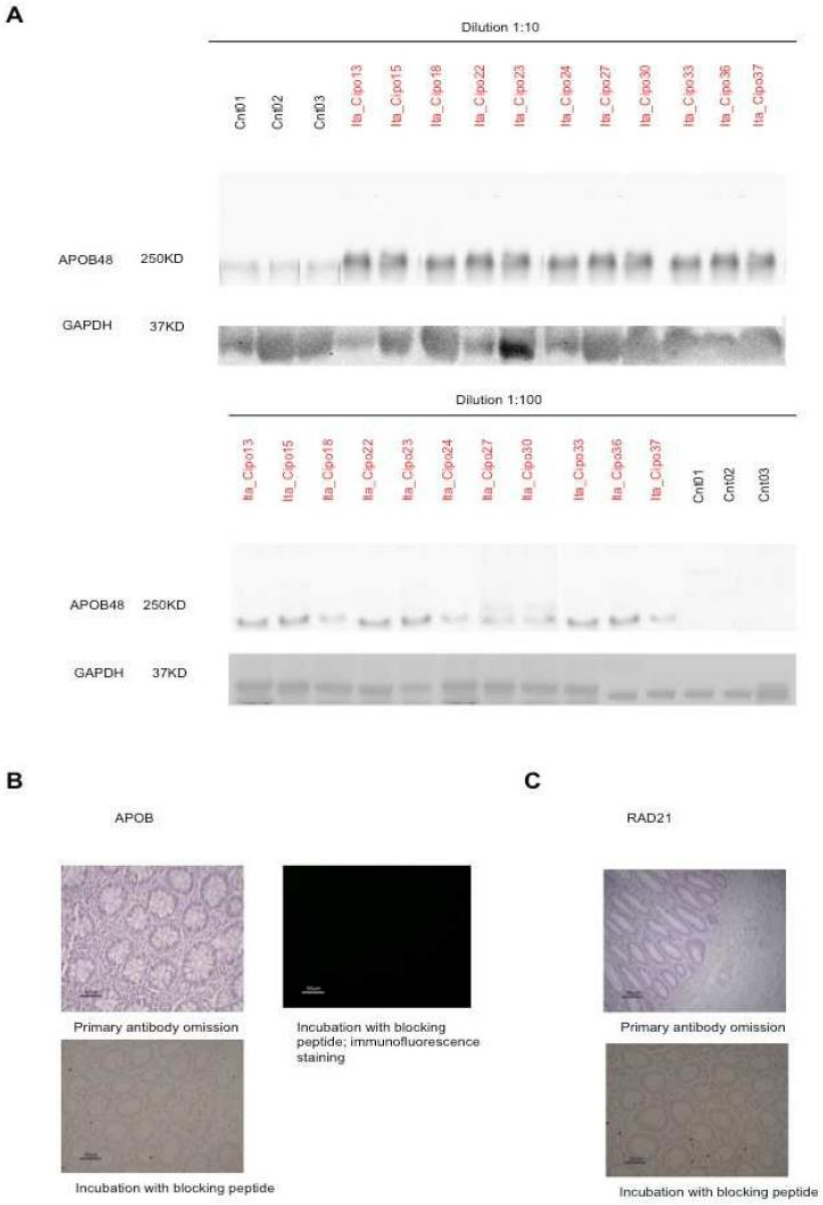

Figure S2. Serial dilutions for APOB48 western blotting and negative controls for immunohistochemistry analysis: (A) serial dilutions of sera from CIPO patients and controls; images are representative of replica experiments; **(B-C)** images of negative controls, including primary antibody omission and blocking with the corresponding peptides for APOB (B) and RAD21 **(C)** are shown.

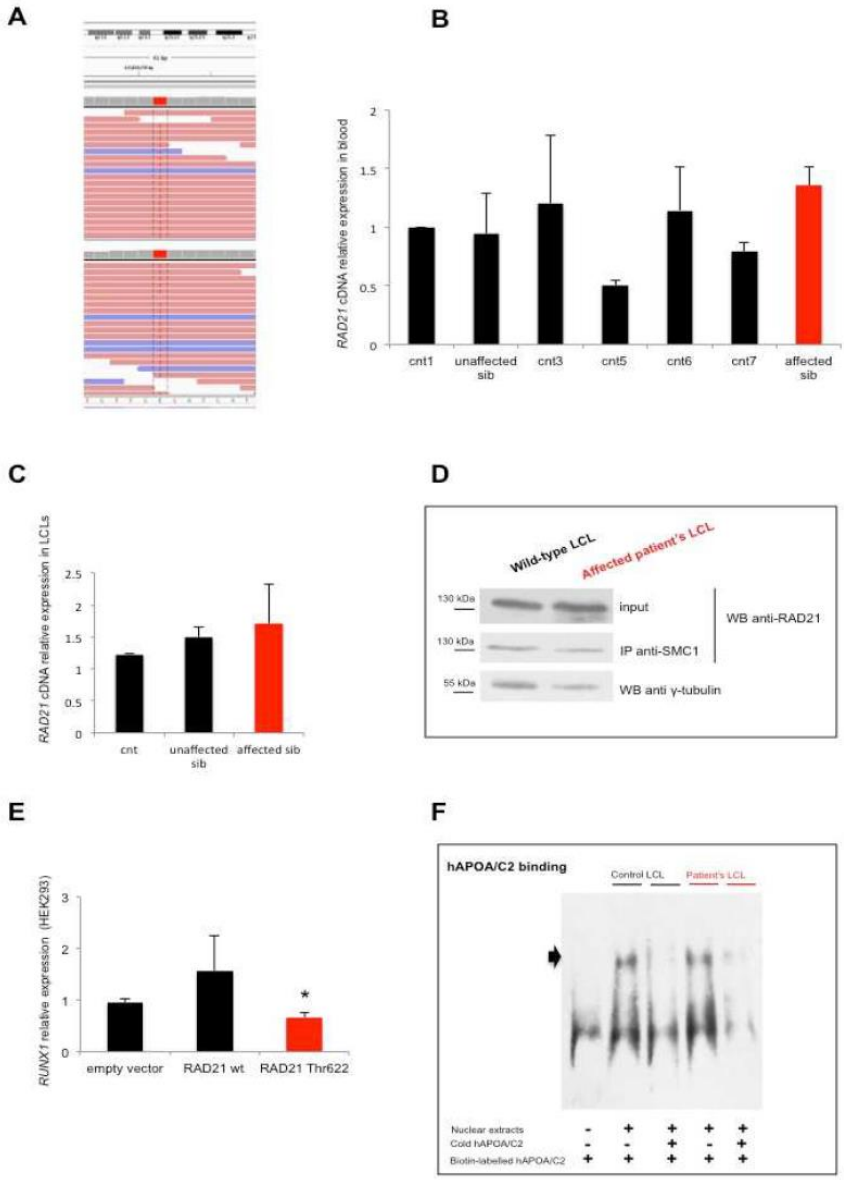

Figure S3. Functional studies of RAD21 mutation p.622Ala>Thr *in vitro*. (**A**) Screenshot of the parallel sequencing output for the mutation in RAD21 (genomic change). (**B,C**) *RAD21* expression is not altered in patient's blood (**B**) and lymphoblastoid cell lines (**C**). RAD21 expression was evaluated by real-time qRTPCR; (**D**) *RAD21* expression immunoprecipitation analysis for SMC1 and RAD21 from protein extracts of LCLs, showing that the binding is retained even in presence of the mutated protein (second lane in each panel). (**E**) RT-qPCR for *RUNX1* expression in HEK293 cells transfected either with empty, RAD21 wild-type, RAD21 mutant p.622 Thr vectors. Data represent the mean values of three independent transfection experiments. Bars represent the standard deviation; black asterisk indicates the

significant p-value. (Representative western blotting analysis of recombinant RAD21 in frame with the DDK epitope in the transfected cells is reported in main Figure 4C). (**F**) EMSA analysis showing that LCL nuclear extracts containing either RAD21 wild-type or mutant protein retain the binding ability for the AC2 site on chromosome 11. Black arrows indicate the gel-shifts.

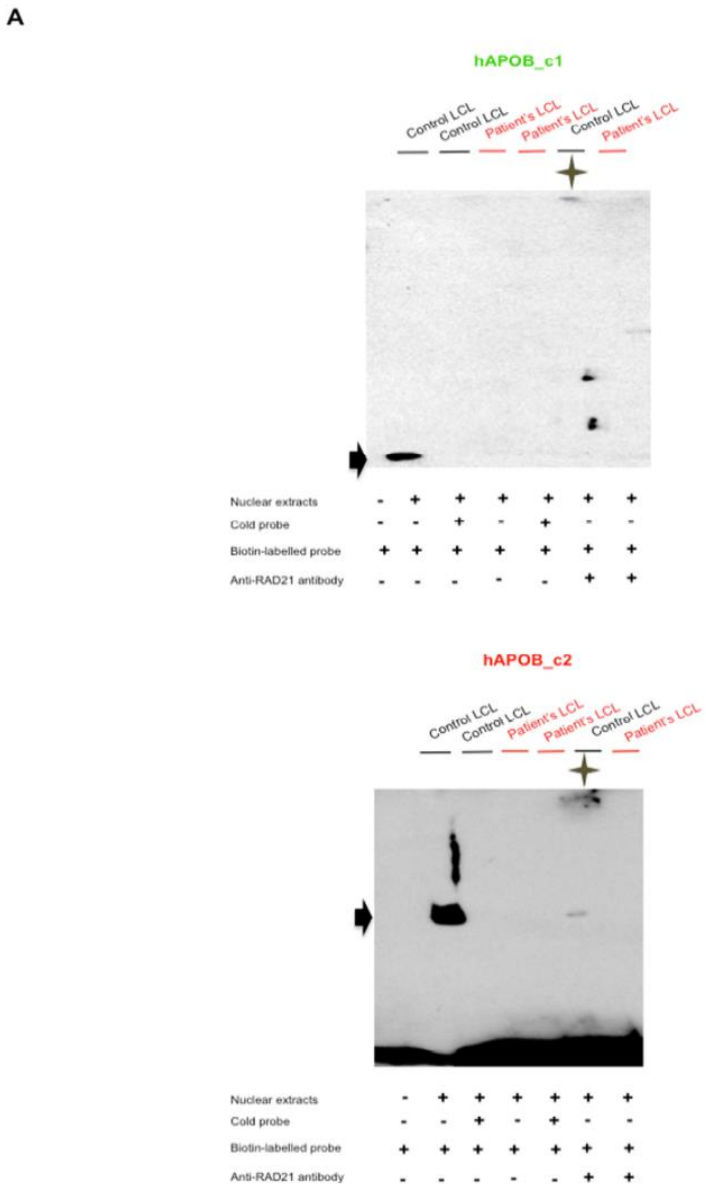

Figure S4. EMSA assays showing the RAD21-specific supershifts for the two regions in human APOB promoter. Black arrows indicate the shift, grey stars indicate the supershift observed only in lanes derived from control's nuclear extracts. For the c_1 region the complexes in the supershift were resolved only after a longer gel run, without keeping into the gel the free biotin-labelled probes. Images are representative of three independent experiments.

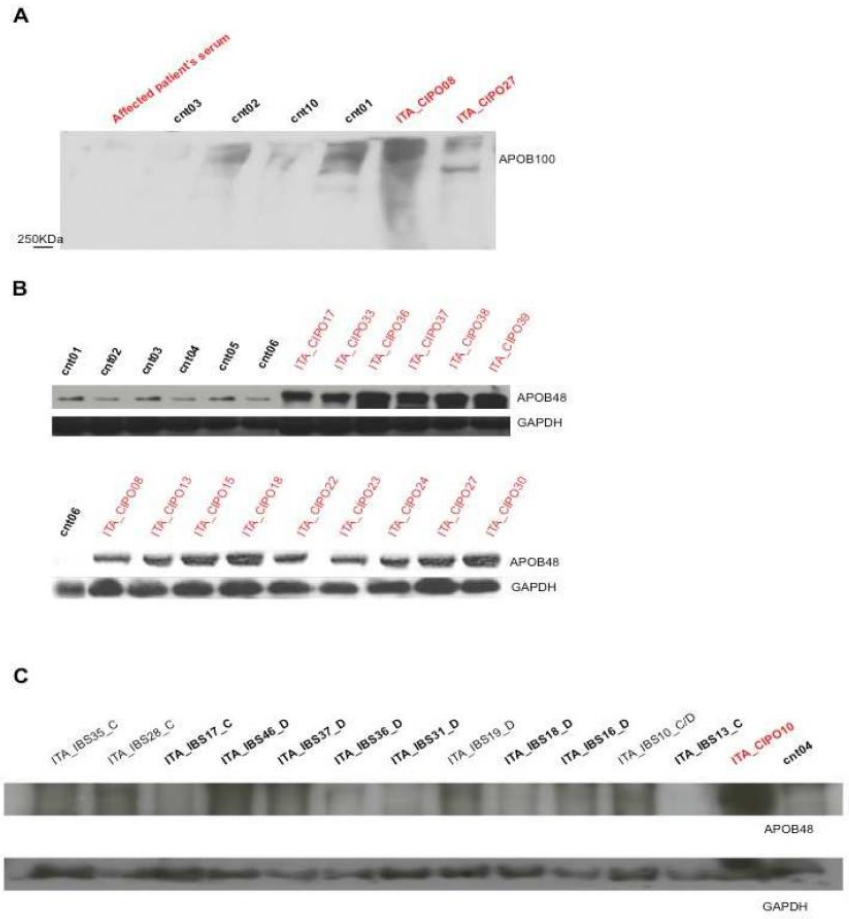

Figure S5. Western blotting for APOB on CIPO and IBS sera. (**A**) Western blotting analysis of APOB100 in sera of CIPO patient carrying RAD21 mutation, controls and CIPO patients; (**B**) Immunoblots showing the increased expression of APOB48 in sera of the CIPO patients included in RAD21 mutation screening, compared to controls; (**C)** Immunoblots for APOB48 in IBS vs CIPO patients and controls. Abbreviations are as shown in Figure 5. In bold are represented the samples loaded in different gels and analysed for APOB48 expression.

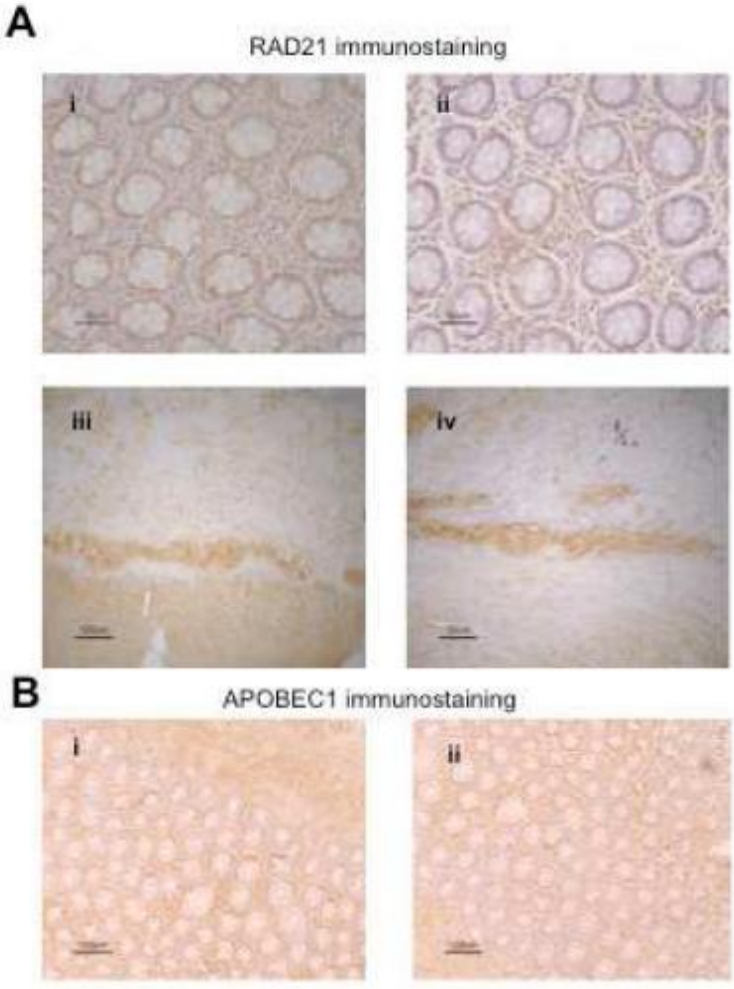

Figure S6: **RAD21 and APOBEC1 immunostaining**. Figures (**A**-i to iv) show RAD21 immunoreactivity in gut biopsies of controls (**A**-i, iii) and CIPO patients (**A**-ii, iv). The representative pictures indicate RAD21 immunolabeling in the nuclei of epithelial and *lamina propria* immunocyte-like cells (**A**-i, ii) as well as myenteric neurons (**A**-iii, iv). Figures (**B**i-ii) illustrate APOBEC1 labeling with an overlapping immunoreactive pattern to that observed for RAD21, showing no changes in controls (**B**-i) and CIPO patients (**B**-ii).

Chapter VII

11. Expression of RAD21 Immunoreactivity in Myenteric Neurons of the Human and Mouse Small Intestine

Modified from

"Expression of RAD21 Immunoreactivity in Myenteric Neurons of the Human and Mouse Small Intestine"

Running title: RAD21 in the enteric nervous system

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Abstract

Background: RAD21 is a double-strand-break repair protein and a component of the cohesin complex with key roles in several cellular functions. A RAD21 loss-of-function mutation was found in cases of chronic intestinal pseudo-obstruction (CIPO) with associated enteric neuronal loss. An analysis of RAD21 expression in the enteric nervous system is lacking, thus we aimed to identify and characterize RAD21 immunoreactivity (IR) in enteric ganglia. Methods: Double labeling immunofluorescence in mouse and human jejunum were used to determine co-localization of RAD21 with HuC/D, PGP9.5, neuronal nitric oxide synthase (nNOS), neuropeptide Y (NPY), choline acetyl transferase (ChAT), Kit, platelet-derived growth factor receptor-α (PDGFRα) and glial fibrillary acid protein (GFAP) IRs. Results: A subset of PGP9.5- and HuC/D-IR neuronal cell bodies and nerve fibers in the myenteric plexus of human and mouse small intestine also displayed

cytoplasmic RAD21-IR. This immunolabeling pattern was found in 42.5% of RAD21/HuC/D-positive neurons/field in adult mouse small intestine and 43% of RAD21/HuC/D-IR neurons/field in young mice. RAD21-IR did not co-localize with nNOS. A subset of ChAT-positive neurons was RAD21-IR. Punctate RAD21-IR was restricted to the nucleus in most cell types consistent with labeling of the cohesin complex. RAD21-IR was not detected in interstitial cells of Cajal, fibroblast-like cells or glia. Subsets of neurons in primary culture exhibited cytoplasmic RAD21-IR. RAD21 signal was knocked-down in cultured neurons (shRNA). Conclusions: Our data showing cytoplasmic RAD21 expression in enteric neurons provide a basis towards understanding how mutations of this gene may contribute to altered neuronal function/survival thus leading to gut-motor abnormalities.

Keywords: chronic intestinal pseudo-obstruction; enteric neurons; immunofluorescence

INTRODUCTION

RAD21 is a transcription factor and a key central component of the multi-protein cohesin complex,1 which functions to protect chromosome separation during the metaphase–anaphase transition of mitosis.2 RAD21 is ubiquitously expressed3 and its critical role was indicated by the lethal early embryonic phenotype in RAD21 knockout mice.4 Studying a familial cluster of chronic intestinal pseudo-obstruction (CIPO), we have recently demonstrated a novel loss-of-function mutation in RAD21 which was associated with enteric neuropathy and severe gut dysmotility, in affected family members. The CIPO-causing RAD21 mutation was found to alter the expression of related genes, including RUNX1 and APOB. Indeed, APOB48, the gut-specific isoform of APOB, was overexpressed in sera of patients with CIPO carrying the RAD21 mutation.5

CIPO is a rare and intractable chronic digestive disease in which clinical symptoms of intestinal obstruction appear without mechanical cause.6,7 CIPO can result from derangement affecting the integrity of a variety of regulatory cells/tissues, i.e. smooth muscle cells (effectors of contractility / relaxation), the interstitial cells of Cajal (ICC) (pace-makers of gut motility and regulators of neuronal input to smooth muscle cells), and neurons (both intrinsic and enteric nervous system).6 Specifically, the RAD21 loss-of-function mutation was associated with a markedly reduced number of enteric neurons, thus implying a neurogenic origin of the gut dysmotility reported in the affected family.8,5 The clinical evidence that the small bowel is most affected in CIPO, provided the background to our set of experiments focusing on RAD21 expression in the small bowel. The

distribution of RAD21 in the enteric nervous system, the intrinsic neural network controlling gastrointestinal (GI) physiology and homeostasis,9,10 is unknown. The enteric nervous system contains different functionally distinct neuronal subclasses, e.g. motor neurons – either excitatory or inhibitory, interneurons and intrinsic primary afferent neurons, which can be identified according to their neurochemical coding.10 Inhibitory motor neurons are immunohistochemically identified by the presence of the neuronal isoform of the enzyme nitric oxide synthase (nNOS) leading to nitric oxide synthesis, whereas most excitatory motor neurons are immunohistochemically identified by choline acetyltransferase (ChAT) (the acetylcholine synthesizing enzyme) or via detection of peptides of the tachykinin family (e.g., substance P or neurokinin A). In addition to these two major subclasses of enteric neurons, a variety of secondary neurotransmitters or modulators, e.g. neuropeptide Y (NPY), can be co-synthesized, stored and released by other enteric neuronal subsets, depending on their localization throughout the GI tract and the mammalian species considered.11 The present study was designed to investigate whether RAD21 expressed in a subset of myenteric neurons in human and mouse small intestine. Furthermore, in order to establish whether RAD21 is influenced by age-dependent mechanisms, we compared the RAD21 expression in myenteric neurons of the small intestine from adult vs. young mice. Our findings clearly demonstrated enrichment of RAD21-IR in the cell bodies and nerve fibers of a subset of myenteric cholinergic, but not nitrergic, neurons in tissues and in primary culture using two different primary antibodies. Silencing (shRNA) of RAD21 expression confirmed specificity of RAD21-IR.

MATERIALS AND METHODS

All authors have access to the study data and have reviewed and approved the final manuscript.

Immunohistochemistry

Single and double labeling immunofluorescence for RAD21 was done using two specific, commercially available rabbit and goat polyclonal antibodies (Abcam, Cambridge MA and Santa Cruz, Dallas TX, respectively see Table 1 for details) directed to RAD21 protein according to previously validated protocols.12 Normal human small bowel (jejunum) was obtained from n=3 (2F, age: 35-48 yrs) patients undergoing surgery for gastrointestinal cancers or having bariatric surgery at Mayo Clinic, MN, USA. The use of human tissue for research was approved by the Institutional Review Board of Mayo Clinic. Small bowel whole mount preparations from adult (n= 4) and young (n= 3) BALB/c (Harlan Sprague-Dawley, IN, USA) mice were processed for RAD21 immunolabeling (Abcam antibody at the same dilution used for human tissues, Table 1). Mice were

killed by CO2 inhalation and all experiments were approved by the Institutional Animal Care and Use Committee of the Mayo Clinic. Immersion-fixed (4% paraformaldehyde) human tissues (10 μm thick) and acetone-fixed mouse whole mounts were incubated over night at 4°C with rabbit RAD21 primary antibody as well as with a number of antibodies to a variety of neuronal, neurochemical, glial, and interstitial cells of Cajal markers (for details see Table 1). After washing, sections and whole mounts were incubated with donkey fluorescein- and rhodamine-conjugated anti-rabbit secondary antibodies as well as secondary antibodies to the previously mentioned markers (Jackson ImmunoResearch West Grove PA, See Table 1 for details) for about 2 h at room temperature. Sections and whole mounts were thoroughly washed in 0.1 M phosphate buffered saline (PBS) and mounted in SlowFade® Gold Antifade Reagent with 4, 6-diamidino-phenylindole,dihydrochloride (DAPI) (Invitrogen, CA, USA), as a nuclear counterstain. Specificity tests to ensure that there was no cross reaction among secondary antibodies included omission and substitution of primary or secondary antibodies. Secondary antibodies directed against IgG from a different species were used to control for the specificity of secondary antibodies in doubly labeled tissues.

Primary cultures from mouse jejunum

Primary cultures were obtained by enzymatic dissociation of mouse jejunum from neonatal mice (post-natal day 3) based on a previously described protocol.13 Utilizing a collagenase-based dissociation cocktail, tissues were dissociated and then plated on a feeder layer of fibroblasts expressing the membrane bound m248 form of Kit ligand (steel factor). Cells were grown in M199 media (Invitrogen) with 1% antibiotic/antimycotic mixture (Gibco). Primary cells were allowed to attach and recover for 24 hours before treatment. Living, single cells (150,000 each coverslip) were plated in a 12 -well plate with 1 ml of media M199 (Thermo Fisher, Waltham, MA, USA). Primary cultures were maintained at 37°C, 20% O2, 5% CO2 in M199 supplemented with glucose (4.5 g l−1, Sigma), antibiotic/antimycotic mixture (pencillin G sodium, 200 i.u. ml−1; streptomycin sulphate, 200 μg ml−1; amphotericin B, 0.5 μg ml−1; Thermo Fisher). Cultures were immunolabeled for RAD21, nNOS, ChAT HuC/D and PGP9.5 antibodies (see Table 1). Nuclei were counterstained with DAPI (Molecular Probes, Eugene, OR, USA).

shRNA lentiviral particles transduction in primary cell cultures

Primary cultures from 3 day old mice were exposed to shRNA lentiviral particles to knock down expression of RAD21 as follows: 50% of the culture medium was replaced with conditioned media and each experimental condition was run in duplicate. Cells were transduced for 24 h by adding to

the culture medium 2 µg/mL polybrene (Santa Cruz) and viral particles. Cultures were treated with non-targeting (NT) and shRNA RAD21 (n = 3) lentiviral transduction particles (Santa Cruz shRNA lentiviral transduction particles) with a multiplicity of infection (MOI) of 1 based on the confluence of the mixed cell culture being 50-70% at the time of treatment. For the next 5 days, cultures were washed and fresh culture medium was added daily. After day 1 and 5, cultures were used for RAD21 immunolabeling.

Confocal microscopy

Images of immunolabeling were collected using an Olympus FV1000 laser scanning confocal microscope. Confocal images were collected with ×60 1.2-NA or × 20 0.95-NA water objectives. Quantitative analysis counted the numbers of immunolabeled neurons using Olympus Fluoview Ver.2.1c viewer program. Neurons were counted from (635x635) µm2 fields per tissue. All images were prepared for individual figures using Adobe Photoshop CS. No 3D reconstructions, deconvolution, surface or volume rendering, or gamma adjustments were performed.

Statistics

Data are expressed as means ± SEM. Statistical significance was determined by GraphPad Prism using Student's t-test. P values of less than 0.05 were taken as statistically significant. The 'N' value identifies the animal count.

RESULTS

Extranuclear RAD21 immunoreactivity in human myenteric neurons from the small intestine

RAD21-IR was present in cell bodies and nerve fibers of a subset of human small intestinal enteric neurons identified by HuC/D-IR (Figure 1 A-C) and PGP9.5 (Figure 1 D-F) respectively. RAD21-IR was not detected in nNOS-IR inhibitory neurons (Figure 1 G-I arrows). RAD21-IR colocalized with ChAT in some neurons (Figure 1 J-L arrowhead). To further characterize this labelling pattern, we used mouse small intestine.

Extranuclear RAD21 immunoreactivity in mouse myenteric neurons from the small intestine

The extra-nuclear labeling pattern of RAD21-IR was confirmed using two different primary antibodies raised against different epitopes in two different species. Rabbit (Figure 2A) and goat (Figure 2B) anti-RAD21 antibodies labeled the same structures (Figure 2C) in thin sections from mouse jejunum. Additional controls were performed to ensure specificity for RAD21 with regard to

each primary antibody. Pre-adsorption of antibody with specific blocking peptide abolished signal intensity suggesting specificity of antibody used (Figure 2D-F). Furthermore, no IR was observed when the tissue preparations were incubated with only secondary antibody, demonstrating that there was no non-specific labeling by the secondary antibody (data not shown).

RAD21-IR is detected in the cytoplasm of a subset of HuC/D-positive myenteric neurons of adult and young mice

Whole mount analyses were conducted in order to determine the mapping of RAD21 expression in adult and young mouse enteric nervous system. We performed RAD21 and HuC/D double labeling immunohistochemical analysis of adult BALB/C mice (n=4; age range: 4-8 weeks). In whole mounts of adult mouse small intestine, RAD21-IR was present in 42.5% of HuC/D positive myenteric neurons. Notably, RAD21-IR was detected in the cytoplasm of a subset of neurons (arrowheads in Figure 3A). Overall, there were 61.56±0.9 RAD21 and 144.7±4.1 HuC/D-positive neurons/field (6 fields per preparation; each field measured 635x635 μm2, n = 4 mice ($P < 0.05$, Figure 3B).

To determine if RAD21 expression in the cytoplasm of enteric neurons is age dependent, we analyzed the expression of RAD21 in young mice (age range: 4 to 10 days old) (Figure 3C). Similar to adult mice, in young mice RAD21-IR was present in 43% of HuC/D positive myenteric neurons. Specifically, 79.56±4.5 RAD21 and 185.0±4.0 HuC/D-positive neurons/field (6 fields per preparation; each field measured 635x635 μm2) (n= 3, $P < 0.05$, t-test) (Figure 3D, $P < 0.05$ compared to adult mice).

Chemical coding of RAD21-IR neurons

Co-labeling with several antibodies were used to clarify the neurotransmitters expressed in RAD21 positive neurons (Table 1). RAD21-IR did not co-localize with nNOS (Figure 4A-C arrows). We also detected RAD21-IR cells that were positive for the neuronal peptide NPY in whole mount preparations (Figure 4D-F). RAD21-IR was present in a subset of neurons that expressed ChAT (Figures 4G-I arrows). Co-labeling of preparations with PGP9.5 demonstrated RAD21-IR in neuronal fibers (Figure 4J-L).

RAD21 did not colocalize with Kit (ICC), PDGFRα or enteric glia (GFAP) in mice

RAD21-IR was not detected outside the nucleus of Kit-IR interstitial cells of Cajal (ICC) (Figure 5A-D) or PDGFRα-IR fibroblast-like cells (Figure 5E-H) in mouse whole mount preparations.

RAD21-IR also did not overlap with glial fibrillary acid protein (GFAP)-IR (Figure 5I-L) in mouse jejunal myenteric ganglia indicating that RAD21 is not expressed in the cell bodies of enteric glia.

RAD21 expression in primary cultures from neonatal mouse jejunum

Primary cultures from mouse small intestinal muscularis propria that contain myenteric neurons contained RAD21-IR similar to that observed in tissues. RAD21-IR was observed in the cell bodies and nerve fibers of a subset of enteric neurons that were identified by PGP9.5 (Figure 6A-D). RAD21-IR was also detected outside the nucleus of a subset of ChAT-IR neurons (Figure 6E-H), but not in nNOS-IR inhibitory neurons (Figure 6I-L). We next used a shRNA lentiviral approach to knock down RAD21 in primary cultures. We first determined if we could maintain intact RAD21 expression in 5-day-old primary cultures. We prepared primary cultures from mouse jejunum (4 days old), grew these cultures for up to 5 days and performed an immunohistochemistry analysis for RAD21. We observed no significant changes in RAD21-IR when labelled after 5 days in primary culture (Figure 7E) compared to 1 day in primary culture (Figure 7B). We next optimized the knock down of RAD21 using Santa Cruz transduction shRNA-RAD21. A lack of RAD21-IR in shRNA treated cultures grown for up to 5 days confirmed the specificity of the RAD21 expression in a subset of enteric neurons (Figure 7H).

DISCUSSION

In the present study we demonstrated that RAD21-IR is expressed in cell bodies and processes of a subset of neurons of the adult human and mouse small intestine. High magnification revealed a predominantly cytoplasmic immunolabeling, although, as expected by its molecular nature (nuclear phospho-protein) and function (cohesin complex / transcription factor),15 RAD21-IR was also detectable in nuclei. No other cell types in the small intestinal muscularis propria displayed cytoplasmic RAD21-IR, including GFAP expressing glial cells, Kit+ ICC and PDGFRα-IR fibroblast-like cells. The specificity of the two anti-RAD21 antibodies was confirmed by a number experimental conditions and controls. First, both anti-RAD21 antibodies identified overlapping immunoreactive patterns, i.e. a selective subset of myenteric neurons (see below); secondly, omission of primary (or secondary) antibodies, substitution of the primary with secondary antibodies and preadsorption control did not yield any immunostaining; thirdly, experiments in which RAD21 was knocked-down by shRNA abolished RAD21-IR. Taken together these tests demonstrate the specificity of RAD21-IR and confirm neuronal expression. Based on this reliable

immunolabeling, we investigated the intrinsic innervation of the human and mouse small intestine and identified RAD21 expression in myenteric ChAT-IR neurons, likely belonging to motor neurons / ascending interneurons.16 By contrast, nNOS expressing myenteric neurons, mainly inhibitory motor neurons / descending interneurons, did not show RAD21-IR in either species. Our data provided the first analysis of RAD21 localization in the mammalian enteric nervous system and clearly showed that this cohesin complex protein is expressed outside the nucleus of cholinergic neurons in a subset of ChAT-IR neurons. RAD21 is known to exert a variety of regulatory functions critical to cellular homeostasis,17 thus our findings suggest a role for extra-nuclear RAD21 in maintenance and survival mechanisms of a subset of cholinergic neurons occurring in the healthy myenteric plexus.

Expression of RAD21 in the cytoplasm of cells has been previously associated with the translocation of a 64 KDa C-terminal fragment of the RAD21 protein from the nucleus following cleavage by caspases.18 RAD21 contains a consensus caspase 3/7 cleavage site19 and it has been proposed that RAD21 translocation is an early indicator of activation of caspase dependent apoptosis.18 Both of the antibodies used in this study were directed against the C-terminal region of RAD21 and therefore would be expected to detect cleaved, cytoplasmic RAD21 if present. Recent studies in mice20 and our own unpublished observations in human tissues found that a surprisingly high proportion (10-20%) of myenteric neurons express cleaved, active caspase-3 leading to the controversial suggestion that there is extensive ongoing apoptosis in the enteric nervous system. The proportion of caspase-3 positive neurons is much lower than the proportion of myenteric neurons that express cytoplasmic RAD21-IR (approximately 40%), thus it is unlikely that the presence of RAD21 in the cytoplasm is a marker for imminent cell death. However, the present study does indicate that RAD21 cleavage and translocation occurs preferentially in ChAT-positive, but not nNOS-positive, myenteric neurons possibly suggesting transient activation of caspase-3 activity in ChAT-positive neurons that does not always progress to cell death. In contrast, low caspase-3 activity or reduced RAD21 cleavage and translocation in nNOS-positive neurons could contribute to the sparing of these cells in healthy aging.12 The impact of the identified loss-of-function mutation in RAD21, such as that reported to be associated with CIPO,5 may be related to altered sensitivity of RAD21 to cleavage and inappropriate translocation of the protein leading to loss of some or all subtypes of myenteric neurons. This mechanism may be responsible for altered motor function in CIPO patients. However, the actual effect of the RAD21 p.Ala622Thr mutation is still to be clarified in an appropriate mouse model. It is worth noting that rad21 homozygous null mice are embryonic lethal.14 In vivo studies in the rad21 morpholino zebrafish model showed a

delayed intestinal transit along with a significant reduction of enteric neurons, a finding indicative of an oligoneuronal hypoganglionosis reminiscent of the neuropathic CIPO phenotype.5 Our data indicate that RAD21 is not expressed by nitrergic inhibitory neurons, a functionally distinct subclass commonly targeted by damaging mechanisms taking place in experimental (nNOS-/- mice) and human enteric neuropathies, and detectable in early stages of achalasia or idiopathic gastroparesis.21 It has not been determined whether nitrergic neurons remain unaffected in pathological conditions such as patients with RAD21-related CIPO.

Finally, in order to understand whether RAD21 distribution in myenteric neurons was altered during development, we investigated the distribution and relative abundance of RAD21-IR neurons in neonatal mice. RAD21-IR neurons were quantitatively comparable in neonatal and adult tissues, a finding implying that there is no major age-dependent cellular redistribution of RAD21-IR in the cytoplasm of neuronal subsets.

In conclusion, our data showed that RAD21 is expressed in a distinct subset of myenteric, i.e. cholinergic, neurons supplying the human and mouse small intestine. These findings, combined with the knowledge that RAD21 mutations occur in distinct CIPO cases, imply that the cholinergic subset may be the primary target in some patients with this pathological condition.

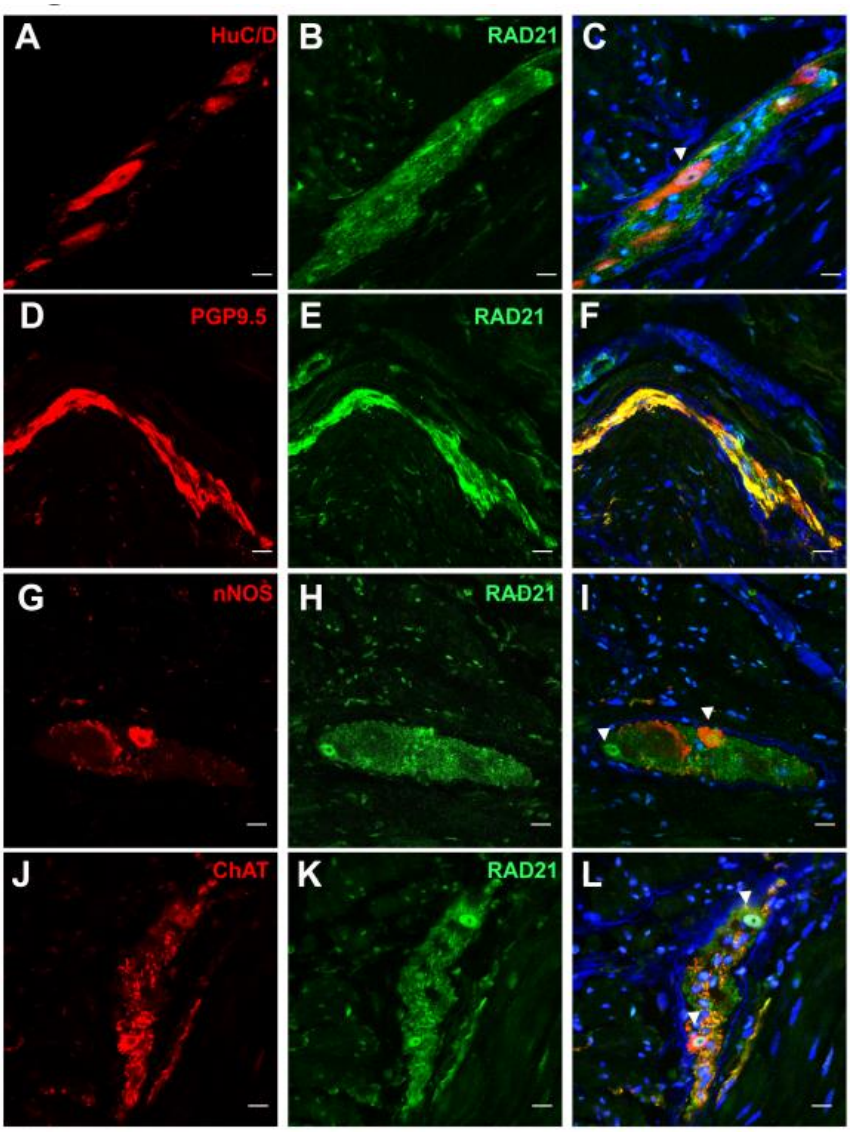

Figure 1. RAD21-IR in human small intestine. RAD21-IR was distributed in the human similarly to the mouse small intestine. RAD21-IR was not found in nNOS-IR inhibitory neurons (**A-C** arrows). RAD21-IR also colocalized with ChAT in some neurons (**D-F**). RAD21 was observed in cell bodies and nerve fibers (arrows) of a subset of enteric neurons identified by HuC/D-IR (**G-I**) and PGP9.5 (**J-L**). Scale bar 50 µm.

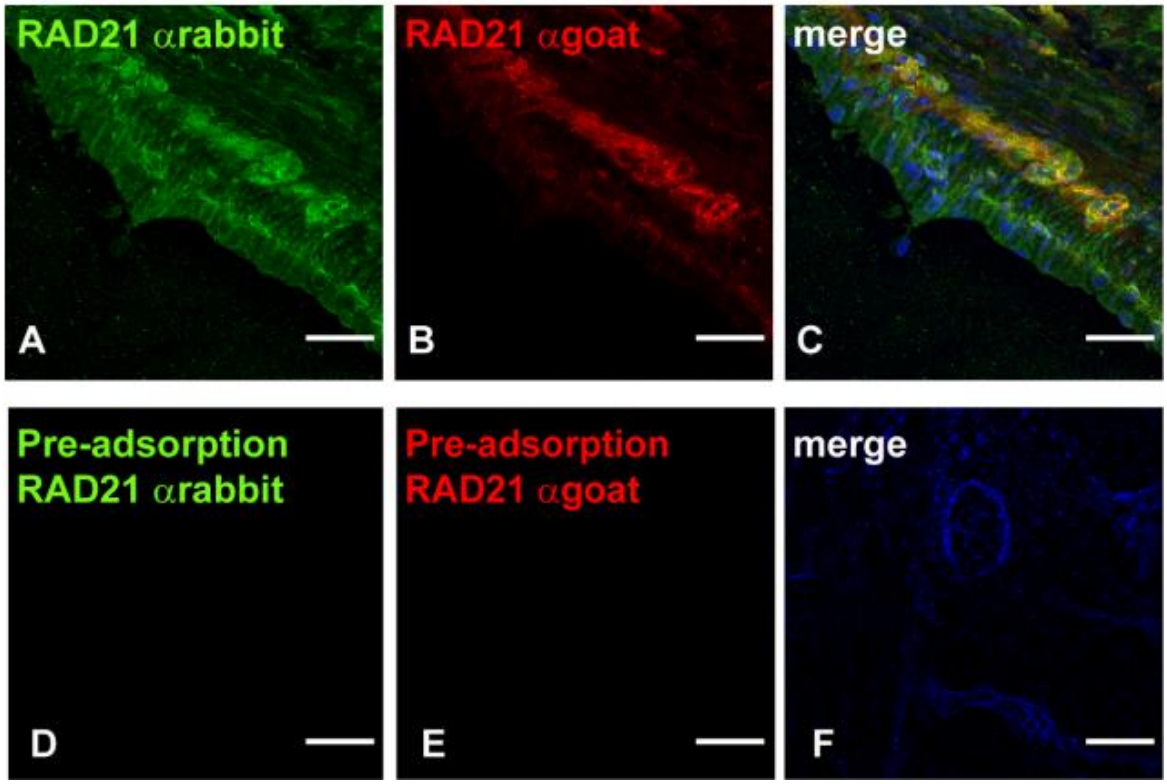

Figure 2. Double labeling with two different RAD21 primary antibodies. Anti-rabbit (abcam ab992) (**A**) and anti-goat (Santa Cruz sc54323) (**B**) anti-RAD21 antibodies labeled the same structures in BALB/C jejunum preparations. Colocalization is shown in yellow (**C**). Pre-adsorption of antibody with specific blocking peptide abolished signal intensity suggesting specificity of antibody used in this study (**D**-**F**). Scale bar 50 μm.

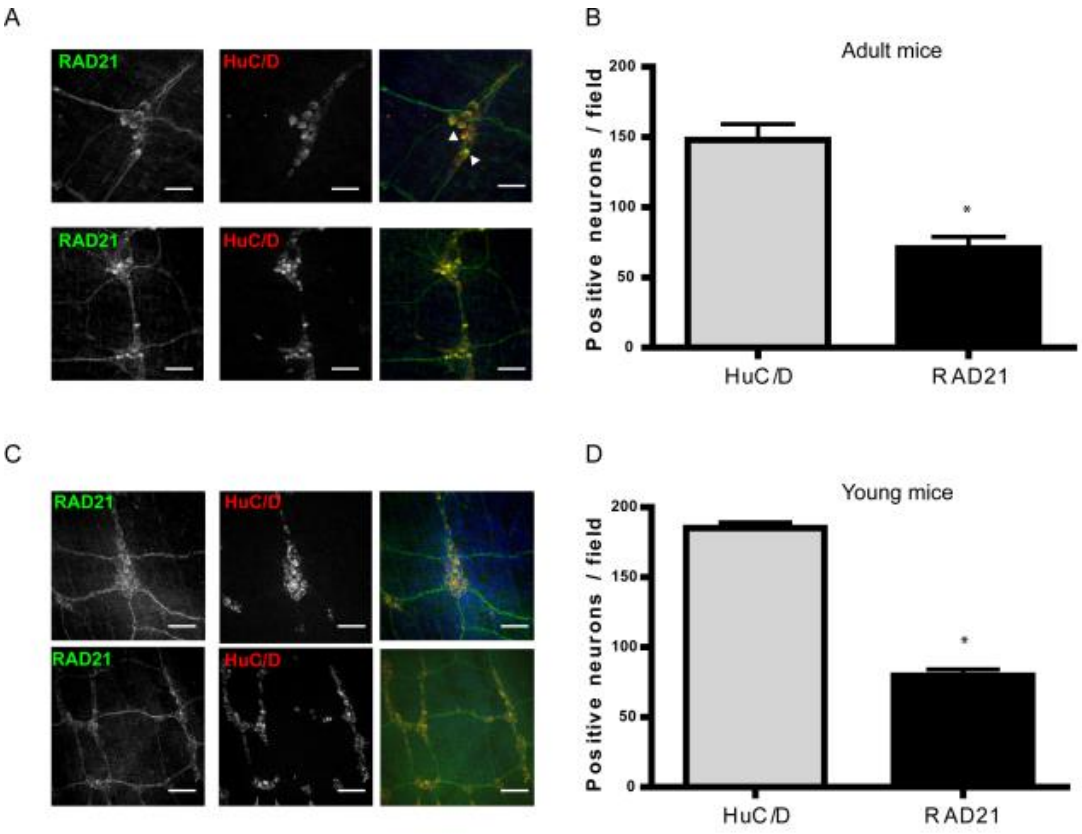

Figure 3. HuC/D and RAD21 Immunolabeling in mouse jejunal whole mounts. (A). Immunolabeling of RAD21 in whole mounts of adult mice. Within the myenteric plexus this pattern was found in 61.56±0.9 and 144.7±4.1 neurons/field for RAD21 and HuC/D, respectively (n=4, P <0.05, t-test). (**B)** Graphs showing the number of neurons per field of RAD21 and HuC/D expression in whole mounts preparations of adult BALB/C mice (n=4, P< 0.05). Scale bar 10 μm (upper panel) and 50 μm (lower panel). (**C)** Immunolabeling of RAD21 in whole mounts of young (4 to 10 day old) BALB/C mice. Within the myenteric plexus this pattern was found in 79.56±4.5 and 185.0±4.0 neurons/field for RAD21 and HuC/D, respectively (n=3 mice, P <0.05, t-test). (**D)** Graphs showing the number of neurons per field of RAD21 and HuC/D expression in whole mounts preparations of young BALB/C mice (n=3 mice, P <0.05). Scale bar 10 μm (upper panel) and 50 μm (lower panel).

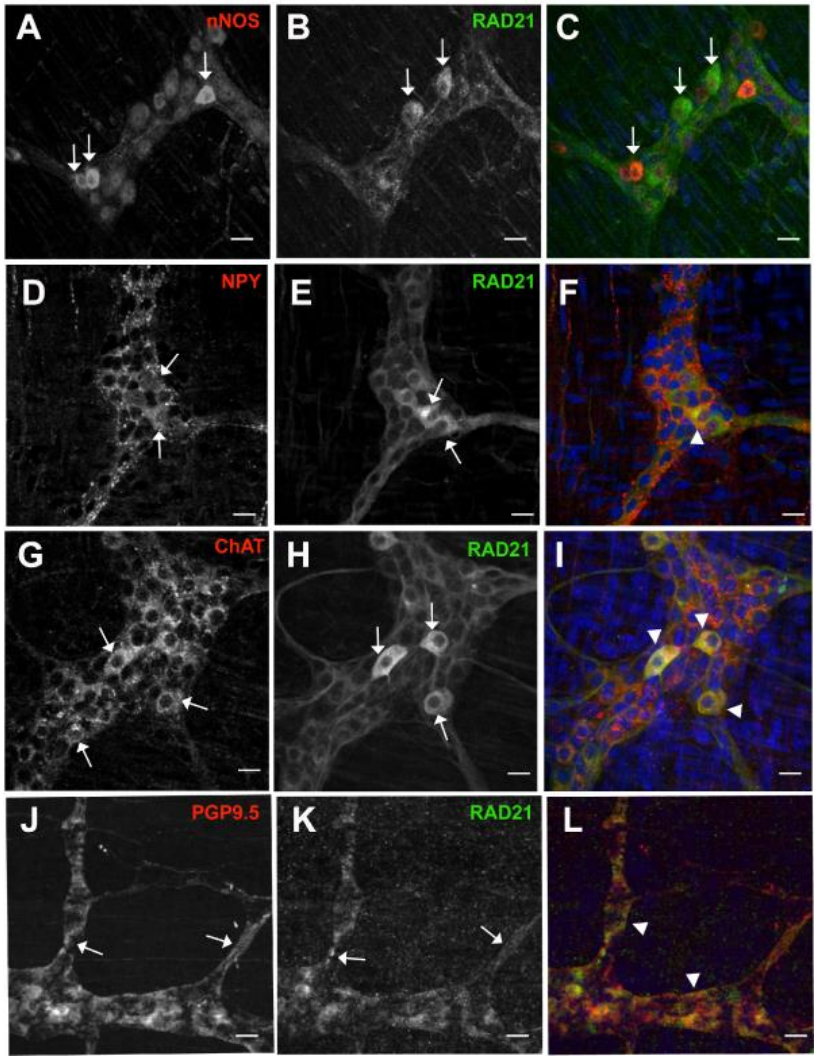

Figure 4. Double labeling of RAD21 with different neuronal markers. RAD21-IR did not colocalize with neuronal nitric oxide synthase (nNOS) (**A-C** arrows). A subset of choline acetyl transferase (ChAT) positive neurons were RAD21-IR (**D-F** arrows). RAD21-IR was also present in neurons that were NPY positive (**G-I**). RAD21-IR was found in a subset of neuronal cell bodies and nerve fibers in the myenteric plexuses of mouse small intestine, labeled by PGP9.5 (**J-L**). Scale bar 50 μm.

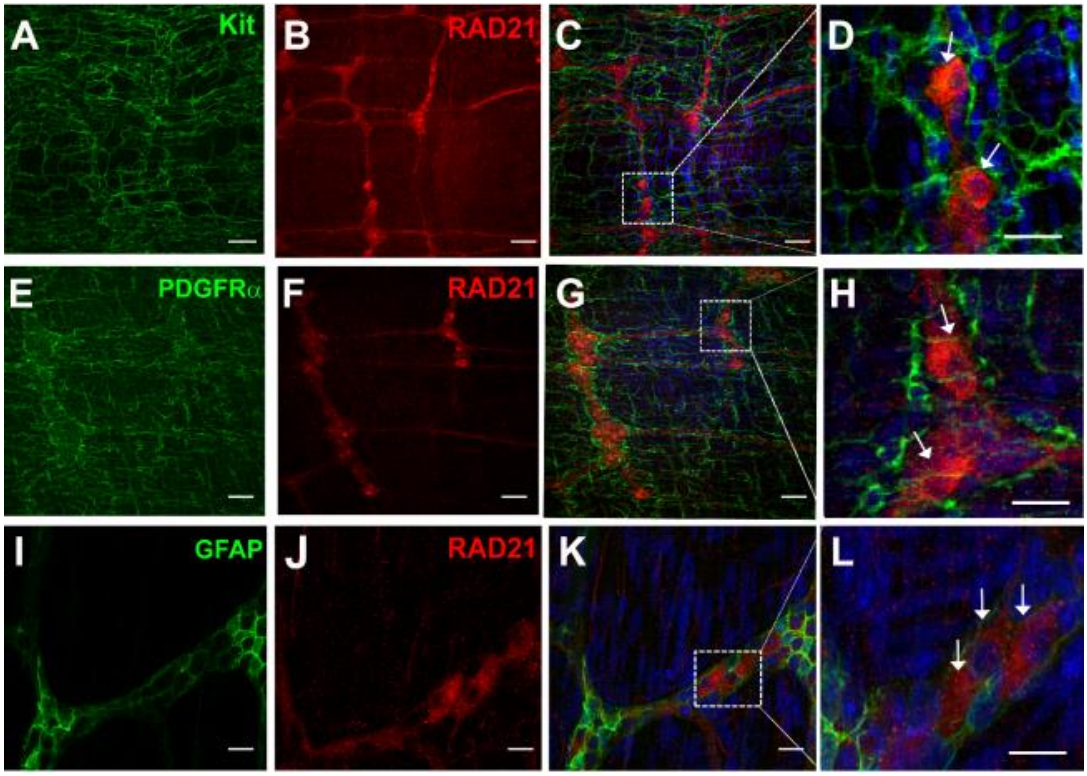

Figure 5. RAD21 did not colocalize with Kit (ICC), PDGFRα and GFAP. RAD21-IR was not detected in whole mounts preparations in the cytoplasm of interstitial cells of Cajal (ICC) with Kit (**A-C**) and in fibroblast-like cells which were positive for PDGFRα (**D-F**). RAD21 was not detectable in glial cells identified by GFAP (**G-I** arrows). Scale bar 50 μm.

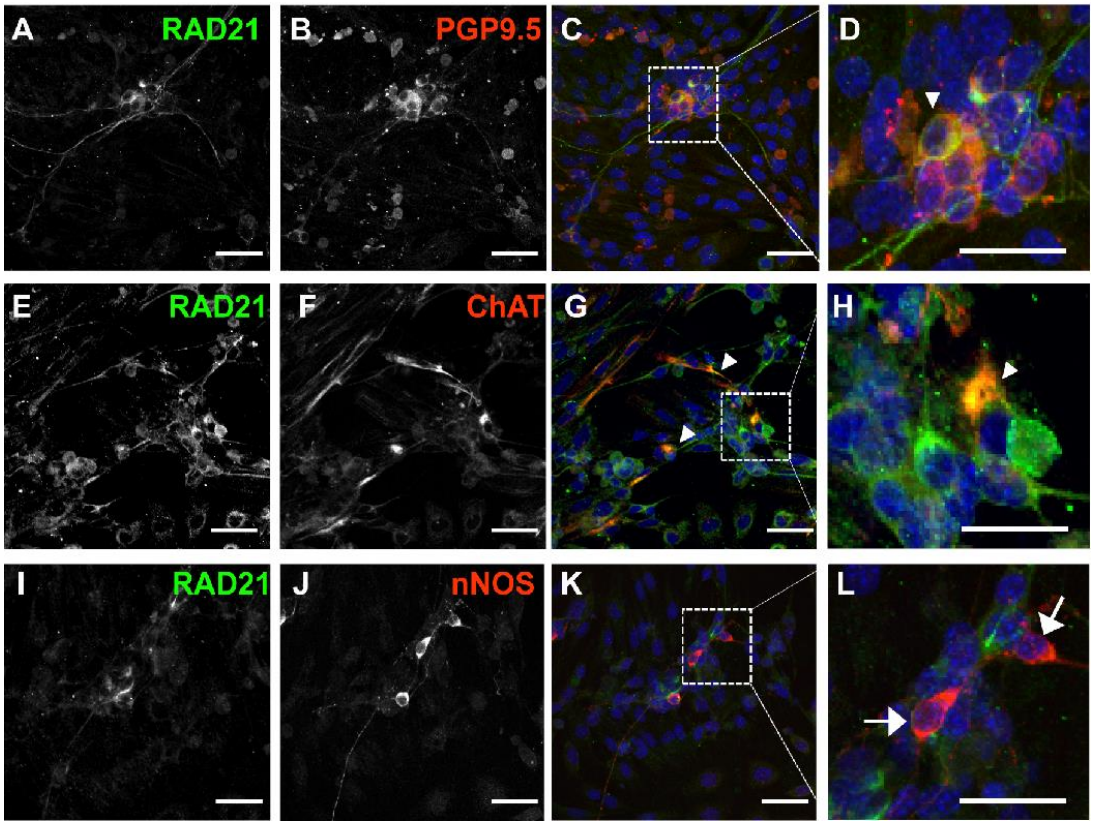

Figure 6. RAD21-IR in primary cultures from small intestine of BALB/C mice. RAD21-IR in primary cultures from mice small intestine showed a similar distribution to that found in tissues. RAD21-IR was observed in cell bodies and nerve fibers of a subset of enteric neurons identified by PGP9.5-IR (**A-C**). RAD21-IR also colocalized with ChAT in some neurons (**D-F** arrows), although none of the nNOS-IR neurons displayed RAD21 immunolabeling (**G-I**). Scale bar 50 µm.

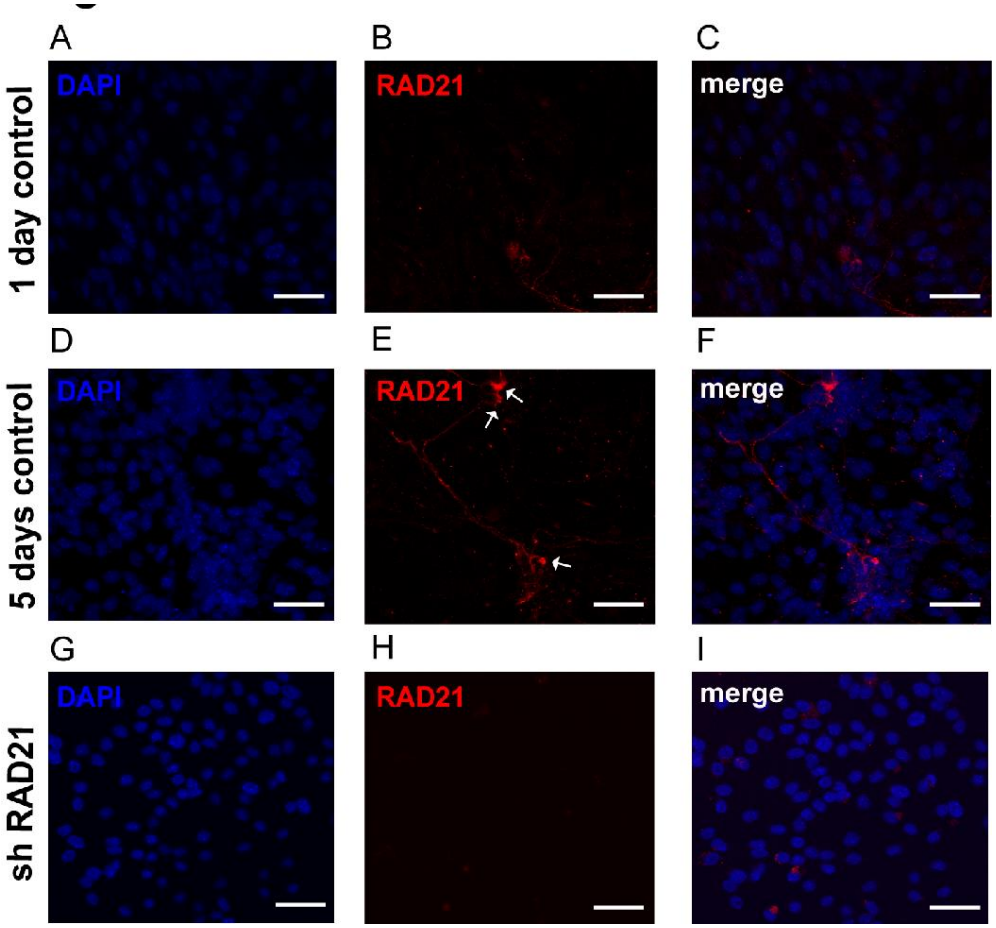

Figure 7. Selective silencing of RAD21-IR. RAD21 knock down in 5 day old primary cultures. Note the absence of RAD21expression in 5 day old primary cultures from the mouse jejunum after shRNA lentiviral particles treatment (**H**). No difference in the density or distribution of RAD21-IR was seen in untreated controls (**B, E**) after 1 or 5 days seeded. Cells were immunolabelled for RAD21 at day 1 and 5 after seeded. Cells were treated with shRNA lentiviral particles at 24 hours after plating for 4 days. Photomicrographs in A, D and G illustrate DAPI for cell nuclei staining. Photomicrographs **C**, **F** and I show merged images. Scale bar 50 μm.

Chapter VIII

12. Protective Actions of Epithelial 5-Hydroxytryptamine 4 Receptors in Normal and Inflamed Colon

Modified from

"Protective Actions of Epithelial 5-Hydroxytryptamine 4 Receptors in Normal and Inflamed Colon"

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Abstract

Background & Aims-The 5-hydroxytryptamine receptor 4 (5-HT4R or HTR4) is expressed in the colonic epithelium but little is known about its functions there. We examined whether activation of colonic epithelial 5-HT4R protects colons of mice from inflammation.

Methods-The 5-HT4R agonist tegaserod (1 mg/kg), the 5-HT4R antagonist GR113808 (1 mg/kg), or vehicle (control) were delivered by enema to wild-type or 5-HT4R knockout mice at the onset of, or during, active colitis, induced by administration of dextran sodium sulfate or trinitrobenzene sulfonic acid. Inflammation was measured using the colitis disease activity index and by histologic analysis of intestinal tissues. Epithelial proliferation, wound healing, and resistance to oxidative stress-induced apoptosis were assessed, as was colonic motility.

Results-Rectal administration of tegaserod reduced the severity of colitis, compared to mice given vehicle, and accelerated recovery from active colitis. Rectal tegaserod did not improve

colitis in 5-HT4R knockout mice, and intraperitoneally administered tegaserod did not protect wild-type mice from colitis. Tegaserod increased proliferation of crypt epithelial cells. Stimulation of 5-HT4R increased Caco-2 cell migration and reduced oxidative stress-induced apoptosis; these actions were blocked by co-administration of the 5-HT4R antagonist GR113808. In non-inflamed colons of wild-type mice not receiving tegaserod, inhibition of 5-HT4Rs resulted in signs of colitis within 3 days. In these mice, epithelial proliferation decreased and bacterial translocation to the liver and spleen was detected. Daily administration of tegaserod increased motility in inflamed colons of guinea pigs and mice, whereas administration of GR113808 disrupted motility in animals without colitis.

Conclusions-5-HT4R activation maintains motility in healthy colons of mice and guinea pigs reduces inflammation in colons of mice with colitis. Agonists might be developed as treatments for patients with inflammatory bowel diseases.

Keywords

IBD; colonic motility; wound healing; mucosal drug action

METHODS

Animal Preparations

All experimental protocols were approved by the Institutional Animal Care and Use Committees of the University of Vermont and the University of Calgary. Animals were euthanized by isoflurane overdose and exsanguination or cervical dislocation. The following animals were used for these studies: 7–8 week Male CD-1 IGS mice from Charles River, Canada; 7–8 week male and female 5-HT4R knockout mice and their litter mates on an SV129 background from Dr. Valérie Compan, Université Montpellier, via Dr. David Linden, Mayo Clinic; 250–300 g male Hartley guinea pigs, Charles River, Canada.

Induction of Colitis

Dextran sodium sulfate (DSS) colitis was induced in mice by administering DSS (w/v in water; 3% for SV129 mice and 4% for CD-1, MW: 36,000–50,000, MP Biomedicals, Solon, OH) for 5 days followed by a return to tap water for 2–10 days. 2,4,6-trinitrobenzene sulfonic acid (TNBS; Sigma-Aldrich, St. Louis, MO) colitis was induced by a single colonic enema

(mice: 7.5 mg/mL in 50% ethanol, 100 μL; guinea pigs: 25 mg/mL in 30% ethanol in 300 μL) delivered under anesthesia.

Colitis Paradigms

For the prevention experiments, mice received DSS for 5 days and were then switched to tap water for 2 days. Alternatively, mice and guinea pigs were given a single enema of TNBS. Enemas with either vehicle (1% dimethyl sulfoxide (DMSO) in 0.9% saline; 0.2 mL/mouse) or drug (tegaserod provided by John McRorie from Proctor and Gamble, GR113808 from Sigma-Aldrich; both delivered at 1 mg/Kg) were administered daily for 5–7 days starting 24 h after induction of colitis. These doses were chosen because they were effective in previous studies of the effects of luminal administration of these compounds on visceral sensitivity (Hoffman JM et al.2012). In a preliminary study, we found that enema treatment did not affect the histological damage score (HDS) (naïve, 0.6±0.25, n=5; vehicle enema, 1.1±0.3; p=0.13, n=7). In another preliminary study, involving enema administration of a vehicle solution containing 0.5% Evans Blue, we found that the solution delivered spread as far orally as the cecum after 10 minutes. Animals were euthanized on day 6 or 7 for TNBS or DSS studies, respectively. In the recovery paradigm drug treatment began on day 6 and lasted for 10 days, with animals euthanized on day 15. The time courses were chosen to test the effectiveness of the treatments leading up to the peak of inflammation, or beginning once the peak had been reached. Transcript levels for the 5-HT4 receptor were not altered in DSS (p=0.56) or TNBS (p=0.9) colitis.

Assessment of Inflammation

Colitis severity was monitored using the disease activity index (DAI), which includes evaluation of weight loss, stool consistency, and presence of fecal blood.11 Fecal blood was assessed using Hemoccult Single Slide testing slides from Beckman Coulter (Brea, CA). After euthanasia, tissue was collected and fixed overnight in 4% paraformaldehyde for immunohistochemistry.

Hematoxylin and eosin stained sections from paraffin embedded tissue were used for histological assessment of colitis. A scoring rubric based on histological features of human inflammatory bowel disease (IBD) was developed (Villanacci Vet al. 2013). This histological damage score (HDS) reflects epithelial damage, altered crypt architecture, infiltration of monocytes and polymorphonuclear cells into the lamina propria and epithelium, and evidence

of ulcers or erosions. Two slides for each experimental group were scored by an observer blinded to the treatment groups.

Intestinal permeability

On day 7 following enema treatment, mice were orally gavaged with FITC-dextran (150 mg/mL, 60 mg/100 g body weight). Four hours following gavage, mice were anesthetized, the chest and abdomen cleaned with 70% ethanol and blood was drawn via cardiac puncture (Savidge TC et al 2007). Blood was allowed to clot, then spun at 2000 × g for 10 minutes, the supernatant was read on a spectrophotometer at 485/535 nm.

Immunohistochemistry

Immunohistochemical staining of sections from paraffin embedded tissue was performed as previously described (Hoffman JM et al. 2012). Immunostaining with a rat anti-mouse Ki-67 primary antiserum (1:100; eBioscience, San Diego, CA) was visualized with a goat anti-rat Cy3 antibody (1:600; Jackson ImmunoResearch, West Grove, PA), in sections counterstained with DAPI (1:1,000; Sigma-Aldrich). The data are presented as proportion of Ki-67 positive cells relative to total epithelial cells per crypt (Joly F et al. 2009). Images were produced on an Olympus AX70 fluorescence microscope and captured using an Optronics MagnaFire digital camera and software.

Motility

Guinea pig distal colon motility was examined using a GastroIntestinal Motility Monitor (GIMM; Catamount Research and Development, St. Albans, VT), and prepared as previously described (Hoffman JM et al. 2010). The most distal 5–10 cm of colon was placed in the organ bath, and equilibrated for 30 minutes in circulating Kreb's solution (37°C). Five trials, spaced by 5 min rest periods, were performed for each colon. Murine colonic transit was assessed as previously described (Nasser Y et al. 2006). Mice were lightly anesthetized using 3% isoflurane, and a small glass bead was inserted 2 cm into the distal colon. Time from the insertion of the bead to expulsion was recorded. This assay was performed before and after treatment with either vehicle, agonist or antagonist. For each time point, measurements were taken on two consecutive days and the two times averaged. For each mouse, bead expulsion time was normalized to the before treatment value.

Intracellular Recording

Intracellular recordings from guinea pig colonic circular muscle were carried out in a Sylgard-lined recording chamber with a circulating, aerated Kreb's solution (37°C) containing nifedipine (5 μM; Sigma-Aldrich) (Roberts JA et al. 2013; Strong DS et al. 2010). Cells were impaled with glass microelectrodes (70 to 120 MΩ, filled with 2M KCl) under visualization with an inverted microscope at 100X magnification. Junction potentials were evoked by transmural stimuli (0.5ms pulse duration, 0.5Hz, 50V). Voltage recordings were obtained with an Axoclamp-2A amplifier (Axon-instruments, Union City, CA, USA) and analyzed with PowerLab Chart (version 5.01; AD Instruments, Castle Hill, NSW, Australia).

Cell lines culture and treatment for oxidative stress

Human Caco-2 cells, a epithelial colorectal adenocarcinoma cell line (ATCC, UK), were maintained in a humidified atmosphere of 95% air and 5% CO2 at 37°C in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin and 100 Ag/mL streptomycin. To produce oxidative stress in Caco-2, cells were treated with 200 μM of H_2O_2 in phosphate-buffered saline (PBS) for 30 min (Clement MV et al. 1998; Strong DS et al. 2010). 24 h after the cells were seeded (Roberts JA et al. 2013).

The sulforhodamine B (SRB) assay was used to determine cell density, as previously described and validated (Vichai V et al. 2006; Clement MV et al. 1998; Strong DS et al. 2010). Cells grown in 24 well plates were treated for 1 hr with vehicle, agonist or agonist plus antagonist. Supernatant was aspirated from wells and cells were fixed with cold trichloroacetic acid solution (30% w/v) at 4°C for 1 h. Fixed cells were washed with H2O and dried, and SRB solution (0.057% w/v) was applied to stain the cellular protein contents. Using spectrophotometry, absorbance was measured at 540 nm with a reference wavelength of 630 nm.

Scratch Assay

Caco-2 cells (provided by Dr. J Turner, University of Chicago) were cultured in 6 well plates to ~90% confluence in DMEM high glucose supplemented with 10% FBS, 1% GlutaMAX, 10 mM Hepes and 100 U/mL Penicillin-Streptomycin (ThermoFisher Grand Island, NY). Once ~90% confluence was reached, three wounds were created using a sterile 200 uL pipet tip dragged perpendicular to a black line drawn on the underside of the plate for reference.

Images were captured of each scratch at time points 0 h and 48 h with a Nikon D7100 camera on a Nikon Diaphot inverted microscope at 4X magnification. Only scratches whose edges could be captured in one frame at time point 0 h were included for final analysis. Measurements were taken from edge to edge at time 0 h and compared to measurements from 48 h using ImageJ software (Schneider CA et al. 2012). The reported values are the difference between 0 h and 48 h, with higher values representing increased cellular migration. Three separate experiments were conducted with all three conditions.

Bacterial Translocation

After euthanasia, mice were wiped with alcohol, and the spleen and liver were removed. Samples were weighed and homogenized in 1 mL sterile PBS. Between samples the homogenizer was rinsed in sterile PBS, water, then 70% ethanol to prevent cross contamination. Homogenates (200 μL) were plated on Columbia and MacConkey agar plates (Dalynn Biologicals, Calgary, AB) and incubated at 37° C in 5% CO2.22 After 48 h, the number of positive plates was determined; contaminated plates were not counted.

Data Analysis

The data are presented as mean ± SEM for n of animals or preparations. Statistical analyses were performed using the GraphPad Prism software application (version 6.0c, GraphPad Software, La Jolla, CA). Data sets were examined prior to analysis to ensure the validity of test assumptions such as similar variability and n-values between groups. In cases in which assumptions were met, data sets were compared using unpaired student's t-test, 1-way ANOVA or 2-way ANOVA with Bonferroni's correction, or Fisher's exact test, when appropriate. The assumptions were not met in two of the experiments: a Welch's correction was used in the case of a t-test and a square root transformation was used to compress variance in the case of an ANOVA. These tests are indicated in the figure legends. Statistical significance was defined as one tailed p-values of less than 0.05.

RESULTS

Effects of Epithelial 5-HT4 Receptor Stimulation on Colitis 5-HT4 Receptor stimulation attenuates the development of colitis—In DSS-inflamed mice, treatment with tegaserod (1 mg/Kg), beginning 24 hr after DSS was introduced, significantly reduced the clinical (DAI,

$p<0.05$) and the histological (HDS, $p<0.001$) damage of the colon compared to vehicle treated DSS inflamed animals (Fig 1A; Supplemental Fig 1). The protective effects of tegaserod were blocked by the 5-HT4 antagonist, GR113808 (Fig 1A; Supplemental Fig 1; DAI, $p<0.05$; HDS, $p<0.0001$).

In TNBS colitis, treatment with tegaserod significantly reduced the DAI as compared to inflamed controls (Fig 1B; $p<0.05$); however, the HDS was not changed. The protective effect of tegaserod on the DAI was blocked by the 5-HT4 antagonist (Fig 1B; $p<0.05$).

To further confirm that tegaserod was mediating its protective action via 5-HT4 receptor activation, experiments were conducted with 5-HT4 knockout mice and their wild type littermates with DSS colitis. Tegaserod failed to improve the DAI or HDS in mice lacking the 5-HT4 receptor (Fig. 1C), but it significantly reduced inflammation in the wild type animals (DAI: vehicle, 7.7±0.5; agonist, 5.5±0.5; $p=0.004$; HDS: vehicle, 10.3±0.6; agonist, 8.2±0.3; $p=0.017$ by t-test; n=7–11 per group).

5-HT4 receptor stimulation accelerates healing from established colitis—To test whether activation of epithelial 5-HT4 receptors in the distal colon affects the recovery from colonic inflammation, animals were treated with tegaserod after colitis was established (days 6–15; Fig. 2). 5-HT4 agonist treatment significantly accelerated the recovery from DSS colitis as compared to vehicle treated animals (Fig 2A; $p<0.0001$ at day 15). Furthermore, DSS-inflamed mice treated with tegaserod showed significant improvement in HDS (Fig 2A; $p<0.0001$). These effects were blocked by the 5-HT4 antagonist (Fig 2A; DAI, $p<0.001$; HDS, $p<0.001$).

5-HT4 agonist treatment in established TNBS colitis accelerated recovery of the DAI (Fig 2B; $p<0.001$ at day 15) and improved the HDS ($p<0.01$), and these actions were also inhibited by antagonist treatment (Fig 2B; DAI, $p<0.001$; HDS, $p<0.05$).

Intraperitoneal administration of the 5-HT4 receptor agonist fails to affect colitis—To test whether the effects of enema-administered 5-HT4 agonist could involve 5-HT4 receptors at other sites in the GI tract or elsewhere, the agonist was delivered daily by intraperitoneal (IP) injection at the same dose (1 mg/Kg) beginning on day 1. In DSS-inflamed animals, IP administered agonist had no effect on the clinical or histological scores (DAI: vehicle, 5.1±0.6; agonist, 4.5±0.5; $p=0.4$; HDS: vehicle, 10.0±0.9; agonist, 7.8±0.9; $p=0.1$ by t-test; n=7–8 per group).

5-HT4 receptor mediated protective mechanisms

Epithelial 5-HT4 receptor activation could mediate the protective effects via a variety of mechanisms, including maintenance or reestablishment of the epithelial barrier through cell proliferation and migration, and also by increasing resistance to epithelial apoptosis induced by oxidative stress. We first assessed epithelial permeability by evaluating FITC-dextran in serum by spectroscopy (arbitrary fluorescence units; AFUs) following gastric gavage. Colitis was associated with a 3–4 fold increase in permeability (control, 3530 ± 125 AFUs, n=17; DSS, 15,690 ± 3222 AFUs, n=22, $P<0.01$). Despite the fact that the cecum and entire colon is affected in DSS colitis, a tegaserod enema in the distal colon demonstrated a tendency to reduce the permeability to FITC-dextran (9,372 ± 944 AFUs, n=16, $P=0.07$). Associated with the increase in epithelial permeability there is a degree of bacterial translocation associated with colitis (4/9 animals with colitis had bacterial translocation to the liver or spleen compared to 0/5 control animals). After tegaserod enema there was again a tendency for this to be reduced (1/8 animals with bacterial translocation). These actions prompted us to examine detailed mechanisms of action of 5-HT4 receptor activation in the epithelium.

The effects of 5-HT4 receptor activation on proliferation were tested in the DSS recovery paradigm, as this condition yielded the most robust response. Effects of 5-HT4 receptor stimulation on migration and resistance to oxidative stress were evaluated in Caco-2 cells, which were found to express the 5-HT4 receptor by rtPCR and immunoblot (Supplemental Fig. 2).

The nuclear protein, Ki-67, is an effective marker of post-mitotic cells (Joly F et al. 2009). In the colons of DSS-inflamed animals that received daily agonist enemas beginning on day 6, there was a significant increase in the percentage of crypt epithelial cells that were Ki-67 positive at day 15 (Fig. 3; $p<0.05$; Supplemental Fig 3), and this effect was blocked by the 5-HT4 antagonist (Fig. 3; $p<0.01$; Supplemental Fig 3). The proportion of epithelial cells immunoreactive for Ki-67 was also significantly higher in colons from animals treated with DSS and agonist beginning on day 1 and euthanized on day 7 (vehicle, 0.5±0.04 vs 0.7±0.02; $p<0.001$; n=5 per group).

An important mechanism of epithelial healing is enhanced epithelial cell migration (Heath JP et al. 1996). To assess this we performed a scratch wound healing assay (Denker SP et al. 2002) and saw a significant increase in the rate of Caco-2 cell migration in cultures treated

with tegaserod (1 μM) (Fig. 4A,C; $p<0.001$), and this effect was inhibited in the presence of the antagonist (Fig. 4A,C; $p<0.05$).

Oxidative stress is a feature of colitis (Shi XZ et al. 2011), and triggers epithelial apoptosis (Novak EA et al. 2015, Becker C et al. 2013). Therefore, Caco-2 cells were exposed to the free radical donor, H_2O_2 (200 μM), and cell survival in response to 5-HT4 receptor stimulation was determined using the SRB assay. H_2O_2 caused a significant reduction in cell survival compared to untreated cells (Fig. 4B; $p<0.001$), and cell survival in H_2O_2 treated cultures was significantly improved by tegaserod (10 μM) (Fig. 4B; $p<0.001$). This agonist-mediated protection was blocked by the specific antagonist, GR113808 (10 nM) ($p<0.001$).

Effects of 5-HT4 Receptor Activation on Propulsive Motility

A central feature of IBD is altered GI motility (Reddy SN et al 1991, Brierley SM et al 2014). We have investigated dysmotility in guinea pig TNBS colitis (Roberts JA et al 2013; Strong DS et al. 2010; Linden DR et al. 2004; Hoffman JM et al. 2011), and have demonstrated that changes in enteric neuronal excitability (Hoffman JM et al. 2011) and purinergic inhibitory neuromuscular transmission (Roberts JA et al 2013) contribute to disrupted motility. We therefore treated TNBS-inflamed guinea pigs daily with tegaserod enemas (1 mg/Kg) for 6 days beginning 24 h after TNBS instillation, and evaluated propulsive motility.

Consistent with previous findings (Roberts JA et al 2013; Strong DS et al. 2010; Hoffman JM et al. 2011; Linden DR et al. 2003) the distal colons of TNBS-inflamed animals exhibited a significant reduction in the rate of propulsive motility (Fig. 5A; $p<0.0001$), and an increase in trials in which fecal pellets became obstructed (Fig. 5B; $p<0.0004$). Tegaserod significantly improved the rate of propulsive motility in TNBS inflamed colons ($p<0.0001$), and eliminated the obstructions (Fig. 5A,B). Antagonist treatment blocked the protective effects of tegaserod on the rate of propulsive motility ($p<0.0001$), and on the occurrence of obstructions ($p<0.0001$). It is worth noting that preparations from animals receiving antagonist treatment were more frequently obstructed than vehicle-treated TNBS inflamed preparations ($p=0.0012$).

Since the disruption of propulsive motility in guinea pig TNBS colitis involves an attenuation of inhibitory junction potentials (IJPs), we measured IJP amplitudes in preparations from animals treated with tegaserod. Daily agonist treatment significantly improved the IJP in TNBS inflamed animals (naïve: −19.1 mV ± 1.3, TNBS with vehicle: −9.4 mV ± 0.9, TNBS with agonist: −18.1 mV ± 0.6; $p<0.0001$ by one-way ANOVA).

Colonic motility was also evaluated in vivo in mice in the 15 day recovery paradigm using the bead expulsion assay. As was detected in the TNBS inflamed guinea pig colon, mice with DSS colitis exhibited a slowing of colonic transit that was inhibited by tegaserod treatment (Fig. 5C). Furthermore, the effect of tegaserod (1mg/Kg) was blocked by the 5-HT4 antagonist, GR113808 (1 mg/Kg).

Epithelial 5-HT4 receptors play a protective physiological role in healthy animals

The findings described above indicate that 5-HT4 receptor activation decreases the extent of colitis as it develops, and accelerates recovery from colitis once it has been established, raising the possibility that 5-HT4 receptors could serve as a novel therapeutic target for the treatment of colitis. These results also suggest that 5-HT4 receptors might play a role in maintaining the integrity of the epithelial layer under physiological conditions.

To test whether 5-HT4 receptor activity influences epithelial integrity, normal mice were treated for 10 days with the 5-HT4 antagonist, GR113808 (1 mg/Kg), administered by enema. Daily treatment of mice with the 5-HT4 antagonist showed a significant increase in the DAI ($p<0.0001$; Fig. 6A) and HDS ($p<0.0001$; Fig. 6B, Supplemental Fig. 4).

Consistent with the effect of pharmacologically inhibiting the receptor, 5-HT4 knockout mice exhibited a significantly higher HDS than wild type littermates ($p<0.05$; Fig. 6C)

The results from colitis paradigms described above demonstrate that 5-HT4 receptor stimulation by agonist administration increases epithelial proliferation, as measured by Ki-67 immunoreactive cells. Therefore, Ki-67 immunoreactivity was evaluated in normal animals treated with GR113808. In animals treated with the antagonist alone, there was a significant reduction in Ki-67 immunoreactivity compared to vehicle-treated controls (Fig. 7A; $p<0.0001$).

Evaluation of bacterial translocation from the gut to either the spleen or the liver has been shown to be an effective assay to assess barrier permeability (MacEachern SJ et al. 2015; Deitch EA et al. 1992). Antagonist treatment in normal mice led to a significant increase in the proportion of cultures that were positive for bacterial translocation as compared to vehicle-treated animals (Fig. 7B; $p<0.02$).

To test whether endogenous 5-HT4 receptor activation influences colonic function, propulsive motility was evaluated in distal colons from guinea pigs treated daily for 10 days with the antagonist alone. Treatment with the antagonist did not have a significant effect on the rate of propulsive motility; however, fecal pellet obstruction was observed in 25% of trials in colons

from animals receiving antagonist treatment, which was significantly different from the control patterns (Fig. 7C; p=0.0035).

DISCUSSION

This study tested the hypothesis that epithelial 5-HT4 receptor activation attenuates the development of colitis, and improves recovery from active colitis. Our findings support this hypothesis by demonstrating that epithelial 5-HT4 receptor stimulation reduced disease activity and histological damage in both DSS and TNBS colitis, supporting an anti-inflammatory effect. The epithelial 5-HT4 receptor stimulation can exert its protective effects through several mechanisms, including increased epithelial proliferation, enhanced epithelial cell migration, and resistance to oxidative stress-induced apoptosis. Furthermore, treatment with the 5-HT4 agonist attenuated inflammation-induced changes in colonic motor function. Importantly, all of these effects were blocked by the 5-HT4 antagonist, GR113808, and protection was not detected in 5-HT4 KO mice. Our findings also indicate that epithelial 5-HT4 receptors serve an important physiological role in maintaining mucosal integrity since inhibition of 5-HT4 receptor activity in normal animals leads to inflammation and disrupted motor function. Collectively, these studies contribute new knowledge regarding the protective actions of 5-HT4 receptor activation, and provide evidence for an anti-inflammatory role of 5-HT4 in normal physiology.

Prior to the current investigation, it had been established that 5-HT can exert a pro-inflammatory influence in the GI tract (Gershon MD 2012). For example, colitis is reduced in mice lacking or deficient in mucosal 5-HT (Ghia JE et al. 2009), and it is worsened in SERT knockout mice, which have elevated mucosal 5-HT availability (Bischoff SC et al 2009). This effect is likely mediated by activation of 5-HT7 receptors on dendritic cells in the lamina propria (Bertrand PP et al. 2004). These previous studies examined the global effect of gut-derived 5-HT on inflammation. The current study specifically examined the role of 5-HT4 receptor activation in the context of inflammation and we found an anti-inflammatory role of 5-HT signaling in the mucosa, supporting previous work from our labs suggested that activation of these receptors may be protective (Hoffman JM et al. 2012). It will be interesting, in future studies, to directly compare the relative pro- and anti-inflammatory effects of mucosal 5-HT signaling. Regardless, during colitis, the protective actions of 5-HT4 stimulation by endogenous 5-HT are dominated by an over-riding influence of pro-inflammatory mediators and mechanisms. On the other hand, this does not preclude the

possibility that stimulation of the 5-HT4 receptor pharmacologically could have a beneficial effect, as has been demonstrated in the current study.

In addition to these protective, anti-inflammatory actions of epithelial 5-HT4 receptors, there is evidence that 5-HT4 receptors play a beneficial, neurogenic effect in the muscularis. Activation of 5-HT4 receptors in primary cultures of enteric neurons promotes neuronal growth and survival, and in vivo, agonist treatment promotes neurogenesis in adult mice (Liu MT et al. 2009). This neuro-protective action has been supported by in vivo studies demonstrating that recovery of the recto-anal reflex is significantly augmented, through neurogenesis and axon outgrowth, by 5-HT4 receptor treatment following rectal transection and anastomosis (Takaki M et al. 2014; Matsuyoshi H et al- 2010; Kawahara I et al 2012). Furthermore, 5-HT4 receptor-mediated enteric neurogenesis occurs in colitis (Belkind-Gerson J et al. 2015) a condition in which bioavailability of 5-HT is increased (Mawe GM et al. 2013). Taken together with the results reported here, it is becoming increasingly clear that the 5-HT4 receptor exerts protective actions in the inner and outer layers of the gut.

Several mechanisms appear to contribute to the protective effects of epithelial 5-HT4 receptor stimulation, and these mechanisms are apparently effective for both Th1-(TNBS) and Th2-predominant (DSS) inflammatory responses. One mechanism by which 5-HT4 receptor stimulation is acting is through enhanced wound healing processes. 5-HT4 receptor stimulation increased both cell proliferation and epithelial cell migration in a 5-HT4 antagonist-sensitive manner. Epithelial erosions, ulcers and decreased epithelial barrier integrity are all common features of active colitis, and these conditions would likely be mitigated by enhanced epithelial proliferation and migration.

The anti-inflammatory effects of 5-HT4 receptor activation may also involve resistance of the epithelium to the harmful effects of oxidative stress. Oxidative stress, and resultant epithelial apoptosis, is a key feature of inflammation and has been demonstrated in both DSS and TNBS colitis. Treatment with a 5-HT4 agonist protected CaCo-2 cells from apoptosis that was elicited by the free radical donor, H_2O_2, in a 5-HT4 antagonist-sensitive manner.

Another unexplored mechanism that likely contributes to the protective effect of 5-HT4 receptor stimulation is secretion of mucus from goblet cells. The mucus layer serves as a protective barrier, and disruption of this barrier with mucolytic agents or deletion of the mucin 2 gene results in colitis (Van der Sluis Met al. 2006; Johansson ME et al. 2014). Goblet cells express the 5-HT4 receptor, and 5-HT4 receptor activation leads to degranulation (Hoffman JM et al. 2012).

The guinea pig distal colon ex vivo model of motility is probably the most extensively characterized animal model of propulsive motility (Hoffman JM et al. 2010; Costa M et al. 1976; D'Antona G et al 2001). We have previously used this assay to investigate changes in motility that are associated with TNBS colitis (Strong DS et al 2010; Linden DR et al. 2003), and we have linked disruptions in motility to inflammation-induced neuroplasticity, particularly intrinsic primary afferent neuron hyperexcitability (Hoffman JM et al. 2011) and attenuated purinergic neuromuscular transmission (Roberts JA et al. 2013). In the current study, treatment of TNBS-inflamed guinea pigs, and DSS-inflamed mice with the 5-HT4 agonist improved propulsive motility and eliminated obstructive motility patterns. Consistent with our previous report linking disrupted motility to IJP attenuation (Roberts JA et al. 2013), the amplitude of the IJP is comparable to that of control animals following 5-HT4 agonist treatment in TNBS colitis animals.

Data from studies reported here involving various models and paradigms of 5-HT4 receptor stimulation in colitis indicate that the 5-HT4 receptor plays a host defense role in inflammatory conditions through a number of actions that support the epithelial barrier and resistance to damage from oxidative stress. These novel findings, and the knowledge that 5-HT released from enterochromaffin cells reaches the lumen (Bertrand PP et al 2004; Patel BA et al. 2007), led to the question of whether 5-HT4 receptor activity exerts a protective influence in the mucosal layer under physiological conditions. Treatment of normal mice with a 5-HT4 receptor antagonist led to increased DAI and HDS scores, bacterial translocation, and reduction in cell proliferation. Furthermore, inhibition of 5-HT4 receptors in the colonic epithelium of normal guinea pigs resulted in obstructed motility patterns, which is not a feature of the healthy colon. Also, antagonist treated mice exhibited slowed colonic transit. Consistent with these results, there were situations in our colitis paradigms in which antagonist treatment not only blocked the agonist, but led to a condition far worse than the vehicle treated inflamed group. This included the histological damage in the DSS mouse colitis prevention paradigm (Fig. 1) as well as the obstructed motility pattern in guinea pigs with TNBS colitis (Fig. 7). These findings suggest that endogenous 5-HT may be acting on epithelial 5-HT4 receptors to dampen physiological inflammation and maintain homeostasis.

It is possible that treatment with the 5-HT4 antagonist mediates its effect by blocking stimulation of epithelial 5-HT4 receptors by 5-HT released from enterochromaffin cells. Another possibility is that the antagonist that was used, GR113808, decreased constitutive activity of the epithelial 5-HT4 receptors. Certain isoforms of the 5-HT4 receptor have low

levels of constitutive activity, which could lead to a steady state activation of the protective pathways stimulated by 5-HT4 receptor activation with an agonist (Claeysen S et al. 2001; Blondel O et al 1998; Claeysen S et al. 2000). The antagonist that was used in the current studies, GR113808, can suppress this constitutive activity by acting as an inverse agonist (Kamel R et al. 2005).

Conclusion

Here we report the discovery of a protective and healing action of epithelial 5-HT4 receptor stimulation in the colon. Translation of these observations could provide a new and safe treatment strategy for the treatment of colitis, and that expand our appreciation of the roles of 5-HT receptor signaling in the GI tract. Treatment in two different models of colitis decreased the extent of inflammation as it was occurring, and accelerated the process of remission once colitis had been established. This beneficial effect likely involves several mechanisms that include enhanced wound healing, resistance to oxidative stress, and improved colonic motor function. Thus these findings demonstrate that luminally restricted, and therefore low risk, administration of 5-HT4 agonists could be beneficial in the treatment of IBD. The discovery that 5-HT4 receptors also contribute to the epithelial integrity in healthy animals reveals a newly identified role for 5-HT signaling in the mucosal layer, and one that physiologically opposes the previously identified pro-inflammatory actions of 5-HT in the colon. Collectively, these findings advance our understanding of colonic physiology and pathophysiology, and provide a new target for the treatment of colonic inflammation.

Figures

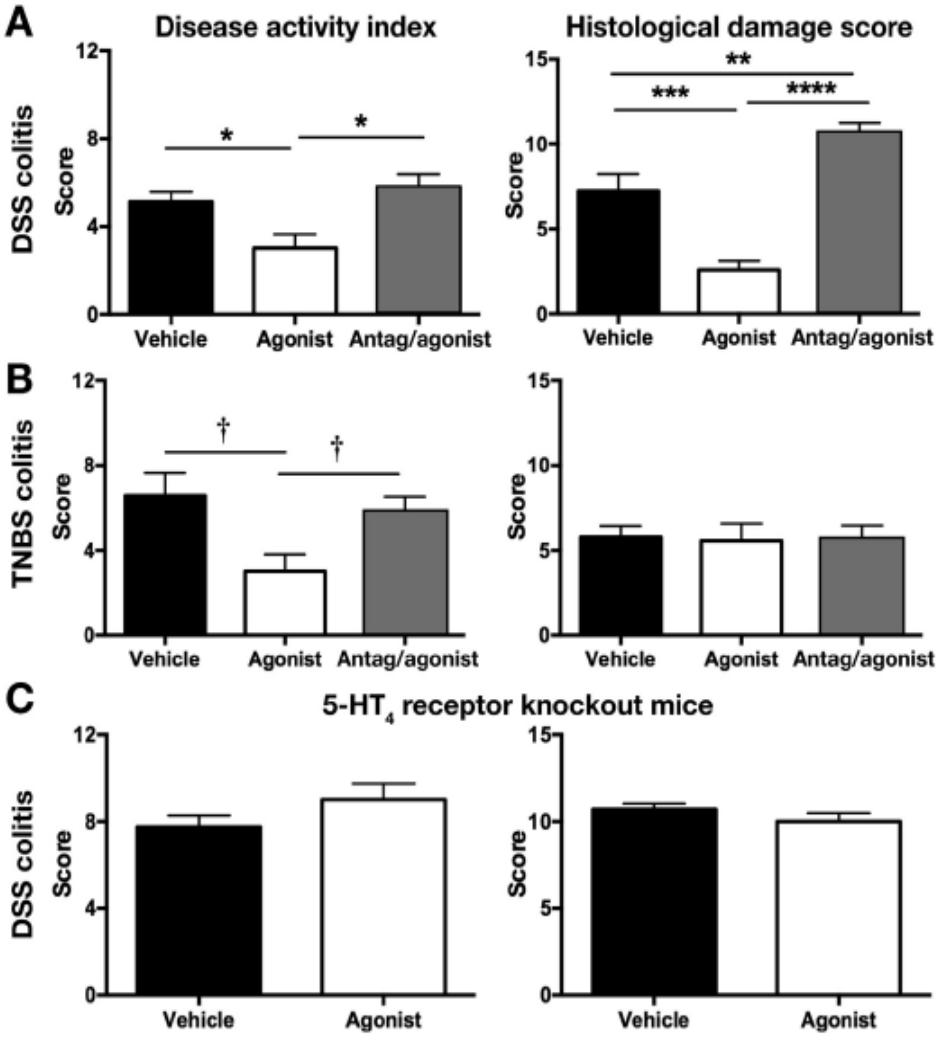

Figure 1: Daily intraluminal treatment with the 5-HT4 agonist, tegaserod (1mg/Kg) reduced the extent of DSS and TNBS colitis. A. In DSS colitis, tegaserod caused an antagonist-sensitive reduction in the DAI and HDS (vehicle, n=9; agonist, n=10; agonist/antagonist, n=5; for HDS, 2 values were obtained from each animal). B. In TNBS colitis, agonist treatment significantly reduced the DAI, but did not affect the HDS (n=7 animals for all groups, with 2 values per animal for HDS). C. Tegaserod failed to improve DAI or HDS in DSS inflamed 5- HT4 knockout mice (n=8–9 animals per group, with 2 values per animal for HDS). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 by one-way ANOVA. †p<0.05 by one-way ANOVA with square root transformation.

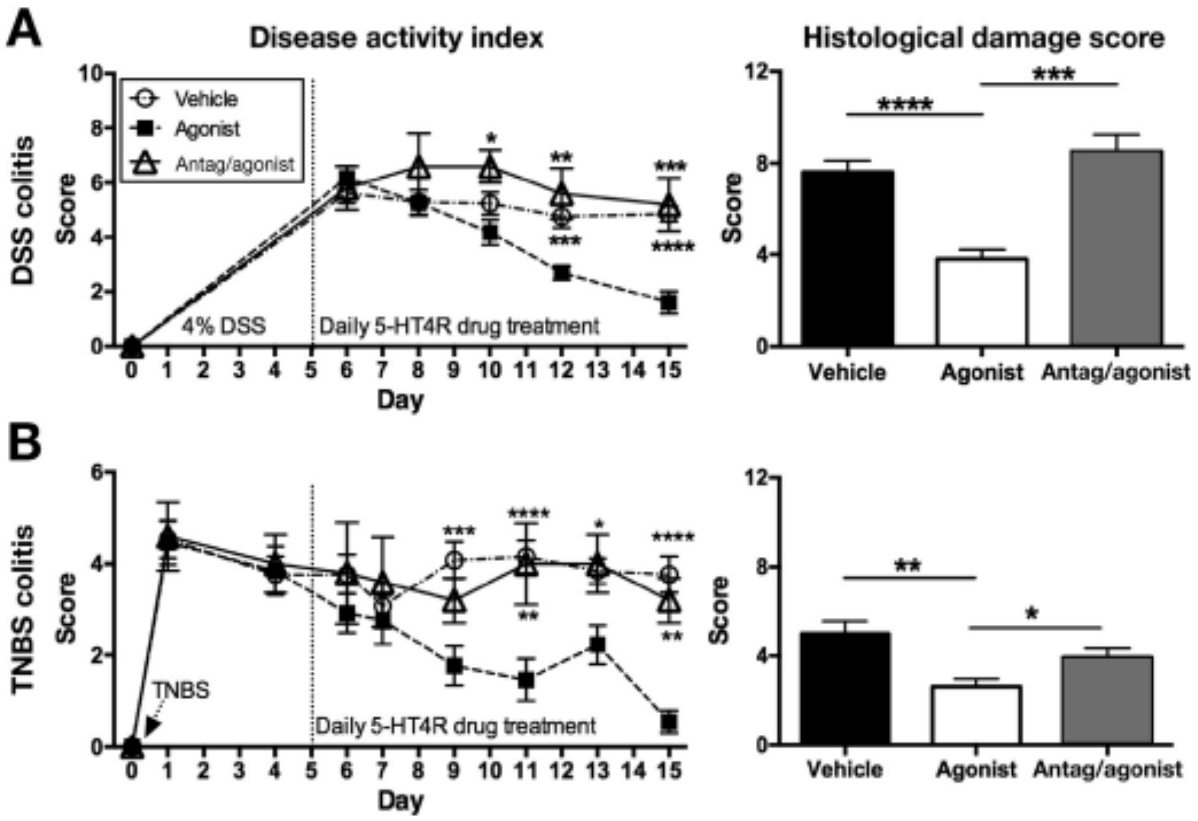

Figure 2: Daily intraluminal treatment with the 5-HT4 agonist, tegaserod (1mg/Kg) beginning on day 6, after colitis had been established, accelerated recovery from DSS and TNBS colitis. A. In DSS colitis, the 5-HT4 receptor agonist significantly improved the DAI by day 12, and at the termination of the experiment on day 15, both the DAI and HDS were significantly improved as compared to the vehicle control and antagonist plus agonist treatment groups. (vehicle, n=21; agonist, n=23; antagonist, n=5 with 2 values per animal obtained for HDS). C. In TNBS colitis, significant improvement in the DAI was detected on Day 9, and at the day 15 time point, both the DAI and HDS were significantly improved (vehicle, n=13; agonist, n=14; antagonist, n=5 with 2 values per animal obtained for HDS). *p<0.05;**p<0.01; ***p<0.001; ****p<0.0001 by two-way ANOVA for DAI and one-way ANOVA for HDS. In the DAI graphs, comparisons were made between agonist treatment and the other groups.

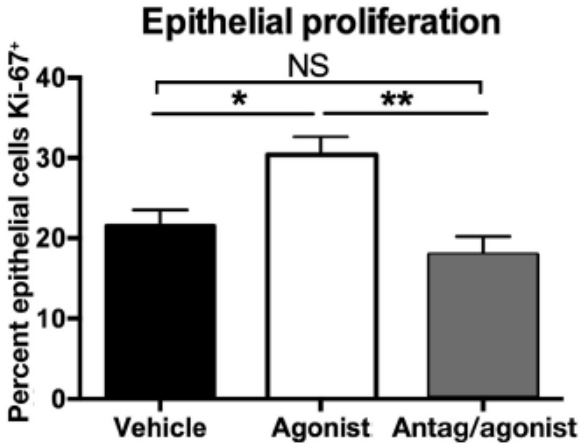

Figure 3: Intraluminal treatment with tegaserod increased epithelial proliferation. Graph demonstrating the proportion of crypt epithelial cells immunoreactive for the proliferation marker, Ki-67 (vehicle, n=10; agonist, n=9; antagonist, n=9). * p<0.05, **p<0.01 by oneway ANOVA.

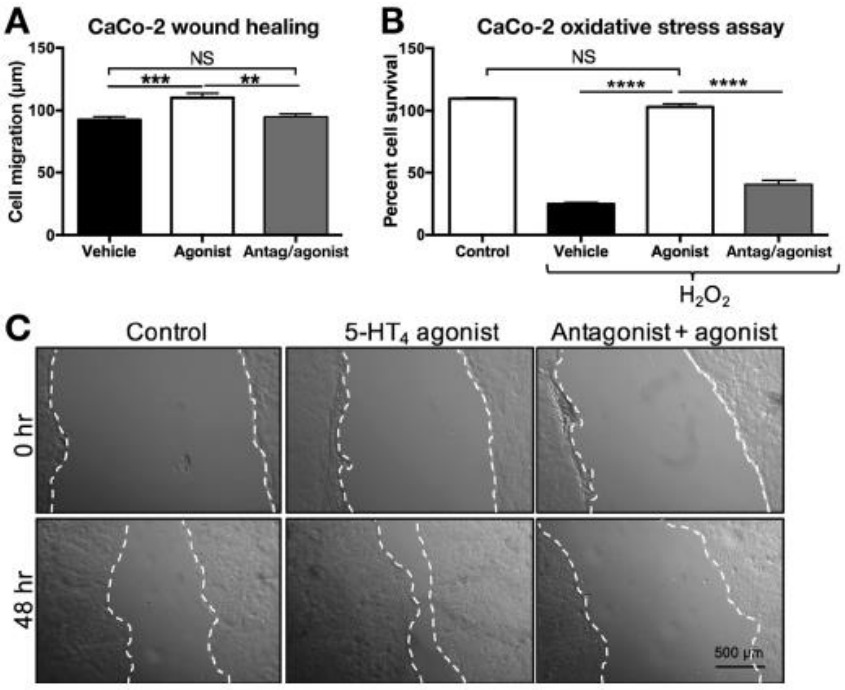

Figure 4: Treatment of Caco-2 cells with tegaserod increased the rate of cell migration, and caused protection from cell loss due to oxidative stress. A. Closure of scratches in Caco-2monolayer cultures after 48 h (1 well per treatment group, 3 scratches per well × 3 experiments). B. Survival data from cultures that were treated with normal medium (control) or 200 µM H_2O_2 (n=4 per group). C. Photomicrographs of Caco-2 cultures showing scratches at the 0 and 48 h time points. ** p<0.05, **** p<0.0001; one-way ANOVA.

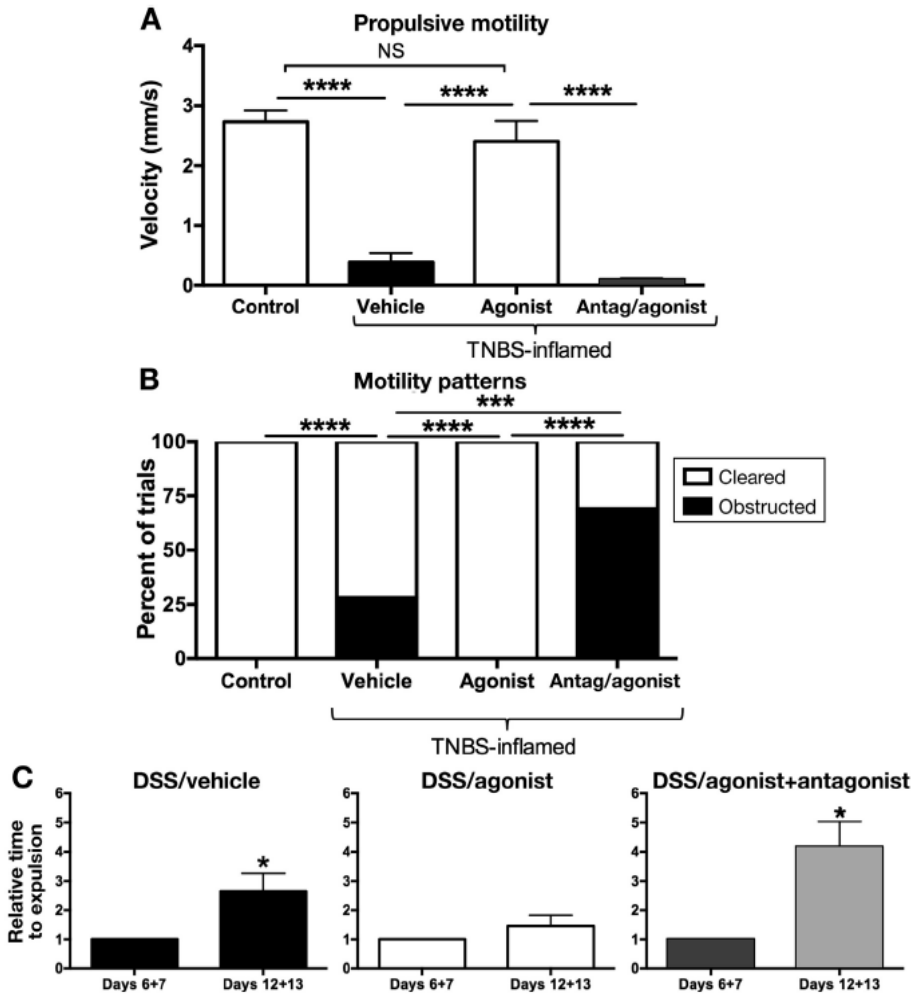

Figure 5: Daily intraluminal treatment with tegaserod (1 mg/Kg) improved distal colon propulsive motility in guinea pigs with TNBS colitis. A. Graph illustrating rate of pellet propulsion along the length of the colon (control, n=6; TNBS/vehicle, n=9; TNBS/agonist, n=6; TNBS/agonist plus antagonist, n=4; ****p<0.0001, one-way ANOVA). B. Graph showing the proportion of trials in which the fecal pellet cleared the colon or was obstructed, and did not clear the colon within 5 min (5–6 trials per animal; ***p<0.001, ****p<0.0001, Fisher's Exact Test). C. Graphs demonstrating results of bead expulsion assays from mice with DSS colitis in the 15 day recovery paradigm that were treated in vivo by enema (DSS/vehicle, n=5; DSS/agonist, n=4; DSS/agonist plus antagonist, n=5; *p<0.05, paired t-test).

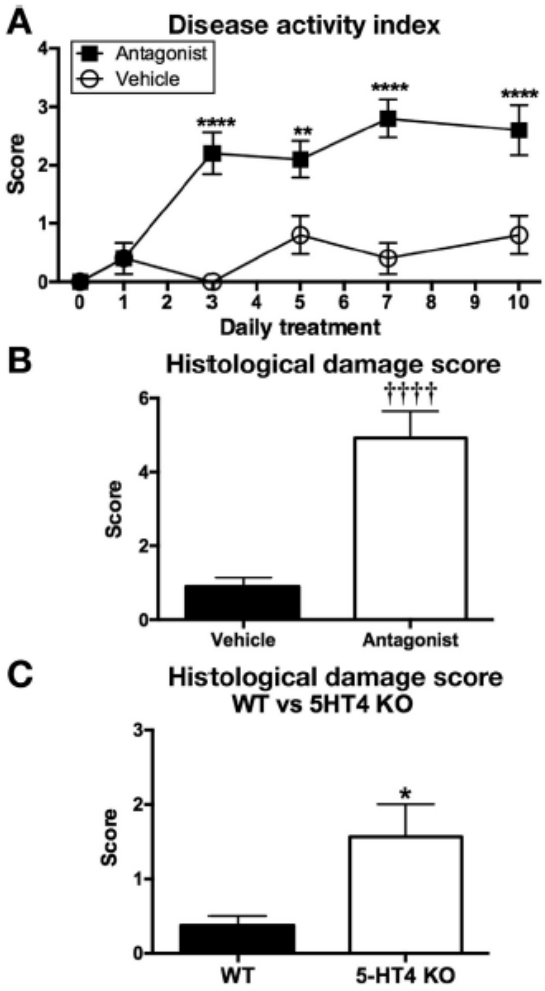

Figure 6:Pharmacological or molecular disruption of 5-HT4 signaling increased inflammatory scores in normal mice. Intraluminal administration of the 5-HT4 antagonist GR113808 (1 mg/Kg) induced colitis in mice. Antagonist treatment resulted in a significant increase in the DAI that was detected as early as day 3 (A), and an increase in the HDS (B). n=10/group. **p<0.01; ****p<0.0001 by two-way ANOVA; ††††p<0.0001 by t-test with Welch's correction. C. The HDS was significantly higher in 5-HT4 knockout mice, as compared to wild type littermates (*p<0.05 by t-test; WT, n=4; 5-HT4 KO, n=8).

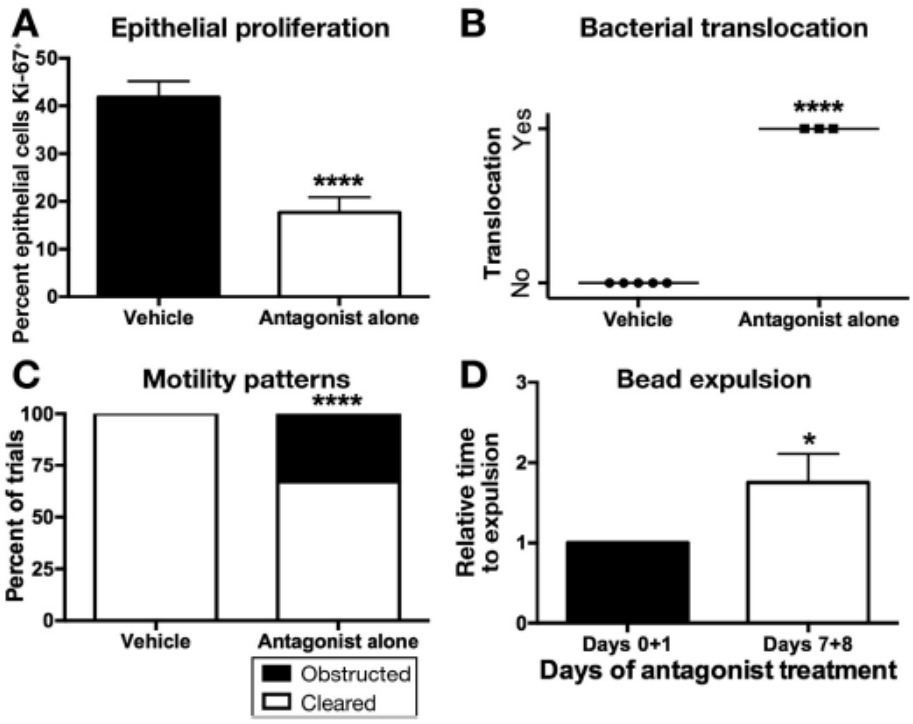

Figure 7:Daily intraluminal administration of the 5-HT4 antagonist GR113808 (1 mg/Kg) disrupted barrier function in normal mice, and motility in guinea pigs, as well as motility in mice. A. The proportion of crypt epithelial cells that were immunoreactive for the proliferation marker, Ki-67 was significantly decreased in animals treated with the antagonist (vehicle, n=8; antagonist, n=10; ****p<0.001 by t-test). B. Proportion of mice in which bacteria were detected in the liver or spleen following 10 days of vehicle or antagonist treatment. Vehicle, n=5; antagonist, n=3; ****p<0.0001, Fisher's Exact Test. C. Proportion of trials in which pellet propulsion was obstructed for at least 5 min. (vehicle, n=31 trials from 4 colons; antagonist, n=30 trials from 5 colons; ****p<0.0001, Fisher's Exact Test). D. Time to bead expulsion at the onset and following daily antagonist treatment in normal CD-1 mice. Data for each mouse were normalized to data collected at onset of treatment (*p<0.05, paired ttest; n=5).

Chapter IX

13. Prucalopride exerts neuroprotection in human enteric neurons

Modified from

"Prucalopride exerts neuroprotection in human enteric neurons"

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Abstract

Background: Serotonin (5-hydroxytryptamine, 5-HT) and its transporters and receptors are involved in a wide array of digestive functions. In particular, 5-HT4 receptors are known to mediate intestinal peristalsis and recent data in experimental animals have shown their role in neuronal maintenance and neurogenesis. This study has been designed to test whether prucalopride, a well known full 5-HT4 agonist, exerts protective effects on neurons, including enteric neurons, exposed to oxidative stress challenge.

Methods: SulfoRhodamine B (SRB) assay was used to determine the survival of SH-SY5Y cells, human enteric neurospheres and *ex vivo* submucosal neurons following H_2O_2 exposure in the presence or absence of prucalopride (1 nM). Specificity of 5-HT4 mediated neuroprotection was established by experiments performed in the presence of GR113808, a 5-HT4 antagonist.

Results: Prucalopride exhibited a significant neuroprotective effect. SH-SY5Y cells pre-treated with prucalopride were protected from the injury elicited by H_2O_2 as shown by increased survival (73.5±0.1% of neuronal survival vs. 33.3±0.1%, respectively; $P<0.0001$) and a significant reduction of pro-apoptotic caspase-3 and caspase-9 activation in all neurons

tested. The protective effect of prucalopride was reversed by the specific 5-HT4 antagonist GR113808.

Conclusions: Prucalopride promotes a significant neuroprotection against oxidative-mediated proapoptotic mechanisms. Our data pave the way for novel therapeutic implications of full 5-HT4 agonists in gut dysmotility characterized by neuronal degeneration, which go beyond the well known enterokinetic effect.

Methods

Neuronal cell lines and neurospheres culture

Murine Neuro2A as well as human SH-SY5Y, SK-N-BE, S-K-NSH, LAN-1 and HEK293 (ATCC, UK) cells were cultured in 95% air and 5% CO2 at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. Primary human enteric neurospheres were generated from human ENS stem cells containing neurosphere-like bodies according to a previously modified method (Bondurand N et al. 2003). In order to produce oxidative stress, H_2O_2 was freshly prepared from 30% stock solution prior to each experiment. Cells were treated with 200 μM H_2O_2 in phosphate-buffered saline (PBS) for 30 min as previously indicated (Clement MV et al. 1998).

Western blotting

Whole-cell lysates were obtained in IP buffer (Sigma-Aldrich, St. Louis, MO, USA), protease inhibitors cocktail (Roche Diagnostics, Indianapolis, IN, USA) and phosphatase inhibitors (Sigma-Aldrich) on ice for 15 min. Protein concentration was evaluated using the DC protein concentration assay kit (Biorad, Hercules, CA, USA). Fresh biopsies were sonicated on Bioruptor Pico Sonication System (V 1.1) (Diagenode, Liege, Belgium) in 100 μL of IP buffer and inhibitors cocktail (Sigma-Aldrich). Bands were visualized using the enhancer chemioluminescence method (GE Healthcare, Little Chalfont, UK). Primary antibodies used were: anti-5-HT4 (1:200; Abcam, Cambridge, UK) anti-HuC/D (1:200; Invitrogen, Carlsbad, CA, USA); anti-p75 (1:200; Thermo Fisher Scientific, Waltham, MA, USA); anti-γ-tubulin and anti114 vinculin (1:10,000 and 1:50,000, respectively; Sigma-Aldrich); anti-caspase-3

and caspase-9 (both 1:200; Cell Signaling, Denver, CO, USA); anti-neuronal nitric oxide synthase (nNOS) (1:50; Santa Cruz, Dallas, TX, USA) and anti-peripheral choline acetyl transferase (pChAT) (1:100; Justus-Liebig University, Giessen, Germany). The experiments on neurospheres were performed on cells cultured for at least 14 days.

Cytotoxicity assay

Sulforhodamine B colorimetric (SRB) assay was used for cell density determination. The method has been optimized for the toxicity screening of compounds to adherent cells in a 24-well plate (Vichai V et al. 2006). To determine the dose-response profiles of prucalopride, the SRB assay was performed by incubating cells for 0 (untreated), 30 and 60 min with prucalopride (Shire, Lexington, MA, USA) at the following final concentrations: 10mM, 10μM, 10nM, 1nM and 10pM to establish a correct dose finding. Controls were exposed to 0.05% (v/v) dimethyl sulfoxide in culture medium while wells containing only culture medium served as blank. The selective antagonist GR113808 (10nM) (1-[2-[(methylsulfonyl)-amino]ethyl]-4- piperidinyl]methyl-1-methyl-1H-indole-3-carboxylate, Sigma-Aldrich), was applied 30 min before prucalopride administration (Figure 1A). Absorbance was measured at λ540 nm using spectrophotometry.

Cell proliferation assay

SH-SY5Y cells (3×105132) were plated in duplicate and incubated with 10μM BrdU (Millipore Corporation, Billerica, MA, USA) for 1-48 hrs. BrdU-labeled cells adhered to the plate by centrifugation (300x*g* for 10 min) and then dried to the plate at room temperature for 1h. Anti mouse HRP-conjugated IgG antibody was used as secondary antibody. Absorbance measured at λ 450 nm was used to calculate the BrdU labeling index.

Submucosal neuron whole-mount preparations from colonic biopsies

A written informed consent (protocol: PaBio-2011) was obtained from enrolled subjects (n= 3 female; age range: 30-55 yrs) undergoing routine screening colonoscopy with biopsy sampling (n=3 each subject from the descending colon). Subjects were recruited at the outpatient clinics of Internal Medicine and Gastroenterology Units of the Department of Medical and Surgical Sciences of University of Bologna. Biopsies were transferred to sylgard-coated Petri dish filled with ice-cold Hank's solution, pinned flat and the mucosa separated from the submucosa under a stereomicroscope (16). Fresh submucosal specimens

were treated with prucalopride (1nM) for 30 min in combination with H_2O_2 maintained in DMEM F12 medium. Biopsies were sonicated for cycles at 20-60 kHz.

Immunofluorescence

Immunofluorescence was performed on neurospheres fixed in 4% PFA, rinsed in PBS and treated with blocking solution for 30 min. Previously described primary antibodies, i.e. rabbit anti pChAT (1:100), mouse anti-nNOS (1:50), rabbit anti-5HT4 (1:200), HuC/D (1:200) were applied to neurospheres overnight at 4°C. Specificity experiments included primary antibody omission and pre-incubation with the respective homologous antigen. After washing with PBS, a donkey anti-rabbit Cy3, anti-rabbit Alexa488, anti-goat Alexa488 and anti-mouse Alexa488 (1:200, Thermo Fisher Scientific) secondary antibodies were applied for 30 min at room temperature. FluoroschieldTM 155 DAPI solution (Sigma Aldrich) was used as mounting solution and to counterstain nuclei. Image capturing was performed on a Nikon microscope using DS-5M digital camera (Nikon Instruments, Düsseldorf, Germany).

Statistical analysis

Statistical analysis was performed using GraphPad Prism software v.5.0 (San Diego, CA, USA). Statistical significance was defined as $P<0.05$ as evaluated by one-way analysis of variance (ANOVA) and Tukey's multiple comparison test. Statistical analysis of densitometry was performed using Image Lab™ Software (Bio-Rad, California, USA).

Results

Evaluation of the 5-HT4 expression in human enteric neurospheres and other neuronal cell lines

The 5-HT4 protein expression was studied by western blot analysis using human neuroblastoma cell lines, SH-SY5Y, SK-N-BE, S-K-NSH, and LAN-1, the human embryonic kidney cell line HEK293, a mouse neural crest-derived cell line, Neuro2A (used as a negative control for antibody specificity) and human enteric neurospheres (Figures 1B and 1C). All the human cell lines analysed showed 5-HT4 expression, whereas no signals were detected in the murine Neuro2A cells since the antibody used was specific for the human 5-HT4 isoform. Western blot analysis of human enteric neurospheres and SH-SY5Y showed that these cells

also expressed the neuronal marker HuC/D and the progenitor/stem cell marker p75 (Figure 1D), thus indicating that these cells were suitable for studies on neuroprotection.

The 5-HT4 agonist prucalopride protects SH-SY5Y cells from oxidative stress insult

We tested different concentrations of the full 5-HT4 agonist prucalopride on SH-SY5Y cells exposed to the oxidative stress agent H_2O_2. The oxidative stress evoked by 200 μM of H_2O_2 resulted in a significant reduction of cell survival (33.3±0.1%) compared to untreated cells (P<0.001).

Prucalopride at 1 nM concentration showed the best protective effect, with a significantly higher cell survival compared to cells exposed to H_2O_2 (73.5±0.1% of neuronal survival *vs.* 33.3±0.1%, respectively; P<0.0001) (Figure 2A). In order to verify the specificity of the 5-HT4-mediated protective effect, cells were exposed to the selective antagonist GR113808 (10nM) applied 30 minutes before prucalopride administration (18). Pre-treatment with GR113808 induced a significant reduction in cell survival, i.e. 23.3±0.1% *vs.* the 73.5±0.1% in the H_2O_2-treated cells exposed to prucalopride (P<0.0001). These data are comparable to those obtained in presence of H_2O_2 alone, indicating that the observed protection was elicited via 5-HT4 activation (Figure 2A). Untreated cells exposed to GR113808 at 10 nM concentration showed a survival rate of 81.3±0.1%, which was comparable to that of vehicle-treated cells, indicating lack of toxicity of the 5-HT4 antagonist (Figure 2A, P<0.001). By contrast, prucalopride becomes toxic at high concentrations with the highest toxic effect observed with 10 mM prucalopride as indicated by reduced survival (32±1.2% and 29±1.2%, at 30 and 60 min, respectively) in cells unexposed to oxidative stress (cell survival being virtually 100%). Prucalopride at the lowest concentration tested (10pM) failed to rescue neuronal cells from damage induced by oxidative stress (survival being 41±0.8 vs. 32±1.2%, respectively) (Figure 2B).

Evaluation of prucalopride in neurogenesis in vitro

In order to determine whether prucalopride induces neurogenesis *in vitro*, we evaluated the bromodeoxyuridine (BrdU) incorporation 24 hours after the treatment with different concentrations of the 5-HT4 agonist. Prucalopride did not evoke a significantly different incorporation of BrdU compared to untreated SH-SY5Y or H_2O_2-treated cells (P=0.384) (Figure 2C). This suggests that the effect of prucalopride treatment of SH-SY5Y cells

exposed to oxidative stress is predominantly neuroprotective, and not due to neuroproliferation.

Effect of prucalopride on caspase expression in neuronal cell lines and neurospheres

To investigate the mechanisms underlying the neuroprotective effects mediated by prucalopride, we examined the expression of two apoptotic markers, i.e. caspase-3 and caspase-9, which are activated by oxidative stimuli. No changes in procaspases expression were found before and after treatments (Figures 2D, E and 3A, B), whereas a significant decrease of the active form of both oxidative stress-activated caspases-3 and caspase-9 was detected in SH-SY5Y cell lines (Figures 2F, G) and in human enteric neurospheres (Figures 3C, D), treated with 1 nM prucalopride.

Effect of prucalopride in different subgroups of neurons

To determine whether the neuroprotective effect evoked by prucalopride occurred in selective subpopulations of enteric neurons, we investigated the effect of prucalopride following the oxidative stress injury on the expression of two major enteric neuron transmitters nNOS (neuronal nitric oxide synthase, the enzyme that produces nitric oxide) and pChAT (peripheral cholinacetyltransferase, the enzyene that catalyzes the synthesis of acetylcholine) in human enteric neurospheres. Both nNOS and pChAT expression was identified by immunofluorescence and western blot (Figures 3E-G). H_2O_2 treatment induced a quantitative reduction of both nNOS and pChAT proteins in neuropheres compared to the total protein. In contrast, prucalopride treatment prevented the oxidative-induced reduction of each subset of transmitters compared to H_2O_2 treatment without prucalopride (Figure 3H, I, $P<0.05$).

Western blot analysis confirmed the expression of 5-HT4 in colonic biopsy-derived submucosae obtained during colonoscopy (Figure 4A). After treatment with prucalopride, submucosal whole mount preparations were exposed to H_2O_2 and the levels of activated caspases were measured. Procaspase expression did not vary with any treatment (Figures 4B, C). However, activated caspase-3 and caspase-9 were significantly reduced in prucalopride-treated biopsies compared to H_2O_2 only (Figures 4D, E, $P<0.001$).

Discussion

The results of this study provide strong evidence for a neuroprotective role of 5-HT4 subtype,which is expressed in human neuronal cell lines, neurospheres and enteric neurons as confirmed by *in vitro* and *ex vivo* experiments. Stimulation of 5-HT4 with the specific, high-affinity full agonist, prucalopride, induced significant protection against the oxidative stress injury elicited by H_2O_2. The protective effect of prucalopride on human neurons, including enteric neurons, was demonstrated by: 1) the increased survival of human neuronal cell lines exposed to oxidative stress; and 2) reduction of apoptosis in neuronal cell lines, human enteric neurospheres obtained from perinatal gut biopsies and whole mount preparation of submucosal neurons from adult colonic tissues, following oxidative stress challenge. The abolition of all of these effects by pretreatment with the 5-HT4 antagonist (GR113808) demonstrated that prucalopride-evoked protective effect was due to specific 5-HT4 activation. Taken together these results expand previous data showing that other 5-HT4 agonists (i.e. tegaserod and renzapride) protected primary cultures of mouse enteric neurons from apoptosis (Liu MT et al. 2009). Notably, our study indicated that the optimal neuroprotective effect was obtained with 1 nM prucalopride, which is at least 10 times lower compared to the doses used by Liu et al. with either tegaserod or renzapride (Liu MT et al. 2009).

The difference in terms of concentrations and related neuroprotective effect can be the result of a diverse efficiency in 5-HT4 251 receptor activation exhibited by the different agonists used in our *vs.* Liu et al. study (Liu MT et al. 2009). The neural network supplying the digestive system, including the ENS, exerts a prominent regulatory role on several digestive functions (De Giorgio R et al. 2007). A tight association links gut dysfunction to ENS abnormalities characterized by neuronal damage and / or loss (Knowles CH et al 2013). In addition to the many effects elicited by serotoninergic pathways, 5-HT4 plays an important role in mediating 5-HT neuroprotective function as supported by the observation that mice lacking 5-HT4 displayed a markedly reduced number of submucosal and myenteric neurons in an age-dependent manner (Gershon MD et al 2007; Liu MT et al. 2009). Oxidative stress is well known to trigger neuronal loss and represents a key target for neuroprotection. A neuroprotective effect of 5-HT against oxidative stress-induced cell death has been supported by the results of a study showing that 5-HT treatment increased the activity of anti- oxidants enzyme and counteracted lipid peroxydation in the brain in a rat model of Parkinson disease (Anderson G et al. 2014). Our study extends the data reported in the brain, by showing that

prucalopride, a specific 5-HT4 receptor agonist protects peripheral neurons against oxidative stress-mediated proapoptotic insults induced by exposure to H_2O_2, as indicated by the significant reduction in caspase-3 and caspase-9 activation, both key regulators of apoptosis in a variety of cells including the ENS (Danial N et al. 2004; De Giorgio R et al. 2003). The prucalopride-mediated neuroprotection in SH-SY5Y cell lines, human neurospheres and *ex-vivo* colonic submucosal neurons, indicates that 5-HT4 counteracts pro-apoptotic pathways by influencing the activation of both initiator and effector caspases. Notably, the finding that prucalopride rescued the neuronal expression of nNOS as well as pChAT that was reduced by oxidative stress challenge suggests that damage of functionally distinct neuronal populations, i.e. nitrergic (inhibitory) and cholinergic (excitatory) neurons (De Giorgio R et al. 2007; Furness JB 2012), can be prevented by 5-HT4 stimulation. The 5-HT4 activity is mediated by PKA / CREB signaling (. Liu MT et al 2009), a system crucial for neurogenesis and neuronal connectivity in the brain and pharmacological modulation of PKA has been proposed as a possible therapeutic target in neuronal degeneration, e.g. Alzheimer's and Parkinson's diseases (Irwin RW et al 2014; Park SY et al 2014). Ideally, 5-HT4-mediated PKA activation might be exploited to restore neuronal circuitry also in enteric neuropathies. Our results provide a pharmacological basis for prucalopride as a compound potentially useful in patients with enteric degeneration and loss of neurons, such as enteric neuropathies. These conditions are characterized by a severe abnormality of gastrointestinal motility and might represent the 'tip of the ice-berg' of functional digestive disorders (Knowles CH et al 2013). Enteric neuropathies can be either primary (i.e. of unknown etiology) or secondary to a variety of diseases including central neurodegenerative disorders. Patients with enteric neuropathies manifest a broad spectrum of digestive symptoms resulting from the severe abnormalities of gut motility. Typical examples of such conditions, which markedly impair the patients' quality of life and can be life threatening, include gastroparesis, chronic intestinal pseudo-obstruction and severe slow-transit constipation/colonic inertia. The treatment of these conditions is mainly supportive and so far lacking a targeted, e.g. receptor-mediated, approach (Furness JB 2012; . Knowles CH et al. 2013). Interestingly, a previous study reported that the 5-HT4 partial agonist tegaserod increased the number of neurons and length of neurites of *in vitro* cultured neural crest-derived enteric neurons, indicating that activation of enteric neural 5-HT4 receptors promotes neurogenesis (Gershon MD et al 2007; Liu MT et al. 2009; Takaki M et al. 2014). By contrast, our *in vitro* experiments did not show neurogenetic effects of prucalopride on human neuronal cell lines. This apparent discrepancy between our data and

the previously reported results may be ascribed to a shorter period of analysis used in our investigation (24 hours) compared to the Matsuyoshi et al. study where mosapride (another partial 5-HT4 agonist) was used for 15 days in animal models (Matsuyoshi H, et al. 2010). Whether a longer period of treatment with prucalopride can also induce an increase in cell proliferation and / or differentiation (i.e., neurite outgrowth) remains to be elucidated.

In conclusion, the present data highlight for the first time that the specific 5-HT4 full agonist prucalopride evokes significant neuroprotective effects in neurons, including human enteric neurons, *in vitro* and *ex vivo*. Prucalopride is currently used for treatment of chronic constipation and has an excellent safety and tolerance profile. These findings widen the role of this compound beyond its well-defined enterokinetic property and support that 5-HT4 activation can be a target for pharmacological intervention in neurodegenerative diseases of the gut. Ad hoc designed clinical trials are awaited to test the efficacy of 5-HT4 in stopping the progression of neuronal damage and loss in patients with severe gut dysmotility.

Figures

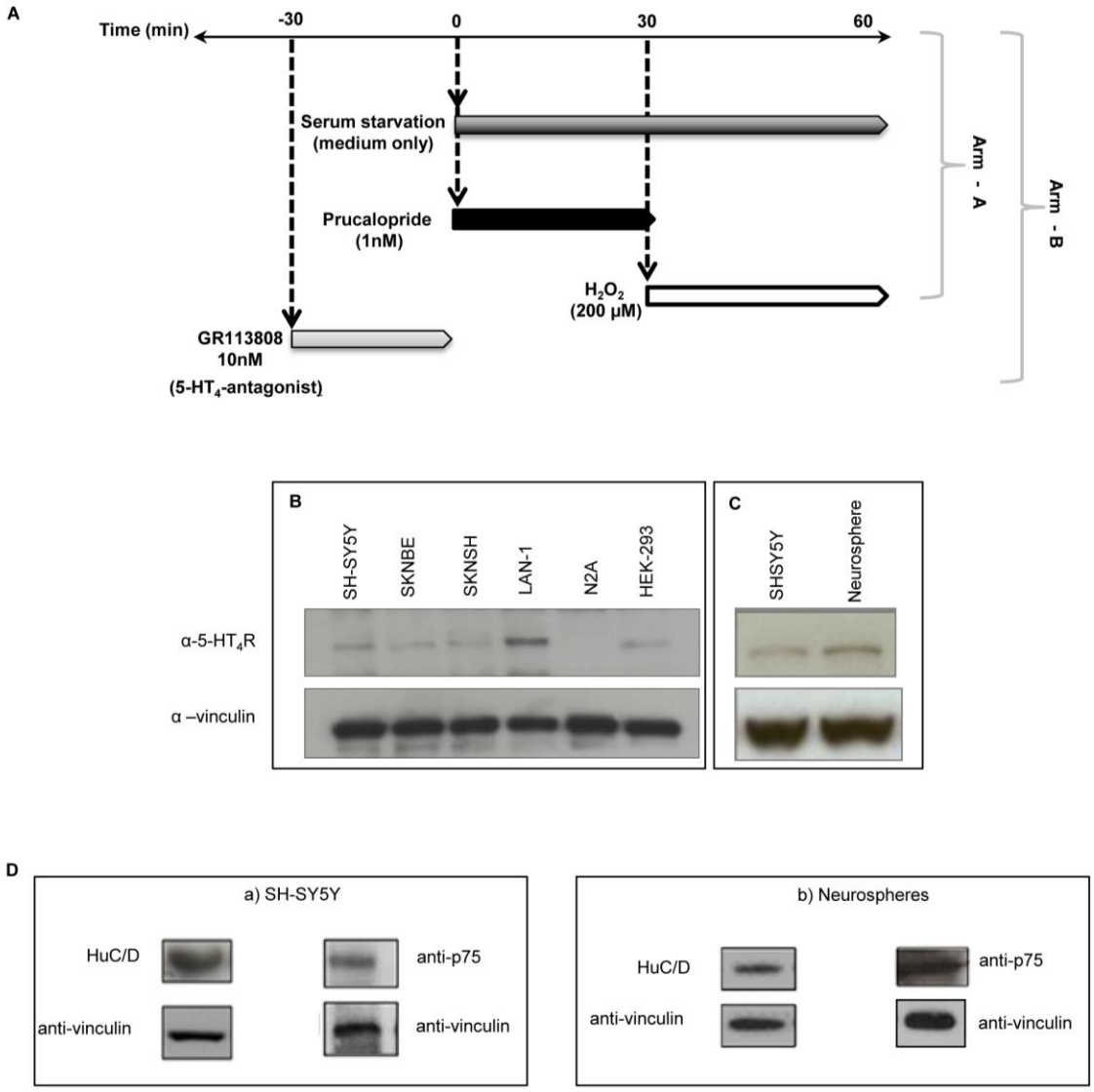

Figure 1: A: experimental design used in the present study. Arm-A: serum starvation (medium only) was applied at time 0 in cell cultures. Prucalopride (1 nM) was applied 30 min after challenge with H_2O_2 (200 µM). Arm-B: the 5-HT4 antagonist GR113808 (10 nM) was applied 30 min before either prucalopride or H_2O_2. B: Western blot of total cell lysates with antibody to 5-HT4 protein and with anti-vinculin in SH-SY5Y (lane 1), SK-N-BE (lane 2), SK-N-SH (lane 3), LAN-1 (lane 4), Neuro2A (N2A, lane 5), and HEK293 (lane 6). Separate Western blot of total cell lysates with antibody to 5-HT4 protein and with anti-vinculin in SH-SY5Y (lane 1) and human enteric neurospheres (lane 2). C: total cell lysates prepared from SH-SY5Y (a) and human enteric neurospheres (b) immunoblotted with anti-HuC/D, anti-p75, and anti-vinculin antibodies.

Figure 2.

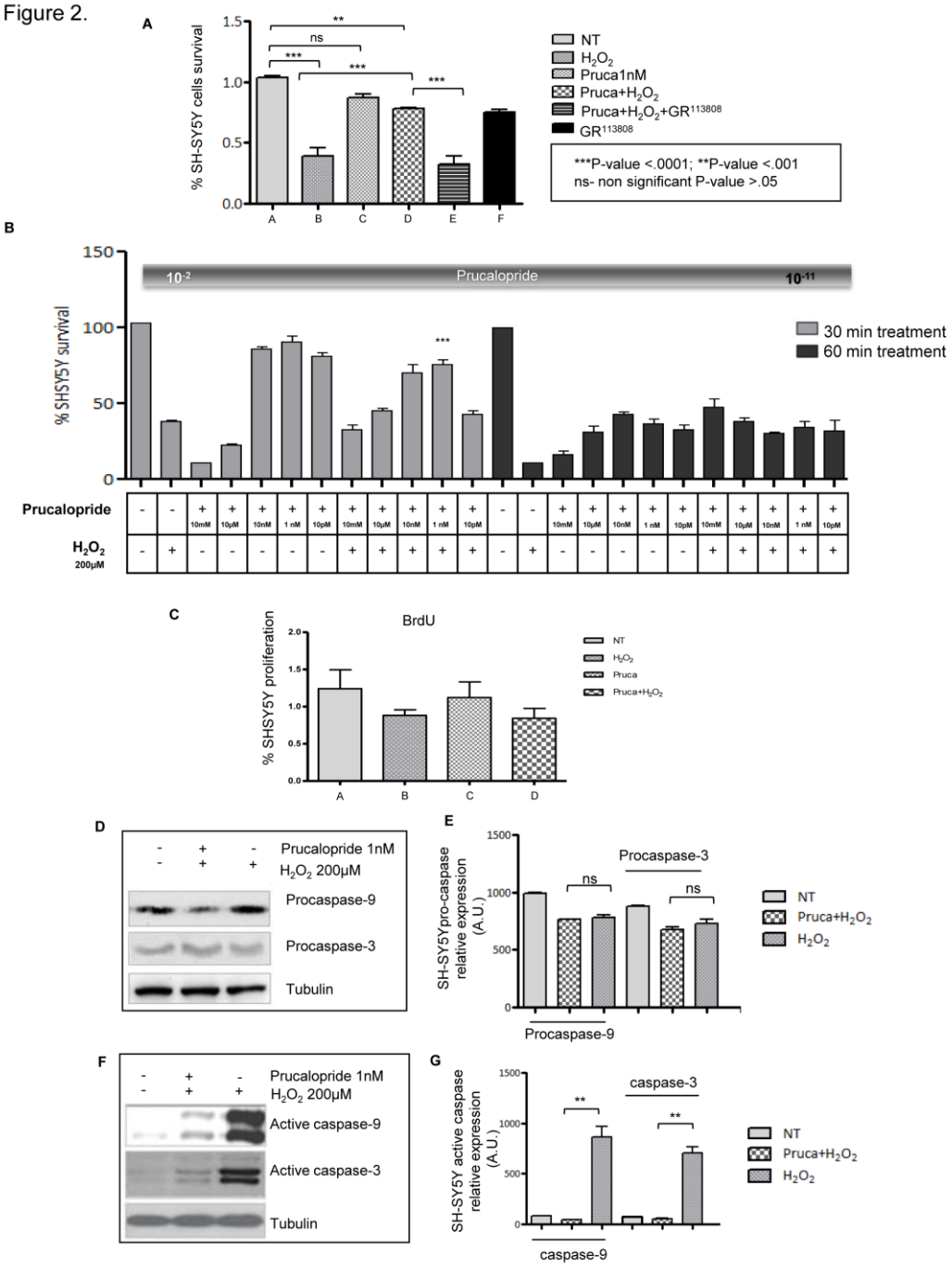

Figure 2: A: percentage of SH-SY5Y survived after treatment with 1 nM prucalopride alone (C bar) or in combination with 200 µM H_2O_2 (prucalopride+ H_2O_2 vs. H_2O_2 **P < 0.0001) for 30 min (D bar) and GR113808 either alone [GR113808 vs. untreated (NT); ns] (F bar) or with 200 µM H_2O_2 (prucalopride+ H_2O_2 vs. prucalopride+GR113808+ H_2O_2 ***P < 0.0001) (E bar). A bar, NT are considered as 100% cell survival; B bar, H_2O_2 alone. B: dose finding was established by treating SH-SY5Y cells for 30 or 60 min with prucalopride at different concentrations (10 mM, 10 µM, 10 nM, 1 nM, and 10 pM). Compared with 30 min, 10 nM prucalopride for 60 min resulted in a reduced neuroprotective effect (cell survival 80 vs. 60%, respectively, P < 0.0001). C: BrdU analysis in the

SH-SY5Y cell line. Percentage of SH-SY5Y proliferation after treatment with 1 nM prucalopride, alone (C bar) and in combination with 200 μM H_2O_2 for 30 min (D bar). A bar, NT; B bar, 200 μM H_2O_2 for 30 min. ANOVA and Tukey's multiple comparison tests did not reached statistical significance (P = 0.384). D and E: Western blotting of SH-SY5Y total lysates did not show differences in procaspase-3 and -9 expression; lane 1: NT; lane 2: 1 nM prucalopride (Pruca) and H_2O_2; lane 3: H_2O_2 alone. F and G: Western blotting of SH-SY5Y showing significant differences in active caspase-3 and -9 expression (**P < 0.001 vs. NT); lane 1: NT; lane 2: 1 nM prucalopride and H_2O_2; lane 3: H_2O_2 alone.

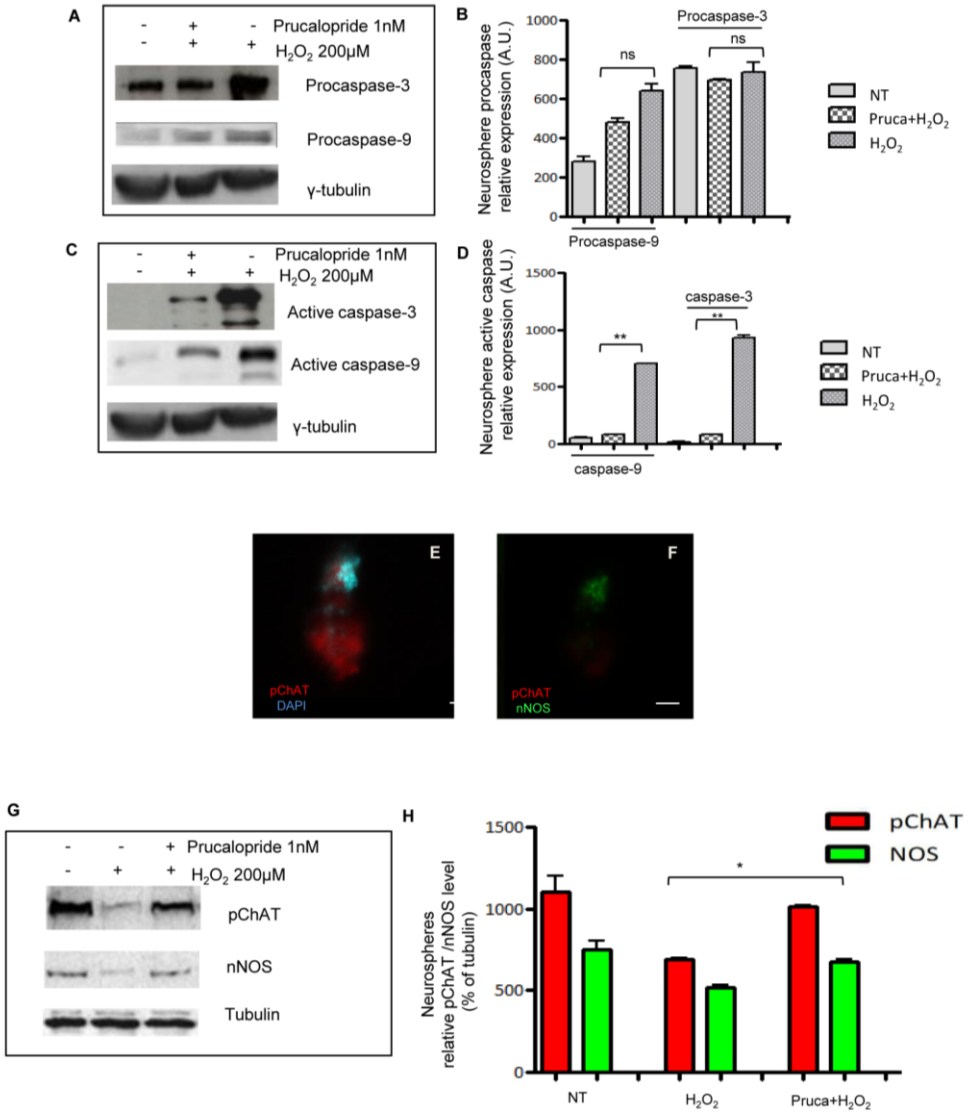

Figure 3: A and **B**: Western blotting of human enteric neurospheres did not show differences in procaspase-3 and -9 expression; lane 1: NT; lane 2: 1 nM prucalopride and H_2O_2; lane 3: H_2O_2 alone. **C** and **D**: Western blotting showed significant differences in active caspase-3 and -9 expression (**P < 0.001 vs. NT); lane 1: NT; lane 2: 1 nM prucalopride and H_2O_2; lane 3: H_2O_2 alone. **E** and **F**: note nitrergic (nNOS) (E) and cholinergic (pChAT) (F) immunoreactive neurons (scale bar 100 µm). **G** and **H**: Western blotting for nNOS and pChAT showed rescue of these 2 neuronal subsets after prucalopride treatment; lane 1: NT; lane 2: H_2O_2 alone; lane 3: H_2O_2+prucalopride (*P < 0.05 pChAT/nNOS prucalopride+ H_2O_2 vs. H_2O_2).

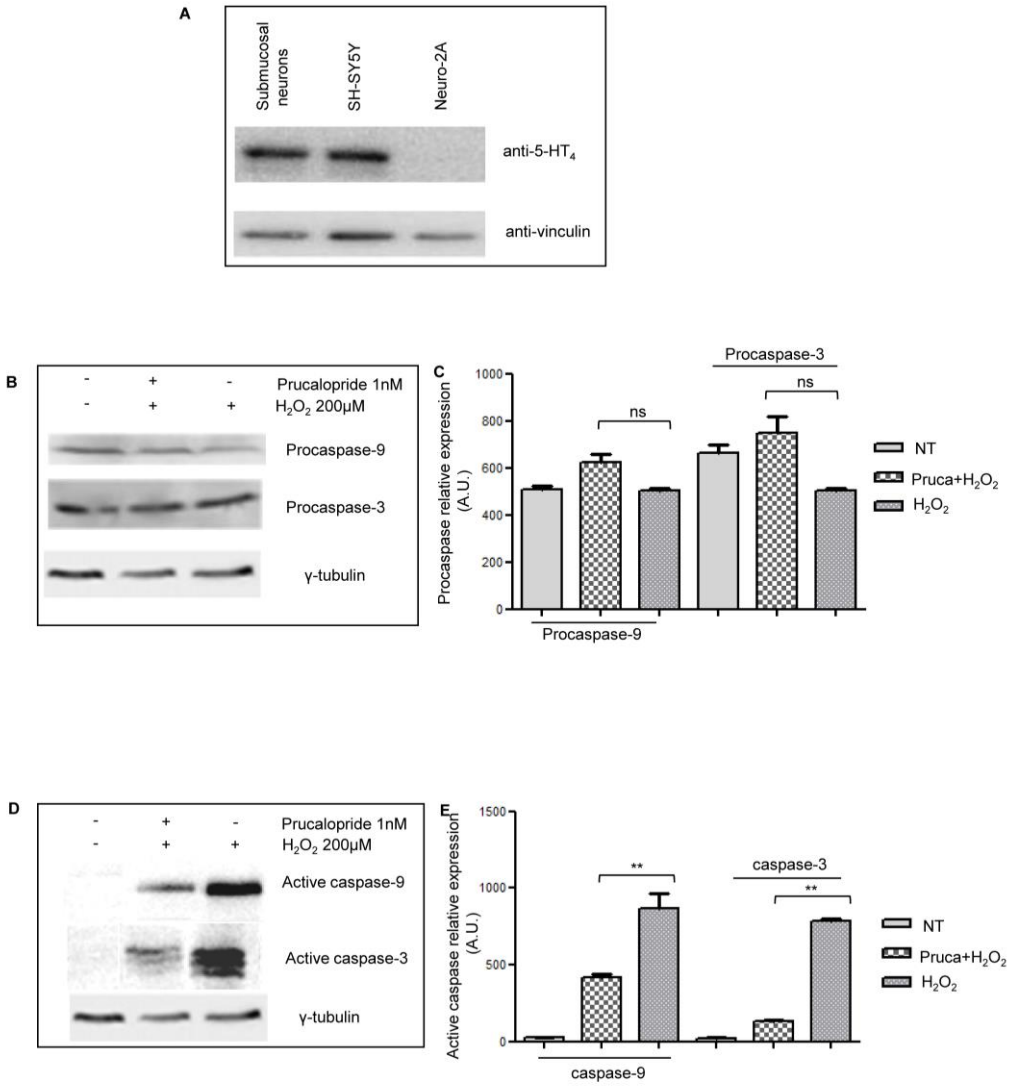

Figure 4: A: Western blot showing 5-HT4 expression in submucosal neuron whole mounts (lane 1) compared with human SH-SY5Y (lane 2, positive control) and murine Neuro2A (lane 3, negative control). B and C: Western blotting did not show differences in procaspase-3 and -9 expression; lane 1: NT; lane 2: 1 nM prucalopride+ H_2O_2; lane 3: H_2O_2 alone. D and E: Western blotting showed significant differences in active caspase-3 and -9 expression (**P < 0.001 vs. NT); lane 1: NT; lane 2: 1 nM prucalopride+ H_2O_2; lane 3: H_2O_2 alone.

CONCLUSIONS

Chapter X

14. Conclusions

The ENS serves a vast array of regulatory functions through which the physiology of the GI tract occurs to preserve body homeostasis and ultimately life. Mounting evidence clearly indicates that the ENS is reminiscent of the CNS organization, not only for the neuro-bioelectrical properties and function, but also for its neurochemical properties and receptor expression, which provides a number of possible targets of therapeutic intervention. In addition to neurons, the ENS possesses a prominent glial cell component, which is, in some extent, comparable to astrocytes for morphological and functional features. Thus, it should not be surprising that changes altering such a finely tuned and highly integrated system, such as the ENS, can lead to neuro-glia abnormalities and loss, thereby ensuing gut dysfunction. In this thesis, I tackled some aspects of enteric neuropathies particularly focusing on genetically-driven molecular abnormalities. A clinical phenotype of enteric neuropathy is given by CIPO a severe dysmotility caharctaerized by a markedly compromised quality of life and poor prognosis for affected patients. Notably, there are no effective treatment for this condition and from a pathophysiological stand-point no established animal models for studying the various pathogenetic mechanisms contributing to neuropathy often underlying CIPO. My research work during this PhD program helped generating initial evidence that genetic mutations are at the base of ENS degeneration and loss.

The first study focused on genetic aspects of a consanguineous family with syndromic CIPO (OMIM 611376). Whole exome sequencing analysis led ud to identified a novel homozygous mutation in the gene RAD21, mapping to the previously identified linkage interval on chromosome 8 (Deglincerti et al, 2007) and leading to the missense substitution A622T. The RAD21 protein is part of the cohesin complex involved in pairing and unpairing of sister chromatids during cell replication and regulates gene expression independently of cell replication (Horsfield et al, 2012). Heterozygous RAD21 mutations have been reported in the Cornelia de Lange syndrome (Deardorff et al, 2012), but none of RAD21 mutation carriers showed the clinical features of that syndrome. A few genes have been so far associated to CIPO and timely identification of genetic variants is essential to predict pathogenic mechanisms and guide appropriate management options for patients.

In this line, my second study was designed to investigate the localization of RAD21 in mouse and human ENS in order to understand how mutations of RAD21 may contribute to altered neuronal function/survival, thus leading to gut motor abnormalities. A thorough tissue analysis of RAD21 expression is lacking, thus we aimed to identify and characterize RAD21-IR in enteric ganglia. Our data indicated that RAD21 was not expressed by nitrergic inhibitory neurons, a functionally distinct subclass commonly targeted by damaging mechanisms in experimental ($nNOS^{-/-}$ mice) and human enteric neuropathies, e.g. in early stages of achalasia or idiopathic gastroparesis. It has not been determined whether nitrergic neurons remain unaffected in pathological conditions such as patients with RAD21-related CIPO. These findings, combined with the knowledge that RAD21 mutations occur in distinct CIPO cases, imply that those cholinergic subset may be the primary target in some patients with this pathological condition. In addition we are currently generate $rad21^{A626T}$ conditional knock-in mouse for understanding the role of RAD21 in gut motility and creating a model for CIPO. This strategy will be relevant to increase our knowledge on ENS through the investigation of other mutations identified in idiopathic CIPO patients.

These studies highlight neurodegeneration and related subsets of enteric neurons as pathogenetic features detectable in patients with CIPO. On the other hand, I posed the question as to how rescue or protect neurons from degenerative processes. Clearly, genetic factors cannot be modified as so far gene therapy did not yet emerge for the neurogastroenterological field. Therefore, I wanted to exlore the relatively less uncommon sporadic CIPO and establish whether neuroprotective mechanisms may be useable. In the attempt to establish whether 5-HT4 can be a possible target of reparative and protective effect, I have been involved in two main studies. The first demonstrated that 5-HT4 activation maintains motility in healthy colons of mice and guinea pigs and reduces inflammation in mice with colitis. The epithelial 5-HT4 receptor stimulation can exert its protective effects through several mechanisms, including increased epithelial proliferation, enhanced epithelial cell migration, and resistance to oxidative stress-induced apoptosis. Furthermore, treatment with the 5-HT4 agonist attenuated inflammation-induced changes in colonic motor function. Importantly, all of these effects were blocked by the 5-HT4 antagonist, GR113808, and protection was not detected in 5-HT4-/- mice. Translation of these observations could provide a new and safe treatment strategy for the treatment of colitis, and that expand our appreciation of the roles of 5-HT receptor signaling in the GI tract. Collectively, these findings advance

our understanding of colonic physiology and pathophysiology, and provide a new target for the treatment of colonic inflammation.

In the second study, I tested whether prucalopride, a full 5-HT4 agonist used for the treatment of laxative resistant constipation, can evoke neuronal protection. The results demonstrated that prucalopride protect enteric neurons against oxidative (H_2O_2-induced) stress *in vitro*. These findings are important because many noxious factors converge to oxidative stress as ultimate mechanism promoting enteric neuron degeneration and loss (Brown et al. 2016; Gulbransen et al. 2012).

As ancillary data during the PhD program I contributed to two other recently published studies by Giancola et al. One of these regarded the localization of 5-HT4 in the equine ENS. This model was investigated as a tool to detect the expression of 5-HT4, a largely known topic, which provides support to the physiological effect mediated by this enterokinetic serotonin receptor. Notably, the 5-HT4 expression occurs in both cholinergic and nitrergic motor neurons as well as in spinal ganglia of the horse. This latter finding open to 5-HT4 as a possible receptor pathway involved in sensory perception, an aspect so far poorly recognised and largely attribute to other serotoninergic receptors.

The second study was designed to shed light on clinical, manometric and neurochemical/molecular findings in constipated Parkinson disease patients. In this study we showed a dowregulation of VIP and related receptors in non-PD and, with a slightly more pronounced compromission, PD constipated patients, which suggests an abnormal secretory function in ths latter group. Moreover, we recently submitted a study by Bonora et al. in which we detected 111 dysregulated genes in primary (idiopathic) achalasia. The identification of gene abnormalities (c-KIT downregulation and INPP4B upregulation) highlights novel signaling pathways involved in the neuro-interstitial cell of Cajal changes underlying achalasia.

In conclusion, changes to the neuro-glial and epithelial aspects driven by genetic and environmental factors in the context of a severe dysmotility, i.e. CIPO, or animal models emerge as new important and fascinating topics fueling the interest of researchers in translational neurogastroenterology. The present thesis clearly manifests my willgness to continue to investigate pathophysiological mechanisms that impact on diseases affecting gut homeostasis. Over the years, I learned that perseverance and enthusiasm in research pave the

way to solving biological problems, which help clinicians in improving patients' management and treatment.

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